

Tropical Medicine Point-of-Care Testing



Supporting the
UN 2030 Agenda



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Tropical Medicine Point-of-Care Testing

**Supporting the
UN 2030 Agenda**

COVID-19

**July 2022
Supplement**

**Available from
Tropical Health Technology
www.tht.ndirect.co.uk**

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Cheesbrough**

Published by Tropical Health Technology
www.tht.ndirect.co.uk

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First published 2020 Update 2022

Typeset by Fakenham Prepress Solutions

Printed by ImprintDigital

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Colour Plates on Front Cover
Courtesy of Laboratory Department, Amref International University, Nairobi, Kenya.

Tropical Medicine Point-of-Care Testing **2022 Update**

Preface

As countries recover from the effects of the COVID-19 pandemic, the importance of point-of-care testing in helping to strengthen community-based healthcare is being highlighted. Of particular importance are those laboratory investigations that can be integrated in primary healthcare services, such as tests for HIV, malaria, tuberculosis, hepatitis, diabetes, anaemia, and tests required during pregnancy.

To meet the requirement for community-based laboratory tests, this 2022 Update of *Tropical Medicine Point-of-Care Testing* includes the most recently developed tests that can be performed both in district laboratories and also in peripheral healthcare facilities by non-specialist personnel. Also included are self-tests for diseases such HIV and hepatitis with accompanying information on the need for confirmatory testing and link to treatment and care.

The text on communicable and non-communicable diseases prevalent in tropical countries has been updated. Like the first edition, the Update also includes laboratory quality management, quality assurance of diagnostic investigations and laboratory safety. District laboratory staff have a key role in supporting community-based laboratory testing to ensure tests are available when and where they are needed and performed correctly, safely and cost-effectively.

As more affordable, stable, point-of-care tests become available it is anticipated that community-base testing and self-testing will increase, bringing *universal health coverage* closer and the Sustainable Development Goal 3 of *ensuring healthy lives and well-being for all*, a nearer reality.

Monica Cheesbrough
September 2022

Tropical Health Technology
www.tht.ndirect.co.uk

Acknowledgements

The author would like to thank all those who have helped in producing *Tropical Medicine Point-of-Care Testing – Supporting the UN 2030 Agenda*, especially:

Jane Carter, Director of Clinical and Diagnostics Programme, Amref International University, Nairobi, Kenya and Orgenes Lema, Manager of the Mission for Essential Supplies, Tanzania for reviewing the Chapters covering the organization of district laboratory services, laboratory quality management and equipping district laboratories. Gratitude is also expressed to Amref International University for providing the ZN photomicrograph used in Section 2.11 and the photographs used on the front cover and to illustrate the text.

Tim O' Dempsey, Senior Lecturer Clinical Tropical Medicine, Liverpool School of Tropical Medicine for updating the clinical information, prevention and control of parasitic diseases described in Section 2 and to Jayne Jones, Laboratory Quality Manager Clinical Diagnostic Parasitology, Liverpool School of Tropical Medicine, for reviewing the laboratory diagnosis of parasitic diseases.

Paul Shears, previously Consultant Microbiologist/Director for Infection Control, Wirral University Teaching Hospital, for contributing the Healthcare associated infections text and the Syphilis Serology Diagnostic Table and for reviewing the pages covering bacterial diseases in Section 2.

Patrick Simms, Senior Biomedical Scientist, Biochemistry Department and Pathology Training Manager, Weston Area Health Trust for suggesting improvements and updates to the text covering diabetes mellitus and lipid disorders and the tests describing the investigation of kidney disease and liver disease.

Adekunle Bashiru Okesina, Member Executive Board IFCC, Consultant Chemical Pathologist, University of Ilorin, Nigeria and staff of the Department of Chemical Pathology, University of Ilorin (Abudulrazaq Adeyinka Akande, Sikiru Abayomi Biliaminu and Ademola Adelekan) for their suggestions covering the clinical chemistry text in Section 3.

Jon Sims, Morphology Lead Scientist, UK NEQAS Haematology for reading through and suggesting improvements to text and artwork covering the investigation of anaemia.

Jaya Shrivastava, Parasitology Scheme Manager and the UK NEQAS team for supplying advice and images of Giemsa stained malaria parasites.

Martin Griffiths for reviewing the text covering the safety of power supplies and to Scott Chapman for suggesting useful resources.

Fakenham Prepress Solutions for originating the PDF files, particularly Terry Money for his valuable expertise, and ImprintDigital for printing the book digitally.

Special thanks are due to my sister Mary for her valuable editorial and computer skills, support and encouragement.

Acknowledgements for the colour plates

The author would like to thank all those who kindly supplied photomicrographs and artwork used to illustrate the publication, including:

The World Health Organization

CDC DPDx Image Library

Anthony Moody

John Williams

Richard W Ashford

Herman Zaiman

The Royal Tropical Institute, Amsterdam

Bernhard-Nocht Institut für Schiffs und Tropenkrankheiten

Tropical Doctor and BOL Dukes and J Anderson

Liverpool School of Tropical Medicine Laboratory

WB Saunders Company and W Jann Brown

Medical Microbiology Dept, University of Leeds

Haematology Dept, Middlesex Hospital School of Pathology

Jane Carter

Jayne Jones

Churchill Livingstone/Elsevier

Balbir Singh

Lee Kim Sung

John Richens

Richard Suswillo

Brian Watt

Elizabeth Gerwing-Adima

Michael Watts

John Burthem

Michelle Brereton

Role of the laboratory in supporting the United Nations 2030 Agenda for Sustainable Development

“*Transforming our World: The 2030 Agenda for Sustainable Development*” is a comprehensive plan of action for people, the planet and prosperity, implemented by the United Nations in 2016. It comprises 17 interdependent Sustainable Development Goals (SDGs)* with targets.

Access from <https://sdgs.un.org>

* SDGs cover poverty, hunger, health and well-being, education, gender equality, water and sanitation, energy, work and economic growth, industry, cities and communities, responsible consumption and production, climate, life below water and on land, peace and social justice.

SDG 3 Health and well-being:

To “ensure healthy lives and promote well-being for all at all ages”

District laboratories have an important role in achieving the targets of SDG 3 which include:

- Achieving universal health coverage and access to quality essential healthcare services. This requires well-managed laboratory services, a laboratory workforce that is well-trained and motivated and ensuring essential investigations are available at point-of-care.
- By 2030, ending the epidemics of AIDS, tuberculosis, malaria and neglected tropical diseases and combating hepatitis, water-borne diseases and other communicable diseases. A network of supported district laboratories is required to diagnose communicable diseases, monitor their eradication and perform disease surveillance.
- By 2030, reducing by one third premature mortality from non-communicable diseases. Laboratory investigations are important in diagnosing and monitoring diseases such as diabetes mellitus, kidney disease, liver disease and anaemia.
- By 2030, ensuring universal access to sexual and reproductive healthcare services. This includes laboratories testing for sexually transmitted infections and performing essential investigations during pregnancy and following delivery.

SDG 6 Clean water and sanitation

To “ensure availability and sustainable management of water and sanitation for all”

District laboratories can help to achieve the targets of SGD 6 which include:

- By 2030, achieving universal and equitable access to safe and affordable drinking water and access to adequate sanitation and health. District laboratories can help to monitor the quality of community water supplies and investigate water, sanitation and hygiene (WASH) related infections.
- Supporting the participation of local communities in improving water quality and sanitation management. Laboratory staff can train community health workers to collect water samples and when indicated, perform water quality analysis using portable water testing kits.

PROGRESS ON SDGs

Progress on achieving the targets of the SDGs, including the impact of the COVID-19 pandemic, can be found in *The Sustainable Development Goals Report 2022*.

Access by entering the title in Google.

Importance of District Laboratories in Point-of-Care Testing

District laboratories are important in helping to achieve universal health coverage by point-of-care testing, also known as near-patient testing.

A network of reliable well-managed district laboratories and a well-trained workforce can improve the quality and cost-effectiveness of community healthcare by:

- Providing earlier diagnoses at point-of-care, optimizing patient management and outcome and reducing the risk of disease progression.
- Helping to identify the causes of infectious diseases and participating in disease surveillance.
- Increasing patient satisfaction and community confidence in its healthcare services.

Unless the importance of the laboratory in generating valid and objective health data is recognized:

- District health programmes will be unable to respond effectively to local healthcare needs, medical emergencies and disease outbreaks.
- Scarce health resources are likely to be wasted on ineffective interventions.
- National health planning will lack a scientific foundation on which to develop and evaluate its health strategies.

For district laboratories to operate effectively, health authorities must allocate the correct proportion of available resources to:

- Laboratory quality management.
- Laboratory accreditation and proficiency testing (external quality assessment).
- Training, continuing education and remuneration of laboratory personnel.
- Equipping district laboratories and ensuring a reliable supply of essential consumables.
- Instructing district clinicians and community health workers in the correct and optimum use of laboratory services, particularly how to select tests appropriately, collect samples correctly and interpret test results correctly.

Section 1 Strengthening the quality of district laboratory services

- 1.1 District laboratory network
- 1.2 Supporting district laboratory personnel
- 1.3 Implementing laboratory quality management
- 1.4 Laboratory safety
- 1.5 Equipping district laboratories

1.1 District laboratory network

A typical district laboratory network is made up of:

- Peripheral laboratories located in health centres, dispensaries and rural clinics.
- Sub-district and sub-county hospital laboratories.
- District hospital laboratory which serves the clinical needs of inpatients and outpatients, patient and sample referrals, public health services, and the training of district laboratory personnel.

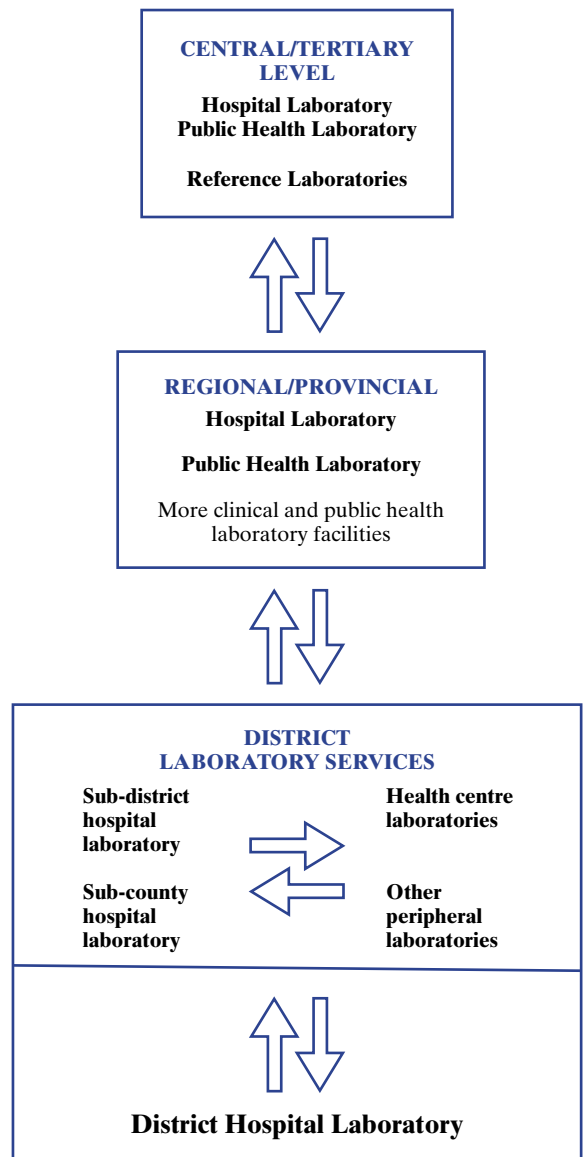
To be effective, district laboratory services must be integrated within a National Laboratory Network as shown in the example opposite.

Network connectivity

Network connectivity refers to how well the tiers of a laboratory network connect with each other to achieve a laboratory service that is reliable, sustainable, and fulfils clinical and public healthcare needs. It also provides for a more equitable use of available resources.

Effective communication within a laboratory network is important to maintain staff motivation and prevent the isolation of peripheral laboratory workers. Mobile phones and e-mail are being used increasingly to facilitate network communications and the speedy transfer of data. In some places drones (unmanned aerial vehicles) are being used to transport samples and medical supplies including blood to places difficult to reach e.g. remote islands.

Note: The referral structure of laboratory services and level of facilities vary in their terminology, complexity, and organization in different countries.



Examples of how the upper tiers of an integrated network provide support to district laboratories

- Helping apply laboratory quality management and compiling a Quality Manual.
- Preparing and implementing Standard Operating Procedures (SOPs).
- Promoting external quality assessment (EQA).
- Validating, calibrating and maintaining equipment.
- Supplying district laboratories with diagnostic test kits, reagents and other essential supplies.
- On-site training of staff.
- Carrying out supervisory support visits.
- Applying health and safety procedures.
- Implementing new point-of-care technologies.
- Improving laboratory communication with clinicians and other healthcare staff.
- Helping to establish quality indicators and customer care targets including acceptable turn around times for laboratory tests.
- Helping district laboratories become accredited.

Examples of how district laboratories communicate with the upper tiers of an integrated network

- Referring samples for tests that cannot be performed at district level.
- Participating in EQA schemes.
- Providing laboratory data that can be used in health planning and monitoring disease trends.
- Providing information on the personnel, equipment, and other resources required for district healthcare.
- Supplying information on drug resistant pathogens, where facilities for culture are available.
- Providing early warning of infectious disease outbreaks.
- Advising on the performance of Point-of-Care Testing (POCT) technologies.

To be effective, district laboratory services must meet acceptable performance standards, be supervised, accessible, affordable and sustainable.

1.2 Supporting district laboratory personnel

District laboratory services are strengthened by having a well trained, motivated and up-to-date workforce. Incorrect test results can be potentially dangerous for patients, lead to loss of confidence in laboratory services and resources being wasted.

Indicators of poor performance include:

- Increase in the number of incorrect tests results.
- Ineffective laboratory quality management.
- Frequent and serious complaints from laboratory users and an increase in requests for repeat tests as confidence decreases.
- Incorrect interpretation and reporting of test results.
- Damage to equipment.
- Laboratory running out of reagents, controls and other consumables.
- Greater incidence of laboratory-acquired infections and laboratory-related accidents.
- Poorly motivated staff and poor cooperation with higher levels of laboratory services (due to lack of confidence).
- Increase in laboratory operating costs.

A competency-based training programme, often referred to as a task-orientated programme, is recommended for the training of district laboratory personnel (see Appendix II). It ensures training is relevant to the working situation. Competency and job-satisfaction are major factors in achieving and retaining quality of service.

The competence of laboratory staff should be assessed and monitored using internal audits, external quality assessment and supervisory support visits. When indicated, the District Laboratory Officer should arrange for retraining and updating. Newly qualified staff require support and supervision in the workplace.

Employment contracts should reflect the qualifications and responsibilities of district laboratory staff and provide career opportunities.

CONTINUING PROFESSIONAL DEVELOPMENT

Practising laboratory personnel have a professional responsibility to participate in continuing professional development (CPD) and keep up-to-date in their profession. CPD is *essential* to prevent a decline in working standards and prevent errors affecting patients. It increases the knowledge, skills, professional effectiveness, motivation and confidence of laboratory workers, and promotes the value of life-long learning.

In some countries, CPD is required for relicensing, and/or continued registration, with a laboratory worker having to gain a required number of professional development credits to maintain their license to practice. A certain number of hours of formal training may be required.

Professional updating may also enable new responsibilities to be undertaken by laboratory personnel, increasing their opportunities for career development and work promotion. Career prospects should be sufficiently attractive to discourage trained staff from leaving their National Health Service.

Important areas of CPD relevant to district laboratory personnel include learning about advances in diagnostic technologies, implementation of quality management, and application of computer-based laboratory programmes.

Effective ways of achieving CPD

Ways of achieving CPD include:

- Participating in external quality assessment.
- Hands-on workshops and practical courses.
- Interaction with senior laboratory staff and clinicians, including case discussions and participation in clinical research work.
- Work-place learning and refresher training, avoiding the need for district staff to be absent from their laboratory.
- CPD provided by National Laboratory Associations and International Organizations, including training days, refresher courses, seminars, conferences, internet-based learning (E-learning), newsletters and journals.

The following organizations provide opportunities for CPD:

- **Amref International University**
<https://amref.ac.ke/academic-programme/laboratory-courses/>
 Courses include malaria microscopy, quality management and laboratory procedures refresher course.

- **African Society for Laboratory Medicine (ASLM)**

www.aslm.org

Launched in 2011, ASLM is a pan-African professional body working to advocate for the critical role and needs of laboratory medicine and networks throughout Africa. ASLM provides CPD through its *Stay Informed* service, publication of a quarterly open-access Newsletter *Lab Culture*, and *African Journal of Laboratory Medicine*. The society also organizes international conferences directed at the latest developments and initiatives for strengthening national laboratory health systems and networks.

- **The Pacific Paramedical Training Centre (PPTC)**

www.pptc.org.nz

PPTC is a Collaborating Centre of the WHO Western Pacific Region based in Wellington Hospital Campus, New Zealand. It provides CPD through its short in-country teaching workshops for Pacific Island laboratory personnel, Distance Learning Programme, and its Wellington-based courses.

- **Centers for Disease Control and Prevention (CDC)**

www.cdc.gov

CDC provides a wide range of E-Learning courses to support CPD including basic microbiology, antimicrobial sensitivity testing, diagnostic parasitology. DPDx provides an excellent parasitology Image Library.

MEDICAL LABORATORY CODE OF ETHICS

Adopting a Code of Professional Ethics helps to remind laboratory workers of their responsibilities to patients, their duty to uphold professional standards and need to work with complete integrity. In most countries it is the National Medical Laboratory Association that formulates the Code of Ethics for practising medical laboratory personnel, sometimes referred to as the Code of Professional Conduct.

The following *Ethical Principles in Laboratory Medicine* have been taken from the ISO 15189 international standard for medical laboratories.

Ethical Principles in Laboratory Medicine

- Laboratories shall uphold the principle that the welfare and interest of the patient are paramount and patients should be treated fairly and without discrimination.
- Every medical laboratory shall provide its services to all users in a manner that respects their health rights and without discrimination.
- Every medical laboratory shall ensure that patient consent is obtained for all procedures carried out on the patient. In emergency situations, if consent is not possible under these circumstances, necessary procedures may be carried out, providing they are in the best interest of the patient.

- Medical laboratories should have in place policy guidelines that address conflicts of interest, undue internal or external pressure, and confidentiality that could influence the credibility of the work conducted and information generated by the laboratory.
- Personnel employed within medical laboratories shall not compromise their organization by engaging in activities that could adversely affect their quality of work, competence, impartiality, judgement or operational integrity.

1.3 Implementing laboratory quality management

Ensuring the quality of district laboratory practice is key to strengthening district laboratory services and providing the route for laboratories to become accredited for the work they undertake. Laboratory accreditation is described on pages 18–19.

Laboratory Quality Management System

A Quality Management System (QMS) ensures that all aspects of laboratory work are integrated, performed to recognized standards, monitored and improvement in quality is continuous. The objectives are to provide test results that are accurate, timely and reproducible and a service that is reliable, efficient, safe and sustainable. An effective QMS achieves optimal use of resources, increases staff motivation and the confidence that patients, clinicians and health authorities have in laboratory test results.

The ultimate goal of a QMS is to provide the best possible service to patients. The WHO observes that health outcomes depend on the accuracy of testing and reporting. Inaccuracies result in increased cost in time and personal effort, waste of resources and often in poor patient outcomes including unnecessary treatment, failure to provide correct treatment and delay in establishing a correct diagnosis.

There are three stages in laboratory testing which require quality management:

- Pre-Analysis*
- Analytical stage*
- Post-Analysis*

* Also referred to as Pre-Examination, Examination, Post-Examination.

- The **Pre-Analytical stage** takes place both inside and outside the laboratory and includes test selection and sample collection, labelling, packaging, transport, and registration checks.
- The **Analytical stage** includes test method selection, testing samples manually or by automated analyzer and quality control.
- The **Post-Analytical stage** includes the recording, reviewing, timely reporting, verification, interpretation, and storing of test results.

To ensure a quality sustainable service to patients and a safe working environment, a range of coordinated activities and procedures is required, referred to as the Quality system essentials.

Quality system essentials*

Page

*Taken from the WHO SLIPTA Checklist, 2:2015

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Occurrence (error) management and Process improvement	13–14
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Pages 7–15 summarize the implementation of the Quality system essentials in district laboratory practice.

FURTHER READING

Ong S K et al. Strengthening the clinical laboratory workforce in Cambodia: a case study of a mixed-method in-service training programme to improve laboratory quality management system oversight. *Human Resources Health*, 2020. <https://doi.org/10.1186/s12960-020-00521-8>

Jayamani J et al. Medical laboratory quality management: Current scenario and challenges in developing nation. *Primary Health care*, 2022. Access by entering the title in Google.

Implementation of the Quality System Essentials

ACTION

OUTCOME

Documents and Records

<ul style="list-style-type: none"> ● Compile written documents detailing: <ul style="list-style-type: none"> – policies (quality goals of the laboratory), – processes (steps taken to carry out policies and organize workflow), – procedures (detailed instructions for carrying out processes, i.e. SOPs, see page 16). 	Written information provided on laboratory direction, activities, and specific work instructions.
<ul style="list-style-type: none"> ● Include policies, processes and procedures in the <i>Laboratory Quality Manual</i>. (see page 19). 	<i>Laboratory Quality Manual</i> compiled.
<ul style="list-style-type: none"> ● Standardize the format for writing documents and ensure they are easily accessible and reviewed regularly. 	Clearly written up-to-date documents are available.
<ul style="list-style-type: none"> ● Keep laboratory records (permanent recorded information) of all activities associated with testing and quality issues, including: <ul style="list-style-type: none"> – request forms and reports, – worksheets, quality control records, – personnel competency and training records, – audit findings and error reports, – user surveys and complaints, – safety reports, – equipment calibration and maintenance reports, – inventory reports. 	Reports available for monitoring quality essentials, tracking patient reports and managing problems.
<ul style="list-style-type: none"> ● Use a reliable system for storing and tracking reports. When using a computerized system, organize a backup system. Ensure the confidentiality of records. 	Reports securely stored and accessible.

Management reviews

<ul style="list-style-type: none"> ● District Quality Officer or district laboratory supervisor reviews at least annually the quality programme, checking: internal audits, corrective actions, EQA, internal QC procedures, quality indicators, sample rejections, equipment, supply chain, workload, test suitability, customer complaints and feedback. 	Important areas of the quality programme are monitored and discussed with staff.
<ul style="list-style-type: none"> ● Discuss with staff any problems and suggestions for improvements. 	
<ul style="list-style-type: none"> ● Document and report findings to staff with time frames for completing corrective actions. 	Staff are motivated and helped to improve their technical performance and service to patients.

Organization and Personnel

<ul style="list-style-type: none"> ● Manage and monitor the activities and resources of the laboratory to meet work requirements and workload. 	An effective and efficient workflow.
<ul style="list-style-type: none"> ● Employ qualified laboratory personnel. 	Staff employed with the necessary skills.
<ul style="list-style-type: none"> ● Manage staff numbers. 	Staffing levels matched to workload.

ACTION	OUTCOME
● Organize staff responsibilities with job descriptions.	Staff receive a job description and are notified of their duty rosters, and responsibilities.
● Regularly assess competence of staff.	Working standards and quality maintained.
● Ensure working conditions are safe and acceptable (see Section 1.4).	Satisfactory working environment.
● Train in the work-place when introducing new technologies.	New technologies applied successfully with on-site problem-solving.
● Provide continuing professional development (see page 5).	Laboratory staff progress in their profession and responsibilities and in some cases, meet the requirements for re-licensing.
● Provide opportunities for career promotion and advancement.	Prevent staff from becoming dissatisfied and leaving their employment and profession.
● Provide appropriate remuneration dependent on qualification and experience.	Staff less likely to leave their job or seek additional employment.
Contract of employment	
Employers should provide laboratory staff with a written contract, detailing employee grade, responsibilities, hours of work, emergency work arrangements, vacation, and salary.	

Client management and Customer service

SERVICE TO PATIENTS

● Provide an ethical service (see pages 5–6). Be courteous and helpful to patients and their relatives.	Patient welfare and health rights respected. Trust and confidence in the laboratory increased.
● Respect patient confidentiality. Do not disclose or discuss patient details or test results with any unauthorised person.	Confidentiality of patient data and test results assured.
● Keep waiting times and laboratory service turnaround times as short as possible.	Patient's test results are available in time for clinical management.
● Provide clear instructions for sample collection (see Appendix IV).	Correct sample provided.
● Handle patients' complaints with as little delay as possible.	Complaints and concerns resolved in a timely manner.
● Conduct patient surveys.	Information provided on how patients rate the quality of laboratory services.

SERVICE TO LABORATORY USERS

● Provide a <i>User Handbook</i> , detailing: <ul style="list-style-type: none"> – working hours of the laboratory, – emergency out of hours arrangements, – tests provided on-site and their turnaround times, – tests available for referral and their turnaround times, – collection of samples, – patient information to accompany test requests, – tests (and conditions) that may be requested as urgent, – procedure for repeating tests when test results do not match clinical findings. 	Improved use of the laboratory by clinicians and other laboratory users. Correct collection of samples accompanied by relevant patient details.
● Notify laboratory users of any anticipated disruption to laboratory services.	Notice given of unavoidable test delays or test withdrawals.

ACTION	OUTCOME
<ul style="list-style-type: none"> ● Laboratory staff provide competent information covering: <ul style="list-style-type: none"> – the selection of tests, – sensitivity, specificity and predictive value of tests, – meaning of test results, – suggestions for further testing and patient monitoring. 	Clinicians can discuss the selection of tests and interpretation of test results with laboratory staff.
<ul style="list-style-type: none"> ● Hold regular meetings with clinical staff to discuss, e.g. patient cases, workload, staffing, participation in clinical research and introduction of new technologies. 	Laboratory staff increase their knowledge on how laboratory tests are used and receive suggestions for service improvement.
<ul style="list-style-type: none"> ● Discuss and resolve complaints as soon as possible. 	Problems solved efficiently with minimum inconvenience.

Equipment

<ul style="list-style-type: none"> ● Inventory the equipment in the laboratory, assigning identification numbers and recording: <ul style="list-style-type: none"> – manufacturer, model, and serial number, – supplier, date purchased, order number, cost, – source(s), expected shelf-life and order codes for replacement parts and consumables, maintenance schedule requirements as recommended by the manufacturer, – service contract requirements, – warranty conditions and expiry dates. 	<p>Manufacturer and supplier identified with equipment specifications.</p> <p>Parts and consumables ordered correctly.</p> <p>Maintenance and servicing performed as scheduled.</p>
<p>Document the information in an <i>Inventory Register</i>.</p>	Equipment inventory list available for inspection.
<ul style="list-style-type: none"> ● Prepare equipment SOPs as described in Appendix III. 	Written information provided on how to use, store and maintain equipment correctly and safely.
<ul style="list-style-type: none"> ● Train laboratory staff on equipment maintenance as detailed in Equipment SOPs. 	Equipment less likely to break down and will have a longer life.
<ul style="list-style-type: none"> ● Investigate faults in the performance of equipment as directed by the manufacturer's troubleshooting guide. 	Causes of equipment malfunctioning identified and corrective action taken.
<ul style="list-style-type: none"> ● Repair equipment on-site when competent or request the help of a qualified engineer. Arrange for equipment replacement during repair (made easier when equipment is standardized). 	<p>Repairs carried out correctly and recorded.</p> <p>When possible, replacement equipment provided.</p>
<ul style="list-style-type: none"> ● Place equipment in a safe and secure place where it cannot be easily damaged or stolen. Protect it from extremes of heat and humidity, direct sunlight, dust, vibration, and damage from rodents and insects. 	Equipment installed correctly and protected.
<ul style="list-style-type: none"> ● When needing to replace equipment, obtain standardized equipment and ensure: <ul style="list-style-type: none"> – conditions for use are satisfactory. e.g. power supplies are reliable, water supply is acceptable, space and ventilation are adequate. – supplier installs, checks and validates the equipment on-site and provides training and on-going support for staff, – parts, standards, and reagents are readily available, preferably from more than one source, – warranty conditions are satisfactory, – date put into service is recorded. 	<p>Performance of equipment validated under conditions of use.</p> <p>Staff adequately trained and supported.</p> <p>Interruptions to service due to unavailability of supplies are avoided.</p> <p>Costs reduced by not having to use a single supplier.</p>

ACTION	OUTCOME
● Organize service contracts or planned preventive maintenance contracts for automated equipment, microscopes, and safety cabinets.	Performance and condition of equipment are monitored.
● Maintain back-up equipment should automated equipment fail or essential consumables become unavailable.	Laboratory service is not disrupted.
● Remove non-functional equipment from testing areas, and request the assistance of the District Quality Officer to dispose of it.	Obsolete equipment managed appropriately.

Evaluations and Audits

<ul style="list-style-type: none"> ● District laboratory supervisor or District Quality Officer: <ul style="list-style-type: none"> – carries out on-site auditing with checklist based on laboratory activities, workload, and staff performance, – assists in identifying and resolving problems, – documents audit findings, establishes timelines for improvements, and arranges checking of the improvements, – reviews and implements recommendations from Regional laboratory supervisors and proficiency testing scheme. 	<p>Need for quality assessment highlighted.</p> <p>Opportunity for on-site training, problem-solving, and checking of equipment and supplies.</p> <p>Staff motivated.</p>
<ul style="list-style-type: none"> ● An experienced senior laboratory officer: <ul style="list-style-type: none"> – carries out at regular intervals internal audits with checklist to determine compliance with laboratory standards, policies and procedures, – identifies areas of non-compliance and factors affecting quality of testing and customer service, – provides an audit report with recommendations and timelines for implementing and monitoring the improvements. 	<p>Quality of performance assessed against designated standards.</p> <p>Problems identified and corrective actions taken.</p> <p>Areas requiring refresher training identified.</p> <p>Staff encouraged to improve quality of performance.</p>
<ul style="list-style-type: none"> ● Participate in an external quality assessment (EQA) scheme (proficiency testing), involving: <ul style="list-style-type: none"> – testing EQA samples of undisclosed known results following the same SOP used for patient's samples, – storing EQA samples for future re-checking, – returning test results promptly, – using EQA feedback to make improvements and provide continuing education for laboratory staff. 	<p>Objective assessment of proficiency with errors identified and improvements suggested.</p> <p>Need to modify or replace a test procedure identified.</p> <p>Opportunities for CPD.</p> <p>EQA samples available for training and quality control.</p>
<ul style="list-style-type: none"> ● District laboratory supervisor or District Quality Officer checks at random a selection of previously reported Giemsa/Field stained blood films, stained sputum smears and Gram stained smears. 	<p>Quality of staining and accuracy of reporting assessed with feedback to microscopist(s).</p>
<ul style="list-style-type: none"> ● When available, participate in an external slide re-checking scheme to improve the accuracy of reporting, e.g. blood films for malaria parasites, sputum smears for AFB. 	<p>Laboratory diagnosis of malaria and tuberculosis improves.</p>

Note: Information on EQA proficiency testing and slide re-checking schemes can be found in the paper of J Y Carter, *External quality assessment in resource-limited countries*. *Biochemia Medica* 2017; 27(1):97–109. Can be accessed from website www.dx.doi.org/10.11613/BM.2017.013

ACTION	OUTCOME
Purchasing and Inventory	
<ul style="list-style-type: none"> ● Manage inventory using a computerized programme or manual system with record cards. Inventory the supplies required to perform laboratory tests, including: <ul style="list-style-type: none"> – consumables, – equipment replacement parts, – diagnostic and clinical chemistry test kits, – controls, standards, ready-made reagents, – stains, – chemicals, – computer supplies. <p>Record and monitor stock quantities.</p>	Inventory of supplies compiled.
<p><i>Note: A manual inventory system for use in peripheral laboratories is described in <i>Practical Laboratory Manual for Health Centres in Eastern Africa</i>.¹</i></p>	
<ul style="list-style-type: none"> ● Decide the levels of stock to hold based on past usage, anticipated requirements, product stability, expected delivery times, and cost considerations. Determine: <ul style="list-style-type: none"> – minimum stock levels to avoid stock running out, – maximum stock levels to prevent unnecessary expenditure and waste due to deterioration or expired shelf-life and space being occupied unnecessarily. 	Stock levels determined and monitored to avoid disruption to services and waste of resources.
<ul style="list-style-type: none"> ● Perform stock-taking at regular intervals to check inventory records match stock quantities and to check the condition of stock. 	Assurance that stock levels match inventory and the condition of stock is satisfactory.
<ul style="list-style-type: none"> ● Track orders and upon receipt, inspect their correctness and condition. Record the delivery date, lot number, expiry date for supplies with limited shelf-life, and current stock levels. Check the performance characteristics of new test kits. 	Orders followed up, checked upon delivery and quality of test kits assessed.
<ul style="list-style-type: none"> ● Store supplies correctly: <ul style="list-style-type: none"> – store consumables, equipment parts, paper items, and computer supplies in dry secure cupboards or stockrooms, – store test kits, controls, standards and ready-made reagents as instructed by manufacturers, – store stock stains out of direct sunlight in cupboards or storerooms. Ensure ready-made flammable stains are stored safely, – store oxidizing, flammable, toxic, harmful and irritating chemicals as described in Appendix III. 	Supplies stored safely and securely under correct storage conditions.
<ul style="list-style-type: none"> ● Ensure stock near expiry are used first. Label stock items clearly and if applicable, highlight expiry dates. 	Stock is not wasted.
<ul style="list-style-type: none"> ● Monitor the use of consumables by recording the quantity of supplies used over a designated period, e.g. over one month. 	Planning and budgeting of stock, facilitated.
<ul style="list-style-type: none"> ● Dispose of expired stock items safely or return items to the supplier at the time of next delivery if disposal cannot be carried out on-site. Be aware of the regulations covering the disposal of chemicals. 	Disposal of stock managed appropriately.

ACTION	OUTCOME
Process control	
<ul style="list-style-type: none"> ● Verify samples prior to testing, checking: <ul style="list-style-type: none"> – the sample is correct for the test requested, – the sample has been collected, stored and transported correctly (see Appendix IV), – patient details on the request form match those on the sample container. 	<p>Correct sample with patient and sample correctly identified.</p> <p>Errors due to incorrect sample collection, faulty sample, or patient misidentification are avoided.</p>
<ul style="list-style-type: none"> ● Package and transport samples according to standard procedures (see Appendix IV). 	<p>Samples packaged and transported correctly and safely.</p>
<ul style="list-style-type: none"> ● Record samples and patient information using a computerized system or a reliable manual system. 	<p>System established for recording and tracking patient data and samples.</p>
<ul style="list-style-type: none"> ● Use a computerized system or keep a register of test referrals and returned test results. 	<p>Referred tests can be tracked.</p>
<ul style="list-style-type: none"> ● Handle urgent test requests as soon as possible. 	<p>Urgent test results are available with the minimum of delay.</p>
<ul style="list-style-type: none"> ● Follow the correct procedure for the decontamination of samples (see pages 29–32). 	<p>Samples disposed of safely within the specified time.</p>
<ul style="list-style-type: none"> ● Use Standard Operating Procedures (SOPs) for all tests (see page 16). 	<p>Tests performed reliably according to designated standards.</p>
<ul style="list-style-type: none"> ● Implement internal quality control for all tests including: <ul style="list-style-type: none"> – control sera and control charts for clinical chemistry quantitative tests, – control slides for microscopical examinations, – reference control strains for the control of antimicrobial susceptibility testing. 	<p>Confidence that test results are correct and can be used in patient care.</p>
<ul style="list-style-type: none"> ● Check and record the quality of new batches of test kits, stains and reagents. 	<p>Test kits, stains and reagents can be relied on to perform reliably.</p>
<ul style="list-style-type: none"> ● Check the performance of new equipment before using it in test analysis. 	<p>Tests performed using calibrated checked equipment.</p>
<ul style="list-style-type: none"> ● Check and record daily the temperature of the laboratory room(s), refrigerators, water-baths, and incubators. 	<p>Room temperature is monitored. Temperature control mechanisms are installed when room temperature is excessive. Temperature of water-baths, incubators and refrigerators is correct.</p>
<ul style="list-style-type: none"> ● Verify patients' test results after reviewing clinical data and checking quality control results. Issue test reports with information to help in their interpretation. 	<p>Verified test reports issued with helpful information e.g. reference intervals.</p>
<ul style="list-style-type: none"> ● Investigate any unexpected abnormal test result. 	<p>Unexpected abnormal result reported after checking quality control and the correct sample and test procedure have been used.</p>
<ul style="list-style-type: none"> ● Ensure test reports reach the persons who have ordered the tests. 	<p>Reports reach the clinician or other health-provider.</p>
<ul style="list-style-type: none"> ● Appoint a district laboratory supervisor or District Quality Officer to oversee the performance of community-based laboratories. 	<p>Work of community laboratories is monitored.</p>
<ul style="list-style-type: none"> ● Designate an experienced person to be responsible for laboratory quality. 	<p>Quality manager evaluates the quality of laboratory work and ongoing improvements.</p>

ACTION	OUTCOME
Information management	
<ul style="list-style-type: none"> ● Use a test reporting system that is accurate, informative, trackable, and timely. ● When using a manual reporting system, ensure results are: <ul style="list-style-type: none"> – clearly written using a standardized form and signed by the person verifying and issuing the report, – recorded in a register with patient details, – despatched confidentially with the minimum of delay to the clinician or other health-provider identified on the request form, – record time of receipt of samples and time of results despatch. ● When using a computerized Laboratory Information System ensure: <ul style="list-style-type: none"> – laboratory and clinical staff are sufficiently trained to use a computerized system and interpret error codes, – security passwords are in place to prevent unauthorized use, – a reliable alternative reporting system is available should the computerized system fail due to power failure or computer malfunction, – a back-up system is in place to prevent loss of data. ● Keep informed on the application of digital technology and use of mobile phones to obtain reports rapidly, discuss their interpretation, and participate in further education. 	<p>Quality of test reporting assured.</p> <p>Reporting system provides accurate, verified, trackable, timely, confidential reports.</p> <p>Written transcription errors avoided.</p> <p>Clinicians can access patient data and reports quickly with the benefit of screen presentation. Patient data, samples, and test results can be easily inter-faced with hospital information systems. Confidentiality of patient data is assured.</p> <p>Automated equipment printout results can be directly linked to the laboratory information system, providing rapid access to test results and their interpretation.</p> <p>Patient data and reports stored securely for future comparison and health planning.</p> <p>Mobile phones provide rapid transfer of data, with opportunities for discussion with senior staff, resulting in better patient care. Phones with high resolution cameras can assist microscopical diagnosis.</p>
Non-conformities, Corrective and preventive actions	
<ul style="list-style-type: none"> ● Investigate and document the cause of any standard procedure or activity not being followed, e.g. due to inadequate documentation or poor training ● Take action to correct the non-compliance and prevent its recurrence. 	<p>Cause(s) of non-compliance identified.</p> <p>Corrective and preventive actions taken.</p>
Occurrence (error) management and Process improvement	
<ul style="list-style-type: none"> ● Identify the causes of errors (see page 17). ● Investigate errors at the time they occur, and monitor the effectiveness of corrective action(s). ● Check for errors when: <ul style="list-style-type: none"> – samples are rejected, – complaints received from laboratory users, – test turnaround times are not met, – test reports are delayed. ● Notify those affected by an error and rectify the problem as soon as possible. ● Document the occurrence and frequency of errors, action(s) taken to resolve the errors and prevent their recurrence. 	<p>Knowing how errors occur.</p> <p>Preventive actions taken, fewer errors made.</p> <p>Errors identified and resolved promptly.</p> <p>Sources of errors identified and managed.</p> <p>Any adverse outcome affecting patient safety, testing process or workflow is contained.</p> <p>Recurrence of errors reduced.</p>

ACTION	OUTCOME
<ul style="list-style-type: none"> ● Review audit recommendations and note whether they have improved: <ul style="list-style-type: none"> – workflow and better use of facilities, – accuracy of test results, – reliability, sustainability and biosafety. 	Audit recommendations to improve quality assessed.
<ul style="list-style-type: none"> ● Regularly review and update SOPs and laboratory policies. 	Quality of test procedures and policies maintained and changes made when required.
<ul style="list-style-type: none"> ● Discuss with laboratory users what changes are needed to improve quality. 	Laboratory users involved in improvements.
<ul style="list-style-type: none"> ● Organize on-site continuing education to maintain and improve the quality of performance. 	CPD promotes quality awareness and provides information for forward planning (see page 5).
<ul style="list-style-type: none"> ● Support community-based laboratories. 	Supervisory support improves the quality of work and motivates staff.
<ul style="list-style-type: none"> ● Use indicators to measure progress in quality improvement, e.g. the number of times: <ul style="list-style-type: none"> – samples have been rejected, – turnaround times for issuing test results have been missed, – tests have not been performed due to equipment malfunction or unavailability of supplies, – laboratory users have complained, – errors in testing have been detected by quality control results, – laboratory-related accidents have occurred. 	Achievable indicators measure quality progress and set improvement targets.
<ul style="list-style-type: none"> ● Looking ahead, plan and budget realistically for improvements and future laboratory needs. 	Improvement plan implemented.
<ul style="list-style-type: none"> ● District Laboratory Quality Officer promotes the right attitude to quality, evaluates quality indicators, and discusses with staff what improvements are needed and timelines for their implementation. 	District Laboratory Quality Officer identifies areas for improvement and motivates staff.

Facilities and Safety

<ul style="list-style-type: none"> ● Use the facilities of the laboratory to provide safe and efficient working conditions by: <ul style="list-style-type: none"> – keeping the main testing area separate from the sample reception area and place where blood samples are collected from patients. – providing a safe well ventilated and secure laboratory premise (see page 21). – organizing work stations and appropriate placement of equipment. – ensuring the laboratory is clean and staff practice personal safety measures (see page 22). 	Safe and secure workplace, safe arrangements for patients, and best possible testing facilities.										
<ul style="list-style-type: none"> ● Carry out a risk assessment to manage hazards including: <table border="0" style="margin-left: 20px;"> <tr> <td style="padding-right: 20px;"><i>Hazards</i></td> <td><i>Page</i></td> </tr> <tr> <td>Microbial (biosafety)</td> <td>22–23</td> </tr> <tr> <td>Equipment and glassware</td> <td>23–24</td> </tr> <tr> <td>Fire</td> <td>24</td> </tr> <tr> <td>Chemical</td> <td>24–26</td> </tr> </table> 	<i>Hazards</i>	<i>Page</i>	Microbial (biosafety)	22–23	Equipment and glassware	23–24	Fire	24	Chemical	24–26	<p>Risks and hazards involving staff, patients, the community, and the environment are identified and addressed.</p> <p>Also hazards addressed that may involve animals, wild and domestic, that often wander about in these environments.</p>
<i>Hazards</i>	<i>Page</i>										
Microbial (biosafety)	22–23										
Equipment and glassware	23–24										
Fire	24										
Chemical	24–26										

ACTION	OUTCOME
● Provide a <i>Safety Manual</i> detailing safety policies and procedures.	Staff are provided with the information to implement safe laboratory practices.
● Train staff in emergency First Aid (see pages 35–39). Provide a booklet in which to record accidents, near misses and preventive actions taken.	Staff know what to do when an accident occurs.
● Appoint a person in charge of monitoring laboratory safety (see page 35).	Safety Officer available to consult on safety issues and monitor laboratory safety.

Checklist to monitor QMS in district laboratories

A Checklist can be used during a supervisory support visit to identify problems contributing to a poor quality laboratory service. It should reflect the work of the laboratory, staff skills and available resources of the laboratory being visited. The information obtained by the supervisor with the involvement of laboratory staff can be used to implement improvements. A suggested Checklist for monitoring quality in district laboratories can be found in Appendix II.

District Laboratory Quality Officer

The Quality Officer can assist district laboratories provide a quality, sustainable, patient-focused service by regularly carrying out supervisory support visits using a Checklist (see Appendix II):

- To assess staff performance e.g. by checking whether SOPs are being followed, quality control results are satisfactory, and checking the quality of microscopical reporting by verifying a selection of previously reported slide samples.
- To discuss with staff the performance of service and help to resolve any problems, particularly those related to workload, test turnaround times, equipment malfunction, supplies shortages, safety issues and communications with those using the laboratory.
- To make recommendations for any immediate changes which may be required and any future improvements with timelines, checking later that improvements have been carried out.
- To arrange CPD and when indicated, refresher training of staff.

Visits by the district Laboratory Quality Officer, motivate staff and indicate to laboratory users a laboratory's commitment to continuing improvement.

EXTERNAL QUALITY ASSESSMENT (EQA)

In EQA, an outside facility is used to assess a laboratory's performance and compare it with other participating laboratories using samples of known but undisclosed content. This method of EQA is often

referred to as proficiency testing. Fluctuations in trends can often be noted over a prolonged period which may not be apparent by internal quality control.

The objectives of EQA are to improve the quality of testing and share best practice, support and educate laboratory staff. EQA enables unacceptable test results to be detected and the EQA Organizing Centre to assist laboratories investigate and resolve problems. Laboratory staff are motivated and kept up to date.

Any problem associated with a particular reagent test kit, specific analyzer or test method is more likely to be detected by EQA. This is particularly important in clinical chemistry when a wide range of analyzers and methodologies are being used by different laboratories.

Clinicians need to be assured of continuing reliability of patient's test results. Participation in EQA at all levels can help to achieve this. EQA is an essential requirement of ISO 15189 and for a laboratory to achieve and maintain accreditation.

The WHO advises that the costs of setting up and running an EQA programme must be viewed against resources wasted in producing inaccurate test results and the improvement in patient care which can be achieved.

EQA materials include:

- Stained thick and thin blood films for reporting and quantifying malaria parasites.
- Fixed thin blood films for staining and reporting blood cell morphology.

- Stained sputum smears for detecting and reporting acid fast bacilli (AFB),
- Preserved faecal suspensions for detecting and reporting helminth eggs and protozoal cysts,
- Preserved urine samples for detecting parasites,
- Stabilized blood lysate for measuring haemoglobin,
- Preserved liquid sera or lyophilized samples for syphilis screening and HIV antibody testing.

Resources permitting, other EQA samples may be added including, control sera for clinical chemistry assays, control cultures for microbiology reporting, stabilized blood samples for blood typing and compatibility testing.

EQA programmes should include questions relating to the clinical and public health significance of test results. EQA samples should be tested and reported at the earliest opportunity and the results with recommendations returned to the participating laboratories with the minimum of delay, e.g. by electronic transfer. EQA programmes help to identify performance deficiencies, evaluate the appropriateness of technologies, and also provide opportunities for the continuing education of staff.

STANDARD OPERATING PROCEDURES

Standard Operating Procedures (SOPs) are written instructions which are required to carry out laboratory activities:

- accurately to an accepted standard,
- the same way each time,
- the same way by each member of staff.

Standardized procedures promote quality and consistency, help to train new members of staff, avoid wasting resources, and make it easier to compare test results. The following are examples of activities that require SOPs:

- Collection, packaging and transport of samples.
- Testing routine and urgent samples.
- Preparation of stains.
- Preparation of reagents.
- Equipment calibration, use and maintenance.
- Safety practices.

SOPs need to be *applicable to the laboratory in which they will be used*. They need to be compiled using relevant, valid sources of information and references. Copies of SOPs should be laminated and kept in the *Quality Manual* or in a separate *SOP Manual*. SOPs need to be reviewed annually to keep up to date with new technologies, health requirements and National guidelines. Any amendments must be authorized, referenced, dated, signed and issued to all members of staff. Users of the laboratory must also receive written amendments to Test SOPs when these involve changes to the collection of samples and reporting of tests. No new test should be introduced without a SOP.

What should be included in SOPs?

All SOPs require:

- a title,
- identification and version numbers,
- name of person who has written the SOP and authorized its use.

SOPs should include:

- Date of preparation, commencement, review.
- Staff qualified or authorized under supervision to perform the SOP.
- Meaning of abbreviations and terms.
- Purpose, i.e. reason(s) for performing the procedure and background information.
- Instructions on how to perform the procedure.
- Procedure notes, i.e. factors affecting the performance of the SOP.
- Safety measures.
- Sources of information and references.
- Details of other linked SOPs.
- When applicable, appendices containing other information relevant to the SOP.

The information provided in the SOP must be sufficient to perform the procedure accurately, reliably, and safely, particularly SOPs for testing samples and measuring analytes, i.e. Test SOPs. These (or a summary SOP) should be located at work-stations. SOPs should be signed by staff indicating the SOPs have been read, understood and accepted.

Note: Examples of suggested SOP formats for Tests, Stain preparation, Reagent preparation and Equipment can be found in Appendix 111.

PREVENTING ERRORS

Serious consequences can result when errors are made in the performance of tests. Incorrect, delayed or misleading results can lead to:

- Patients receiving inappropriate or no treatment.
- Late detection and ineffective management of a disease outbreak.
- Misleading laboratory data resulting in incorrect healthcare planning.
- An increase in laboratory operating costs due to resources being wasted unnecessarily.
- Confidence by laboratory users being adversely affected.

Reliable and timely test results depend on:

- Knowing how to prevent errors.
- Identifying and correcting the source of errors with the minimum of delay.

Most errors in district laboratory practice are due to:

- Patient misidentification or faulty samples (pre-analytical), often the result of clerical errors, incomplete identification data, or lack of a check-in system when samples reach the laboratory.
- Imprecision (inconsistent errors), e.g. equipment malfunctioning, variable pipetting or dispensing, inadequate mixing of samples with reagents, or using glassware or plastic-ware that is not clean or dry.
- Inaccuracy (consistent errors), e.g. due to setting the volume of an automatic pipette incorrectly, using a wrong calculation factor, or using a reagent that has been prepared or stored incorrectly, is contaminated or out-of-date.
- Test results reported on the wrong request form or given to the wrong patient (post-analytical).
- Adverse working conditions, e.g. workload is too high or too low, working area is poorly lit, there is excessive noise or frequent interruptions.

Further information on preventing errors can be found in subunit 2.4 in *Part 1 District laboratory practice in tropical countries*. Access on-line by entering the title in Google and clicking on PDF entry <https://medicallabtechno.weebly.com>

Summary of how to prevent errors

- Follow exactly SOPs for testing samples (see Appendix III).
- Carry out internal quality control and participate in external quality assessment, particularly proficiency testing schemes.
- Perform on-site and internal audits.
- Equip district laboratories adequately, validate equipment, check it is in good working order, and is used and maintained correctly as detailed in equipment SOPs (see Section 1.5).
- Prepare and store stains and reagents as detailed in SOPs for preparing stains and reagents (see Appendix III).
- Ensure essential consumables are available and stock levels monitored (see page 11).
- Carry out performance checks on rapid test kits, store and use kits correctly as instructed by manufacturers.
- Monitor laboratory working conditions and workload.
- Implement competency-based training for laboratory personnel (see Appendix II), assess staff competency and provide continuing professional development (see page 5).
- Hold regular meetings with clinical staff and other laboratory users to prevent errors due to the incorrect collection and transport of samples (see Appendix IV).
- Use a test reporting system that is accurate, informative, trackable, and timely (see page 13).
- Use indicators to measure improvements in the quality of laboratory services (see page 14).

Note: The management of errors is an essential component of QMS, (see pages 13–14. Occurrence, i.e. error, management).

POINT-OF-CARE TESTING

Point-of-care testing (POCT) refers to testing that can be performed at the site of patient care using POCT diagnostics and POCT equipment.

WHO criteria for POCT diagnostics in resource-limited settings

These are diagnostic POCT technologies that are affordable, sensitive, specific, user-friendly, rapid and robust, equipment free and deliverable to end users, summarised in the word ASSURED.

To these criteria can also be added:

- can be controlled by the user at the site of use,
- provide results that are easy to interpret and communicate, with the option for electronic transfer,
- have the potential to be manufactured in the country of use.

In resource limited countries POCT diagnostics need to be assessed by their impact on relevant clinical outcomes (test efficacy) and their cost-effectiveness.²

POCT in district hospital laboratories

POCT diagnostics and POCT equipment are being used to:

- Obtain rapid test results to initiate early treatment and reduce follow-up hospital visits.
- Simplify testing procedures, enabling a wider range of investigations to be undertaken, often at lower cost.

POCT in district laboratories includes microscopy, lateral flow immunoassays (LFIA) to diagnose infectious diseases such as malaria, HIV disease and syphilis. POCT equipment that is being used, depending on work-load, includes devices that measure blood glucose and HbA1c, small haematology and clinical chemistry analyzers, and instruments that quantify CD4+ T-cells.

In disease outbreaks, rapid POCT can help to limit an epidemic by preventing onward transmission, e.g. rapid tests for cholera, meningitis, Ebola Virus Disease.

Future of POCT

Increased use of POCT technologies in resource-limited countries is dependent on:

- reduction in manufacturing costs,
- technologies becoming more specific, sensitive, and accessible.

Emerging technologies include molecular-based tests, multiplex technologies enabling several analytes to be tested using a single device, and microfluidic-based tests in which all the analytic steps and reagents are miniaturised in a small device.

A key requirement when considering introducing a POCT technology is to obtain independent evaluations and whether the technology has been validated and meets the requirements of ISO 22870 standard for POCT systems.

Note: Further information on the use of POCT technologies in low resource countries, with examples of present and emerging technologies, can be found in the Open-access paper: *Point-of-care diagnostics in low resource settings: Present status and future role of microfluidics*. Shikha Sharma et al. *Biosensors* 2015, 5, pp. 577–601. (Access the paper electronically by entering the title in Google).

Task shifting

In situations of limited laboratory access and lack of trained personnel, POC tests such as rapid malaria tests, HIV testing and CD4+ T-cell counting are being performed increasingly by non-laboratory personnel such as nurses, HIV counsellors, and community health workers. To be reliable and safely carried out, task shifting must be implemented within systems that contain checks and balances that are sufficient to protect both health workers and the people receiving healthcare.

Task shifting requires proper training using an approved national curriculum, regular supervision using observational structured checklists, attention to safety and waste disposal, supply chain management and staff able to accommodate POCT within their busy schedules, e.g. nurse in an antenatal clinic.³ The POCT provider must be trained to perform essential control procedures, maintain a POCT instrument where instrumentation is required, and be able to interpret test results correctly and act upon them appropriately.

Note: Examples of the outcomes of task shifting for rapid malaria testing and HIV testing can be found under Further Reading.

LABORATORY ACCREDITATION

An effective QMS is essential for laboratory accreditation. The process of accreditation uses independent external assessment to determine whether a laboratory meets the requirements of recognized standards for quality management and competency. Accreditation is a mark of quality,

demonstrating objectively to patients and health authorities that laboratory tests are performed by competent personnel and the laboratory practice is safe, efficient, consistently reliable, and patient-focused.

The recognized international standard for accrediting medical laboratories is ISO 15189 “*Medical Laboratories – Particular requirements for quality and competence*”. In several countries National Accreditation Organizations have developed and successfully implemented national standards based on ISO 15189 that are attainable and adapted to country-specific conditions.⁴

Note: Information on the technical and management requirements for the introduction of ISO 15189 standard can be found on the website of the International Organization for Standardization (ISO), www.iso-15189.com

Assessment for accreditation is performed in countries by independent National Accreditation Organizations or Agencies with the authority to inspect a laboratory and provide written evidence of its competency and compliance to ISO 15189 standard or national designated standard. To ensure continued compliance, accredited laboratories are assessed annually and required to demonstrate continuing improvement and participation in proficiency testing programmes.

Difference between accreditation and licensing

Whereas accreditation is recognition of a laboratory's compliance to ISO 15189 standard or national standard for quality and technical competence, licensing is the permission to operate granted to a laboratory by a National Healthcare Authority. It is based on the laboratory meeting specified standards that protect health and safety as determined by an on-site inspection and proof that laboratory staff are qualified to practice. Licensing is a legal requirement to operate.

Attaining and maintaining accreditation is challenging, requiring long-term commitment, leadership, teamwork and the support of clinical hospital management, careful planning, and the resources to train laboratory personnel in implementing and maintaining Quality system essentials.⁵

To assist countries with limited resources in their progress towards laboratory accreditation, the WHO and other organizations have produced QMS programmes, working guides and publications which describe a stepwise approach to accreditation. These include:

WHO Stepwise Laboratory Improvement Process Towards Accreditation (SLIPTA) in the African Region, 2015

SLIPTA is a framework for auditing and monitoring using the SLIPTA Checklist which specifies requirements for quality and competency aimed at improving laboratory services and raising quality to established international

standards, based on ISO 15189. It is a *stepwise* user-friendly process that identifies non-conformities and provides recommendations for corrective actions. Audits are performed by the African Society for Laboratory Medicine (ASLM) or other approved auditors.

Further information on SLIPTA can be found in the 2020 paper of Datema T A M *et al. Review of the stepwise laboratory Quality Improvement Process Towards Accreditation (SLIPTA) version 2.2015*⁶.

Checklist Version 2:2015

The Checklist contains 12 main sections, corresponding to the Quality system essentials listed on page 6. The Checklist is based on the requirements of ISO 15189 used in auditing laboratories for accreditation.

Full details of the Checklist can be found by entering SLIPTA in Google.

Strengthening Laboratory Management Toward Accreditation (SLMTA) Programme

SLMTA is a training and mentoring programme aimed at developing the management skills required to implement QMS. It is designed to bring about immediate and measurable improvements using available resources. The programme is closely linked to SLIPTA, using the SLIPTA checklist for baseline and exit audits.

Several countries have adapted the SLMTA programme to meet local requirements while retaining its main components (workshops with improvement projects and monitoring).

Further information on SLMTA can be found on website: www.slmta.org

Laboratory Quality Manual

It is the responsibility of the person in charge of district laboratory services, with the help of the district laboratory supervisor, or Laboratory Quality Officer, to compile a Quality Laboratory Manual. It should be clearly written, accessible, accepted, and implemented by all members of staff. It forms part of the laboratory's QMS, and should be written for the laboratory in which it will be used.

Quality Manual based on ISO 15189 standard

“A quality manual should be available that summarizes the laboratory's quality programme, includes policies that address all areas of the laboratory service, and identifies the goals and objectives of the quality programme. The quality manual should include policies, processes and procedures for all areas of the laboratory service and should address all of the quality system essentials.”

The Quality Manual should be signed, dated, and reviewed at regular intervals. It is the framework for the entire quality management system and therefore it must always be correct and up-to-date.¹

Note: Further information on writing a Quality Manual based on ISO 15189 standard can be found in the WHO, CLSI Supplement to Module 16 of the LQMS Training Toolkit, entitled *Quality Manual Template*. It can be accessed from the WHO website www.who.int using the Search facility.

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FURTHER READING

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EQA SCHEMES IN AFRICA, ASIA, PACIFIC REGION

- **East African Regional External Assessment Scheme**
www.eareqas.org
Developed to establish standards and strengthen clinical and laboratory diagnostic services at primary healthcare level, EA-REQAS is an integrated programme, focusing on local diseases and educating laboratory staff.

• Pacific Paramedical Training Centre Regional EQA Programme

www.pptc.org.nz/regional-external-quality-assurance-programme

Actively helps and suggests ways to improve the quality of laboratory service to patients in 20 Pacific Island countries, Lao PDR, Cambodia, Timor, and Bhutan.

• Asian Network for Clinical Laboratory Standardization and Harmonization

www.ancls.org

Organizes an EQA Programme for more than 70 clinical laboratories in 13 countries in Asia, focusing on laboratory quality control.

Note: Several countries also provide EQA (proficiency testing, rechecking of slide samples, on-site evaluations) for individual diseases, e.g. malaria, tuberculosis, HIV. Details can be found in the paper of **Carter J Y**. External quality assessment in resource-limited countries. *Biochem Med (Zagreb)*, 2017, Feb15;27(1): pp 97–109.

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1.4 Laboratory safety

A safety programme relevant to the work of district laboratories is required to protect staff from harmful pathogens (biosafety), provide safe working conditions and ensure laboratory activities do not harm the community or damage the environment. Safety should be included in laboratory audits. Implementing an effective safety programme involves:

- identifying hazards* in the workplace,
 - * A hazard is anything that can cause harm, e.g. any biological agent, hazardous substance, activity or situation.
- assessing, reducing and managing the risks associated with each hazard,
- appointing a district laboratory safety officer to oversee and monitor the safety programme,
- compiling a *Laboratory Safety Manual*.

The following are important hazards which require risk assessment and management:

- **Premise hazards**
- **Microbial hazards**
- **Equipment and glassware hazards**
- **Fire hazards**
- **Chemical and reagent hazards**

Note: Other hazards which a laboratory may need to include in its safety programme will depend on its work activities, laboratory layout and workflow, climatic considerations and other local factors. The district laboratory safety officer should be able to advise on National laboratory safety guidelines.

PREMISE HAZARDS

- Unsafe laboratory design and construction,
- Inadequate water supply,
- Poor ventilation,
- Lack of safe facilities for staff.

Assessing, reducing, and managing premise hazard risks

- Separate laboratory testing areas from areas where patients wait, blood is collected, and samples are received.

- Make the laboratory premise as safe as possible by ensuring:
 - Bench surfaces are washable, impermeable to liquids, and resistant to the disinfectants and chemicals in use. Keep benches as clear as possible to provide maximum working area and facilitate cleaning.
 - Floor surfaces are non-slip, impermeable to liquids, chemically resistant, bevelled to the wall, and the entire floor is accessible for washing. Floor drains are recommended.
 - Walls are smooth, crack-free, impermeable to liquids and painted with washable light coloured paint.
 - Doors open outwards, internal doors are self-closing and external doors are fitted with secure locks. When possible there should be a door at each end of the laboratory to facilitate exit in the event of fire.
 - There is sufficient storage space to avoid benches being cluttered and floors being obstructed.
- Provide adequate ventilation supplied by wall vents and windows that can be opened. Fit windows with sun blinds, insect proof screens and when indicated, with secure window bars. Monitor air flow direction to ensure air flows away from testing areas.
- Make sure there is a safe plumbing and waste disposal system. Whenever possible site the handbasin near to the door with taps that can be operated by wrist levers. When there is a water shortage, provision must be made for the collection and storage of water, e.g. rain water when available.
- Provide as good illumination as possible. Low energy tube lights are recommended.
- Ensure the electricity supply is safe (check with an electrician), with sufficient electric wall points sited away from water taps.
- Provide a staff room that is separate from the working area where refreshments can be taken and personal belongings stored safely. Fit wall pegs *in* the laboratory for hanging protective clothing. Provide toilet and hand washing facilities near to the staff room. These should be separate from the toilet facilities for patients.
- Ensure only authorised staff have access to the laboratory. Display a “No entry” sign on the entrance door of the laboratory (see later text). Ensure door locks are secure.

MICROBIAL HAZARDS (BIOSAFETY)

- Ingestion of pathogens from contaminated fingers,
- Accidental inoculation of pathogens,
- Inhalation of pathogens in aerosols (airborne droplets),
- Not placing “Sharps” in puncture resistant-containers,
- Not decontaminating infectious waste safely.

Assessing, reducing, and managing microbial hazard risks

- Practice personal hygiene and safe working practices to avoid pathogens being ingested:
 - Never mouth-pipette. Use safe dispensing and pipetting devices (see later text).
 - Wash hands with soap and running water after handling samples and infected material, before and after attending patients, and when leaving the laboratory.
 - Do not eat, drink, chew gum, or smoke in the working laboratory. Do not store food or drink in laboratory refrigerators.
 - Wear protective clothing over normal clothing or instead of it. Ideally use a fabric such as poly-cotton that can be bleached, laundered frequently and is suitable for tropical climates. Do not take protective clothing home. Soak protective clothing in 0.1% v/v bleach solution overnight before laundering it.
 - Wear protective gloves when taking blood samples and handling samples or cultures that may contain infectious pathogens.
 - Avoid using mobile phones, tablets and laptops when these are not required for laboratory work.
- Avoid accidental inoculation of pathogens by:
 - Not recapping needles. Needle pricks are a common source of laboratory-acquired infection. After use, do not leave a needle or lancet in a collecting tray with cotton wool and other articles. Place it in a puncture proof “Sharps” container.
 - Cover any cuts, scratches, insect bites, open sores or wounds with a water-proof adhesive dressing. Irritating insect bites should be treated.
 - Wear rubber gloves when handling broken contaminated glassware.

- Avoid the formation, dispersal and accidental inhalation of pathogens in aerosols by:
 - Pouring supernatant fluids down the side of a funnel into a container of disinfectant with the end of the funnel submerged in the disinfectant.
 - Avoiding vigorous tapping of a tube to resuspend a sediment.
 - Opening cultures and ampoules of stock cultures safely. Hold the ampoule in a cotton wool swab when cutting the glass and wait a few minutes to allow air to enter the ampoule and destroy the vacuum before extracting the contents.
 - Not heating a contaminated wire loop in an open Bunsen burner. When available use a hooded burner as shown in Plate 1.1. After flaming a wire loop, allow it to cool before reusing it. Do not use long springy and improperly closed loops (when available, use disposable sterile loops).
 - Avoiding vigorous mixing and shaking of cultures and materials containing microorganisms. Make sure containers are tightly closed. When available, use a Vortex mixer.



Plate 1.1 Hooded Bunsen burner.

- Use correct procedures for decontaminating infectious waste, e.g. using TST control strips when autoclaving, using disinfectants correctly, not overfilling discard containers.
- Balance centrifuges to avoid breakages. Follow the manufacturer’s instructions particularly when using multi-tube carriers.

Note: When a breakage occurs, wait at least 30 minutes, preferably 1 hour before opening the centrifuge to allow time for aerosols to settle. Wear rubber gloves to remove the buckets and debris (autoclave before discarding the debris).

Swab the centrifuge bowl with a suitable disinfectant such as 2% *Virkon*, wipe with clean water and dry.

- Cap tubes used for centrifuging infectious material and samples. Do not fill the tubes more than three-quarters. When available, use sealed buckets.
- Never open a centrifuge until the motor has stopped and the rotor is no longer spinning (a safe lid interlock will prevent this).
- When there is a spillage of a sample or culture, avoid inhaling air close to the spillage and use a safe cleaning up procedure. Cover the spillage with a cloth soaked in disinfectant and leave for 30 minutes before cleaning the area. Alternatively, apply disinfectant granules to disinfect the spillage in a shorter time.
- When withdrawing a syringe and needle through the rubber cap of a vaccine-type bottle containing microorganisms, withdraw the needle through a cotton wool swab held over the cap. This prevents needle vibration and any fluid leakage which may create aerosols.

Important: SOPs must be available for the immediate handling and follow-up care of accidents involving “sharps”, e.g. needle-stick injuries, and possible exposure to HIV, VHF viruses, HBV or HCV.

Health checks should be arranged for all new members of staff. A medical officer should decide which vaccinations are required.

Further information on biosafety

This can be found in the new 4th edition of the *WHO Laboratory Biosafety Manual*.¹ It includes the requirements for biosafety, performing a risk assessment, implementing control measures, descriptions of biological safety cabinets, transportation of infectious samples and laboratory biosecurity.

EQUIPMENT AND GLASSWARE HAZARDS

- Electric shock from unreliable earthing/grounding,
- Fire from unsafe connections and circuitry overload,
- Injury from broken glass.

Assessing, reducing and managing equipment hazard risks

- When installing electrical equipment, check:
 - The voltage of the equipment is the same as the electricity supply of the laboratory and the voltage selector switch is set correctly.

- The power required does not exceed the power supply of the laboratory. Do not overload the electric circuit.
- The equipment is wired correctly, using fuses of the correct rating and the system has a grounded conductor. Circuit-breakers* and earth-fault interrupters* should be fitted to all laboratory circuits.

* Circuit-breakers protect wiring from being overloaded with electric current. Earth-fault interrupters protect people from electric shock.

- Avoid using adapters and extension leads.
- Do not cover ventilation openings. To prevent rodents from entering, place wire mesh over ventilation grills. Ensure manufacturers’ ventilation instructions are followed.
- Always ensure hands and the floor underfoot are dry before using electrical equipment. Keep water and chemicals well away from equipment.
- Do not locate equipment in a place where access for cleaning and maintenance will be difficult. Do not place items on top of equipment.
- Should a fuse “blow”, obtain the help of an electrician to check the circuitry.
- Fit voltage stabilisers to equipment to avoid damage due to voltage surges and spikes.
- Inspect equipment regularly for corrosion, worn components, damaged insulation, frayed cable, loose connections, fungal growth, insect and rodent damage.
- When unable to obtain replacement parts from a manufacturer, make sure the specifications of alternatives are correct, e.g. fuse ratings, dimensions, voltage and wattage of lamps, etc.
- Always turn off and disconnect electrical equipment (except refrigerators and incubators) during an electrical storm and at the end of the day’s work. Disconnect equipment when cleaning and maintaining it.
- Minimize glassware breakages by:
 - using items made from plastic instead of glass,
 - using racks to hold bottles,
 - avoiding lifting bottles by their caps,
 - using separate containers for decontaminating pipettes, slides, tubes and other glassware, and not overfilling the containers.
- Before use, inspect tubes, pipettes, sample containers, flasks, cylinders and beakers for cracks and chipped ends.

- Never leave used haematocrit (capillary) tubes on the bench from where they can easily roll to the floor. Place them in a “Sharps” container.
- Unpack glassware in a safe place where any broken glass from packing cases can be easily contained. Regularly inspect places where glassware is stored for glass fragments. Use forceps to pick up glass fragments. Discard broken glass into a puncture-resistant “Sharps” container.

FIRE HAZARDS

- Burns,
- Smoke inhalation,
- Explosive reactions.

Assessing, reducing, and managing fire hazard risks

- Reduce fire risks from flammable chemicals by following safe practices:
 - When using a spirit burner or Bunsen burner, check there is no flammable chemical or reagent nearby and never leave it unattended.
 - When heating carbol fuchsin on slides, use only a small lighted swab. Keep the flame well away from acid alcohol, acetone, methanol, Leishman and Giemsa stains and other highly flammable reagents which may be close by.
 - Do not flame the neck of a bottle containing a flammable liquid.
 - Keep the laboratory well ventilated to prevent any build up of flammable vapours and gases.

Note: A fire caused by a flammable chemical is best controlled by smothering the flames. Cover a small fire on a bench with a lid or fire blanket. When on a floor, smother the fire with a fire blanket, sand or dry earth, or use a dry chemical fire extinguisher.

- Keep emergency exits from the laboratory unlocked during working hours. Keep fire exit routes clear and ensure the routes are clearly marked. When fire doors are fitted, keep these closed.
- Locate fire-fighting equipment at accessible points, including:
 - Buckets of water to extinguish paper, fabric and wood fires. NEVER use water to extinguish an electrical fire or one caused by a flammable liquid.
 - Buckets of sand or dry soil (kept free of

refuse) to smother flames, contain and extinguish a free flowing liquid fire.

- Fire blanket(s) made from heavy cotton twill treated with a fire retardant chemical or preferably a manufactured fire blanket made from woven fibre glass and fire retardant material, to extinguish fire on personal clothing or a small fire on the floor or bench.
- Dry powder chemical fire extinguisher to extinguish electrical fires and fires caused by flammable liquids and gases.
- Ensure staff *know what to do in the event of a fire*, and know how to use the fire fighting equipment. Carry out regular fire drills.
- Fit a battery operated smoke detector and test the alarm at regular intervals. To alert those adjacent to the laboratory of a fire danger, keep a loud hand bell or gong in a prominent place. Whenever possible, fit a fire alarm system.
- Do not allow smoking in or adjacent to the laboratory. Display “No smoking” signs (see page 34).
- Whenever possible, avoid using compressed gasses. When necessary, follow the safety measures described on pages 26–27.

Fire hazard of some commonly used chemicals

CHEMICAL	FLASH POINT*	CLASSIFICATION
Diethyl ether	–40°C	Extremely flammable
Acetone	–18°C	Extremely flammable
Toluene	4°C	Highly flammable
Methanol	12°C	Highly flammable
Ethanol, absolute	13°C	Highly flammable
Isopropanol	12°C	Highly flammable
Ethanol, 70%	21°C	Highly flammable
Xylene	25°C	Flammable
Acetic acid, glacial	40°C	Flammable

***Flash point of a chemical:** This is the lowest temperature at which the vapour above a liquid can be ignited in air. The lower a chemical’s flash point, the higher is the risk of an ignition source igniting it. The most hazardous flammable chemicals are those with flash points below ambient temperature because they evaporate rapidly from open containers.

CHEMICAL AND REAGENT HAZARDS

- Poisoning and chronic ill-health from ingesting,

inhaling or absorbing toxic and harmful chemicals,

- Fire hazard,
- Chemical burns, skin irritation, allergic reactions,
- Eye injuries,
- Explosive reactions.

Assessing, reducing and managing chemical hazard risks

- Keep accurate records of all the chemicals used in the laboratory. Know which chemicals are hazardous and how to handle and store them correctly (see Appendix III). Read carefully the safety and risk phrases written on container labels.
- Before opening a bottle containing a flammable liquid, make sure there is no open flame within 2 metres (3 metres for acetone and ether). If needing to heat a flammable liquid, use a water bath (never a hot plate).
- Keep only small quantities of flammable chemicals and reagents on benches and shelves (not over 400 mL), and out of direct sunlight. Store stock supplies in a flame protected container at ground level in a cool and well ventilated location. Display a Flammable notice on the outside.
- Do not store flammable chemicals in a refrigerator.
- Store oxidising chemicals* well away from flammable substances, organic materials, and reducing agents. Oxidising substances are fire-promoting, impede fire-fighting and are dangerous to eyes and skin.

* Include hydrogen peroxide, sodium nitrite, perchloric acid, sodium chlorate, chromic acid, potassium dichromate, calcium hypochlorite bleach powder, potassium permanganate.

- Handle toxic, harmful and irritating chemicals with great care (see Appendix III). Wear protective gloves, goggles or visor and wash the hands immediately after handling these chemicals. Ensure an eyewash bottle is accessible to rinse the eyes should a toxic or harmful chemical enter the eyes (see Plate 1.2). Store such chemicals in secure cupboards, not in excess of 400 mL on open shelves. Lock away highly toxic chemicals such as potassium cyanide and sodium azide.

Note: First Aid treatment for poisoning and injuries caused by toxic, harmful and irritating chemicals is described on page 37.

- Store corrosive chemicals* at low level. Pour corrosive liquids at below eye level, slowly and with great



Plate 1.2 *Left:* 500mL emergency eye wash bottle. Squeezing the bottle gently sprays the eye. The waste liquid flows through a tube to the sink or collecting vessel. Instructions for use are printed in green on the bottle. *Right:* Protective eyeshields with closed in sides. The goggles are made from polycarbonate.

care to avoid splashing. Wear protective gloves and a face visor (or at least protective goggles).

* Include phenol, concentrated sulphuric acid, nitric acid, glacial acetic acid, trichloroacetic acid, o-phosphoric acid, sodium hydroxide, potassium hydroxide, and some concentrated disinfectants.

- Dissolve a solid corrosive chemical such as sodium hydroxide in water with care, mixing in small amounts at a time to dissipate the heat produced.
- When diluting concentrated acids, particularly sulphuric acid, *always add the acid to the water*. NEVER ADD WATER TO CONCENTRATED SULPHURIC ACID (the heat produced can shatter a glass container).
- When weighing corrosive chemicals, e.g. phenol, remove the weighing container from the balance pan when adding or removing the chemical. This will avoid damaging the balance pan.
- Read carefully the manufacturer's instructions for storing and handling explosive chemicals such as picric acid, sodium azide, diethyl-ether (see Appendix III). Heat, flame, knocks or friction can cause explosive chemicals to explode.
- After use, make sure bottle tops are tightly closed. Do not use rubber liners in the caps of

bottles containing iodine, ether, xylene or other chemical that attacks rubber. Do not use ground glass stoppers in bottles containing sodium or potassium hydroxide. These chemicals absorb carbon dioxide from the air forming carbonates which can cement the stopper in the bottle.

- Do not dispose of hazardous chemicals and reagents onto open ground or discharge them into open drains. The sewer system must not be used to dispose of highly toxic chemicals, water-immiscible chemicals or substances that can react with metal drainage pipes to produce dangerously reactive products, e.g. sodium azide or picric acid (see Appendix III).

Important: Always obtain advice from a qualified safety officer regarding the correct procedure for disposing of environmentally dangerous chemicals.

- Know how to handle chemical spillages (see following text).

Note: Appendix 111 contains a sheet, entitled Chemical Hazards, which lists which chemicals are extremely and highly flammable and which are oxidizing (fire-promoting), corrosive, harmful, toxic, irritant and explosive.

Allergenic, carcinogenic, mutagenic and teratogenic substances (read container label)

Some toxic chemicals are known to cause or are suspected of causing specific types of disease or of affecting particular organs or functions of the body as follows:

Allergen: Causes in some people allergic or hypersensitivity reactions, e.g. skin contact resulting in dermatitis and inhalation resulting in asthma or related conditions.

Carcinogen: Causes or increases the risk of cancer usually after repeated or long term exposure. Examples include benzidine, *o*-toluidine, *o*-toluidine, *o*-dianisidine, *alpha*- and *beta*-naphthylamine, nitrosophenols, nitronaphthalenes, and selenite.

Mutagen: Capable of producing mutations of germ cells leading to genetically induced malformations, spontaneous abortion or death of the offspring of an exposed individual. Exposure of a mother to certain mutagenic chemicals during pregnancy may result in cancer developing in her offspring.

SPILLAGE CONTROL GUIDELINES

In the event of a serious chemical spill, evacuate non-essential personnel from the affected area and proceed as follows:

- If there is personal injury or a hazardous chemical has been spilled on clothing, remove the clothing and immediately wash and immerse

the affected part of the body in water. If the injury is serious, apply appropriate First Aid measures and seek medical advice (see later text).

- If liquid chemicals (non-flammable) are spilled, place sufficient dry sand or absorbent paper around the spillage to prevent its spread and to soak up the chemical. Wearing chemical resistant gloves and using a plastic dustpan, collect the material, neutralize it if a strong acid or alkali and dispose of it safely.

Neutralization

Use 50 g/L (5% w/v) sodium bicarbonate or sodium carbonate to neutralize acid spills, and 10 g/L (1% v/v) acetic acid to neutralize strong alkali spills. Clean the spillage area with water and detergent.

- If the spillage is a volatile flammable chemical, immediately extinguish all flames, e.g. from Bunsen and spirit burners. Open the windows and doors to allow the spillage to evaporate. Clean the area with water and detergent.
- If the spillage is a solid chemical, wearing chemical resistant gloves and dust mask if appropriate, collect the chemical in a plastic dustpan and dispose of it safely by dissolving it in an adequate volume of water and flushing it down the drainage system. If water-immiscible or unsuitable for flushing down the plumbing system, mix the chemical with sand and dispose of it in a deep covered waste disposal pit. Clean the spillage area with water and detergent.

Important: Follow any special spillage control measures recommended by the manufacturer of a chemical and display charts showing how to manage chemical spills.

SAFETY GUIDELINES FOR COMPRESSED AND LIQUIFIED GASES

The use of compressed gases should be avoided whenever possible. Where necessary, the following safety measure should be applied:

- Make sure all cylinders are clearly labelled and correctly colour coded.
- Display warning notices on the doors of rooms where cylinders containing flammable gases are used and stored.
- Do not keep more than one cylinder of a flammable gas in a room at any one time. Store spare cylinders in a locked identified weatherproof store at some distance from the laboratory.

- Fix securely (e.g. chain) a gas cylinder to the wall or a solid bench to avoid it being dislodged.
- Do not locate compressed gas cylinders or liquified gas containers near to radiators, naked flames or other heat sources, sparking electrical equipment, or in direct sunlight.
- Always turn off the main high-pressure valve when the equipment is not in use and when the room is unoccupied.
- Use a trolley to support compressed gas cylinders when they are being transported (transport with caps in place).
- Do not incinerate single use gas cylinders.

Note: Gases for fixed items of equipment should be connected by a drop level safety cock to permanent pipework using screwed union connectors. Bunsen burners should also be controlled by a safety cock. Installation of piped compressed air, vacuum or gas system must be undertaken by a *qualified* person.

DECONTAMINATION, DISPOSAL OF INFECTIOUS WASTE

Laboratory staff have a responsibility to protect themselves, patients, the community and environment from injury or damage originating from infectious or toxic laboratory waste and to minimize the hazards involved in decontamination, disposal and recycling. Decontamination means the making safe of infectious material or articles that have been in contact with infectious microorganisms. Safe processing of infectious laboratory waste and contaminated articles requires the separation of such waste and articles into clearly labelled colour-coded discard containers according to method of decontamination, disposal, and associated hazard.

Decontamination methods used in district laboratories include:

- Sterilization by autoclaving
- Disinfection by boiling
- Disinfection using chemical disinfectants

Sterilization

This is the most reliable way of achieving decontamination because it destroys microorganisms, including bacterial spores.

Disinfection

This aims to destroy or at least reduce the number of contaminating microorganisms to levels that are no longer regarded as harmful to health. It implies the destruction of

vegetative (non-sporing) infectious microorganisms but not necessarily bacterial spores.

Methods used to dispose of laboratory waste include:

- Incineration
- Burial in a deep covered waste pit or landfill

The decontamination, cleaning and sterilization of reusable laboratory items are described on page 33.

Note: Each country will have its own guidelines covering the management of infectious waste from healthcare activities.² District laboratories should consult their district laboratory safety officer regarding the application of any district health authority regulations covering the decontamination of infectious laboratory waste and safe local methods for its disposal. There should be clearly written SOPs covering laboratory infectious waste handling.

AUTOCLAVING

Autoclaving, when performed correctly, is the most effective and reliable means to sterilize laboratory materials and decontaminate infectious waste by killing or inactivating biological agents, including spores.¹

During autoclaving, pressure is used to produce high temperature steam. A temperature of 121 °C and holding time of 15 minutes, timed from when 121 °C is achieved in the load, is used to sterilize infectious waste.

A district hospital laboratory must have access to a reliable autoclave of adequate capacity. The autoclave should be thermostatically controlled and fitted with a thermometer, pressure gauge, pressure release safety valve and an automatic overheat cutout. It should be supplied with a metal tray and wire mesh baskets. Accessories which are recommended to purchase at time of ordering include a spare lid seal gasket, pressure release safety valve, spare washers for valves and TST (Time, Steam, Temperature) control indicator strips.

Important: Laboratory staff must know how to use and maintain an autoclave correctly and be aware of the dangers of its misuse.

SI unit for pressure: Most pressure gauges on autoclaves are calibrated in pounds per square inch (lb/in², or psi) or in bars. The SI unit for pressure force is the pascal (Pa). In SI units, 1 psi is equivalent to 6.9 k Pa and 15 psi to 104 k Pa. Using bars, 1.1 bar is equivalent to 104 kPa or 15 psi.

Temperature

To obtain the correct temperature for sterilization all the air must first be removed from the autoclave. A

mixture of hot air and steam will not sterilize. It has been estimated that if about 50% of air remains in the autoclave the temperature will only be 112 °C and heat penetration will be poor.

At high altitudes, atmospheric pressure is reduced and therefore the pressure required to achieve 121 °C will need to be increased. At 2 100 m (7 000 feet) a pressure of 18.5 psi is required to raise the temperature to 121 °C. The pressure should be raised 0.5 psi for every 300 m (1 000 feet) of altitude. Alternatively, a lower temperature and longer sterilizing time can be used (see later text).

Timing

Before commencing timing, sufficient time must first be allowed for the saturated steam to permeate the entire load and for heat transfer to occur. The heat-up time will depend on the type of autoclave and items being sterilized.

To prevent accidents and injury it is important to allow sufficient time after sterilization for the pressure to return to zero and for the load to cool. If the steam discharge tap is opened before the pressure gauge is reading zero, any fluid in the load will boil and bottles may explode. Several hours may be required for agar to cool to 80 °C for safe handling.

The correct use of an autoclave is essential to protect against injury and to ensure sterilization. Always follow carefully the manufacturer's instructions and recommendations. Locate the autoclave in a safe place, particularly if using a primus stove or gas burner to heat the autoclave. Do not mix loads, i.e. autoclave infectious material separate from non-infectious articles. Always use the correct volume of water in the autoclave each time it is used (it must not be allowed to boil dry).

Decontamination of samples and infectious waste

Place the articles for decontamination in wide, shallow, leak proof, solid bottomed containers, not more than 200 mm deep. Do not cover the containers.

When decontaminating infectious material in plastic bags, do not over-pack the bags. Leave them unsealed with their tops folded back.

Procedure

- 1 Add the correct amount of water to the autoclave as directed in the manufacturer's *User Manual*.

Note: Not all manufacturers recommend using distilled water or deionised water.

- 2 When loading the autoclave, leave sufficient space between articles for the steam to circulate freely. Do not allow articles to touch the sides of the chamber or stand in the water. Use a tray or wire stand in the bottom of the chamber.
- 3 Place a control TST (Time, Steam, Temperature) indicator strip in the centre of the load where steam penetration is likely to be slowest (see later text, *Control of an autoclave*).
- 4 Secure the lid of the autoclave as instructed by the manufacturer.
- 5 Open the aircock (air outlet) and close the draw-off cock.
- 6 Heat the autoclave. Switch on the power to maximum setting. When the water begins to boil, air and steam will be expelled through the aircock.
- 7 Allow the correct length of time for all the air to be expelled as instructed by the manufacturer.

Checking whether all the air has been expelled

It is possible to check whether all the air has been removed from an autoclave by connecting one end of a length of tubing to the air outlet and immersing the other end in a container of water. All the air has been expelled when no more bubbles can be seen emerging from the tubing into the water.

- 8 Close the aircock. This will cause the pressure to rise and with it the temperature of the saturated steam.
- 9 When the required pressure has been reached (15 psi) as shown on the pressure gauge, and the excess steam begins to be released from the safety valve, reduce the heat and begin timing using a timer. The heat should be sufficient to maintain the required pressure for the duration of the sterilizing time.

Depending on altitude, set the timer as follows:

Altitude metres (m)	Time minutes
0	15 (121 °C)
500	20 (120 °C)
1 000	25 (119 °C)
1 500	30 (118 °C)
2 000	35 (117 °C)
2 500	40 (116 °C)

- 10 At the end of the sterilizing time, turn off the heat and allow the autoclave to cool naturally. This will usually take several hours particularly for agar culture media to cool sufficiently for safe handling.
- 11 When the thermometer reads below 80 °C and the pressure gauge registers zero, slowly open

the draw-off cock to vent the autoclave. Open the aircock and wait for a few minutes before opening the lid.

Important: To avoid fluids boiling and injuries from burns or exploding bottles, NEVER open the draw-off cock or aircock, or attempt to open the lid of an autoclave until the temperature has fallen to below 80 °C and the PRESSURE READS ZERO.

Some models of electric autoclave are fitted with a safety device that prevents opening when the autoclave is still under pressure or thermal lock.

- 12 Open the lid of the autoclave as instructed by the manufacturer and carefully unload it.

Caution: Wear full face and hand protection when opening and unpacking an autoclave, particularly when it contains bottled fluids which may still be over 100 °C even when the temperature inside the autoclave is below 80 °C. On contact with air at room temperature the bottle may explode. If possible, leave the autoclave to cool overnight before opening it.

- 13 Check the TST control strip to ensure sterilization has been satisfactory (see later text).

Control of an autoclave

An autoclave thermometer, depending on its location, gives an indication of the temperature of the steam in the chamber or in the drain of an autoclave, not in the load. Ideally, therefore, all autoclaves should be tested with a thermocouple to find the time it takes to bring the load to 121 °C. The thermocouple should be connected to a recorder to produce a graph of the time/temperature conditions during the sterilizing cycle.

When a thermocouple and recorder are not available, an indication of the effectiveness of a sterilizing cycle can be obtained by incorporating in the load a chemical indicator that monitors time, steam, and temperature, such as an TST control strip.

Use of TST control strips

TST (Time, Steam, Temperature) control strips for use in autoclaves and pressure cookers incorporate a yellow chemical indicator in an area on the end of each strip. The control strip should be placed in the load where heat penetration is likely to be slowest (usually in the centre).

Only when the conditions for sterilization have been met, i.e. correct heat/time ratio in saturated steam, will the yellow colour of the indicator change to

match the darker colour of the reference area shown on the strip. The change is distinctive, avoiding any misinterpretation of the result.

TST sterilizer control strips must be stored in the dark because direct sunlight will cause deterioration of the chemical indicator. In conditions of high relative humidity, unused strips should be sealed in an airtight plastic bag. Providing they are stored correctly, the strips have a 3 year shelf-life from date of manufacture.

Other methods of monitoring the performance of autoclaves

Adhesive sterilization tape: Compared to TST sterilizer control strips, this is not such a reliable way of testing whether a sterilizing cycle has been effective. The heat/moisture sensitive ink which forms the bands on the tape and changes from a green to a yellow colour after autoclaving, does not record accurately the conditions during the sterilizing cycle. The tape is useful in showing when an article has been autoclaved.

Care and maintenance of autoclaves

- Read and follow carefully the manufacturer's instructions. Prepare a stock record card and written SOP covering the use, care, and maintenance of the autoclave.
- Hold in stock a spare lid gasket, safety valve, and spare valve washers (seals) and spare heater.
- Clean the inside of the autoclave after use and also around the valve and stopcocks. Make sure the vent is not blocked. Do not use a corrosive cleaning chemical. Lightly lubricate the screw clamps.
- Check regularly for signs of wear and damage. Examine particularly whether:
 - the lid seal gasket is flexible and unperished and is not allowing steam to escape from the lid,
 - the pressure gauge is in working order and reading zero at atmospheric pressure,
 - the safety valve is free moving and in working order (read the *User Manual* on how to check and clean the safety valve),
 - the aircock and draw-off cock are working correctly with no leakage from seals.
- When required, descale the inside of the autoclave using a weak acid descaler.
- Do not use an autoclave if it is defective. Obtain the help of a qualified engineer to repair a faulty autoclave and test it before reuse.
- Use a TST control strip to check the performance of an autoclave (see previous text).

Microprocessor controlled autoclaves

Microprocessor controlled, touch-screen operated autoclaves with optional programmable sterilizing cycles are becoming widely available. They vary however in their complexity of use with some models requiring passwords or a card to make adjustments to set times or temperature.

Additional safety features such as thermal or cooling locks and built-in systems to meet laboratory quality standards have led to the lengthening of sterilizing cycles. To improve performance, many new laboratory autoclaves have vacuum air removal, venting systems and assisted air cooling options. Installation, preventive maintenance, and servicing can be complex and costly.

In most instances it will not be possible for a laboratory to arrange a demonstration of a microprocessor controlled autoclave prior to purchase. It is therefore important to obtain in advance detailed specifications, installation requirements, and a copy of the *User Manual*.

BOILING

Heating in boiling water at 100°C for 20 minutes at altitudes below 600 metres (2 000 feet) is sufficient to kill all non-spore bacteria, some bacterial spores, fungi, protozoa, and viruses including hepatitis viruses and HIV. Adding 20 g sodium carbonate to every litre of water increases the effectiveness of the disinfection.

Boiling at higher altitudes

Water boils at 100°C at sea level. The temperature of boiling water is reduced by approximately 1°C for every 500 metres above sea level. A boiling time of 30 minutes is therefore recommended when the altitude is greater than 600 metres.

When there is no mains electricity, a large pan or other metal container can be used and the water heated using a charcoal stove, kerosene or gas ring burner (located in a safe place outside the laboratory working area). Choose the coolest time of the day to boil items. When boiling, all the items must be completely submerged in the water.

DISINFECTANTS

When compared with autoclaving and boiling, chemical disinfection is the least reliable and controllable method for the treatment of laboratory infectious waste. No single disinfectant is likely to kill all microorganisms in any sample of infectious waste.

The use of chemical disinfectants should be restricted to discard containers on the bench, disinfecting equipment, protective clothing, bench surfaces, and floors, and the treatment of spillages.

Correct dilutions must be used and the dilutions must not be kept beyond their useful life. When used in discard containers, disinfectant solutions must be renewed daily.

All disinfectants are to some extent inactivated by protein (particularly hypochlorite solutions), plastics, rubber, hard water, and detergents. Disinfectant solutions must not be overloaded because there is a limit to the amount of material which they can disinfect effectively. The article to be disinfected must be in contact with the disinfectant and not protected by air bubbles, immersion oil, or films of grease.

When emptying discard containers of disposable items, use a plastic strainer to collect the items. If containing glass, e.g. haematocrit tubes or other sharp items, transfer the waste to puncture-resistant containers.

Caution: Care must be taken when using disinfectants, particularly when preparing dilutions. Disinfectants are toxic and mostly corrosive and irritant. Safety goggles, plastic apron, and chemical resistant rubber gloves must be worn. When not in use, discard containers should be covered using lids that can be removed easily. The containers should not be placed in direct sunlight.

The following are the most commonly used chemical disinfectants in district laboratories:

- chlorine-releasing disinfectants
- phenolics
- peroxygen compounds
- alcohols

Note: None of these substances will kill or even disable microorganisms unless they are properly used and renewed regularly.

Weak disinfectants unsuitable for laboratory use

These include *Dettol*, chlorhexidine products such as *Hibitane*, *Hibisol*, cetrime compounds such as *Cetavlon*, and hypochlorite solutions sold for treating baby feeding bottles.

Chlorine-releasing disinfectants

These disinfectants are the most widely used in district laboratories. They work by giving off free chlorine which is highly active against Gram positive and Gram negative bacteria, bacterial spores, and viruses including HIV, hepatitis viruses and VHF viruses. They are less effective against fungi. Chlorine disinfectants are strongly alkaline, and may corrode

metals and damage rubber. In an acid environment, chlorine release is accelerated. Chlorine solutions must be prepared daily in a well ventilated area and protected from sunlight and excessive heat. Store the solutions in plastic containers. The disinfectant power of chlorine-releasing agents is expressed as parts per million (ppm) or percentage of available chlorine (av Cl) as follows:

- 1 000 ppm = 0.1% av Cl: used for disinfecting working surfaces and floors and decontaminating soiled hands and protective clothing.
- 5 000 ppm = 0.5% av Cl: used for discard containers but do not use for containers into which urine supernatant fluids are discarded because acid accelerates the release of chlorine gas.
- 10 000 ppm = 1% av Cl: used for treating spillages of infectious material.

Chlorine-releasing disinfectants include:

- Sodium hypochlorite solutions sold as bleach solutions for domestic and laundering purposes, e.g. *Chlorox*, *Jik*, *Domestos*, *Presept* and other trade names. These solutions generally contain 5% available chlorine but always read the label on the container as some 'thin' bleach solutions contain less than 5%. Check the strength of solution using a high chlorine test paper.³ Bleach solutions are easily inactivated by organic matter, e.g. blood, faeces, pus.

Instability of hypochlorite solutions

Liquid bleach has the disadvantage that in heat and strong light, it rapidly deteriorates and loses its chlorine content. Starch/iodide indicator papers can be used to check the oxidising power of solutions in discard containers. The

paper will turn blue-black when there is insufficient oxidising power, indicating overloading of the disinfectant.

- Calcium hypochlorite powder which usually contains 35% available chlorine and calcium hypochlorite granules and tablets which usually contain about 70% available chlorine.
- Sodium dichloroisocyanurate (NaDCC), available as powder, granules and tablets. The available chlorine in NaDCC products depends on the manufacturer, e.g. one tablet containing 1.7g (1.67g) NaDCC dissolved in 1 litre of water produces 1 000 ppm (0.1%) av Cl (5 tablets in 1 litre produces 0.5% av Cl). Information on the concentration of NaDCC in products and instructions on how to prepare solutions are provided by manufacturers.

Stability and other advantages of using NaDCC

Compared with hypochlorite solutions, solid NaDCC products are more stable (3–5 y shelf-life) even at high temperatures as long as they are stored in a dry environment. Solutions of NaDCC are less inactivated by protein than hypochlorite solutions. They lower pH, giving a greater microbicidal capacity than equivalent bleach solutions.

When treating spillages, the contact time of NaDCC granules with the spillage can be reduced to 2–3 minutes compared to the 30–60 minutes required when using bleach solutions. NaDCC tablets are easier and safer to use in the laboratory. They are readily soluble in water (must be renewed daily).

Phenolics

Because of safety concerns, phenolics are less used as disinfectants. They are active against all non-spore-forming bacteria including mycobacteria. Phenolics do not kill spores and are poorly active against viruses. They are, however not markedly inactivated by

Table 3.1 Preparation of chlorine-releasing disinfectant solutions

<i>Product</i>	1 000 ppm av Cl (0.1%) Used for benches, floors, clothing	5 000 ppm av Cl (0.5%) Used in discard containers
Sodium hypochlorite bleach solution 5% av Cl	Add 20 mL to 1 litre of cold water and mix well	Add 100 mL to 1 litre of cold water and mix well
Calcium hypochlorite bleaching powder, 35% av Cl	Dissolve 2.9 g in 1 litre of cold water	Dissolve 14.3 g in 1 litre of cold water
NaDCC 1.7 g tablet* * Read manufacturers' instructions	Dissolve 1 tablet in 1 litre of cold water	Dissolve 5 tablets in 1 litre of cold water

Preparing working bleach solutions from commercial bleach

Example: Prepare 0.5% solution from 3.5% Jik bleach

$3.5 \div 0.5 = 7$ Dilute 3.5% Jik 1 in 7 in water

e.g. Add 100 mL 3.5% Jik bleach to 600 mL water.

Preparing working chlorine solutions from bleaching granules

Example: Prepare 1 litre 0.1% solution from 70% bleaching granules

$\frac{1000}{70} \times 0.1 = 1.4$ g Dissolve 1.4 g bleaching granules in 1 litre of water.

70

proteins. At an alkaline pH, the activity of phenolics is reduced (do not make up solutions with hard water). Phenolics are non-corrosive. They are toxic and irritating to the skin and eyes. They can be absorbed through latex gloves and penetrate the skin. Wear heavy duty rubber gloves and protective goggles when preparing solutions.

In district laboratories *Lysol* is frequently used because it is widely available and inexpensive. It is used at a 5% v/v concentration or according to manufacturers' instructions for the "dirtiest conditions". Solutions should be renewed daily. *Lysol* is usually available as a neat solution or at dilutions of e.g. 50%, 12%, 6%, depending on manufacturer.

Peroxygen compounds

An example is *Virkon* which when used at 1% w/v concentration has a wide range of bactericidal, virucidal and fungicidal activity. It has variable activity against bacterial spores. A 3% w/v concentration is recommended for *Mycobacterium* species. Neat powder is used for spillages.

Virkon has a built-in colour indicator and combines cleaning with disinfection. When diluted it is non-irritant and has low dermal toxicity. It can be used to clean centrifuges providing it is washed off. Dilutions are stable for 7 days. It is however expensive when compared to hypochlorite disinfectants.

Alcohols

Ethanol and isopropanol at 70–80% v/v concentration in water are useful for disinfecting skin and surfaces (methanol is less effective). Penetration of organic matter is poor. Alcohols are highly active against mycobacteria, non-sporing Gram positive and Gram negative bacteria. HIV and hepatitis B and C viruses are inactivated. Activity against non-lipid (naked) viruses and bacterial spores is poor. Alcohol solutions are flammable and evaporate rapidly.

Aldehydes

These less used disinfectants include:

- Formaldehyde
- Glutaraldehyde (glutaral)

Formaldehyde gas is an effective disinfectant against all microorganisms including viruses, except at temperatures below 20°C. A 5% v/v formalin solution (diluted from concentrated formalin solution) is occasionally used as a disinfectant but formalin is too irritant for general use as a laboratory disinfectant. When heated, formaldehyde gas is used to decontaminate safety cabinets. It is non-corrosive. Formalin solution is toxic with an irritating vapour, and may cause sensitivity reactions. It is also a suspected carcinogen. Use only in a well ventilated laboratory.

Glutaraldehyde preparations include *Cidex*, *Clinicide*, *Glutarex 3M*, *Asep*, *Totacide* and *Triocide*, usually supplied at

working concentrations of 2%. It is an expensive disinfectant. An alkaline activator must be added which is usually supplied with the product.

In the laboratory, glutaraldehyde is used mainly to disinfect metal surfaces, e.g. instruments and centrifuge parts where corrosive chlorine products cannot be used and viral contamination is possible. It rapidly inactivates bacteria and viruses including HIV and hepatitis B virus. It is also active against mycobacteria, but its penetration of organic matter is poor. Most glutaraldehyde products remain active for 7–14 days after activation. Cloudy solutions however should be discarded. Glutaraldehyde is toxic, irritant, and mutagenic. Contact with skin, eyes, and respiratory tract should be avoided.

INCINERATION

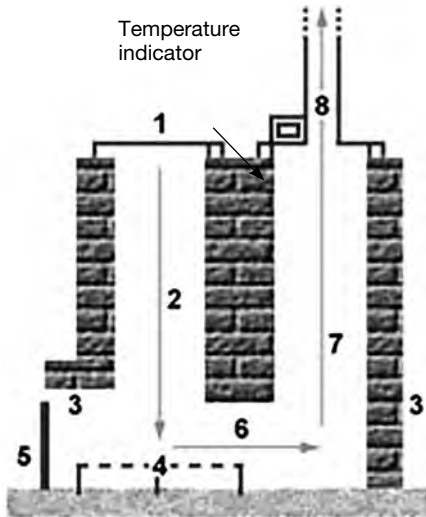
Incineration, i.e. destruction by burning, is a practical and effective method of disposing of infectious and contaminated laboratory waste. Access to purpose built incinerators is often not possible at district hospital level (except where there is health authority collection of medical waste) and even less likely for health centres. In most situations a locally constructed incinerator is used. A deep pit is used for burying the ash.

Local construction of an incinerator

A dual combustion chamber incinerator is recommended. An example is the De Montfort incinerator which can be constructed locally without the need for specialized tools. It is made from firebricks, fire-resistant cement and pre-fabricated metal parts. It can be used with coconut shell, wood or kerosene.

A diagram of a basic De Montfort incinerator is shown in Fig. 1.3. The waste is warmed, dried and melted in the primary combustion chamber before being burnt at 600–900 °C in the grate. Partially burned flue gas and particulates are drawn from the primary area into the second chamber, where additional air results in secondary burning before the flue gases are released into the atmosphere through the chimney. Pathogens pass through two high temperature zones, one in the grate and the other in the second chamber. A careful balance between the rate of loading the incinerator and maintaining the operating temperature is required to minimize smoke emissions and toxic emissions.

Important: Site an incinerator in a safe place. Place a roof over it to protect it from the rain and surround it with a wire fence to prevent entry of unauthorized persons and animals. Supervise the incineration at all times. Wear a face mask and protective gloves. Bury the ash as soon as it has cooled. Do not use the incinerator to destroy broken thermometers, IV



1 Loading door 2 Primary combustion chamber 3 Air inlets
4 Fire grate 5 Ash door 6 Gas transfer tunnel 7 Secondary combustion chamber 8 Chimney, not less than 4 metres high.

Fig. 1.3 Cross-section of a basic De Montfort incinerator.
Courtesy of De Montfort Medical Waste Incinerators.

fluid bags, PVC plastic bags, wet waste, closed glass vials or ampoules.

Note: Further information on the construction of a De Montfort incinerator can be obtained from: https://mw-incinerator.info/en/101_welcome.html

Construction of a deep pit

Locate a deep pit in a safe fenced off area that is not liable to flood, is sufficiently deep (4–5 metres) and wide (1–2 metres) and has a strengthened rim. Keep the pit covered. Infectious waste should be incinerated before it is discarded into a pit.

Important: Always follow local health authority guidelines on the location and use of deep disposable pits and local landfill sites.

REUSE OF LABORATORY ITEMS

In district laboratories the following articles are frequently reused following decontamination:

- Sample containers
- Microscope slides
- Test tubes and centrifuge tubes
- Pipettes
- Glassware including beakers, flasks, funnels, cylinders

These items are decontaminated, cleaned and when required, sterilized before reuse as follows:

Sample containers

- 1 Empty fluid samples (urine, blood, serous fluids) into the sewer system through the sluice.
- 2 Soak the containers, caps and cap liners for at least 1 hour in 0.5% chlorine solution or alternatively boil them for 30 minutes, making sure the containers are fully submerged.
- 3 Wash each container, cap and cap liner in detergent, rinse *well* in water* and drain dry.

* Water used for rinsing reusable items

For the final water rinse, use distilled water. When this is not available, use filtered water.

- 4 When sterile containers are required, autoclave them with caps loosened (tighten after autoclaving) at 121 °C for 15 minutes. Only polypropylene or heat-resistant glass containers can be autoclaved.

Note: Do not reuse glass containers into which sputum has been collected.

Microscope slides

- 1 Wipe any oil from slides using a piece of rag or tissue soaked in disinfectant (wear protective gloves).
- 2 Soak the slides overnight in a plastic container of 0.5% chlorine solution.
- 3 With care, wash each slide with detergent using a soft brush.
- 4 Rinse well with water and dry the slides between cotton cloths.
- 5 When *completely dry*, store the slides in boxes. Discard any slides that are scratched or chipped.

Note: Do not reuse slides that have been used to stain sputum for acid fast bacilli (AFB).

Test tubes and centrifuge tubes

- 1 Immerse the tubes for at least 1 hour in 0.5% chlorine solution.
- 2 Wash the tubes in detergent using a test tube brush.
- 3 Rinse well in water and dry the tubes facing downwards.
- 4 When sterile tubes are required, autoclave them at 121 °C for 15 minutes. Only polypropylene or heat-resistant glass tubes can be autoclaved.

Pipettes

1 Immediately after use, soak pipettes for at least 1 hour in 0.5% chlorine solution in a sufficiently tall container to allow complete immersion of each pipette and the expelling of air bubbles. Do not overcrowd the container. Use separate containers for glass pipettes and micropipettes.

Note: Rinse micropipettes (20 μ L, 50 μ L) in water to avoid blood from clotting in the pipettes.

- 2 Use a vacuum pump to clean the pipettes with detergent and rinse well with water.
- 3 Allow the pipettes to drain and dry completely. Use a rubber bulb to expel the water from each pipette.
- 4 Before use, check the tip of the pipette is not chipped and the pipette is completely clean and dry.

Glassware

- 1 Soak any contaminated items of glassware in 0.5% chlorine solution for at least 1 hour.
- 2 Carefully wash the glassware in detergent and rinse in several changes of water.
- 3 Allow to drain dry, facing downwards in a draining rack. Before storing, examine the glassware for any cracks or chipped surfaces.

MONITORING SAFETY

Providing a safe working environment is the responsibility of everyone working in the laboratory. Technical and auxiliary staff require training in implementing safety procedures based on work activities and the risks associated with the hazards described previously. An effective safety programme is an essential requirement for accreditation. The person in charge of the laboratory should promote safety awareness, ensure safe working practices are followed and safety equipment and a First Aid box are accessible to all members of staff (see later text).

Use of signs to promote safety awareness

Displaying appropriate signs and symbols is one of the ways of promoting safety awareness. Examples of prohibition (do not) signs are shown in Fig. 1.4. They can be easily made locally. Prohibition signs are always crossed by a red line. *No smoking* signs should be displayed in the laboratory and adjacent patient waiting area.

The international *Biohazard* sign as shown in Fig. 1.5 should be displayed on the laboratory door. It indicates that the laboratory handles samples that may contain pathogenic microorganisms and therefore access is restricted to authorised persons.



Fig. 1.4 Prohibition signs for wall display



Fig. 1.5 International Biohazard sign

Recommended signs for labelling chemicals and reagents, e.g. *Flammable*, *Toxic*, *Corrosive*, *Harmful*, *Irritant*, *Oxidizing* and *Explosive* are shown in Fig. 1.6. The hazards associated with different chemicals are listed in Appendix III.

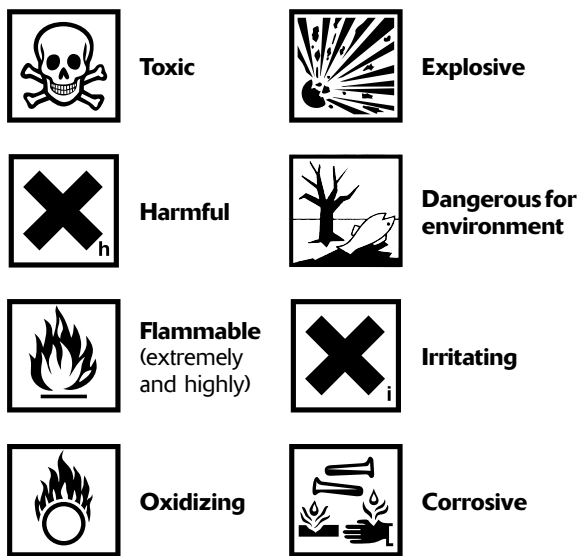


Fig. 1.6 Signs for labelling chemicals and reagents.

Laboratory safety officer

Laboratory staff should consult the safety officer on district safety policies and procedures. Provision should be made for continuing training of laboratory and auxiliary staff in safety procedures.

Staff should be informed of any National waste management guidelines and local health regulations covering the decontamination and disposal of infectious waste.

The district safety officer should establish a system for monitoring safety and helping district laboratories achieve the best possible level of safety. Regular (but random) visits should be made by the safety officer using a checklist which covers safety practices relevant to the laboratory being visited.

During safety visits the safety officer should take the opportunity to:

- demonstrate safety procedures, particularly those which help to prevent infection from aerosols, injury from “sharps”, and accidents from flammable and toxic chemicals,
- find out whether the laboratory is receiving essential biosafety supplies, e.g. disinfectants,
- discuss safety improvements with staff,
- investigate the cause of any recent laboratory-related accident as detailed in the Laboratory Accident Book.

Laboratory accident book

The following information is required:

- Place, date and time of the accident.
- Person or persons involved and injuries sustained.
- Emergency First Aid given and by whom.
- Details of follow-up actions.
- Staff comments on the possible reasons for the accident and what should be done to prevent a recurrence.

Following a safety inspection, the safety officer should provide a written report detailing any non-compliance with safety procedures and suggesting steps which should be taken to ensure compliance, to be checked at the next visit.

EMERGENCY FIRST AID

Knowing what to do immediately an accident occurs can help to reduce suffering and the consequences of serious accidents. In some situations, First Aid can be life saving, e.g. by resuscitation or the control of bleeding. It can also prevent an injured person's condition from worsening, e.g. by protecting and treating wounds, placing a person in the best possible position, offering reassurance, and seeking immediate assistance.

Training in First Aid

All laboratory workers should receive a practical training in First Aid, with particular attention being paid to the types of accidents that may occur in the laboratory. They should also know what emergency action to take if an outpatient collapses in the laboratory. Training must be given by a person qualified to teach First Aid. A certificate of competence should be issued to those who complete the course successfully. Refresher courses should be held every 3–4 years.

First Aid equipment

An adequately equipped First Aid box and eyewash bottle should be kept in the laboratory in a place that is known and accessible to all members of staff. The contents of a First Aid box are listed on page 39.



The First Aid box should be clearly labelled. It should be made of metal or plastic to prevent it being damaged by pests and to protect the contents from dust and damp. The contents should be inspected regularly.

EMERGENCY FIRST AID PROCEDURES

First Aid applied to the laboratory should include emergency management of the following:

- Cuts, needlestick injuries
- Bleeding
- Resuscitation
- Fainting
- Heat exhaustion
- Electric shock
- Heat burns and scalds
- Chemical burns
- Swallowing chemicals or infected material

Emergency treatment of cuts and bleeding

If the cut is small:

- 1 Wash with soap and water.
- 2 Apply pressure with a piece of cotton wool.
- 3 Disinfect the area with a skin antiseptic.
- 4 Cover with a water-proof dressing.

Note: If the cut has been caused by contaminated glassware or is a needlestick injury, encourage bleeding before washing well with soap and water. Apply a skin antiseptic and cover the area with a waterproof dressing. Seek medical attention if bleeding fails to stop, a foreign object is embedded in the cut or especially if a needlestick injury has occurred.

If there is serious bleeding from a limb:

- 1 Raise the injured limb to reduce the bleeding.
- 2 Apply pressure with a clean dressing backed with cotton wool. Do not dislodge blood clots.
- 3 Bandage the dressing in position.
- 4 Immediately seek medical assistance.

Bleeding from the nose:

- 1 Seat the person upright with the head slightly forward.
- 2 Tell the person to pinch firmly the soft part of their nose for about 10 minutes and breathe through their mouth.
- 3 If the bleeding does not stop, seek medical assistance.

Emergency treatment for heat exhaustion

Carry out the following:

- 1 Assist the person to a cool shady place .
- 2 Lay the person down and raise the legs above the level of the head (see Fig. 1.7 on page 37). Support the legs with a stool or chair.

- 3 Loosen clothing and cool the skin by dabbing with cool water.
- 4 Give clean water to drink.
- 5 Gradually raise the person to the sitting position.
- 6 Seek medical assistance if the person continues to feel unwell.

Emergency treatment of heat and chemical burns

Heat burns and scalds

- 1 If clothing is alight, smother the flames using a fire blanket. Prevent the person from moving around (any movement or breeze will fan the flames).
- 2 Remove the person from the danger area and place on the ground.
- 3 Immediately cool the burnt area by flooding it with cold water for at least 10 minutes. Alternatively, apply a pad soaked in cold water.
- 4 Remove any smouldering material but DO NOT remove anything sticking to the burnt area (to avoid the risk of introducing infection).
- 5 Gently remove any constricting articles such as rings or bracelets before the affected area starts to swell.
- 6 Cover with a dry dressing or apply cling film lengthwise over the burnt area (do not restrict the affected area by wrapping around the cling film).
- 7 Provide frequent small cold drinks.

Note: If more than a minor burn, seek medical treatment immediately. Reassurance of the casualty is important.

Chemical burns of the skin

- 1 Wash the skin immediately in running water for several minutes. Gently remove any contaminated clothing.
- 2 Neutralize with a suitable chemical as follows:
If an *acid* burn, neutralize with sodium bicarbonate powder.
If an *alkali* burn, neutralize with boric acid powder.
- 3 Seek medical attention.

Note: Respiratory symptoms may develop if fumes from strong acids and alkalis have been inhaled. Transfer the patient urgently for medical care.

Chemical injury to the eye

- 1 Wash the affected eye as quickly as possible under running tap water or with water from an eye wash-bottle for 10 minutes.
- 2 Neutralize with a suitable chemical as follows:
If an *acid* injury, neutralize with 5% sodium bicarbonate solution.
If an *alkali* injury, neutralize with 5% acetic acid or vinegar diluted 1 in 5.
- 3 Keep both eyes still as any movement could damage the affected eye.
- 4 Immediately seek medical attention.

Swallowing chemical or infected material**Swallowing acid or alkali**

- 1 Immediately rinse the mouth well with water.
- 2 Neutralize with a suitable chemical as follows:
If acid has been swallowed, neutralize by drinking 8% w/v magnesium hydroxide suspension (milk of magnesia) or milk.
If an alkali has been swallowed, neutralize by drinking lemon juice.
- 3 Drink three or four cups of water.
- 4 Seek medical attention.

Note: When an acid or alkali has been swallowed, do not encourage vomiting.

Swallowing other poisonous chemicals

- 1 Rinse out the mouth well with water.
- 2 Depending on the chemical swallowed, take a suitable chemical antidote under medical supervision.

Note: Always seek medical advice and treatment after swallowing toxic or harmful chemicals.

Swallowing infected material

- 1 *Immediately* seek medical treatment.
- 2 When required provide follow-up tests.

Emergency treatment when someone is electrocuted

Carry out the following:

- 1 Immediately turn off the electricity from the mains if it can be reached easily, otherwise remove the plug or wrench the cable free. **DO NOT TOUCH THE PERSON'S FLESH WITH YOUR HANDS** until the contact has been broken.

Important: On no account try to free an electrocuted person from the electrical contact without using some form of insulation such as a wooden broom handle, wooden or plastic stool or chair. If insulation is not used, the person rescuing will also be electrocuted.

- 2 If the person has collapsed, send immediately for medical help and if the person is not breathing, start cardiopulmonary resuscitation (CPR), see pages 38–39, until assistance arrives.

Emergency treatment when someone faints

Emergency treatment of a faint is as follows:

- 1 Lay the person down and raise the legs above the level of the head (see Fig. 1.7).

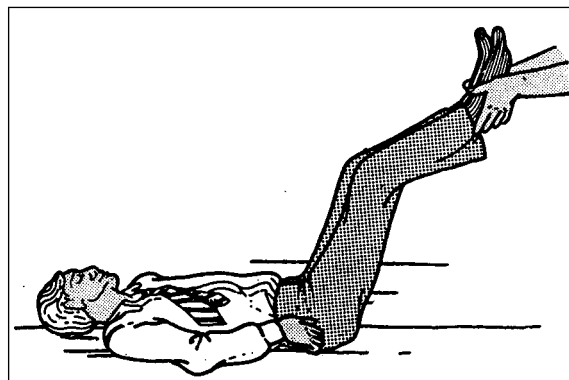


Fig. 1.7 Position to place a person who has fainted.

- 2 Loosen clothing at the neck, chest, and waist.
- 3 Cool the skin with dabs of water.
- 4 Make sure the room is well-ventilated.
- 5 Reassure the person as consciousness is regained.
- 6 Gradually raise the person to the sitting position. Sips of drinking water may be given.

Note: If breathing becomes difficult, place the person in the recovery position shown in Fig. 1.8.

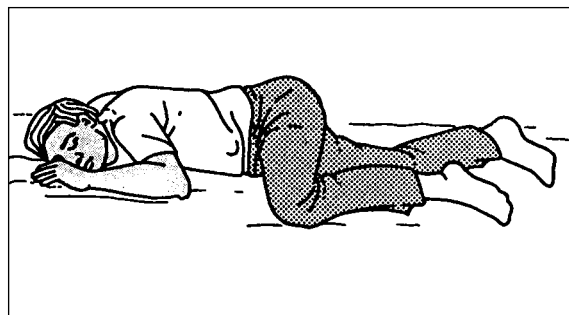


Fig. 1.8 The recovery position.

Emergency resuscitation when a person stops breathing

Breathing in oxygen and its circulation to all parts of the body are essential to sustain life. When brain cells are deprived of oxygen for more than 3–4 minutes, permanent brain damage will occur. (To check for breathing, listen for normal signs of breathing, look for chest movements, and feel for breaths). When a person stops breathing, emergency medical assistance is required and life-saving cardiopulmonary resuscitation (CPR) to provide circulation and oxygen to the body *must be started immediately*.

How to perform CPR

- 1 With the person lying on the floor, take a few seconds to ensure the airway is open by tilting the head backwards and lifting the chin (see Fig. 1.9).

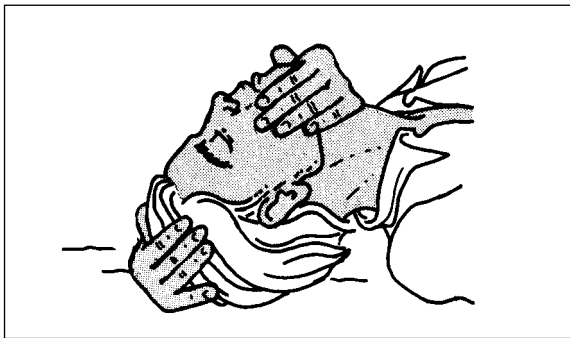


Fig. 1.9 Ensuring the airway is open.

- 2 When there is no breathing, immediately begin chest compressions:
 - Place the heel of one hand on the breastbone at the centre of the person's chest (see Fig. 1.10).

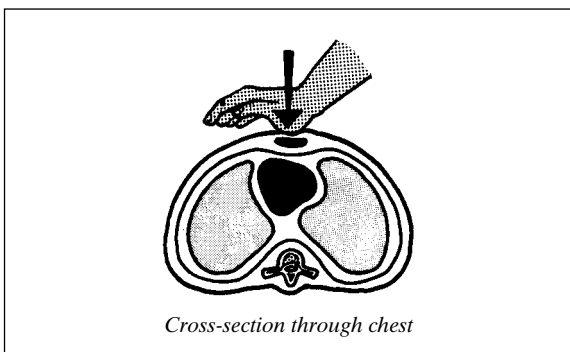


Fig. 1.10 Placing the hand on the breastbone.

- Place the heel of the other hand on top of the first hand and interlock the fingers, making sure the fingers are kept off the ribs (see Fig. 1.11).



Fig. 1.11 Interlocking the fingers

- Leaning over the person, straighten the elbows and press down on the breastbone and depress the chest 5–6 cm.
 - Release the pressure without removing the hands and allow the chest to come back up fully before giving the next compression.
- 3 Give 30 quick chest compressions (100–120 compressions per minute).
 - 4 Check whether breathing has restarted. If not, continue chest compressions and provide rescue breaths as follow:
 - Take a breath and place the lips around the person's mouth to make a good seal.
 - Blow into the person's mouth until the chest rises. A complete rescue breath should take 1 second. If the chest does not rise, check the head position to make sure the airway is still open.

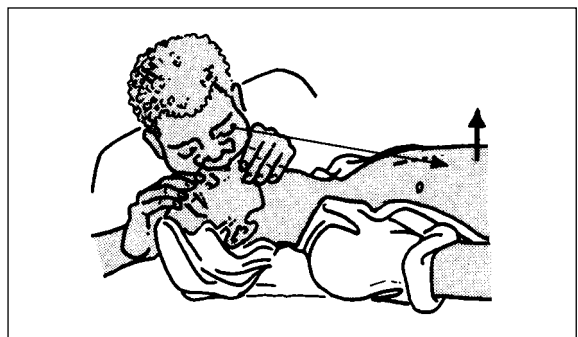


Fig. 1.12 Providing rescue breaths

- Provide 2 rescue breaths after 30 chest compressions.

Note: In the first few minutes after the heart has stopped, the oxygen level remains constant and therefore chest compressions are more important than rescue breaths. After 2–4 minutes, the oxygen level falls and rescue breaths become important.

- 5 Continue the cycle of 30 compressions followed by 2 rescue breaths until the person shows signs of becoming responsive e.g. speaking, opening the eyes, coughing, and starts to breath normally. Check the heart is beating by feeling the carotid pulse (see Fig 1.13).

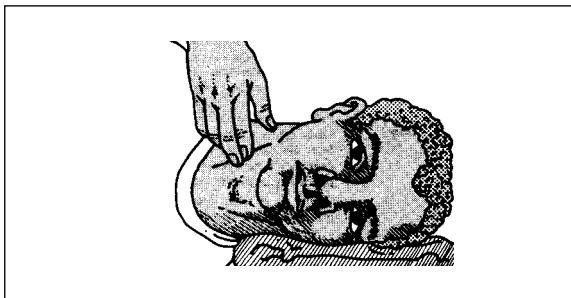


Fig. 1.13 Feeling for the carotid pulse

- 6 Once natural breathing has been restored and the heart is beating, place the person in the recovery position (see Fig 1.8). This is important because the person may vomit and in this position there will be no danger of choking.

Important: Medical assistance should be obtained at the earliest opportunity.

FIRST AID BOX CONTENTS

- Clear instructions on how to apply emergency treatment of cuts, bleeding, heat burns, chemical burns, chemical injury to the eye, swallowing of acids, alkalis, and infected material, treatment of fainting, electric shock, and how to perform emergency resuscitation (illustrated).
- Sterile unmedicated dressings to cover wounds.
- Absorbent cotton wool, triangular and roll bandages, safety pins.
- Sterile adhesive waterproof dressings in a variety of sizes.
- Cling film for burns.
- Sterile eyepads with attachment bandages.

- Roll of adhesive tape.
- Scissors and forceps.
- Sodium bicarbonate powder, boric acid powder, and sterile saline.
- Equipment for person giving First Aid (mouth-piece, gloves).

Eyewash bottle

An eyewash bottle of the type shown in plate 1.2, page 25, is suitable for district laboratories. Clean tap water, sterile water or isotonic saline should be kept next to the eyewash bottle.

Acknowledgement: Illustrations are reproduced by permission of St John Ambulance Association.

PIPETTING AND DISPENSING SAFELY

Highly infectious pathogens can be found in samples sent to the laboratory for testing and harmful and corrosive chemicals are frequently used to analyze samples. For these reasons mouth-pipetting is banned in medical laboratories. This text describes devices used to pipette and dispense samples and reagents safely. Factors that need to be considered include:

- level of accuracy and precision required,
- how well the device performs and ease of use,
- workload,
- whether the device will need to be autoclaved,
- cost of the device and consumables.

Most district laboratories will probably find that they need to use several different devices.

CAPILLARY AND MICROPIPETTE FILLERS

Bulb capillary filler

A small bulb aspirator is suitable for use with calibrated capillaries when collecting and dispensing blood and serum. The bulb has a hole which is left uncovered when drawing blood or serum into the capillary and covered with the finger when expelling and rinsing the sample from the capillary.

Micropipette fillers

The micropipette filler (controller) shown in Plate 1.3 on page 40 can be used with both calibrated capillaries and micropipettes, e.g. 20 μ L and 50 μ L

micropipettes. It has an ejection mechanism which allows contaminated capillaries to be discarded safely into a "Sharps" container. The device is autoclavable. The micropipette filler has a thumb wheel for filling, setting the fluid level and discarding the fluid. When used with micropipettes calibrated to deliver, a venting button is pressed to allow all the fluid to be dispensed.

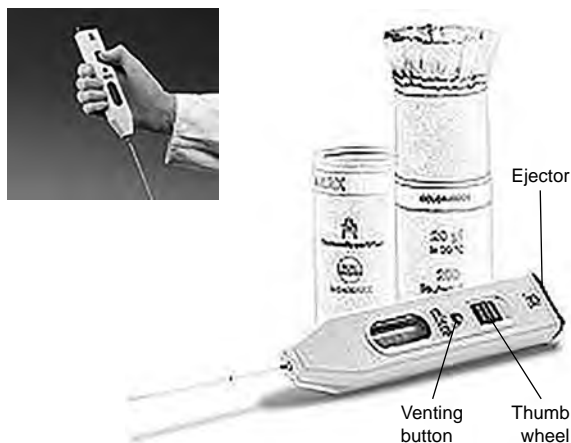


Plate 1.3 Micropipette with thumb wheel.
Courtesy of Brand GmbH. www.brand.de

The micropipette and capillary filler shown in Plate 1.4 is the *Micro-Classic Controller* manufactured by Brand GmbH. It is operated from a thumb wheel and can be used with micropipettes up to 1 mL. The adapter and suction tube are autoclavable at 121 °C.



Plate 1.4 *Micro-Classic Controller*.
Courtesy of Brand GmbH. www.brand.de

DEVICES FOR USE WITH PIPETTES

Examples of effective, easy to control devices that can be used to draw up and dispense fluid from pipettes include a *Pi-pump* pipette filler, a *RF 1000 Pipette controller*, a *Pipette pump 111*.

All these devices are manually operated. Electronic pipette fillers are also available but these operate from rechargeable batteries and are expensive.

Pi-pump pipette filler

This polypropylene thumb-wheel device for use with glass and plastic pipettes, is lightweight and very easy to operate. It gives excellent control of the level of fluid both when aspirating and dispensing. All the fluid can be dispensed by depressing the side lever or dispensed in measured volumes using the thumb-wheel as shown in Fig. 1.14.

The end of the green 10 mL *Pi-pump* is flexible and tapered, enabling it to be used with most pipettes up to 10 mL, including 1 mL and 2 mL pipettes (it is not necessary to purchase the smaller volume *Pi-pump*). Always hold the upper end of the pipette when inserting it into the device.

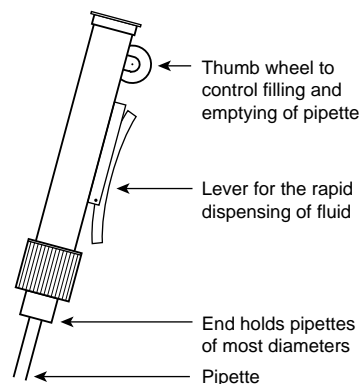


Fig. 1.14 *Pi-pump* (green) for use with pipettes of 1 mL to 10 mL.

Pipette Pump 111

The *Pipette Pump 111* has a comfortable hand grip and is operated using a plunger and thumb wheel. To use the device, press the plunger fully down before inserting the pipette. Holding the upper end of the pipette, insert it into the silicone chuck using a slight rotating movement. Use the thumb wheel or plunger to fill the pipette and the thumb wheel to dispense the fluid.



Plate 1.5 Green *Pipette Pump III* for pipettes up to 10 mL.
Courtesy of Bel-Art Products. www.belart.com

The device can be used with glass or plastic pipettes. The green colour *Pipette Pump 111* as shown in Plate 1.5 can be used for 1–10 mL pipettes. The device can be disassembled for cleaning.

RF 1000 Pipette Controller

Lightweight and easy to use, the *RF 1000 Pipette Controller*, shown in Plate 1.6, can be used for glass and plastic pipettes, including 1 – 10 mL. In use, the bulb is squeezed and a toggle knob is moved forward to draw fluid into the pipette and backward to dispense the fluid. The blowout bulb is used to dispel any remaining fluid.

The *Pipette Controller* polypropylene nozzle is autoclavable. Replacement parts are available, including the silicone pipette adapter, nose cone, and pack of 5 filters (uses readily available 0.45 µm Whatman filters). It is manufactured by Heathrow Scientific and stocked by many international distributors.



Plate 1.6 *RF1000 Pipette controller*.
Courtesy of Heathrow Scientific Company.
www.heathrowscientific.com

Caution: When inserting a pipette into the end of a pipette filling device, always hold the *upper end*, not the middle or lower tapered end of the pipette. Serious injuries to the hand or wrist can occur when a long glass pipette is held lower down and snaps when it is being pushed into a device.

AUTOMATED PIPETTES (PIPETTORS)

Automated pipettes are hand-held single or multi-channel liquid handling devices. They include fixed and variable volume pipettes that are operated manually or electronically. Depending on their principle of operation, they are described as air displacement pipettes or positive displacement pipettes.

Air displacement and positive displacement pipettes

Pipettes operate by means of a piston (plunger) with liquids being transferred and dispensed by displacement.

When there is a layer of air separating the piston from the liquid, the term air displacement pipette is used. To dispel the last drop of liquid from the pipette, the plunger needs to be fully depressed, i.e. the fluid is expelled in two stages.

When the piston is in direct contact with the liquid the pipette is referred to as a direct, or positive, displacement pipette and special glass or plastic capillaries are used instead of plastic tips.

Both air and positive displacement pipettes are manufactured to high standards of accuracy and precision. The use of positive displacement pipettes is recommended when measuring particularly viscous fluids and when high levels of accuracy and reproducibility are required.

For routine district laboratory work air displacement pipettes are adequate and generally more available.

Fixed volume pipettes

A fixed volume pipette measures a single volume of liquid. It is more convenient and less complicated to use than a variable volume pipette and costs less. Fixed volume pipettes are usually available in the following sizes:

10 µL	200 µL
20 µL	250 µL
50 µL	500 µL
100 µL	1000 µL

Note: Different pipette tips will usually be required for 10–200 µL pipettes and 250–1000 µL pipettes depending on manufacturer.



Plate 1.7 Pipetting safely using an automatic pipette.
Courtesy Amref International University, laboratory unit.

Variable volume pipettes

A variable volume pipette as shown in Plate 1.8, is calibrated to measure and dispense μL volumes over a set range, usually digitally although electronic devices are also available. Changing from one volume to another requires care. A thorough knowledge is required of μL measurements and for some pipettes, also how vernier scales work.

The range of variable volume pipettes usually includes the following:

20–100 μL	1000–5000 μL
40–200 μL	2000–10 000 μL
200–1000 μL	

Note: Always use the tips recommended by the manufacturer for a particular pipette.



Plate 1.8 Variable volume pipette.
Courtesy of Rainin Mettler Toledo. www.mt.com

How to use an air displacement pipette

Pipetting aqueous solutions using normal forward pipetting:

- Fill and empty the tip twice with the liquid. Press the plunger to the first stop.
- With the pipette held vertically and the tip about 5 mm below the surface of the liquid, release the plunger smoothly and slowly to aspirate the fluid.
- To dispense, touch the tip at an angle against the side of the receiving vessel, press to the first stop, wait a few seconds, then press the plunger to the second stop to expel all the fluid.

Pipetting plasma, high viscosity fluids and very small volumes, using reverse pipetting:

- Fill and empty the tip twice with the liquid. Press the plunger to the second stop.
- With the pipette held vertically and the tip about 10 mm below the surface of the liquid, release the plunger slowly and smoothly to fill the tip. Wipe the outside of the tip.
- With the tip against the side of the receiving vessel, press the plunger to the first stop to dispense the required volume. Do not press to the second stop (the liquid remaining in the tip should be discarded).

Diluting whole blood using repetitive pipetting.

- Fill and empty the tip twice with the blood. Press the plunger to the first stop.
- With the pipette held vertically and the tip well below the surface of the blood, release the plunger smoothly and slowly to aspirate the blood.
- Remove the pipette and wipe carefully the outside of the tip. Dip the tip into the diluent well below the surface. Press the plunger slowly and smoothly several times to fill and empty the tip until all the blood is expelled. Depress the plunger to the second stop to empty the tip completely.

Important

- Always hold the pipette in an upright position when aspirating.
- Always read carefully the manufacturer's instruction regarding the use and care of a pipette.
- When not in use, always store a pipette upright in a stand. Never leave a pipette on its side with tip attached containing fluid.
- Keep the pipette clean, particularly the nozzle.
- Every few months, depending on usage, arrange for the pipette to be checked for accuracy and precision. Re-calibration of a pipette requires the use of an analytical balance of readability 0.0001 g (not normally found in a district laboratory) and specialist training.
- Do not use a tip unless it forms a complete seal with the pipette.

Reuse of plastic tips

Most manufacturers of pipettes will not recommend the reuse of plastic tips. In some situations, however, tips may need to be decontaminated, washed, and reused. Most plastic tips are made from polypropylene and are therefore durable and autoclavable. To reuse, the tips must be cleaned well to remove all traces of protein and disinfectant, rinsed well, and dried completely. Only reuse a tip when it fits the pipette perfectly.

REPEAT DISPENSING USING A REPEATER PIPETTE

An example of a simple to use, accurate, manual repeater pipette is the *Finnpipette Stepper* shown in Plate 1.9. It consists of a lightweight handle into which is inserted a syringe tip. Five delivery volumes are possible for each syringe tip size, covering a volume range 10 μL to 5000 μL . The information is written on the side of the *Stepper* as follows:

Wheel position	1	2	3	4	5	Tip/mL
No. of strokes	44	22	15	11	9	
Delivery Volume in μL	10	20	30	40	50	1.25
	25	50	75	100	125	2.5
	50	100	150	200	250	5.0
	100	200	300	400	500	12.5
	250	500	750	1000	1250	25.0
	500	1000	1500	2000	2500	50.0
	1000	2000	3000	4000	5000	50.0

To obtain the required delivery volume, the appropriate syringe tip is selected and attached, and the volume selector dial adjusted. The pipette is primed by dipping the syringe tip into the fluid and depressing the filling lever to the down position and raising the lever completely several times. The required volume of fluid is dispensed by depressing the dispensing lever. The *Finnpipette Stepper* is a Thermo Fisher Scientific product.

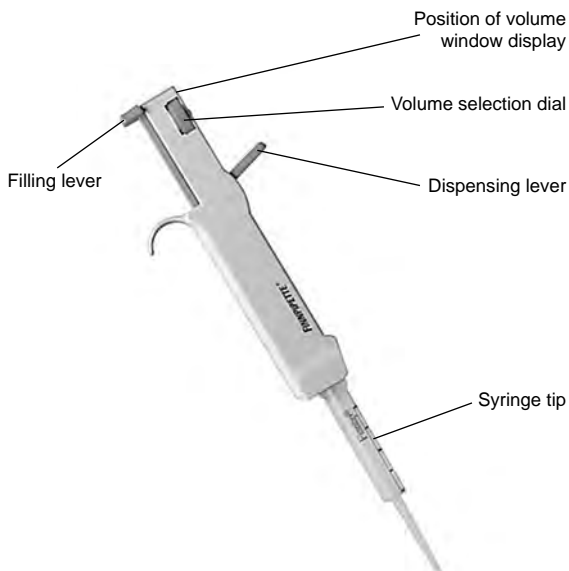


Plate 1.9 *Finnpipette Stepper* repeater pipette.
Courtesy of Thermo Fisher Scientific. www.thermofisher.com

BOTTLE TOP DISPENSERS

A wide range of bottle top dispensers is available usually covering volume ranges:

- 0.25 – 2.50 mL, in increments of 0.05 mL
- 0.50 – 5.0 mL, in increments of 0.1 mL
- 1.0 – 10 mL, in increments of 0.2 mL

The bottle dispenser shown in Plate 1.10 is the 1.0 – 10 mL *LabMax* model which is drip-free and allows air and bubbles to be eliminated when priming with no loss of reagent. Most bottle top dispensers are supplied with a range of adaptors to fit the commonly used reagent bottles.



Plate 1.10 *Labmax* 1.0–10 mL bottle top dispenser.
Courtesy of Labnet International Inc.
www.labnetinternational.com

GRADUATED PLASTIC BULB PIPETTES

Graduated plastic bulb pipettes (*Pastettes*) can be used for measuring body fluids such as urine in most basic clinical chemistry tests performed in district laboratories. The following *Pastettes* are useful:

- 1 mL *Pastette* graduated at 0.5 mL and 1 mL.
- 3 mL *Pastette* graduated from 0.5–3.0 mL in 0.5 mL divisions.

Ungraduated *Pastettes* of varying length are also available and are useful for transferring serum and other fluids from tubes and sample containers. *Pastettes* are manufactured from polyethylene and cannot therefore be heat sterilized. Examples of plastic bulb pipettes are shown in Fig. 1.15 overpage.

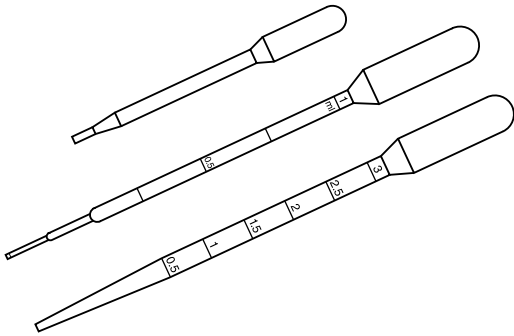


Fig. 1.15 Examples of useful graduated and non-graduated plastic (polyethylene) bulb pipettes (*Pastettes*).

Glass Pasteur Pipettes

These are needed when a sterile Pasteur pipette is required. Glass Pasteur pipettes can be made in the laboratory or bought ready-made. *Volac* glass Pasteur pipettes are recommended because they are made with a slightly heavier wall thickness to reduce breakages in use and in transit.

REFERENCES

- 1 *Laboratory Biosafety Manual*, 4th ed, 2020 World Health Organization. Access by entering the title in Google.
- 2 *Safe management of wastes from health-care activities*. WHO, 3rd ed, 2014. Can be accessed by entering the title in Google.
- 3 *Preparing and measuring high chlorine concentration solutions for disinfection*. USAPHC Technical Information Paper No. 13-034-1114, 2014. To access electronically, enter the title in Google.

FURTHER READING

Laboratory Biosafety Manual, see Reference 1.

Tuberculosis Laboratory Biosafety Manual, WHO, 2012. Access by entering the title in Google.

First Aid Manual St John Ambulance, St Andrew's First Aid, British Red Cross, 11th ed, 2021. Published by Dorling Kindersley, ISBN 978-0-2414-4630-0. Copies from St John Ambulance www.sja.org.uk

Sadia Shakoore *et al.* Barriers to implementation of optimal laboratory biosafety practices in Pakistan. *Health Security*, 2016, Aug 1;14(4):214–219. Access by entering the title in Google.

1.5 Equipping district laboratories

Equipping district laboratories with equipment that is fit for purpose is essential to strengthening district laboratory services. It requires equipment harmonization and standardization.¹

Harmonization and standardization enable equipment to be selected and validated for each level of the laboratory network. It involves collaboration of laboratory personnel from each tier of the network, clinical staff, programme planners, procurement officers and biomedical engineers with expertise in local power supplies. The *Harmonization and standardization team* can help to ensure:

- Equipment is procured using standardized policies and detailed specifications, avoiding whenever possible procuring from a single supplier which may result in monopolization, price rises and possible risk of equipment spares and consumables becoming unavailable.
- Equipment specifications are reviewed regularly and equipment updated when more effective technologies become available or there is a change in healthcare needs, and/or workload.
- Equipment manufacturers are encouraged to increase local representation, provide reliable after sales support and develop battery operated equipment and open system analyzers*.

***Open system analyzers** are able to use consumables from several sources providing the user validates the use of the non-proprietary products and checks that they do not invalidate the equipment warranty. Closed system analyzers, mostly haematology analyzers, can only be used with reagents supplied by the manufacturer, often available from a single source and therefore more expensive and difficult to obtain.

Equipment selection

Selecting equipment requires consideration of the following:

- healthcare needs and priorities,
- affordability,
- availability and reliability of power supplies,
- staff experience and skills,
- complexity of equipment and clarity of *User Manuals*,

- consumables required and supply chain,
- on-site training, after sales support and servicing,
- safety considerations,
- climatic considerations,
- suitability of interfacing with laboratory information systems.

The following also need to be considered when selecting and validating equipment for district laboratories:

- electricity supplies,
- printer interface,
- microprocessor operation.

Electricity supplies

At district level, national grid power supplies are often unreliable or unavailable. Electrical equipment may need to operate from batteries, rechargeable from a generator or photovoltaic system (see pages 46–47). Equipment with low power consumption will be required.

Printer interface

Whenever possible select analyzer equipment that has print-out facilities to avoid transcription errors. Most equipment with a printer interface, e.g. haematology and clinical chemistry analyzers can also be interfaced with computerized laboratory information systems (LIS).

Microprocessor operation

Many items of laboratory equipment are microprocessor controlled with the user being able to programme different functions (operating modes), e.g. balances, autoclaves, colorimeters, centrifuges, water baths, incubators, heat blocks and mixers. Such microprocessor-based digital display equipment is usually more accurate, versatile, efficient, and has more built-in safety features than analogue electronic equipment.

Some microprocessor controlled equipment, however, can be expensive and complex to use, necessitating a longer training to use it correctly. It can also be more difficult and expensive to repair in the field. For some equipment, manufacturers make available videos on the internet, demonstrating equipment assembly and operation.

Note: Guidelines on the procurement of laboratory equipment can be found in the 2017 WHO publication, *Guidance for procurement of in vitro diagnostics and related laboratory items and equipment*.²

Benefits of equipment harmonization and standardization

These include:

- Correct equipment selected for each tier of the laboratory network.
- Cost savings from negotiating better prices with suppliers for:
 - purchasing the same equipment for several laboratories,
 - bulk-purchasing of consumables,
 - purchasing maintenance and service contracts.
- Less complex and more efficient supply chain with fewer different products to order, check, and maintain stock levels. Also, improved forecasting of supply needs.
- Ability to share consumables between laboratories having the same equipment.
- Equipment installation and staff training are facilitated.
- Equipment can be shared between laboratories, of value when equipment is being repaired.
- Test results can be compared and interpreted between laboratories. This is particularly important when monitoring patient treatment.
- The results of EQA can be more easily compared when test procedures and equipment are standardized. Staff can share experiences of using equipment and trouble-shooting.

Keeping equipment in working order

While equipment design faults and the inappropriate purchasing of equipment can lead to premature equipment failure, the commonest causes for equipment malfunctioning in district laboratories are:

- incorrect use of equipment,
- no one trained to maintain equipment and implement equipment standard operating procedures and service contracts,
- lack of the correct replacement parts,
- no regular cleaning, inspection, and maintenance of the equipment,
- untrained personnel attempting unsuccessfully to correct a fault or replace a component,
- damage to electrical equipment caused by unstable power supplies, e.g. surge of current when power is restored after a power failure, or damage caused by lightning during storms (see following text).

Up to 70–90% of equipment breakdowns are caused by users of the equipment. Non-functioning equipment represents not just a loss of important laboratory services to patients, but also a waste of scarce health resources. Incorrect use can incur high equipment repair costs.

Important: Damage to equipment caused by incorrect use and poor maintenance can be avoided by adequate training and following equipment Standard Operating Procedures (SOPs).

Damage to equipment from voltage fluctuations

Even when a laboratory is served by AC mains electricity, the supply may be intermittent and subject to voltage fluctuations. Significant and sudden increases in voltage (spikes and surges), e.g. when mains supplies are restored following a power failure (outage), can damage equipment that is not voltage protected. When an outage occurs, turn off and disconnect electrical equipment and wait a few minutes after the power returns before reusing the equipment.

Significant reductions in voltage (sags or brown-outs) e.g. when the electrical system becomes overloaded, can affect the performance of equipment such as centrifuges, colorimeters, spectrophotometers, electrophoresis equipment, and pH meters. Some microprocessor controlled equipment are programmed to cut out when voltage fluctuations become greater than the built in voltage tolerance, e.g. $\pm 10\%$.

Damage to equipment by lightning

Lightning may damage electrical equipment and also injure the operator. Whenever there is an electrical storm, electrical equipment (except fridges and incubators) should not be used and electrical plugs should be disconnected from wall sockets (and kept 30 cm from the sockets).

VOLTAGE PROTECTION DEVICES

When mains AC electricity supplies are unreliable, electrical equipment can be protected using a voltage protection device such as:

- A voltage protection unit which cuts off the power supply to the equipment when the voltage is significantly increased (usually over 260 V) or decreased (usually below 190 V). Power is restored to the equipment when the voltage returns to acceptable levels. The equipment plugs into the voltage protection unit which is

inserted in the wall socket. Such a device is the least expensive way of protecting equipment from damage due to major voltage fluctuations.

- A voltage stabiliser (surge suppressor plus filter) which regulates the voltage supply so that equipment is not damaged particularly by voltage spikes and surges. Although more expensive than a voltage protection unit, a voltage stabiliser is recommended when mains electricity supplies are subject to frequent major changes in voltage. The cost of a stabiliser will depend on its output power, i.e. volt ampere (VA) rating.
- An uninterruptible power supply (UPS) on line unit, which in addition to regulating the voltage output in a similar way to a voltage stabiliser, also provides power for a limited period (from a battery) when a mains power failure occurs. This may be sufficient time to complete tests, e.g. microscopy, colorimetric tests, or centrifuging samples. Like a voltage stabiliser, a UPS is costed by its VA rating.

Important: Always consult a qualified electrician or biomedical equipment engineer regarding power supply problems.

USE OF SOLAR ENERGY TO PROVIDE ELECTRICITY SUPPLIES

In recent years the cost of photovoltaic technologies has fallen and their performance improved, enabling more solar systems to be installed and used to improve grid electricity supplies, and provide off-grid electricity to district hospitals and health clinics, particularly where fuel for generators has become less available or unaffordable. Unlike generators, solar systems are quiet, do not produce harmful emissions, and their running and maintenance costs are lower over time. The components of a solar system are:

- Photovoltaic panel(s) made from silicon which uses light energy (photons) from the sun to generate DC electricity. Each panel is covered with a transparent waterproof material and mounted in a frame. It is positioned in a sunny location at a tilt and angle correct for the site's latitude. The panels require regular cleaning with a wet cloth to prevent any build-up of debris, particularly in dusty environments.
- Deep cycle 12V lead-acid battery to store the electricity and provide it when required.

Important: Avoid using shallow discharge 12V lead-acid vehicle (automotive) batteries that have thin plates. They are not designed for stationary use but for supplying a heavy current for a short period followed by immediate recharge from a running vehicle. If such a battery has to be used, no more than 20% of its charge should be removed. It should be charged as soon as possible after discharge, and not overcharged. Such batteries will require replacing about every 2 years.

Sealed and low maintenance 12V lead-acid batteries

Sealed batteries are commonly available for use in vehicles. Deep cycle sealed gel (captive electrolyte) and absorptive electrolyte type batteries are also available but expensive. Sealed batteries do not leak or spill and require no topping up of electrolyte.

Compared with vented 12V lead-acid batteries, sealed 12V batteries have a shorter life and their discharge and charge cycle require more careful control. A special regulated charger matched to the battery must be used to avoid damage from overcharging. Self-discharge will be rapid in temperatures over 40 °C.

What are referred to as low maintenance 12V lead-acid batteries are also available which require little or no maintenance if discharged and charged correctly. Unlike a sealed battery, the vent caps can be removed to check the level and density of the electrolyte.

- Charge controller which regulates the voltage and current from the solar panel to the battery to prevent over charging or over discharging, thereby prolonging the life of the battery. The correct size of charge controller to use is determined by the amps produced by the photovoltaic panel.
- Inverter which converts the DC current produced to AC current which can be used to power AC operating equipment. Equipment should be turned off when not in use. Inverters like batteries are affected by high temperatures and humidity which reduce their life-span.

Important: An experienced solar energy supplier is required to install a solar powered system based on location and energy requirements.

Note: Further information on the different technologies used to produce electricity supplies to power laboratory equipment in resource-limited countries can be found in *A review of sustainable energy access and technologies for healthcare facilities in the Global South*, Franco A et al, Elsevier, 2017.
<https://doi.org/10.1016/j.seta.2017.02.022>

Safe use and maintenance of a 12V lead-acid battery

- Connect the leads to a battery correctly (positive lead to the positive (+) terminal, negative lead to the negative (-) terminal).
- Whenever possible use secure clamped battery connections.
- Before disconnecting a battery, turn off the equipment the battery is powering.
- When disconnecting, always remove one lead at a time and DO NOT ALLOW the positive and negative leads or connections to touch. *Touching will cause a dangerous shorting between the terminals.*
- When in use, make sure the lead from one battery terminal does not lie across the opposite terminal as this will cause the lead to burn.

Shorting between battery terminals.

When an accidental connection is made between the positive and negative terminals of a battery, a path of low resistance is created through which a dangerously high current of electricity can flow which can cause burning, fire, and even explosion of the battery.

Caution: As a safety precaution, before touching a battery, always remove any metal objects from the hands, wrists, and neck such as watch straps or jewellery. Also remove metal spatulas and scissors etc, that may fall out of pockets. If using a tool, make sure it has an insulated handle. Never leave metal objects on top of a battery.

Maintenance

- To prevent tracking of electricity on the surface of a battery between terminals, keep the top of the battery dry and dirt-free. Tracking can be dangerous and will increase the self-discharge of a battery.
- To prevent corrosion of the battery terminals and connections, lightly coat the metal surfaces with petroleum grease, e.g. vaseline (do not use oil). If corrosion forms, clean it off using a weak solution of sodium bicarbonate, i.e. about 5 g dissolved in 50 mL of water. Wear rubber gloves and eye protection. Wash off the neutralized acid with water, dry, and apply vaseline to the metal surfaces.
- Inspect regularly (at least once a week) the level of electrolyte in each cell of the battery. The correct level is the top of the level indicator (normally marked). For most 12V DC vented

batteries the electrolyte level should be 9–12 mm above the plates but follow the manufacturer's instructions.

- If the level of the electrolyte is below that recommended, top it up using deionized or distilled water (from a non-metallic container) or special "battery water" available from most garages. Do not overfill as this will cause the electrolyte to overflow during charging. Topping up should be carried out when the battery is fully charged.

Important: Never use tap water to top up a battery as this will contain impurities that will alter the electrolyte and seriously reduce the efficiency and life of the battery. If the level of electrolyte is consistently low, as shown by excessive gassing (bubbling), this indicates that the battery is being overcharged.

DONATED EQUIPMENT

While much laboratory equipment is successfully donated, inappropriate equipment can be donated when there is little communication between donor and recipient. It is important to establish exactly what is required and to ensure that the equipment supplied is medically, technically, economically, and environmentally appropriate and can be operated safely. Technical documents, including the *User Manual* must be supplied with donated equipment.

Full specifications of the equipment must be e-mailed in advance to the recipient including:

- power supply and other infrastructure requirements, e.g. quality of water supply,
- installation, operation, maintenance requirements,
- recommended replacement parts, and any consumables required.*

* Equipment should not be accepted when there is no national supplier of replacement parts or consumables and no person/manufacturer to contact for installation and user training.

Importation of donated equipment: It is important for the recipient to check in advance, import regulations and charges, documentation and procedure required to import and clear the donated equipment through customs.

Standardization may be difficult to achieve when equipment is donated. Donated products should be refused if they do not conform to existing national harmonization and standardization policies.²

WHO guidelines regarding equipment donations⁴

If a donation is to go ahead, the donors should send the equipment specifications to the laboratory ahead of delivery to ensure the necessary physical infrastructure is present.

Donated equipment and reagents should have at least 50% of their useful life remaining at the time of donation. Donated equipment should follow the usual procurement process to ensure adequate reagents and supplies are procured and installation, maintenance (preventive and corrective), and training are also procured.

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Section 2 Laboratory investigation of communicable diseases

- | | |
|---------------------------------------|--|
| 2.1 Malaria | 2.17 Amoebic dysentery, giardiasis, <i>Cryptosporidium</i> , <i>Cyclospora</i> , <i>Cystoisospora</i> infections |
| 2.2–2.10 Neglected tropical diseases* | 2.18 Healthcare associated infections |
| 2.11 Tuberculosis | 2.19 Acute febrile illness
Measuring CRP and PCT |
| 2.12 Meningitis | 2.20 Measuring whole blood lactate |
| 2.13 Sexually transmitted infections | |
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| 2.15 Hepatitis | |
| 2.16 WASH-related infections | |

Neglected tropical diseases (NTDs) are a diverse group of 20 diseases and disease groups* that disproportionately affect people living in poverty, predominantly in tropical and subtropical countries¹.

* *Buruli ulcer, Chagas' disease, dengue and chikungunya, dracunculiasis, echinococcosis, foodborne trematodiasis, human African trypanosomiasis, leishmaniasis, leprosy, lymphatic filariasis, mycetoma, chromoblastomycosis and other deep mycoses, onchocerciasis, rabies, scabies and other ectoparasites, schistosomiasis, soil-transmitted helminthiasis, snakebite envenoming, taeniasis/cysticercosis, trachoma, yaws.*

Many NTDs are vector-borne, have animal reservoirs and are associated with complex life cycles making public health control challenging¹. Action against NTDs is essential to achieve universal health coverage.

NTD 2021–2030 road map²

In 2020 WHO published its NTD 2021–2030 road map highlighting the need for an integrated approach:

- to achieve better health outcomes, greater cost efficiency and effectiveness and better programme management,
- to promote the development of new tools for disease prevention, diagnosis and treatment.

The NTD 2021–2030 road map is a blueprint for preventing, controlling and where feasible eliminating and eradicating NTDs.

NTDs and the COVID-19 pandemic

In April 2020 WHO recommended postponing NTD activities to help control the transmission and spread of COVID-19³. Community healthcare workers were unable to carry out MDA for schistosomiasis, lymphatic filariasis, onchocerciasis and soil-transmitted helminth infections. COVID-19 restrictions also disrupted NTD diagnosis, treatment, routine surveillance, epidemiological surveys, vector

control and rehabilitation services. The transport and distribution of NTD medicines and other essential supplies were also affected. Financial resources were redirected to COVID-19 activities.

For some NTDs the COVID-19 pandemic has increased morbidity and mortality and delayed previously set goals of eradication and elimination of transmission. The restarting of NTD services since July 2020 has been gradual. Resuming NTD activities is essential to achieving the targets of the 2021–2030 NTD road map.

Antimicrobial resistance

Antimicrobial resistance (AMR) is a major factor in the spread of community-acquired infections and the re-emergence of diseases previously under control. The misuse and overuse of antimicrobials in humans and animals combined with a lack of effective prevention and control measures, are accelerating AMR.

Commonly available and affordable antimicrobials previously used to treat and prevent infections

such as urinary tract infections, sexually transmitted infections, sepsis, pneumonia, and tuberculosis are becoming less effective. For some multi-resistant pathogens, currently available antimicrobials have become ineffective.

Patients with infections caused by drug-resistant pathogens are at greater risk of prolonged and often severe illness, disability and death.⁴ AMR increases healthcare costs due to patients requiring longer care, extra investigations and more expensive and often less available drugs.

An increasing number of district laboratories are being supported and staff trained to perform microbial identification and antimicrobial susceptibility testing (AST) in response to:

- An increase in AMR, requiring:
 - guidance in the use of antimicrobials in patient care,
 - AMR surveillance to monitor antimicrobial use and the emergence of drug-resistant pathogens.
- The need for clinicians and health authorities to be better prepared to:
 - respond and contain disease outbreaks rapidly,⁵
 - manage infectious diseases caused by climate change, conflict and/or natural disasters,⁶
 - prevent and control healthcare associated infections more effectively.
 - identify and treat opportunistic infections in patients with HIV infection.
- The need to differentiate bacterial and non-bacterial causes of fever in patients testing negative for malaria.

Using biomarkers to assess antimicrobial treatment

In the absence of bacterial culture and antimicrobial susceptibility testing facilities, measuring biomarkers of systemic inflammation can be helpful in deciding whether to prescribe antimicrobials to treat suspected bacterial infections. Biomarkers that have been found to be particularly useful at point-of-care are C-reactive protein (CRP) and procalcitonin (PCT).⁷

With clinical findings, CRP and PCT levels can help to differentiate bacterial infections requiring antimicrobial treatment from viral and other non-bacterial causes of acute febrile illness for

which the use of antimicrobials is inappropriate. Levels of CRP and PCT rise in response to bacterial infection and inflammation and can also help in monitoring response to treatment. In most viral infections, CRP and PCT levels are normal or only slightly raised.

Section 2.19 describes point-of-care testing of CRP and PCT in the diagnosis and management of sepsis, respiratory infections and non-malaria fevers and in differentiating bacterial from viral meningitis (see pages 223–227).

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The Weekly Epidemiological Record (WER), provides information on disease outbreaks and emerging or re-emerging infections.

2.1 Malaria

In 2020 WHO estimated there were 241 million cases of malaria worldwide with an estimated 627 000 deaths. In 2021 the highest number of deaths from malaria for nearly a decade was reported mainly due to the COVID-19 pandemic disrupting essential malaria supplies, diagnosis and treatment.

Human *Plasmodium* species

Four human *Plasmodium* species cause malaria:

Plasmodium falciparum *Plasmodium malariae*
Plasmodium vivax *Plasmodium ovale*

Subdivision of *Plasmodium*

The genus *Plasmodium* is subdivided into the subgenus *Laverania* and the subgenus of the same name, i.e. *Plasmodium*. *P. falciparum* belongs to the subgenus *Laverania* and *P. vivax*, *P. ovale* and *P. malariae* belong to the subgenus *Plasmodium*.

Plasmodium falciparum distribution

Variants of *P. falciparum*

The species *P. falciparum* contains several variants which show differences in geographical distribution, vector susceptibility, human infection pattern, drug susceptibility, morphology and antigenic composition.

P. falciparum is found in the hotter and more humid regions of the world accounting for up to 80% of malaria worldwide. It is the main species found in tropical and subtropical Africa and parts of Central America and South America, Bangladesh, Pakistan, Afghanistan, Nepal, Sri Lanka, South East Asia, Indonesia, Philippines, Haiti, Solomon Islands, Papua New Guinea and many islands in Melanesia.

Plasmodium vivax distribution

P. vivax strains

Strains of *P. vivax* show differences in distribution, incubation time, relapsing pattern, morphology, number of parasites in red cells and response to antimalarials.

P. vivax is capable of developing in mosquitoes at lower temperatures than *P. falciparum* and therefore has a wider distribution in temperate and sub-tropical areas. It is the main *Plasmodium* species in South America, Mexico, the Middle East, Asia, and the Western Pacific. In Angola, Sudan, Ethiopia, Eritrea, Equatorial New Guinea and other African countries, *P. vivax* infections are being reported

in persons lacking the Duffy antigen suggesting *P. vivax* has evolved alternative red cell invasion pathways.

Plasmodium malariae distribution

P. malariae and *P. brasilianum*

In South America, *P. malariae* has been found to be genetically identical to *P. brasilianum* in monkeys and morphologically almost indistinguishable.

P. malariae has a lower prevalence than *P. falciparum* and *P. vivax*. It is found in Africa, South America and Asia. In tropical Africa it accounts for up to 25% of *Plasmodium* infections.

Plasmodium ovale distribution

Subspecies of *P. ovale*

There are two genetically distinct subspecies of *P. ovale*: *P. ovale wallikeri* and *P. ovale curtisi*. Morphologically the subspecies are identical. *P. ovale wallikeri* is reported as having a shorter period between infection and the onset of disease.

P. ovale wallikeri and *P. ovale curtisi* have a similar distribution being found in West Africa where they account for up to 10% of malaria infections. Low prevalence rates are found in other parts of Africa, the Philippines, Indonesia, Cambodia, Thailand, Vietnam, Myanmar, Papua New Guinea, and parts of the Far East, India, Bangladesh and South America.

Zoonotic *Plasmodium* species

The following *Plasmodium* species that infect monkeys have been reported as causing malaria in humans:

Plasmodium knowlesi
Plasmodium simium

Zoonotic malaria

Molecular testing has made it possible to detect the emergence and extent of zoonotic malaria. Microscopically *P. knowlesi* is difficult to distinguish from *P. malariae* and *P. simium* from *P. vivax*. Accurate identification of species is essential for the correct treatment of patients, effective malaria control and future eradication.

Other *Plasmodium* species infecting monkeys that have been reported as causing malaria in humans include *P. cynomolgi* (morphologically indistinguishable from *P. vivax*).

Factors contributing to humans becoming infected with malaria species that infect monkeys, include humans increasingly working and living in or near to forest monkey habitats and the presence of mosquito vectors able to feed on both monkeys and humans.

Plasmodium knowlesi distribution

P. knowlesi is the predominant cause of malaria in South East Asia with macaques being the reservoir

hosts. Countries reporting *P. knowlesi* infections include Borneo, Malaysia, Cambodia, China, Indonesia, Lao PDR, Myanmar, the Philippines, Singapore, Thailand and Vietnam, in areas where mosquito vectors that feed on both humans and macaques.

Plasmodium simium distribution

P. simium has a limited distribution being found in the Atlantic Forest region of south east Brazil with natural hosts including howler, woolly spider and capuchin monkeys.

Transmission and life cycle of malaria parasites

Malaria parasites are transmitted by the bite of an infected female *Anopheles* mosquito. Sporozoites contained in the saliva of the mosquito are inoculated into the blood of a human host when the mosquito takes a blood meal. Infection can also occur by transfusion of infected donor blood, by injection through the use of needles and syringes contaminated with infected blood, and very occasionally congenitally, usually when a mother is non-immune.

The life cycle of malaria parasites is summarized in Fig. 2.1. It involves:

- Asexual reproduction in a human host, i.e. liver cell schizogony and repeated red cell schizogony cycles and the formation of gametocytes (gametogony).
- Sexual multiplication (sporogony) in the anopheline mosquito in which male and female gametes fuse to form zygotes which develop into oocysts. Each oocyst produces many sporozoites. The sporozoites are found in the salivary glands of the mosquito and are infective to humans.

Development in humans

Following inoculation, the sporozoites rapidly (within 8 h) leave the blood and enter liver cells. Within 5–15 days (depending on species) they develop into liver schizonts and are referred to as pre-erythrocytic (PE) schizonts. Mature PE schizonts contain many merozoites.

Hypnozoites: Some of the sporozoites of *P. vivax* and *P. ovale* after invading liver cells delay their development into PE schizonts. They become dormant forms called hypnozoites, becoming active and developing into PE schizonts only at a later date, causing relapses.

When mature, a PE schizont ruptures from the liver cell, releasing its merozoites into the blood circulation. The merozoites infect red cells (binding to receptors on the red cell membrane). A proportion are

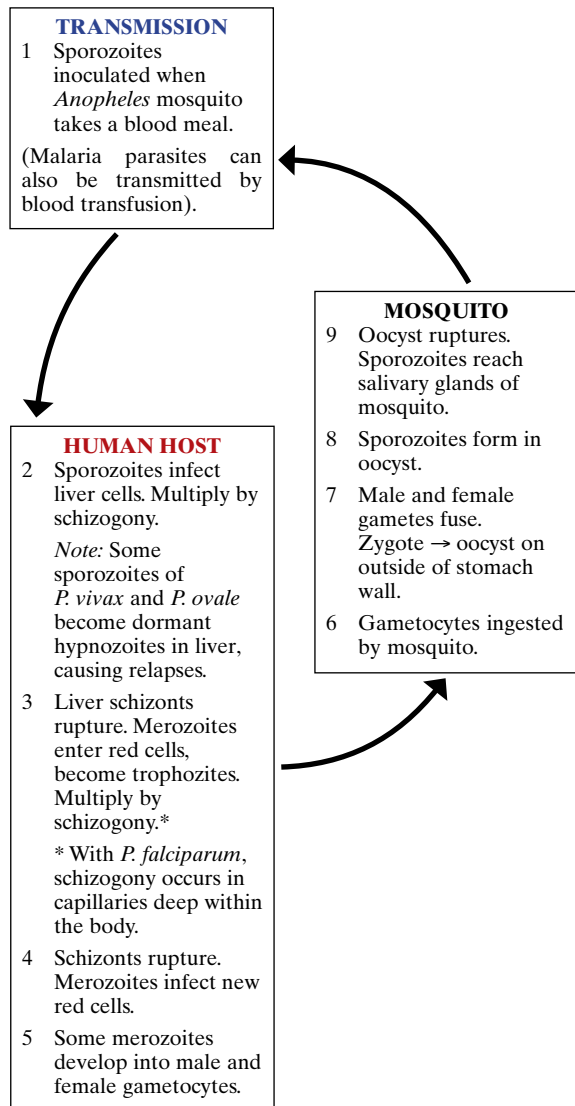


Fig 2.1 Transmission and life cycle of malaria parasites.

phagocytosed and destroyed. Entry of the parasites into red cells starts a cycle of schizogony in the blood which to complete takes 24 hours for *P. knowlesi*, 48 hours for *P. falciparum*, *P. vivax* and *P. ovale* and 72 hours for *P. malariae*. During this time the intracellular merozoites develop into trophozoites ('ring forms') which feed on the contents of the red cells.

As the trophozoite feeds, malaria pigment (haemozoin) is produced as an end product of haemoglobin breakdown. This accumulates in the trophozoite (appearing in blood films as brown-black granules). When the trophozoite is fully developed, the nucleus begins to divide, resulting in the

formation of a schizont containing 8–24 merozoites (depending on species). The mature schizont ruptures from its red cell releasing merozoites, malaria pigment, and toxins into the plasma (cause of a typical malaria attack).

Cytoadherence (sequestration) of *P. falciparum*: As *P. falciparum* trophozoites mature, antigens form on the surface of infected red cells which adhere to receptors on endothelial cells lining capillaries in various organs and tissues of the body. Falciparum trophozoites therefore complete their development into schizonts in the capillaries of deep tissues, not in the circulating blood in contrast to *P. vivax*, *P. ovale*, and *P. malariae*.

Merozoites released from schizonts enter the blood circulation and those which are not destroyed by the host's immune system infect new red cells, beginning a further cycle of schizogony with more red cells being destroyed. After several erythrocytic schizogony cycles, some of the merozoites entering red cells develop into male and female gametocytes. For the life cycle to be continued, the gametocytes must be ingested by a female *Anopheles* mosquito in a blood meal (males do not feed on blood). If they are not taken up by a mosquito they die.

Endemicity of malaria

The intensity of malaria transmission is described from the lowest level to the highest as hypoendemic (lowest), mesoendemic, hyperendemic, holoendemic (highest).

Hypoendemic and mesoendemic malaria are found in areas of unstable transmission with low malaria transmission, affording little protective immunity to communities. Malaria outbreaks can occur following changes in conditions which favour transmission such as mosquito numbers increasing following heavy rains.

Holoendemic and hyperendemic malaria are found in areas of stable transmission with little change in intensity throughout the year. This affords protective immunity with severe malaria attacks decreasing with increasing age. Most malaria deaths occur in young children.

CLINICAL FEATURES AND PATHOLOGY OF MALARIA

The characteristic feature of malaria is fever caused by the release of toxins (when erythrocytic schizonts rupture) which stimulate the secretion of cytokines from leucocytes and other cells. In the early stages of infection the fever is irregular or continuous. As schizogony cycles synchronize, fever begins to recur at regular intervals particularly in quartan malaria (every 72 h), vivax and ovale malaria (every 48 h) and knowlesi malaria (every 24 h).

Splenomegaly occurs in all forms of malaria with repeated attacks causing a greatly enlarged spleen. Infections can be uncomplicated or severe.

Uncomplicated malaria

The symptoms of uncomplicated malaria are non-specific. They include fever, chills, sweats, headache, nausea and vomiting, body aches and general malaise. There is no damage to vital organs.

Severe malaria

Severe malaria is a serious life-threatening condition with complications which include cerebral malaria, severe anaemia, high levels of parasitaemia, haemoglobinuria, acute kidney injury, hypoglycaemia, metabolic acidosis, acute pulmonary oedema and blood coagulation abnormalities.

Malaria caused by *P. falciparum*

P. falciparum is the most pathogenic of the human malaria species with untreated infections causing severe disease and death, particularly in infants, young children, pregnant women and non-immune adults. The pathogenicity of *P. falciparum* is mainly due to:

- The cytoadherence of falciparum parasitized red cells (see previous text). Sequestration of parasitized cells in the microcirculation causes congestion, hypoxia, blockage and rupturing of small blood vessels.
- High levels of parasitaemia resulting in the activation of cytokines and the destruction of many red cells. Up to 30–40% of red cells may become parasitized.

Severe falciparum malaria is associated with cerebral malaria, haemoglobinuria, severe anaemia, parasitaemia >10%, jaundice, kidney damage, bleeding, acidosis, hypoglycaemia, pulmonary oedema, shock, and complications in pregnancy.

Cerebral malaria

This is the commonest cause of coma and death in falciparum malaria. Many parasitized cells can be found in the capillaries of the brain and other organs and in the late stages, haemorrhaging from small blood vessels can occur with convulsions.

Malaria haemoglobinuria

There is rapid and massive intravascular haemolysis of both parasitized and non-parasitized red cells (including transfused cells), resulting in haemoglobinaemia, haemoglobinuria, and fall in haemoglobin. The parasites are difficult to find in the blood. The urine appears dark red to brown-black due to the presence of free haemoglobin.

Anaemia and acidosis

Anaemia can be severe and occur rapidly, particularly in young children (≤ 5 g/L). It is due mainly to the destruction of parasitized red cells. Parasitized cells lose their deformability and are rapidly phagocytosed and destroyed in the spleen. The production of red cells in the bone marrow is also reduced. Immune destruction of non-parasitized red cells also

occurs. In young children, tissue anoxia can develop, leading to acidosis with respiratory distress.

Hypoglycaemia (blood glucose < 2.2 mmol/L)

This is a common finding particularly in children and pregnant women with severe falciparum malaria. Glucose levels require monitoring.

Falciparum malaria in pregnancy

Normal immune responses are reduced during pregnancy. In areas of stable malaria transmission, a pregnant woman will have acquired partial immunity to malaria. This will protect her against serious clinical falciparum malaria but not prevent heavy parasitic infection of the placenta and anaemia (often severe) which can result in a low birth weight baby which may not survive. First pregnancies are at greatest risk.

In areas of unstable malaria transmission, pregnant women lack protective immunity and are at serious risk of developing severe life-threatening falciparum malaria, particularly in the last few months of pregnancy and for several weeks following delivery. Untreated infections can result in abortion, still-birth, premature labour or low birth weight. Cerebral malaria, pulmonary oedema, and hypoglycaemia frequently occur. The situation is similar in all pregnancies.

Malaria and HIV

In untreated HIV with immunosuppression, malaria may be more frequent and severe and patients may respond less well to treatment. In areas of stable endemic malaria, HIV infected patients who are partially immune may have more frequent higher-density infections. In areas of unstable transmission there is an increased risk of severe malaria and death¹.

Malaria caused by *P. vivax*, *P. ovale*, *P. malariae*

Infections caused by *P. vivax*, *P. ovale* or *P. malariae* are rarely life-threatening. Cytoadherence of parasitized cells does not occur and parasitic densities are lower. In vivax and ovale malaria, parasite numbers rarely exceed 50 000/μL or 2% of cells infected, and in quartan malaria (*P. malariae*) parasite numbers are usually less than 10 000/μL with only up to 1% of cells becoming infected.

P. vivax is a common cause of anaemia in infants and young children and can cause severe malaria. Increasingly *P. vivax* infections are being reported in people who are Duffy negative (see previous text).

Relapses are a feature of vivax and ovale malaria due to the delayed development of PE schizonts from hypnozoites in liver cells (see previous text).

Primaquine is used to treat vivax and ovale malaria (targets hypnozoites). Prior to treatment, patients

require screening for deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) to avoid acute haemolysis which can occur in those being treated with primaquine.

Repeated malaria attacks (often over several years) are a feature of *P. malariae* but these are not relapses caused by dormant hypnozoites but recrudescences caused by small numbers of erythrocytic forms of the parasite persisting in the host (recrudescences can also occur in falciparum malaria for up to 1 year following the first attack).

A serious complication of infection with *P. malariae* is nephrotic syndrome which may progress to renal failure. It occurs more frequently in children and is caused by damage to the kidneys following the deposition of antigen-antibody complexes on the glomerular basement membrane of the kidney. It produces oedema, marked proteinuria, and a low plasma albumin level.

Malaria caused by *P. knowlesi* and *P. simium*

P. knowlesi has a 24 h asexual cycle in the blood causing fever, chills and rigor, 9–12 days following infection. Most infections are without serious complications. Heavy parasitaemia, however can develop rapidly particularly when infections are not treated due to late or incorrect diagnosis. Because of its morphological similarity to *P. malariae*, infections with *P. knowlesi* are frequently misdiagnosed microscopically.

Severe *P. knowlesi* infections (parasite density > 100 000/μL) can be life-threatening due to acute respiratory distress syndrome, acute renal failure, liver failure with jaundice, hypotension and acidosis (coma does not occur). *P. knowlesi* malaria is non-relapsing and without recrudescences.

P. simium is closely related genetically and morphologically to *P. vivax*. Prior to the identification of *P. simium* as the cause of malaria in 2015 and 2016 in south east Brazil, most previous infections in the same area were probably misdiagnosed as *P. vivax*. Most of those reported with *P. simium* malaria had periodic tertian fever. Other clinical features and whether *P. simium* can cause relapsing malaria, are yet to be determined.

Hyperreactive malaria splenomegaly

In this rare condition there is a defective regulation of immune responses associated with recurrent *P. falciparum* infection. Those affected are immune adults in malaria endemic areas.

The condition is characterized by massive and chronic splenomegaly with high levels of IgM, malaria antibody, and circulating immune complexes, and a moderately enlarged liver with hepatic sinusoidal lymphocytosis. The patient is usually anaemic (normocytic) and has low white cell and platelet counts. Malaria parasites are rarely found in blood films.

Note: Further information on the clinical features and treatment of malaria can be found in the 2022 *WHO Guidelines for Malaria*¹.

Examples of genetic factors that protect against malaria

- *P. vivax* is rarely found in West Africa or other places where the red cells of the population lack the Duffy blood group antigens Fy^a and Fy^b. The glycoprotein receptors which *P. vivax* needs to attach to and invade red cells are missing on Duffy negative red cells. However, cases of *P. vivax* have been identified in Duffy-negative individuals, suggesting *P. vivax* has evolved alternative red cell invasion pathways.
- Persons with the haemoglobin genotype HbAS (sickle cell trait) are protected against severe falciparum malaria. Sickle cell anaemia (HbSS) is not protective.
- Persons with ovalocytosis have lower parasite densities of *P. falciparum* and *P. vivax*. Elliptical red cells resist parasitic invasion. In areas of South East Asia and Papua New Guinea where falciparum malaria is endemic there is a high prevalence of ovalocytosis.
- Newborn infants have protection against malaria in their first few months of life when there is a high concentration of HbF in their red cells. Malaria parasites do not develop well in red cells containing HbF.
- β (beta)-thalassaemia trait also appears to protect against severe falciparum infection.

Note: With genes that confer resistance to falciparum malaria but can cause death, such as HbS, there occurs what is called balanced polymorphism in which the death of homozygotes (HbSS) is offset by the survival advantage of heterozygotes (HbAS) in falciparum malaria endemic areas.

Measures to prevent and control malaria

- Sleeping under long-lasting pyrethroid insecticide treated nets (using two different insecticides) to avoid being bitten by night indoor feeding mosquito vectors.
- Screening windows and doors with mosquito netting, wearing clothing which protects against mosquito bites and when appropriate, using mosquito repellents. Non-immune persons travelling to malaria areas should seek advice on effective malaria prophylaxis.
- Carrying out indoor residual insecticide spraying and monitoring emerging insecticide resistance.

- With knowledge of local *Anopheles* vectors, preventing mosquito breeding by removing surface water, filling in ponds, draining ditches and covering water containers. When possible, spraying breeding sites and destroying adult mosquitoes as part of a control programme.
- Providing accessible malaria diagnostic testing facilities and affordable effective drugs to treat promptly active infections and prevent malaria in those at risk such as pregnant women, young children and non-immune persons travelling into malaria endemic areas for work or following population migration.
- Using intermittent malaria preventive treatment for pregnant women living in malaria endemic areas and seasonal malaria chemoprevention for infants living in areas of moderate to high transmission.
- Acting rapidly and effectively to control malaria epidemics in complex emergency situations.

WHO Global Technical Strategy for Malaria 2016–2030

The *Strategy* has the following global targets:

- Reducing malaria case incidence and malaria mortality rates by at least 90% by 2030.
- Eliminating malaria in at least 35 countries by 2030.
- Preventing a resurgence of malaria in all countries that are malaria-free.

Malaria vaccines

Since October 2021, WHO recommends broad use of the RTS,S/AS01 malaria vaccine among children living in regions with moderate to high *P. falciparum* malaria transmission. The vaccine has been shown to significantly reduce malaria and deadly severe malaria among young children*.

* Questions and answers on the RTS,S vaccine can be found in the WHO Malaria Fact Sheet, 2022 (see Further Reading).

Malaria updates and news

Readers are referred to the WHO malaria website: https://www.who.int/health-topics/malaria#tab=tab_1

LABORATORY DIAGNOSIS

The laboratory diagnosis of malaria is by:

- Detecting malaria parasites and identifying *Plasmodium* species microscopically in stained blood films.
- Using a rapid diagnostic test (RDT) to detect malaria specific antigens in blood samples.

In all cases of suspected malaria, WHO recommends parasitological confirmation of malaria using quality assured microscopy or rapid diagnostic testing. Treatment should only be given to those with or at high risk of severe malaria when diagnostic testing is not immediately accessible within 2 hours of patients presenting for treatment.¹

Nucleic acid amplification techniques

In specialist laboratories, techniques to detect malaria parasitic nucleic acid, e.g. polymerase chain reaction (PCR), are being used to confirm infections that are difficult to diagnose microscopically or by RDT such as *P. knowlesi* and *P. simium*. Because of their high sensitivity and specificity, nucleic acid amplification techniques are also being used in malaria eradication programmes, drug efficacy trials and to detect and identify mixed low parasitaemia infections.

WHO advises that at present, molecular diagnostic tests based on amplification techniques (e.g. loop-mediated isothermal amplification or PCR) do not have a role in the clinical management of malaria¹.

Likewise malaria antibody tests (used in epidemiological studies) are not of value in diagnosing patients with suspected malaria.

Other tests

- Measurement of haemoglobin or packed cell volume when there is malaria with heavy parasitaemia particularly in young children and pregnant women, see Section 3.1.
- Measurement of blood glucose to detect hypoglycaemia, particularly in young children and pregnant women with severe falciparum malaria. The test is described in Section 3.2.
- Total and differential white cell count and platelet count (severe malaria), see Section 3.1.
- Coagulation tests if abnormal bleeding is suspected in falciparum malaria.
- Testing urine for free haemoglobin when malaria haemoglobinuria is suspected (see Section 3.3).
- Measurement of serum creatinine (see Section 3.3) to monitor renal complications.

- Testing urine for protein when nephrotic syndrome is suspected (see Section 3.3).
- Screening for G6PD deficiency before treating a patient with an oxidant drug such as primaquine.

Blood transfusion in severe falciparum malaria

Blood transfusion may be necessary to correct severe anaemia or to replace deficient clotting factors (fresh blood required). In patients with severe malaria, there may be difficulty in determining the blood group because of autoagglutination of the person's red cells. Testing patient's serum as well as red cells is necessary.

MALARIA MICROSCOPY

Advantages of malaria microscopy

Well performed microscopy is both sensitive and specific for diagnosing malaria. It enables:

- *Plasmodium* species to be identified.
- Hyperparasitaemia to be identified.
- Parasite numbers to be quantified.
- Malaria pigment in white cells to be reported, particularly when parasites are not detected.
- Responses to treatment to be monitored.
- Gametocytes (mosquito infective forms) to be identified and reported.

Note: Examination of stained blood films can also help to suggest an alternative diagnosis when parasites are not detected (see p. 65), diagnose anaemia (see p. 243), and detect increases in white blood cell numbers (see p. 241).

Requirements for malaria microscopy

- Collect blood as soon as malaria is suspected clinically *before the patient is treated with antimalarials*. It may be necessary to collect blood on several occasions to detect the parasites.
- Process and report malaria blood films with the *minimum of delay*.
- Use standardized operating procedures for collecting blood samples, preparing, staining and reporting blood films.

- Follow safe procedures and good laboratory practice when handling blood samples and disposing of infectious material.
- Provide adequate training of staff, regular assessment of competency, supervision at all levels, good supply management, maintenance of microscopes and an adequate budget.³
- Participate in external quality assessment to improve the quality of testing, receive regular support and keep up-to-date (see pages 15–16).

PREPARING MALARIA BLOOD FILMS

Thick and thin blood films are required for the microscopical diagnosis of malaria. They are best prepared directly from capillary blood.*

*Venous blood collected into ethylenediaminetetraacetic acid (EDTA) can also be used providing the blood is not refrigerated and the blood films are made soon after collecting the blood (within 1 hour) to avoid EDTA changes which can make the parasites difficult to identify. Thick blood films made from venous blood are more easily washed from slides than films made from capillary blood.

Thick blood film: Compared with a thin blood film, a thick film is 30–40 times more sensitive (detecting about 20 parasites/ μL) and therefore the most suitable for the rapid detection and quantification of malaria parasites, particularly when they are few in number.

In a thick film, blood cells are not fixed. The red cells are lysed during staining, allowing parasites and white cells to be seen in a larger volume of blood than in a thin film. The thick film is used to report the number of parasites/ μL of blood which can be used to monitor response to treatment.

Thin blood film: This is used to identify *Plasmodium* species. Blood cells are fixed in a thin film, enabling the parasites to be seen in the red cells and malaria pigment to be detected in white cells. Parasitized red cells may become enlarged, oval in shape or stippled. Examination of a thin film can help to detect mixed infections. In heavy infections the percentage of parasitized red cells can be estimated. Thin film examination also gives the opportunity to report red cell morphological changes associated with anaemia and white cell changes such as neutrophilia or eosinophilia.

Thick and thin blood films on the same slide

- 1 Using a clean *grease-free* microscope slide, write the patient's identity number and date on the frosted end of the slide using a lead pencil.*

* If using a slide without a frosted end, the patient's number and date can be written on the end of the dried thin film using a lead pencil.

- 2 Wearing protective latex gloves, cleanse the lobe of the finger (or big toe if an infant) using a swab moistened with 70% v/v alcohol (avoid using a cotton wool swab). Massage the area with the swab if the finger or toe is cold.
- 3 Using a sterile lancet, prick the finger or toe and squeeze gently. Wipe away the first drop of blood using a dry swab. Apply a small drop of blood to the centre of the slide and a larger drop 10–15 mm from the small drop.
- 4 Using a *smooth* edged slide spreader, immediately spread the thin film as shown in Fig. 2.2. Blood from anaemic patients needs to be spread more quickly and the spreader held at a steeper angle.

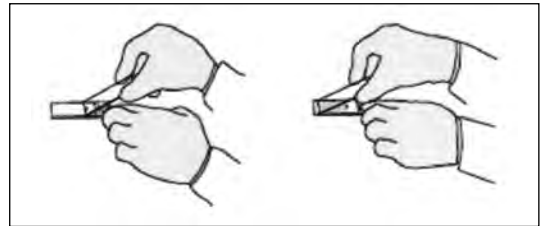


Fig. 2.2 Spreading a thin blood film.

Left: Holding the spreader at an angle of 45°, allow the blood to spread along the edge of the spreader.

Right: Using a rapid smooth movement, push the spreader forwards to make an evenly spread thin blood film.

Figures: Credit DPDx Image Library.

- 5 Without delay, spread the larger drop of blood to make the thick film using the corner of the spreader. Cover evenly an area 10–15 mm in diameter.

Note: When spreading the thick film, mix the blood as little as possible to avoid the red cells forming marked rouleaux which can cause a thick film to be easily washed from the slide. Do not make the film too thick. It should be possible to see newsprint through the film but not read it.

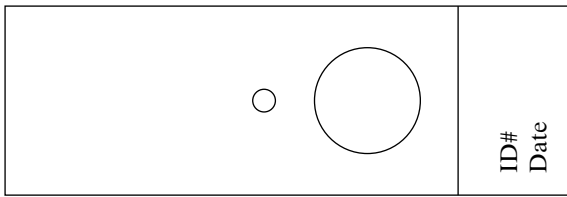


Fig. 2.3 A template which can be used by those collecting blood to show the position of blood drops on a slide and area to be covered by the thick film.
Figure from: WHO Malaria Microscopy MM-SOP-05A.

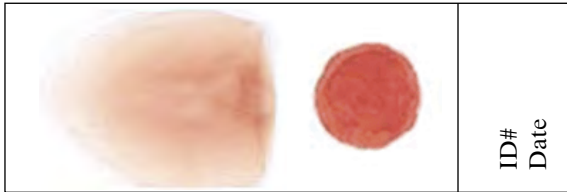


Plate 2.1 Well made thick and thin blood films on a slide with a frosted end for writing patient information and the date.

- 6 Allow the blood films to air-dry with the slide in a horizontal position and in a safe place protected from insects and dust. Do not use excessive heat to dry the films.

Fixing thin blood films

Use absolute methanol (methyl alcohol) to fix thin blood films.*

* The methanol *must be moisture-free* otherwise it will not fix the cells properly. Methanol is toxic and highly flammable. Use it well away from any open flame.

Procedure

- 1 Place the slide on a level staining rack.
- 2 Apply a *small* drop of absolute methanol to the thin film making sure the methanol or its vapours do not touch the thick film as this will prevent red cell lysis, making the thick film unsuitable for staining and reporting. Fix the film for 1–2 minutes.

STAINING MALARIA BLOOD FILMS

The recommended stain for the routine staining of malaria parasites is Giemsa which is an alcohol-based Romanowsky stain.*

* A Romanowsky stain is composed of oxidized methylene blue (azure) and eosin Y. It stains the malaria parasite nucleus dark red and the parasite cytoplasm blue.

Giemsa stain: This is freshly diluted before use in pH 7.2 buffered water and staining takes about

10 minutes at high stain concentration (10%) and 45–60 minutes at lower stain concentration (3–5%). In thin blood films, parasites and stippling in red cells are well stained.

GIEMSA STAINING

Prepare an SOP: *Giemsa staining of malaria blood films*. A suggested SOP format can be found on the WHO website.*

* *Giemsa staining of malaria blood films. Malaria Microscopy Standard Operating Procedure –MM-SOP-07A* 2016. Access from http://www.wpro.who.int/mvp/lab_quality/mm_sop/en/

Reagents

Giemsa stain	Reagent No. 22
Buffered water, pH 7.2	Reagent No. 10
or Buffered saline, pH 7.2*	Reagent No. 9

* Buffered physiological saline is recommended because it provides a cleaner background to blood films.

10% Giemsa procedure

- 1 Immediately before use dilute Giemsa stain 1 in 10 in buffered pH 7.2 water.*

* Measure 45 mL of buffered water, add 5 mL of Giemsa stain and mix gently. This is sufficient to stain 15 slides. If fewer slides, e.g. 3 slides, add 1 mL of stain to 9 mL of buffered water.

- 2 Place the slides *face downwards* in a shallow tray supported on two rods (this will prevent fine particles of stain being deposited on the blood films). Alternatively, place the slides in a Coplin jar or in a staining rack for immersion in a staining trough.

Note: Thick blood films must be *completely dry* and thin blood films require methanol fixing prior to staining.

- 3 Pour the diluted stain into the shallow tray, Coplin jar, or staining trough. Stain for 8–10 minutes*.

* Staining times may need to vary depending on the batch of stain being used (determined previously by using control malaria blood films).

- 4 Wash the stain from the staining container using clean water (need not be buffered unless the water is acidic).

Important: Flushing the stain from the slides and staining container is necessary to avoid the films being covered with a fine deposit of stain.

- 5 Wipe the back of each slide clean and place it in a draining rack for the films to air-dry, thick film facing downwards.

Results

Chromatin of parasite	Dark red
Cytoplasm of parasite	Blue
Schüffner's dots	Red
Maurer's dots (clefts)	Red-mauve
Malaria pigment	Brown-black
Red cells	Grey to pale mauve
Reticulocytes	Grey-blue
Nuclei of white cells	Dark purple
Granules of neutrophils	Mauve-purple
Granules of eosinophils	Red
Cytoplasm of mononuclear cells	Blue-grey

Quality control

Use known malaria control slides to check the performance of newly prepared Giemsa stain and to adjust staining times.

Modified Giemsa staining when blood films may contain viral haemorrhagic fever viruses

When blood samples may contain viral haemorrhagic fever viruses such as Ebola, the WHO recommends using a modified Giemsa staining procedure in which thick and thin blood films are stained on separate slides and Triton X-100 is used to inactivate the virus. An SOP is provided by the WHO: *Ebola virus inactivation during staining of blood films with Giemsa stain – MM-SOP-07B*, 2016. It can be accessed from website: http://www.wpro.who.int/mvp/lab_quality/mm_sop/en/

FIELD STAINING

Field stain: In district laboratories Field stain, a water-based Romanowsky stain is also used because it stains malaria parasites rapidly. It is less expensive, more stable than Giemsa stain and does not require dilution before use. It is composed of two separate stains, Field stain A and Field stain B. The stains are intrinsically buffered at pH 7.2. Parasites in fresh thick blood films are particularly well stained. Field staining is used to stain blood films from waiting outpatients and when reports are required urgently.

Prepare an SOP: *Field staining of malaria blood films*. (A suggested SOP format can be found in Appendix III).

Collect blood when malaria is suspected clinically, before the patient is treated with antimalarials and preferably during times of fever. For Field staining, prepare and stain thick and thin blood films on separate slides. The films are best made from capillary blood.*

* EDTA anticoagulated venous blood can also be used providing the blood is not refrigerated and the blood films are

made within 1 hour of collecting the blood. Thick blood films made from EDTA blood are more easily washed from the slide than films made from capillary blood.

Reagents

Field stain A *	Reagent No. 19
Field stain B *	Reagent No. 20

* Required: Approximately 100 mL of each stain in screw-cap containers. Filter the stains regularly. Keep the stains closed between use and change them frequently depending on use.

Two containers of clean water. If tap water is unsuitable, use boiled filtered water.

Thick and thin blood films on separate slides

- 1 Using clean *grease-free* slides, write the patient's number and date on the frosted end of each slide using a lead pencil.
- 2 Wearing protective latex gloves, cleanse the lobe of the finger (or big toe if an infant) using a swab moistened with 70% v/v alcohol. If the finger or toe is cold, massage the area with the swab.
- 3 Using a sterile lancet, prick the finger or toe and squeeze gently. Wipe away the first drop of blood using a dry swab. Apply a drop of blood to the centre of one of the slides to make the thick blood film and a smaller drop to the second slide.
- 4 Using a smooth edged slide spreader, immediately spread the thin blood film (see Fig 2.2).

Without delay, spread the larger drop of blood to make a thick film covering an area 15 – 20 mm in diameter. Mix the blood as little as possible. *Do not* make the film too thick. It should be possible to see newsprint through the film but not read it.

- 5 Allow the blood films to dry in a horizontal position in a safe place protected from insects and dust. Do not use heat to dry the blood films. Use a fan if available.
- 6 Fix the thin blood film by applying a few drops of absolute methanol.* Fix for 1–2 minutes.

* The methanol must be moisture-free. Keep the dispensing container tightly close between use.

Caution: Methanol is toxic and highly flammable. Use it well away from any open flame.

Thick blood film staining

- 1 Holding the slide with the thick film facing downwards, dip the slide into Field stain A for 5 seconds. Drain off the excess stain by touching the corner of the slide against the side of the container.
- 2 Wash gently in water for about 5 seconds. Drain off the excess water.

- 3 Dip the slide into Field stain B for 3 seconds. Drain off the excess stain.
- 4 Wash gently in water. Wipe the back of the slide clean and place it upright in a draining rack for the film to dry.

Thin blood film staining

Stain the film using a reverse Field technique.⁴ Staining times must be correct:

- 1 Holding the slide with the thin film facing downwards, dip the slide into Field stain B for 4 seconds. Drain off the excess stain by touching the corner of the slide against the side of the container.
- 2 Wash in water for 5 seconds. Drain off the excess water.
- 3 Dip the slide into Field stain A for 6 seconds. Drain off the excess stain.
- 4 Wash in water for 5 seconds. Wipe the back of the slide clean and place it upright in a draining rack for the film to dry.

Results

Chromatin of parasite	Dark red
Cytoplasm of parasite	Blue
Schüffner's dots	Red
Maurer's dots (clefts)	Red-mauve
Malaria pigment	Brown-black
Red cells	Pale pink-mauve
Reticulocytes	Grey-blue
Nuclei of white cells	Dark purple
Cytoplasm of mononuclear cells	Blue-grey
Granules of eosinophils	Red

Quality control

Use known malaria control slides to check staining reactions.

REPORTING MALARIA BLOOD FILMS

The treatment of a patient with suspected malaria is dependent on an accurate laboratory diagnosis of malaria. It is essential that laboratory staff spend *sufficient time* examining blood films to avoid missing an infection or not detecting mixed species infections. Blood films require examination and reporting with the minimum of delay particularly when there is high parasitaemia. Appropriate treatment is dependent on *Plasmodium* species being identified correctly particularly *P. falciparum* and *P. knowlesi* which can cause severe malaria and *P. vivax* and *P. ovale* which require treatment with primaquine to

prevent malaria relapses. Accurate malaria reporting is also required for malaria surveillance, control and monitoring drug resistance.

Malaria reporting of blood films should include the following:

- Species of *Plasmodium* detected in the thick film, confirmed by examining the thin film. It is important to detect and report mixed infections.
- Forms of parasite present, i.e trophozoites, schizonts (asexual forms) and gametocytes (sexual forms).

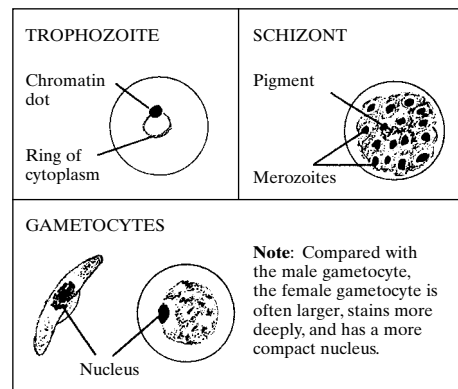


Fig. 2.4 Morphology of trophozoite, schizont and gametocytes of malaria parasites.

- Number of parasites/ μL of blood, calculated from the thick film and white blood cell (WBC) count.
- Percentage of red cells parasitized, calculated from the thin blood film when more than 100 parasites/microscope field are detected in the thick film.
- Presence of malaria pigment in white blood cells. This usually indicates severe malaria infection.
- Infection with other pathogens, e.g. trypanosomes, microfilariae, borreliae, *Babesia*, *Bartonella*.
- Abnormal red blood cell morphology, e.g. sickle cell disease blood picture, hypochromic or macrocytic red cells detected in a well-stained thin film.
- WBC changes e.g. neutrophilia, lymphocytosis, eosinophilia.

When using an automated haematology analyzer, abnormal scatter grams and extra mid-cell peaks in WBC histograms may be seen.

Procedure for examining malaria blood films

- 1 When the thick film is *completely dry*, apply and spread a drop of immersion oil over an area of the film which appears correctly stained and is not too thick. This enables the film to be examined first at lower magnification (there is no need to add a cover glass).

- 2 Scan the film with the 10× objective to select an area of the film that is well stained and where the white cells are evenly distributed. Selecting the correct area of the film to examine is important if parasites are to be detected and quantified reliably.

Note: When scanning at low power magnification, it is usually possible to detect large parasites such as microfilariae.

- 3 Examine the film with the 100× objective, moving systematically from one field to the next. Examine at least 100 high power fields. When parasites are seen, examine a further 100 fields to identify the malaria species and avoid missing a mixed infection. Look for malaria pigment in WBCs.
- 4 Use the colour plates and text shown on pages 62–64 to identify the *Plasmodium* species.

Changes in parasitized red cells as seen in thin films

- Enlargement and irregular shape, characteristic of *P. vivax*.
- Oval cells with ragged ends as may be seen with *P. ovale*.
- Cell stippling (Schüffner's dots) as seen with *P. vivax* and *P. ovale* and less distinct irregular stippling (Maurer's clefts) as seen with *P. falciparum* and (Sinton dots) as seen with *P. knowlesi*.
- Parasites forming a band across the red cell, a feature of *P. malariae* and *P. knowlesi*.
- Several parasites in a single red cell as is common with *P. falciparum*, *P. knowlesi* and some strains of *P. vivax*.

Note: Parasites can often be found along the edges and in the tail end of thin blood films.

- 5 When asexual forms of parasites are seen, count their number against 200 WBCs using two hand tally counters or a WBC differential counter. When there is a mixed *Plasmodium* infection, count the total number of asexual parasites of both species.

Report the presence of gametocytes but do not include them in the parasite count. It may be difficult to differentiate the gametocytes of *P. vivax* and *P. malariae* from the asexual forms.

- 6 Using the patient's known WBC count or an estimated count of 8000 WBCs/μL, calculate the number (Nos.) of parasites/μL as follows:

$$\frac{\text{Parasites}/\mu\text{L} = \text{Nos of parasites counted} \times \text{WBC count}}{\text{Nos of WBCs counted}}$$

Example

Parasites counted against 200 WBCs = 140
Estimated WBC count = 8000/μL

$$\frac{140 \times 8000}{200} = 5600$$

Parasite count: 5600/μL

- 7 When parasitaemia is high i.e. when more than 100 parasites are present in each high power field, calculate the percentage of parasitized red blood cells (RBCs). Do not include gametocytes. Cells containing multiple parasites are counted as one. Select an area of the film where the microscope field contains about 250 RBCs. Using a hand tally counter, count the parasitized cells in 8 microscope fields (approx 2000 cells). Divide by 20 to give the percentage (%) of parasitized RBCs.

Important: High parasitaemia should be reported as soon as possible. Measure also the patient's haemoglobin and blood glucose.

- 8 Report the *Plasmodium* species, forms of parasite, presence of malaria pigment in WBCs, parasites/μL and where indicated % of parasitized cells.

Example

P. falciparum trphozoites 6600/μL.

P. falciparum gametocytes and malaria pigment in WBCs.

- 9 When no parasites are found after examining at least 100 high power microscope fields, report: Malaria thick film: No parasites seen.

Patients with malaria but no parasites are seen

The commonest causes for not finding parasites (given the reliability of laboratory examination) are that the patient has taken antimalarial drugs or the parasites are too few to be detected microscopically due to the stage of infection, patient is from a low malaria transmission area, or the patient is infected with a malaria species normally having a low parasitaemia. Blood film examinations should be repeated particularly during times of fever.

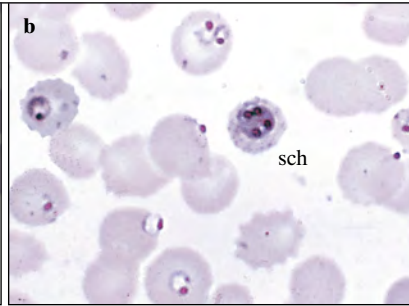
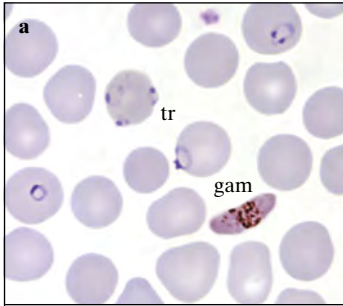
Causes of severe non-malaria febrile illness

Readers are referred to the paper of **Crump et al.** Etiology of severe non-malaria febrile illness in northern Tanzania – a prospective cohort study. *PLoS NTD* 7(7):e2324, 2013.

Worldwide e-Learning Course on Malaria Microscopy

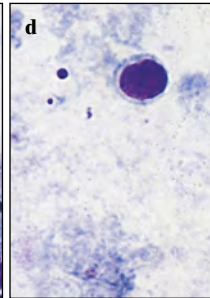
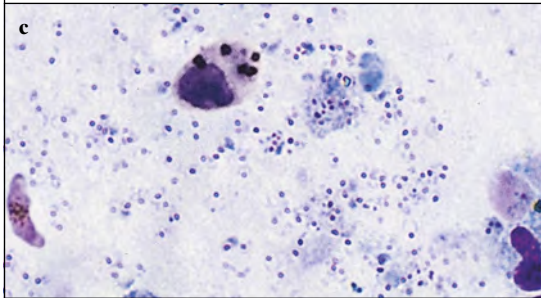
This is a malaria computer course provided on a USB disc that uses virtual microscopy. There are 5 modules covering: 1) Introduction to malaria, 2) Blood specimen processing, 3) Blood film examination, 4) Other laboratory diagnostic methods for malaria, 5) Quality Management System (QMS) elements.

The *Worldwide e-Learning Course on Malaria Microscopy* is available from Amref Health Africa. To access further information, including course fees and contact details, enter the Course title in Google.



P. falciparum in Giemsa stained thin blood films. 100× objective.

a Trophozoites and gametocyte. **b** Trophozoites showing Maurer's dots and schizont.



P. falciparum in Field stained thick blood films.

c Many trophozoites, gametocyte and white cell containing malaria pigment.

d Film from a patient with malaria and sickle cell disease. *Note:* Blue staining reticulum and nucleated red cell (above trophozoite).

Plasmodium falciparum

Only trophozoites and gametocytes are usually seen. Parasites may be many.

Host cell

- Not enlarged.
- Irregular mauve-red Maurer's dots (clefts) often seen in cells with mature trophozoites.

Trophozoites

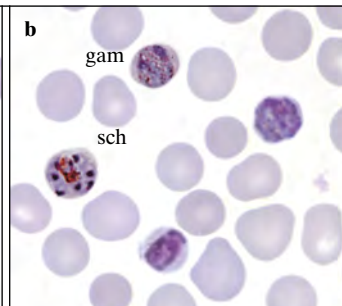
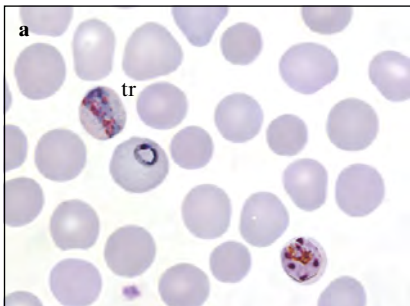
- Mainly small delicate rings, larger rings in heavy infections.
 - Double chromatin dot is common and also several parasites in a cell.
 - May lie on red cell membrane (accolé form).
- Note:* Trophozoites of resistant strains may appear thick and distorted.

Schizonts

- Rarely seen.
- When seen they usually indicate a severe infection and appear small with few merozoites and a single clump of dark pigment.

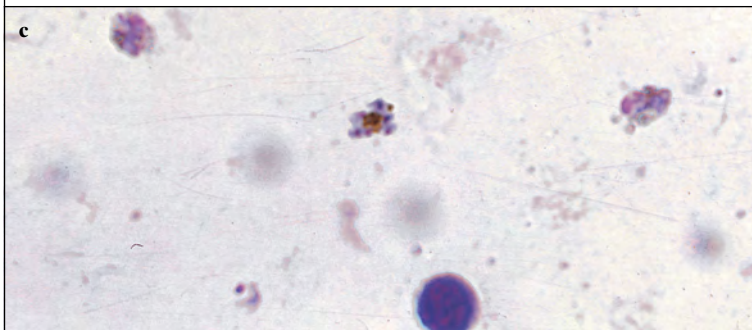
Gametocytes

- Crescent-shaped with round or pointed ends. Oval forms may also be seen.
- Pigment granules scattered around the nucleus or in a single mass.



P. malariae in Giemsa stained thin blood films. 100× objective.

a Trophozoites including band form and immature schizont. **b** Schizont, gametocytes, band form trophozoite.



c *P. malariae* in thick blood film showing trophozoites, gametocyte and schizont.

Plasmodium malariae

Trophozoites, schizonts, gametocytes may be seen. Parasites may be few.

Host cell

- Not enlarged.
- No stippling in cells.

Trophozoites

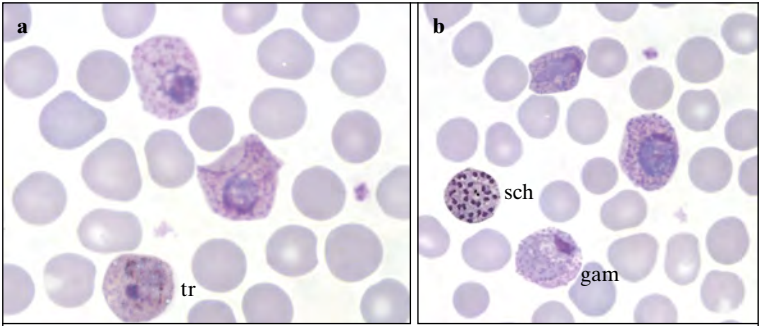
- Thick, compact, densely staining with brown pigment.
- Occasionally band forms can be seen in thin films.
- Sometimes the chromatin dot is central (bird's eye view).
- Double chromatin dots are rare.

Schizonts

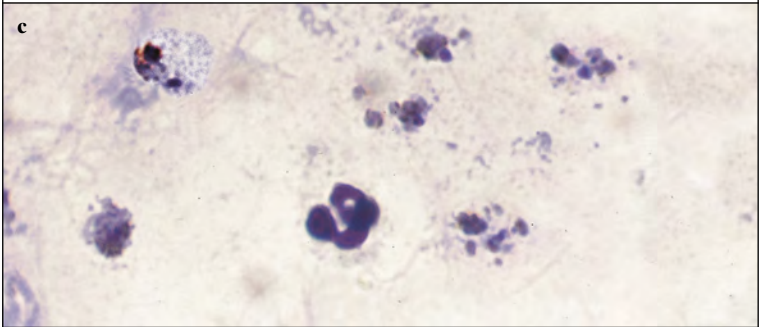
- Small, compact with neatly arranged merozoites surrounding pigment.
- Contain up to 12 merozoites.

Gametocytes

- Small, round or oval and compact.
- Not usually filling the red cell.
- Scattered brown pigment.
- Nucleus usually lies to one side.
- May be difficult to distinguish from mature trophozoites.



P. vivax in Giemsa stained thin blood films. 100× objective.
a Amoeboid trophozoites in enlarged cells with Schüffner's dots. **b** Schizont, gametocytes and trophozoites in enlarged cells with Schüffner's dots.



c *P. vivax* in Field stained thick film showing irregular trophozoites surrounded by Schüffner's dots.

Plasmodium vivax

Trophozoites, schizonts, gametocytes may be seen.
 Parasites invade young red cells.

Host cell

- Becomes enlarged and irregular in shape.
- Schüffner's dots are present and can also be seen surrounding parasites in thick films.

Trophozoites

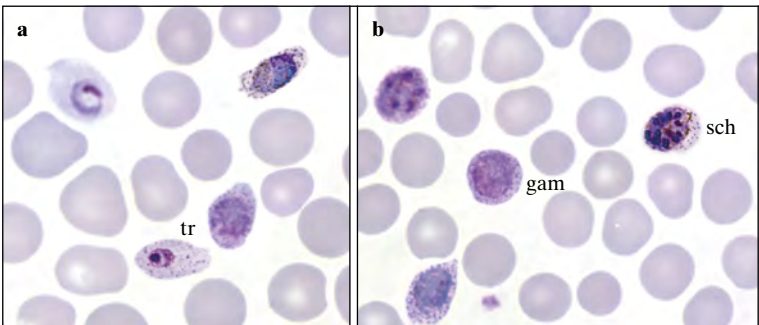
- Most are irregular in form (amoeboid) in enlarged cells.
- Mature trophozoites can fill the cell.

Schizonts

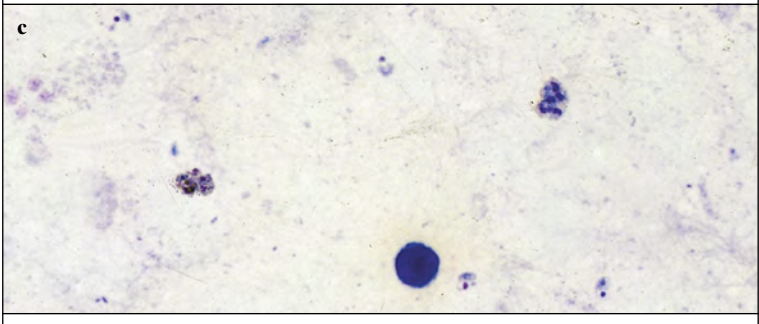
- Large, round or irregular in form.
- Contain up to 24 merozoites with granular yellow-brown pigment.

Gametocytes

- Large, round or irregular in form with scattered brown pigment.
- May fill the red cell.
- Difficult to distinguish from mature trophozoites.



P. ovale in Giemsa stained thin blood films. 100× objective.
a Trophozoites, parasite in fimbriated cell showing Jame's dots, and gametocyte.
b Schizont, gametocytes and trophozoite with Jame's dots.



c *P. ovale* in thick blood film showing trophozoites, schizonts and lymphocyte.

Plasmodium ovale

Trophozoites, schizonts, gametocytes can be seen. Parasites may be few.
 Parasites invade young red cells.

Host cell

- 20–30% of infected cells may become oval or irregular in shape, some with ragged ends (fimbriated).
- Schüffner's dots, (often referred to as Jame's dots) are present.

Trophozoites

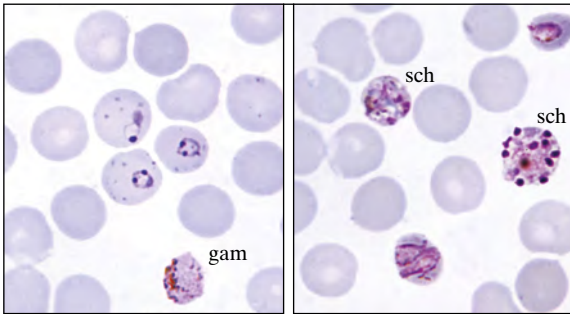
- Compact or irregular.
- Clearly seen chromatin dot and dark brown pigment.

Schizonts

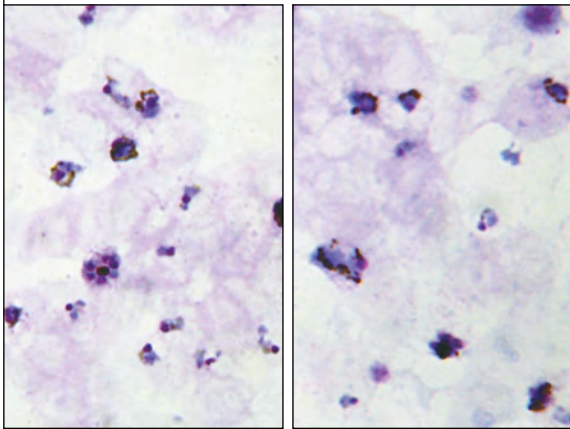
- Compact or irregular.
- Contain up to 12 merozoites with brown pigment.

Gametocytes

- Round or oval, may fill the cell.
- Pigment is scattered and nucleus often lies to one side.
- Difficult to distinguish from mature trophozoites.



Giemsa stained thin films showing *P. knowlesi*.
 Left: Trophozoites showing Sinton stippling and a gametocyte (gam).
 Right: Band form trophozoites, gametocyte and schizonts (sch).



Giemsa stained thick blood films showing trophozoites, schizonts and gametocytes of *P. knowlesi*.

Plasmodium knowlesi

Low parasitaemia is common, however in severe infections many parasites can be present.

Morphologically *P. knowlesi* resembles *P. malariae* and in the early stages, *P. falciparum*.

Trophozoites, schizonts, gametocytes can be seen.

Host cell

- Not enlarged.
- May contain indistinct stippling (Sinton dots).
- Occasionally cell appears oval and fimbriated.

Trophozoites

- Early trophozoites resemble *P. falciparum*.
- Double chromatin dot and multiple cell infections are common.
- Band forms resembling *P. malariae* can be seen.

Schizonts

- Resemble those of *P. malariae* with clumped dark brown pigment.
- Contain up to 16 merozoites (12 for *P. malariae*).

Gametocytes

- Round with scattered pigment, similar to those of *P. malariae*.
- Fill most of the cell.

Reporting blood films

P. knowlesi infections can be life-threatening. Because it is not possible to accurately identify *P. knowlesi* microscopically it is recommended that in areas where *P. knowlesi* occurs, *P. malariae* positive blood films be reported as: *P. malariae/P. knowlesi*.

***Plasmodium simium*⁷**

Morphologically *P. simium* resembles *P. vivax*.

Trophozoites, schizonts, gametocytes may be seen.

Host cell

- Becomes enlarged and irregular in shape.
- Coarse prominent Schüffner's dots.

Trophozoites

- Less amoeboid than those of *P. vivax*.
- Compact cytoplasm and large mass of chromatin.

Colour plates

Excellent images of *P. simium* in Giemsa stained blood films can be found in the paper of Brasil P. et al: Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. *The Lancet Global Health*, 2017; S:e1038-46.

The paper and colour plates can be accessed by entering the title of the paper in Google.

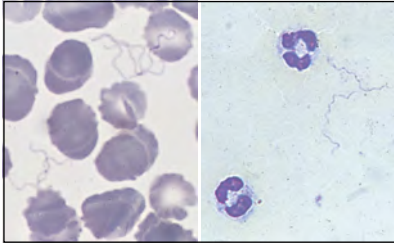
Schizonts

- Similar to those of *P. vivax*.
- Contain up to 14 merozoites (*P. vivax* up to 24).

Gametocytes

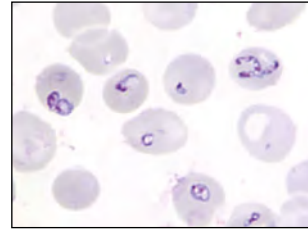
- Round with compact cytoplasm.
- Clearly seen pigment.

Other pathogens that may be detected in thick and thin Giemsa stained blood films



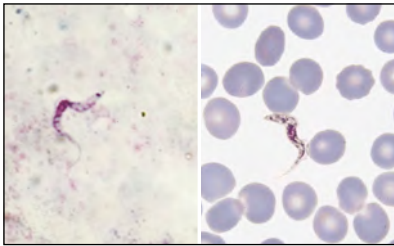
Relapsing fever

Left: Thin film showing *Borrelia* spirochaetes measuring 10–20 $\mu\text{m} \times 0.5 \mu\text{m}$ with uneven coils. *Right:* Borreliae as seen in a thick film. *B. recurrentis* causes louse-borne relapsing fever which is common in Ethiopia, Sudan and Burundi. Lower prevalences are found in other parts of Africa, South America and parts of India, Asia and China. *B. duttoni* causes tick-borne relapsing fever primarily in East Africa, Central Africa and South Africa.



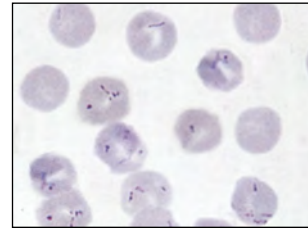
Babesiosis

Babesia parasites as seen in a thin film. Unlike malaria parasites, only trophozoite rings, pear-shaped forms (piroplasm), paired parasites and cross- shape forms (tetrads) can be seen and no pigment is present in *Babesia* parasitized cells. Parasites often appear vacuolated. Red cells are not altered. Babesiosis is found worldwide. It is tick-borne. Recently, infections have been reported from South Africa, DR of Congo and Mozambique. *Photo credit: CDC DPDx Image Library.*



African trypanosomiasis

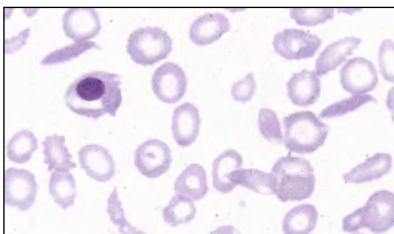
Left: Trypanosome as seen in a thick film. *Right:* Trypanosome as seen in a thin film. The trypanosomes measure 13–42 μm long. African trypanosomiasis is caused by *Trypanosoma brucei*, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense*. Further information on African trypanosomiasis can be found in Section 2.2.



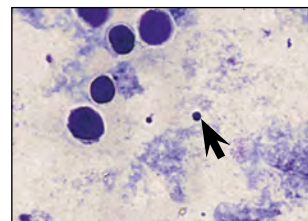
Bartonellosis

Thin film showing pleomorphic *Bartonella bacilliformis* organisms in red cells. They are small organisms occurring as small rods, oval and coccidial forms, X and Y forms. Many organisms may be seen, often with severe haemolytic anaemia. *B. bacilliformis* is found in South America particularly in the foothills of the Western Andes.

Sickle cell disease thick and thin Giemsa stained blood films



Thin film showing sickle and elongated cells, nucleated red cell, target cells and polychromasia. Sickle cell disease is an inherited haemoglobinopathy affecting people from Africa, India, Middle East and the Mediterranean region (see also page 244).



Thick film showing blue stippling in the background (reticulum of reticulocytes). The arrow shows a nucleus of a nucleated red cell. Further tests include measurement of haemoglobin, a sickle cell test and haemoglobin electrophoresis.

MALARIA RAPID DIAGNOSTIC TESTS

Malaria rapid diagnostic tests (RDTs) can help to diagnose and treat malaria in peripheral health facilities where quality assured microscopical diagnosis is not possible. Also to diagnose malaria in non-immune migrant workers and in displaced communities, e.g. refugee camps.

Most RDTs are not as sensitive as microscopy in diagnosing malaria and cannot be used to quantify parasites, provide information on the stages of malaria parasites and check the effectiveness of antimalarial treatment. Specific RDTs for the diagnosis of malaria caused by *P. malariae*, *P. ovale* and *P. knowlesi* have yet to be developed.

Malaria RDTs use monoclonal antibodies to detect the antigens produced by malaria parasites in the blood of those infected. The antigens produced are:

- Histidine rich protein 2 (HRP2) produced by the asexual stages of *P. falciparum* and found in the cytoplasm and membrane of infected red cells.
- Aldolase (pALD), a metabolic enzyme produced by the asexual and sexual stages of all *Plasmodium* species and is therefore pan-specific.
- Lactate dehydrogenase (pLDH), a metabolic enzyme which can be pan-specific detecting a form (isomer) of pLDH produced by all *Plasmodium* species or species-specific detecting a pLDH isomer produced by a particular species such as *P. falciparum* or *P. vivax*. pLDH is produced by asexual and sexual parasitic stages.

P. falciparum *P. vivax* *P. ovale* *P. malariae*

Tests

HRP2	+			
pLDH-Pf	+			
pLDH-Pv		+		
pLDH-Pvom		+	+	+
pLDH-pan	+	+	+	+
pALD-pan	+	+	+	+

Tests may incorporate two bands, e.g. HRP2 and the control band, or three bands e.g. HRP2, a pLDH band and the control band.

Note: Some HRP2 tests have been shown to be more sensitive at detecting *P. falciparum* than pLDH-Pf and pLDH-pan tests. Not all strains of *P. falciparum* produce HRP2 (see later text). HRP2 tests cannot be used to monitor parasitaemia because HRP2 antigen can remain in the blood for up to 4 weeks following treatment. pLDH and pALD antigens clear more rapidly from the blood. When only an HRP2 test is used, mixed infections can be missed.

Based on the antigens targeted, specific RDTs can be used to detect *P. falciparum* and *P. vivax*. When HRP2 and pan-specific RDTs are used in combination they can distinguish between non-*P. falciparum* and *P. falciparum* infections. Concentration of antigen must be adequate to be detected by the RDT. Low parasite densities increase reader variability in reporting tests.

Test selection depends on the prevalence and transmission of a *Plasmodium* species in a geographical area, e.g. in sub-Saharan Africa where the predominant malaria species is *P. falciparum*, HRP2 and pLD-Pf tests are used. In Asia and South America where *P. falciparum* and *P. vivax* co-circulate, a RDT is used to differentiate falciparum and vivax infections. Currently available RDTs have limited application for the detection of *P. knowlesi* and poor sensitivity for the detection of *P. malariae* and *P. ovale*.

Implementing malaria RDTs requires consideration of the situations in which the tests will be used, the persons who will be performing the tests, how test results will be used, local availability of tests, supply chain management, and affordability of RDTs including cost of training, supervision and quality assurance. Tests should be selected that have been evaluated for their quality of performance (see WHO malaria RDT product testing programme page 67).

Method of performing malaria RDTs

RDTs are simple to perform, use capillary blood, take 15–30 minutes to complete and most do not require refrigeration. Some tests can also be used with EDTA-anticoagulated venous blood. Most RDTs use a cassette or card format.

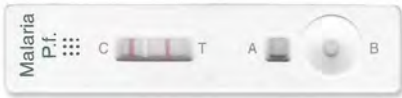
RDTs are lateral flow immunochromatographic tests:

- A buffer solution is used to lyse the blood.
- Malaria antigen from the lysed sample is reacted with anti-malaria monoclonal antibody conjugated to colloidal gold (pink-mauve) particles.
- The antigen-antibody colloidal gold complex migrates along the nitocellulose membrane where it becomes bound (captured) by a line of specific monoclonal antibody, producing a pink band in the test area.
- A further pink band, i.e. inbuilt positive control, is produced above the test band indicating that the reaction mixture has migrated satisfactorily (it is not a malaria antigen control).

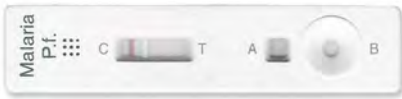
The intensity of colour of the test band is dependent on the concentration of antigen. The

intensity of colour of the control band may decrease with high antigen concentration (most of the dye labelled antibody will have been captured at the test area).

An example of an HRP2 test is shown below. The blood sample is added to the well marked A and buffer solution to well B.



Positive test for *P. falciparum*: Pink band appears in both the C (Control) and T (Test) viewing windows.
Courtesy of Orchid Biomedical Systems.



Negative test for *P. falciparum*: Pink band appears in the C (Control) viewing window only.



Performing a RDT to diagnose malaria in a child.
Courtesy of Fio Corporation (Malaria prevention work in Nigeria with Africare and Fio Corporation, 2017).

There exists significant variation between the performance of RDTs in the field particularly with regard to sensitivity and product stability. Not all RDTs have been evaluated for all transmission areas. Lot to lot variation in test quality can also occur. To assist health authorities in procuring malaria RDTs that meet quality standards, the WHO has issued guidelines (2018) covering selection criteria based on the WHO malaria RDT product testing programme.⁵ The guidelines also cover post-purchase lot-verification.

WHO malaria RDT product testing programme

The programme is coordinated by the Global Malaria Programme and FIND (Foundation for Innovative New Diagnostics) with the collaboration of CDC. It provides comparative data on the performance of the RDTs available on the market to guide procurement. Using a panel detection score, each RDT is evaluated at both lower and higher parasite density. Also evaluated are thermal stability, ease of use, quality of instructions, blood transfer device, kit completeness and blood safety.

The following selection criteria are recommended:

- For the detection of *P. falciparum* in all transmission settings, the detection score against *P. falciparum* samples should be at least 75% at 200 parasites/ μ L.
- For the detection of *P. vivax* in all transmission settings, the detection score against *P. vivax* samples should be at least 75% at 200 parasites/ μ L.
- The false-positive rate should be less than 10%.
- The invalid rate should be less than 5%.

Note: The 2022 WHO list of prequalified in vitro diagnostic products including malaria RDTs can be found on the WHO website. To access, enter in Google, WHO list of prequalified in vitro diagnostic products.

Negative malaria RDT test results

A negative RDT does not always exclude malaria with certainty. A negative test result may be due to:

- Insufficient antigen for detection by the test being used particularly in areas of low transmission.
Note: When a 3 band RDT shows a positive HRP2 band and a negative Pf-pLDH band it cannot be assumed that the test is negative for an active *P. falciparum* infection. The Pf-pLDH band may be negative because it is not sufficiently sensitive.
- Malaria being caused by a *Plasmodium* species not detected by the test being used, e.g. when an HRP2 test is used and the malaria is not a *P. falciparum* infection or when a pan pLDH test is used that is not sufficiently sensitive, e.g. to detect *P. malariae* or *P. ovale*.
- Very high parasitaemia (antigen overload), resulting in difficult to read faint colour bands. The test should be repeated after diluting the sample 1 in 10 or more if indicated.
- The RDT having been damaged because it has not been transported or stored at the temperature recommended by the manufacturer or the test has been exposed to direct sunlight or humid conditions. Enzyme-based RDTs are more susceptible to damage by heat and humidity than HRP2 tests.
- Incorrect testing procedure or problems associated with the buffer solution or accessories provided in the test kit.
- Test band is not detected due to a strong background colour which may occur when a

stored blood sample is used or a poor quality RDT.

- A *P. falciparum* strain not producing HRP2 due to Pfhrp2/Pfhrp3 gene deletions, resulting in a negative HRP2 test.*

* False negative RDT results due to *P. falciparum* strains not producing HRP2 have been reported from Peru, India, Mali, Senegal, Eritrea and several other African countries. The WHO advises that a false negative HRP2 test should be suspected and reported in a patient with suspected malaria when two quality assured HRP2 tests are negative and a subsequent pLDH-Pf or pan pLDH test is found to be positive and when falciparum malaria is confirmed by malaria microscopy.⁶ Prequalified alternative RDTs to HRP2 tests are available⁵ (may be less sensitive).

Positive malaria RDT tests results

A positive RDT does not always signify a diagnosis of active malaria infection because:

- Antigen may persist in the blood following treatment, e.g. HRP2 antigen can be detected for up to 4 weeks following antimalarial treatment.
- There may be an alternative cause of fever in a patient with high immunity.
- Occasionally other substances present in a patient's blood may cause a false positive RDT result, e.g. heterophile antibodies or rheumatoid factor.

Quality assurance of malaria RDTs

An effective integrated quality assurance programme for malaria rapid diagnostic testing is essential if health workers are to have the confidence to act on the results of RDTs and health authorities are to be assured of the value and cost-effectiveness of RDTs in patient care. Malaria RDT quality assurance includes the following:

- Using quality RDTs that have been evaluated by the WHO malaria RDT testing programme and lot-verified (see previous text).
- Ensuring test kits are transported and stored at the manufacturer's recommended temperature and not exposed to direct sunlight, particularly enzyme-based RDTs.
- Preventing kits from being damaged by high humidity. A test should only be removed from its moisture-proof envelope/pouch when it is ready to be used.
- Following exactly the manufacturer's instructions for performing an RDT, reading and interpreting

test results. It is important to read tests in a good light to avoid missing faint colour bands.

- Ensuring those performing RDTs have sufficient training and continuing supervision to perform, report and interpret tests competently.
- Using antigen controls (where available), i.e. dried antigen that can be reconstituted in water and used to check the quality of performance of newly purchased test kits.
- Providing adequate training and the materials needed to ensure the safe handling of blood samples and the safe disposal of lancets and items used to perform RDTs.
- Keeping accurate records, including RDT stock levels, RDT test results, and the number of damaged test kits, invalid test results, and repeat tests.
- With the minimum of delay reporting any RDT kit performance problem to the supervisor of the malaria rapid diagnostic testing programme.

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- 2 WHO *Malaria Fact Sheet*, WHO, 2022. <https://www.who.int/news-room/fact-sheets/detail/malaria>
- 3 WHO *Universal access to malaria diagnostic testing – An operational manual*, WHO, Feb 2013. Access by entering the title in Google.
- 4 AMREF *Standard Operating Procedures. Essential Laboratory Tests*, 2008, The African Medical and Research Foundation.
- 5 WHO *Recommended selection criteria for procurement of malaria rapid diagnostic tests*, WHO, 2018. Access by entering the title in Google.
- 6 WHO *False-negative RDT results and implications of new reports of P. falciparum histidine-rich protein 2/3 gene deletions*. May 2016 (Rev Sept 2017). Global Malaria Programme, Information Note. WHO, 2017. Access by entering the title in Google.

FURTHER READING

Brasil P et al. Outbreak of human malaria caused by *Plasmodium simium* in the Atlantic Forest in Rio de Janeiro: a molecular epidemiological investigation. *Lancet Glob Health*, 2017;5:e1038-46. [dx.doi.org/10.1016/S2214-109X\(17\)30333-9](https://doi.org/10.1016/S2214-109X(17)30333-9) or access the article by entering the title in Google.

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Aidoo M, Incardona S. Ten years of universal testing: How the rapid diagnostic test became a game changer for malaria case management and improved disease reporting. *Am J Trop Med Hyg*, 2022 Jan;106(1):29-32. 10.4269/ajtmh.21-0643

2.2 African trypanosomiasis Chagas disease

Human African trypanosomiasis, also known as sleeping sickness (a reference to the comatose state found in the late stages of the disease), is caused by:

Trypanosoma brucei rhodesiense, causing acute trypanosomiasis.

Trypanosoma brucei gambiense, causing chronic trypanosomiasis.

The parasites are closely related and belong to the *Trypanosoma brucei* group, or complex.

Trypanosoma brucei complex

Trypanosoma brucei brucei, infective to livestock (causing nagana) but not to humans. The human pathogens *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense* are morphologically indistinguishable. Each trypanosomiasis endemic area has associated with it several strains of biochemically and antigenically distinct trypanosomes.

Distribution

Human African trypanosomiasis occurs in rural parts in the tsetse fly areas of sub-Saharan Africa between 14 °N and 20 °S. Tsetse flies are found only in Africa.

T. b. gambiense is found in 24 countries in west and central Africa, accounting for up to 97% of cases of sleeping sickness. *T. b. rhodesiense* is found in 13 countries in eastern and southern Africa, accounting for the remaining 3% of cases. In Uganda both species are found (in separate areas).

Following successful control measures, the number of new cases of human African trypanosomiasis has been falling. In 2020 there were 663 new cases reported (lowest number recorded). Interruption of transmission (zero cases) has been targeted by 2030.¹

Transmission and life cycle

Human African trypanosomiasis is transmitted by tsetse flies belonging to the genus *Glossina*. Both male and female tsetse flies suck blood and can therefore transmit the disease. Once infected a tsetse fly remains a vector of trypanosomiasis for the remainder of its life. The life-span of a tsetse fly is about 3 months.

T. b. rhodesiense

T. b. rhodesiense is transmitted by woodland and savannah tsetse flies. Rhodesiense trypanosomiasis is a zoonosis with many game animals being naturally infected. Infection can persist for long periods in animals such as the bushbuck, reedbuck, warthog, hartebeest, goat, hyena, giraffe, and lion. Persons at greatest risk are therefore game wardens, honey collectors, fishermen, hunters, wood collectors, tourists, and

other persons who enter tsetse fly infested game country in endemic areas. During epidemics, humans are the main sources of infection. Cattle movements increase the risk of infection.

T. b. gambiense

T. b. gambiense is usually transmitted by lakeside and riverine tsetse flies. Humans are the main reservoirs of infection. Semi-domestic animals may act as reservoir hosts.

Note: Trypanosomiasis can also be transmitted by blood transfusion (fresh blood). Congenital transmission can also occur with trypanosomes crossing the placenta and infecting the foetus but this is rare. Transmission through sexual contact has also been reported.

Life cycle

The life cycle of *T. b. rhodesiense* and *T. b. gambiense* is summarized in Fig. 2.5.

Metacyclic trypomastigotes are inoculated through the skin when an infected tsetse fly takes a blood meal. The parasites develop into long slender trypomastigotes which multiply at the site of inoculation and later in the blood, lymphatic system, and tissue fluid. They are carried to the heart and various organs of the body and in the later stages of infection they invade the central nervous system (CNS).

Trypomastigotes are ingested by a tsetse fly when it sucks blood. In the midgut of the fly, the

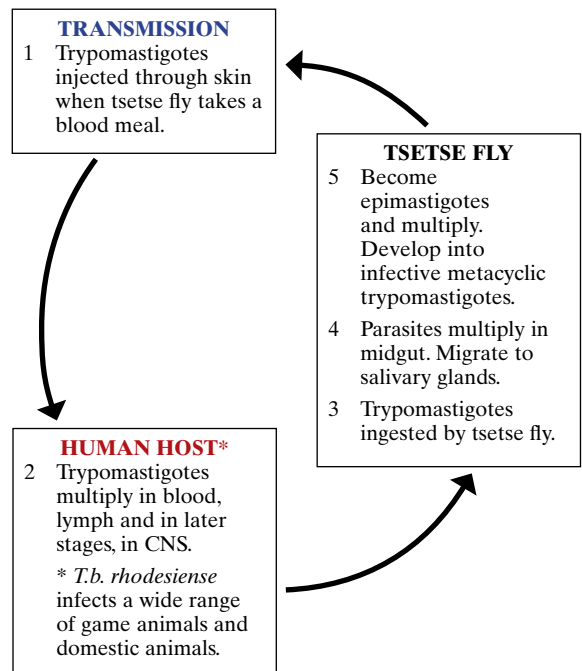


Fig 2.5 Transmission and life cycle of *T. b. rhodesiense* and *T. b. gambiense*.

parasites develop and multiply. After 2–3 weeks, the trypomastigotes migrate to the salivary glands of the tsetse fly where they become epimastigotes, multiply and develop into infective metacyclic trypomastigotes.

The main immune response in African trypanosomiasis is a humoral one with stimulated B lymphocytes producing large amounts of Ig M followed in the later stages of infection by Ig G.

African trypanosomiasis is usually fatal unless treated. In rhodesiense trypanosomiasis and less commonly in gambiense infections, a painful swelling called a chancre develops and can be seen at the site of inoculation of the trypomastigotes. It contains multiplying trypomastigotes. It usually heals in 2–3 weeks.

Differences between gambiense and rhodesiense trypanosomiasis

Gambiense sleeping sickness tends to be a more chronic type of disease showing early lymphatic involvement with swollen glands. Other symptoms develop slowly over several months. The parasites are usually difficult to find in the blood and are more commonly found in aspirates from enlarged lymph glands.

Rhodesiense trypanosomiasis tends to be more acute with a rapid development of encephalitis and symptoms leading to early death from toxæmia or heart failure.

Note: Occasionally infection with *T. b. gambiense* can be acute and infection with *T. b. rhodesiense* may be chronic. Asymptomatic carriers of both *T. b. gambiense* and *T. b. rhodesiense* have been found in some areas.

Measures to prevent and control African trypanosomiasis

- Detecting and treating human infections at an early stage¹.
- Screening populations at risk and monitoring drug resistance. During the COVID-19 pandemic active screening of gambiense trypanosomiasis was suspended.
- Siting human settlements in tsetse fly infested areas only when there is adequate vector control.
- Reducing tsetse fly numbers by:
 - Using and maintaining insecticide impregnated tsetse fly traps.
 - Identifying and studying the breeding habits of local vectors.
 - Selectively clearing the bush and wooded areas, especially around game reserves, water-holes, bridges, and along river banks.
 - Sterilizing male flies by radiation.
- Spraying vehicles with insecticide as they enter and leave tsetse fly infested areas.

- Identifying animal reservoir hosts in endemic areas.
- In rhodesiense trypanosomiasis areas, restricting the movement of game animals to within fenced reserves.

LABORATORY DIAGNOSIS

Because of the risks associated with the drug treatment of African trypanosomiasis, particularly if needing to treat for CNS (central nervous system) involvement, it is essential to confirm the diagnosis parasitologically before commencing treatment. The laboratory *must* examine samples carefully and for a sufficient length of time. Several samples may need to be examined before detecting the trypanosomes (trypomastigotes).*

* Under laboratory diagnosis, the more familiar term trypanosome is used.

In district laboratories, the diagnosis of African trypanosomiasis and investigation of the stage of infection is by:

- Examining blood for trypanosomes.
- Examining chancre fluid or aspirates from enlarged lymph glands for trypanosomes, particularly when gambiense trypanosomiasis is suspected.
- Examining cerebrospinal fluid (CSF) for trypanosomes, cells (including Morula cells), raised protein and when available, IgM. Testing CSF will provide information on the stage of the disease, i.e. whether the CNS has been involved and further treatment with more toxic drugs is indicated.

Important: To avoid the accidental introduction of trypanosomes into the CNS should the lumbar puncture be traumatic, CSF should not be collected until treatment to kill the parasites in the blood has been started.

- *T. b. gambiense* antibody test.

Other tests

- Measurement of haemoglobin. Rapidly developing anaemia with reticulocytosis is a feature of African trypanosomiasis.
- Total white blood cell count and differential. A moderate leucocytosis with monocytosis, lymphocytosis and presence of plasma cells are common findings in African trypanosomiasis.

- Checking urine for protein, cells and casts once treatment has started.

Blood grouping and crossmatching of blood from patients with trypanosomiasis

The grouping (typing) and crossmatching (compatibility testing) of blood from patients with African trypanosomiasis may cause difficulties due to autoagglutinins and rouleaux formation of red cells. When blood grouping, it is essential to test both the cells and serum of the patient and to check for autoagglutination. When crossmatching, auto-controls must be used and an antihuman globulin crossmatch should be performed.

Transmission of trypanosomes in infected donor blood

When blood is stored for a few days at 2–8 °C, the risk of transmitting trypanosomes is removed because when stored at this temperature the parasites rapidly lose their infectivity. A rapid sedimentation of red cells should alert laboratory staff as to the possibility of infected blood when taken from a donor living in a trypanosomiasis area.

EXAMINATION OF BLOOD FOR TRYPANOSOMES

Repeated examinations and concentration techniques are often required to detect trypanosomes in the blood because parasite numbers vary at different stages of the infection. At times no parasites will be found.

The following techniques are recommended:

- Examination of a thick stained blood film.
- Lyzed blood concentration technique.
- Microhaematocrit concentration technique (requires a microhaematocrit centrifuge).

Miniature anion exchange centrifugation (MAEC) technique

This technique is considered by most workers to be the most sensitive for detecting small numbers of *T. b. rhodesiense* or *T. b. gambiense* trypanosomes, but it is an expensive technique and requires careful control.

In the MAEC technique, the patient's heparinized blood is passed through a buffered anion (negatively charged) exchange column of diethyl-aminoethyl (DEAE)-52 cellulose. As the blood is eluted through the column, the strongly charged blood cells are adsorbed onto the cellulose while the less strongly charged trypanosomes are washed through the column with the buffered saline. The eluate is then collected, centrifuged, and the sediment is examined microscopically for motile trypanosomes.

The cellulose column requires careful preparation and the

pH of the buffer is critical to ensure adsorption of the cells and elution of the trypanosomes.

Note: For further information readers are referred to the Foundation for Innovative New Diagnostics (FIND). Email info@finddx.org

Examination of a thick stained blood film

Although trypanosomes, when in sufficient numbers, can be detected by their motility in fresh unstained wet blood preparations, examination of a thick stained blood film is recommended because more blood can be examined in a shorter time.

Procedure

- 1 Collect a drop of capillary blood on a slide and spread it to cover evenly an area 15–20 mm in diameter.

Rouleaux: Red cells from patients with trypanosomiasis will tend to form rouleaux. To avoid excessive rouleaux, mix the blood as little as possible when spreading it. Marked rouleaux will cause the preparation to be easily washed from the slide during staining. For the same reason it is also best to make thick films from fresh non-anticoagulated blood.

- 2 Allow the smear to dry completely in a safe place, protected from flies, ants and dust.
- 3 Stain the film using Field rapid technique for thick blood films or Giemsa staining technique (described in Section 2.1). Allow the preparation to air-dry.
- 4 Spread a drop of immersion oil on the film and examine it microscopically for trypanosomes using the 40× objective.

Use the 100× objective to confirm that the organisms are trypanosomes.

Note: If no trypanosomes are seen, always check the thick film for other possible causes of a patient's fever such as malaria parasites or borreliae.

Lyzed blood concentration technique

Using a lyzed blood concentration technique increases significantly the chances of finding parasites when trypanosomes are few in number.² The technique described is the Hoff concentration method using 0.87% w/v ammonium chloride to lyze the red cells. The sample should be examined as soon as possible after collecting the blood to increase the chances of finding motile trypanosomes.

Procedure

- 1 Collect 2.5 mL of venous blood into EDTA

anticoagulant and mix gently.

Caution: Wear protective gloves and handle the sample with care to avoid accidental infection with viable trypanosomes.

- 2 Dispense 3 mL of ammonium chloride lysing solution (Reagent No. 7) into a conical centrifuge tube.
- 3 Add 1 mL of blood and mix. Leave for 3 minutes to allow time for the red cells to lyse.
- 4 Centrifuge for 10 minutes at RCF 500–700× g.
- 5 Discard the supernatant fluid. Resuspend the sediment and prepare a wet preparation and a smear for staining.

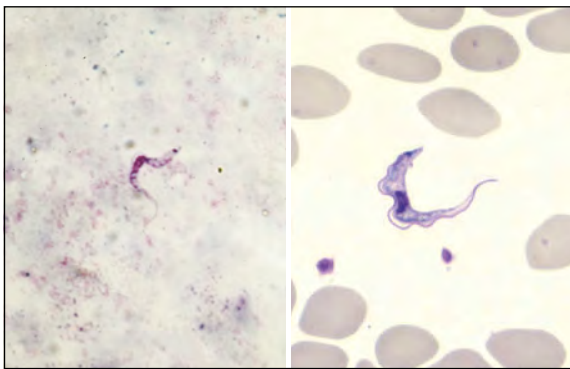


Plate 2.2 Left: *T. b. gambiense/rhodesiense* in Field stained thick blood film. Right: *T. b. gambiense/rhodesiense* in Giemsa stained thin blood film.

Right image: Photo credit CDC DPDx Image library.

Wet preparation to detect motile trypanosomes

Transfer a drop of the sediment to a slide, cover with a cover glass and examine microscopically for motile trypanosomes first using the 10 × objective to focus the sample followed by the 40 × objective.

Ensure the condenser aperture is closed sufficiently to give good contrast. Too bright an illumination will make it difficult to see the trypanosomes.

Preparation of smear for staining

Transfer approximately 20 µL of sediment to a slide and spread to make a thin smear. Allow to air-dry.

Fix the smear with absolute methanol for 2 minutes and allow to dry. Stain the smear using Giemsa stain or Field rapid staining technique as described in Section 2.1. Place a drop of immersion oil on the smear, cover with a cover glass and examine microscopically for trypanosomes using the 40 × objective.

For updates on lyzed blood trypanosome concentration techniques: Readers are referred to the Foundation for Innovative New Diagnostics (FIND) website www.finddx.org

E-mail info@finddx.org

T. b. rhodesiense and *T. b. gambiense* trypanosomes

- Trypanosomes measure 13–42 µm in length and may show a variety of forms (pleomorphic).*

* In the early stages of acute African trypanosomiasis, long slender trypanosomes (often seen dividing) can be found. In the later stages, intermediate and short trypanosomes, some having no free flagellum, may be seen.

- Single flagellum arises from the kinetoplast. It extends forwards along the outer margin of the undulating membrane and usually beyond it as a free anterior flagellum.
- Small dot-like kinetoplast stains darkly.
- Nucleus stains dark-mauve and is usually centrally placed but posterior nuclear forms may also be seen.
- Cytoplasm stains palely and contains granules.

Note: Microscopically, the trypanosomes of *T. b. rhodesiense* and *T. b. gambiense* are morphologically indistinguishable.

Using *Primo Star iLED* fluorescence microscope to detect trypanosomes

The *Primo Star iLED* microscope enables lyzed blood samples to be stained using acridine orange and examined by fluorescence microscopy which has been reported as improving the detection of trypanosomes with low parasite concentration in endemic areas.³ The microscope is described in Section 2.11.

Using acridine orange, trypanosomes and white cells fluoresce orange and can be easily seen against a dark background as shown in Plate 2.3. The lyzed blood technique described previously can be used to concentrate the parasites prior to staining. Because the *Primo Star iLED* microscope is both a brightfield transmitted light and fluorescence microscope, both an acridine orange and a Giemsa or Field stained smear can be examined.

Acridine orange staining technique

- 1 Prepare a 20 µL smear for staining using the sediment from a lyzed blood concentration technique, described previously.
- 2 Fix the smear with absolute methanol for 2 minutes. Allow to air-dry.

- 3 Stain the smear with working acridine orange stain for 3 minutes.*
 - * Prepare a stock acridine orange solution at 1 mg/mL in distilled water. Dilute the stock solution 1 in 10 in phosphate buffered saline, pH 7.4 (obtainable as a powder from Sigma-Aldrich, cat. No P3813).
- 4 Briefly wash off the stain with phosphate buffered saline, pH 7.4 (do not over-wash).
- 5 Wipe the back of the slide clean and place it in a draining rack for the smear to air-dry.
- 6 Examine microscopically for orange fluorescing trypanosomes using the 40 × objective.
- 7 Examine also a Giemsa or Field stained preparation using brightfield illumination to confirm the morphology of the trypanosomes.

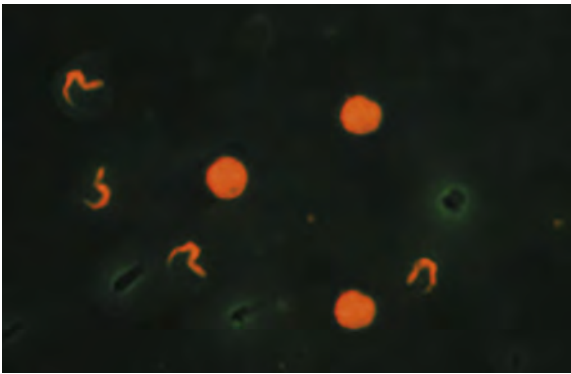


Plate 2.3 *T. b. gambiense*/*T. b. rhodesiense* as seen in an acridine orange stained lyzed blood preparation using fluorescence microscopy and 40 × objective. The trypanosomes and white cells fluoresce orange.

Microhaematocrit technique to concentrate trypanosomes

Although *T. b. rhodesiense* trypanosomes are usually easier to find in the blood than *T. b. gambiense* trypanosomes, they can be very few and difficult to detect in unconcentrated preparations.

The microhaematocrit technique is rapid and recommended for detecting motile trypanosomes in blood when a microhaematocrit centrifuge is available. It also enables the packed cell volume (PCV) to be measured to check whether the patient is anaemic.

Note: The capillary tube technique is not suitable in areas where *Mansonella* or other species of microfilariae are likely to be present with trypanosomes in the blood. In such areas it is best to examine a stained preparation from a lyzed blood concentration technique.

Procedure

- 1 Fill to about 10 mm from the top, two heparinized capillary tubes with capillary blood or two plain capillary tubes with fresh EDTA anticoagulated blood.

Note: Examination of four or more capillary tubes is recommended to improve detection of the parasites.

- 2 Seal the dry top end of each capillary tube by rotating it in a suitable sealant, e.g. *Cristaseal*.
- 3 Centrifuge the capillaries in a microhaematocrit centrifuge for 3–5 minutes.
 - Caution:** Handle the blood and capillary tubes with care. Infection can occur if viable organisms penetrate the skin or mucous membranes.
- 4 Wipe clean the area of each capillary where it will be viewed, i.e. where the red cell column joins the white cell layer (buffy coat) and plasma.
- 5 Mount the two capillaries on a slide, supported on two strips of plasticine or *Blutak* as shown in Fig. 2.6. Using a cloth or tissue, gently press to embed the capillaries in the plasticine.
- 6 Fill the space between the two capillaries with clean water and cover with a cover glass.
- 7 Examine immediately the plasma just above the buffy coat layer for motile trypanosomes. Use a 20× objective or if unavailable use a 10× objective, making sure the condenser iris is closed *sufficiently* to give good contrast, or preferably use dark-field microscopy. Use the 40× objective to confirm that the motility is due to trypanosomes.

The trypanosomes are very small but can be detected with *careful* focusing and providing

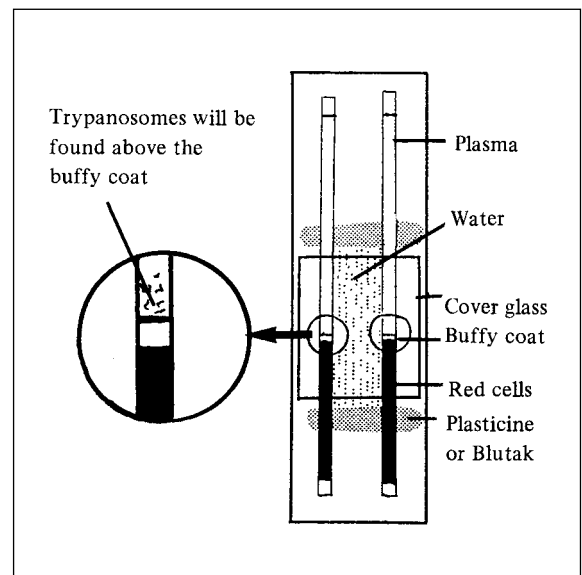


Fig. 2.6 Microhaematocrit centrifugation technique showing capillaries mounted on a microscope slide with close up of the area (circle) in which to look for trypanosomes.

the light is not too intense and the glass of the capillaries and cover glass is *completely* clean.

Important: The preparation must be examined within a few minutes of the blood being centrifuged otherwise the trypanosomes will migrate into the supernatant plasma and be missed. Also, the trypanosomes will gradually become less active and therefore more difficult to detect.

Presence of microfilariae

Motile microfilariae will make it impossible to see whether there are also trypanosomes. When this occurs it is best to examine a stained preparation from a lyzed blood concentration technique described previously. Breaking capillaries to obtain a preparation for staining is *NOT* recommended for safety reasons.

EXAMINATION OF LYMPH GLAND ASPIRATES

In the early stages of African trypanosomiasis, especially in gambiense infections, trypanosomes can often be found in fluid aspirated from a swollen lymph gland (sensitivity of 40–80%).

- 1 Prepare a small syringe by rinsing it out with sterile isotonic saline. Pull back the plunger half way, ready for use.

Note: Do not use sterile water to rinse the syringe because trypanosomes are rapidly destroyed in water.

- 2 Wearing sterile protective gloves, locate the swollen gland and holding it firmly, gently pull it towards the skin surface.
- 3 Cleanse the swollen area with a spirit swab. Insert a sterile, size 18 gauge needle (without the syringe attached) into the centre of the gland (see Plate 2.4).
- 4 Without moving the needle, gently massage the gland to encourage the fluid to enter the needle. Avoid stirring the needle as this will cause unnecessary pain.
- 5 Remove the needle, and holding it over a slide, carefully attach the syringe. With care, slowly expel the contents of the needle onto the slide. Immediately cover the sample with a cover glass to prevent it from drying on the slide.
- 6 Place a small sterile dressing over the needle wound.
- 7 With the minimum of delay, examine the entire preparation microscopically for motile trypanosomes using the 40× objective with the

condenser iris adjusted to give good contrast.

Note: If only a small amount of fluid is aspirated which is insufficient to examine as a wet preparation, allow the fluid to dry on the slide, fix it with two drops of absolute methyl alcohol and stain by Field rapid technique for thin films or by Giemsa method (see Section 2.1). Examine the stained preparation as described previously.

Examination of chancre fluid for trypanosomes

When a chancre is present (more commonly seen in early *T. b. rhodesiense* infections than in *T. b. gambiense* infections), trypanosomes can often be detected in fluid aspirated from the swelling. The method of collecting and examining chancre fluid is as follows:

- 1 Cleanse the chancre with a spirit swab. Wearing sterile protective gloves, puncture the chancre from the side with a small sterile needle. Blot away any blood.
- 2 Gently squeeze a small amount of serous fluid from the chancre and transfer it to a clean slide. Cover with a cover glass. Examine microscopically for motile trypanosomes as described previously for lymph gland aspirates.
- 3 Place a sterile dressing over the needle wound.

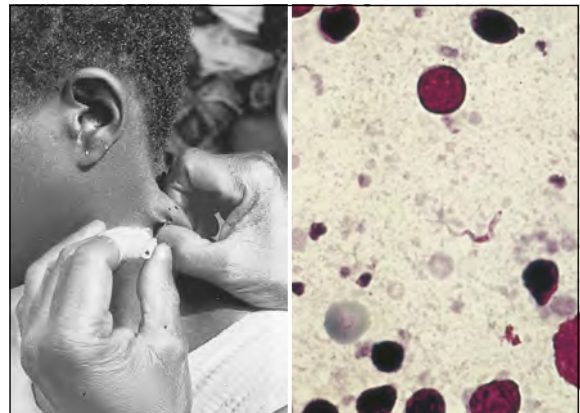


Plate 2.4 *Left:* Collecting lymph fluid to examine for *T. b. gambiense*. *Right:* *T. b. gambiense* in Field stained smear of lymph fluid.

EXAMINATION OF CSF IN AFRICAN TRYPANOSOMIASIS

When there is involvement of the CNS in late stage African trypanosomiasis, the following may be found in CSF:

- few trypanosomes
- more than 5 white cells/ μL
- morula (Mott) cells (IgM producing plasma cells)
- IgM (when measurement is available), usually more than 10% of the total CSF protein
- raised total protein

Caution: As previously mentioned, CSF should only be collected after treatment to kill the trypanosomes in the blood has been started otherwise a traumatic lumbar puncture may introduce trypanosomes into the CNS.

Procedure

Important: The CSF must be examined *as soon as possible* after it has been collected. This is because the trypanosomes are unable to survive for more than 15–20 minutes in CSF once it has been removed. The organisms rapidly become inactive and are lysed.

- 1 Report the appearance of the fluid.
A normal CSF is clear and colourless. In meningoencephalitis due to trypanosomiasis the CSF usually appears clear to slightly cloudy (large numbers of pus cells are not found as in bacterial meningitis).
- 2 Gently mix the CSF and perform a white cell count.
When trypanosomes have invaded the CNS, the CSF will usually contain more than 5 cells/ μL and these will be mostly lymphocytes.
- 3 Centrifuge the CSF at medium to high speed (RCF about 1 000 \times g) for 10 minutes to sediment the trypanosomes and cells.
- 4 Using a plastic Pasteur pipette, carefully transfer the supernatant fluid to another tube. This can be used for measuring the IgM and total protein concentration.
- 5 Mix the sediment and if a microhaematocrit centrifuge is available, collect the sediment in a capillary tube. Place 1 drop on a slide (to make a stained smear) and centrifuge the remainder for 2 minutes. Mount the capillary on a slide as described for the blood microhaematocrit technique and examine microscopically near to the sealed end for motile trypanosomes (as soon as possible after centrifuging).
Alternatively, if unable to concentrate the trypanosomes using a microhaematocrit centrifuge, transfer the sediment to a slide, cover with a cover glass and examine for motile trypanosomes using the 10 \times and 40 \times objectives with the condenser iris reduced to give good contrast. A careful search of the entire preparation is required because only a few trypanosomes will be present. Do *not* use too intense an illumination.
- 6 If no trypanosomes are found but cells are present, examine a stained smear prepared

from the wet preparation. Remove the cover glass. Allow the preparation to dry, fix the smear with methanol for 2 minutes, and stain using Field technique for thin films or Giemsa technique (see Section 2.1). When dry, spread a drop of immersion oil on the preparation and examine for trypanosomes and morula cells.

Appearance of morula cells

Morula (Mott) cells are larger than small lymphocytes. The nucleus stains dark mauve, and the cytoplasm (may be scanty) stains blue.

Characteristic vacuoles can be seen in the cytoplasm as shown in Plate 2.5. When found in CSF, morula cells usually indicate trypanosome infection of the CNS.

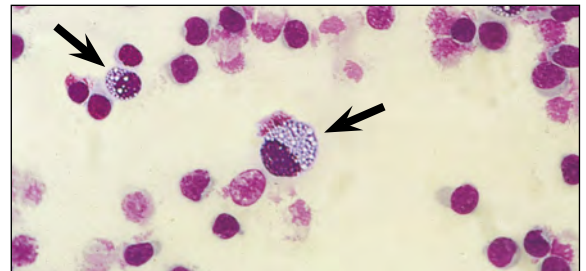


Plate 2.5 Morula cells (IgM producing plasma cells) in Giemsa stained CSF (marked with arrow).

- 7 If no trypanosomes have been found, measure the total protein concentration of the CSF and if possible test also for IgM.

When trypanosomes have invaded the CNS, the CSF total protein will be raised. Very occasionally in the early stages of CNS involvement, trypanosomes can be found without a rise in CSF protein but this is rare.

CSF results in late stage trypanosomiasis

Pressure.....	Increased
Appearance.....	Clear to slightly cloudy
Cell count.....	More than 5 cells/ μL
IgM.....	Positive (>10% total protein)
Total protein.....	More than 0.4 g/L (40 mg/dL)
Pandy's test*.....	Positive
Wet preparation.....	Trypanosomes may be seen
Stained smear.....	May contain few trypanosomes, lymphocytes, morula cells

* Pandy's test is of value only if unable to measure total protein.

Note: IgM may also be detected in the CSF of patients with viral meningitis, tuberculous meningitis and neurosyphilis but usually in amounts less than 10% of CSF total protein.

GAMBIENSE TRYPANOSOMIASIS ANTIBODY TESTS

Tests to detect antibody in those infected with *T. b. gambiense** include the Card Agglutination Trypanosomiasis Test (CATT) and recently developed lateral flow immunochromatographic (ICT) tests.

* The tests cannot be used to detect antibodies in those infected with *T. b. rhodesiense*.

CATT/*T. b. gambiense*

The CATT/*T. b. gambiense* is a sensitive latex agglutination antibody test which is used to screen communities for gambiense trypanosomiasis. The test detects antibodies against *T. b. gambiense* parasites expressing variable antigen type LiTat 1.3.

Occasionally false negative tests occur when patients are infected with strains of trypanosomes that do not express the LiTat 1.3 gene. False positive reactions can occur in patients with other parasitic diseases such as malaria and filariasis.

In the CATT/*T. b. gambiense*, one drop of whole blood (screening test) or 25 µL of diluted plasma/serum (confirmatory test to exclude cross-reactions) is mixed with one drop of reconstituted antigen. When antibodies are present, agglutination occurs after 5 minutes rotation at 60 rpm. Antibodies are present in a patient's blood for several months following treatment.

The freeze dried antigen and control sera should be kept at between 2–8 °C during transport and storage. The shelf-life of the antigen, control sera, and buffer is 1 year when refrigerated at below 10 °C. The reconstituted reagents can be used for up to 7 days when stored at 2–8 °C or up to 8 hours under field non-refrigerated conditions. The antigen suspension must not be frozen.

ICT *T. gambiense* antibody tests

Unlike the CATT, the recently developed lateral flow ICT tests are rapid diagnostic tests (RDTs) that can be stored at ambient temperature and are stable for up to 24 months. This enables testing to be more easily carried out in endemic sleeping sickness areas. Test results are read after 15 minutes.

The ICT tests incorporate more antigens to detect

T. gambiense antibody than the CATT, enabling the disease to be detected in patients infected with parasite strains not expressing the LiTat 1.3 gene. Currently two *T. gambiense* lateral flow ICT tests are commercially available:

- HAT Sero K-SeT, developed by Coris Bio Concept in collaboration with the Institute for Tropical Medicine in Antwerp. Preliminary studies report the test as having high sensitivity and specificity among disease suspects in field conditions.⁴ A second generation of tests is being developed using recombinant parasitic antigens to reduce the unit cost of the test.
- SD Bioline HAT 2 Test, developed by Alere/Standard Diagnostics in collaboration with the Foundation for Innovative New Diagnostics (FIND). This second generation RDT uses recombinant antigens to reduce cost and facilitate standardization and control. Test evaluations are underway in sleeping sickness endemic areas. The first generation test was reported as being optimal for case detection in both passive and active screening with a higher sensitivity than the CATT.⁵ The specificity of the test was reported as greater than 97%, slightly less specific than the CATT.

Note: As highly sensitive tests will be required in the elimination stage of sleeping sickness, it is expected that higher performance ICT tests will be developed. Other technologies are also becoming available such the loop-mediated isothermal amplification (LAMP) technique for staging the disease which can be performed in laboratories with facilities for DNA amplification.

CHAGAS DISEASE

Trypanosoma cruzi causes Chagas disease, also referred to as American trypanosomiasis.

Distribution

T. cruzi is found in tropical and subtropical South and Central America from Mexico to Argentina and also in other parts of the world due to people migrating from endemic areas. In 2017 the WHO estimated that there were between 6 to 7 million people infected with *T. cruzi* worldwide with approximately 7000 deaths occurring annually.⁶

Transmission and life cycle

T. cruzi is mainly transmitted through contact with the faeces of an infected blood-sucking bug of the family Reduviidae, subfamily Triatominae. The faeces

containing infective trypomastigotes are deposited on the skin or mucous membranes as the bug feeds from its host. Infection occurs when irritation at the bite site causes the infected faeces to be rubbed in to the wound.

Vectors of *T. cruzi*

More than 80 species of triatomine bugs, are capable of transmitting *T. cruzi* but the most important vectors are those that are well adapted to living in human dwellings.

Adult triatomine bugs are winged and invade houses at night, often attracted by light. All bug stages can also be carried in to houses on roofing or with personal belongings. In houses colonized by bugs, several thousand may be found in the cracks of walls and in roofs, feeding from humans, domestic animals, and rodents. Chickens, although not infected with *T. cruzi*, often provide a source of blood for large numbers of triatomine bugs.

Animal reservoirs of *T. cruzi* include dogs, opossums, cats, small marsupials, rabbits, armadillos, mice, guinea pigs, and bats.

T. cruzi can also be transmitted by:

- blood transfusion from infected donors,
- transfer of *T. cruzi* from an infected mother to her newborn child during pregnancy or childbirth,
- ingestion of food or fruit juice contaminated with *T. cruzi* infected faeces or urine e.g. from triatomine bugs, rodents or marsupials,
- organ transplants from infected donors,
- laboratory-acquired infection.

The life cycle of *T. cruzi* is summarized in Fig. 2.7. Following penetration, the metacyclic trypomastigotes invade muscle and other tissue cells near the point of entry and multiply intracellularly as amastigotes. Structurally the amastigotes of *T. cruzi* resemble those of *Leishmania* species. The amastigotes develop into trypomastigotes which are released into the blood. No multiplication of the parasite occurs in its trypomastigote stage in the blood.

The trypomastigotes reach tissue cells, especially those of heart muscle, nerves, skeletal muscle, and smooth muscle of the gastrointestinal tract. The trypomastigotes become amastigotes and multiply, forming pseudocysts.

The amastigotes develop first into epimastigotes and then into trypomastigotes which are released into the blood when the host cell ruptures. Some of these trypomastigotes continue to circulate while the majority infect further tissue cells.

The life cycle is continued when a triatomine bug ingests trypomastigotes in a blood meal. In the vector, the trypomastigotes develop into epimastigotes

which multiply by binary fission in the gut of the bug. Within 10–15 days, metacyclic trypomastigotes are formed and can be found in the hindgut of the bug, ready to be excreted when the vector defaecates as it takes a blood meal.

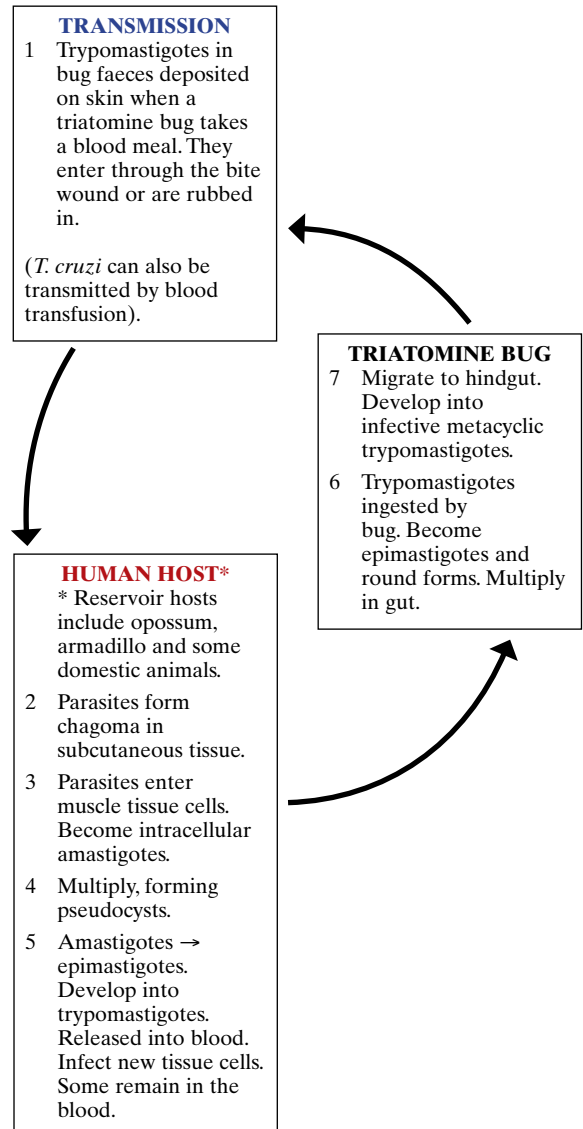


Fig 2.7 Transmission and life cycle of *Trypanosoma cruzi*.

Multiplication of *T. cruzi* at the site of infection, together with a host cellular immune response, can produce an inflamed swelling known as a chagoma which usually persists for several weeks. If the site of infection is the eye, usually the conjunctiva becomes inflamed and oedema forms. This is known as Romana's sign.

Geographically, there are differences in the clinical features and pathology associated with *T. cruzi* infection which may be due to variations in the strains of *T. cruzi* found in different areas and to host factors.

Clinically there are two phases of Chagas disease:

- Acute Chagas disease
- Chronic Chagas disease

Acute Chagas disease

The initial acute phase lasts for up to 2 months following infection. It is usually asymptomatic and often passes unnoticed or there may be fever, headache, enlarged lymph glands, muscle pain, difficulty in breathing, abdominal or chest pain. Lymphocytosis is common and peripheral blood films often resemble those seen in glandular fever.

During the acute phase, trypomastigotes circulate in the blood and can be detected microscopically. The parasites spread throughout the body, invade many cells and multiply intracellularly as amastigotes. Activation of the immune system leads to parasite numbers reducing to undetectable levels. Early detection of *T. cruzi* infection with effective treatment reduces the risk of chronic Chagas disease developing.

Acute disease is most commonly seen in young children. Occasionally an acute attack can cause damage to the heart or result in other serious complications which may be life-threatening.

Chronic Chagas disease

Chronic Chagas disease may be asymptomatic and last for several years during which time the infected person can transmit the disease to others. The parasites are present mainly in heart and intestinal muscle.

In up to 30% of infected persons, symptomatic chronic Chagas disease can develop with cardiac disorders including weak and irregular heart beat, enlargement of the heart and oedema. Severe damage to heart muscle and nerves leads to progressive heart failure. In some areas Chagas disease cardiopathy is the leading cause of cardiac disease in young persons.

In up to 10% of persons with chronic Chagas disease, parasite infection of intestinal muscle can damage nerves in the intestinal wall leading to loss of muscular action. The accumulation and slow movement of food lead to enlargement of the oesophagus and colon.

Coinfection with HIV

This can lead to severe myocarditis, central nervous system involvement with meningoencephalitis and in the absence of antiretroviral treatment, the reactivation of latent infections. *T. cruzi* may be detected in CSF. Frequently meningoencephalitis caused by *T. cruzi* is misdiagnosed as toxoplasmosis. Serological tests for Chagas disease are frequently less sensitive due to weak antibody responses in those infected with HIV.

Measures to prevent and control Chagas disease

- Spraying houses, sheds, latrines and surrounding areas with residual insecticides and painting houses with slow-acting insecticide paint.
- Improving rural housing, especially the plastering of cracked wall surfaces with cement or a strong local clay-mud plaster that will not crack.
- In areas where the vector is *Rhodnius prolixus*, replacing palm roofing with corrugated sheeting.
- Removing animal reservoirs of *T. cruzi* from houses and also chickens (from which bugs feed).
- Using bed nets to protect against vector bites.
- Improving food hygiene to prevent food and drink from becoming contaminated.
- Screening pregnant women and newborns in endemic areas to reduce the risk of mother to child transmission. Other children of a mother found to be infected should also be tested for *T. cruzi* infection.
- Carrying out serological surveys to detect new endemic foci in areas under control.
- Educating communities on transmission and early symptoms of *T. cruzi* infection, the importance of early treatment and ensuring diagnostic facilities and effective treatment are accessible.
- Screening donor blood for *T. cruzi* infection.
- Testing donors of organs for infection prior to transplantation.

Further information on the control of Chagas disease: Readers are referred to the paper of Sosa-Estani S, Segura E L. Integrated control of Chagas disease for its elimination as public health problem – A Review. 2015 Vol 110(3), 289–298. *Mern Inst Oswaldo-Cruz Rio de Janeiro*. The Review can be accessed electronically by entering the title in Google.

LABORATORY DIAGNOSIS

Acute Chagas disease

- Examination of a wet blood preparation for motile trypomastigotes.
- Lysed blood concentration technique.
- Microhaematocrit concentration technique.

Chronic Chagas disease

- Serological techniques to detect *T. cruzi* antibody.

Note: Other techniques used in specialist laboratories to diagnose Chagas disease include blood culture, artificial xenodiagnosis and PCR.

EXAMINATION OF BLOOD FOR TRYPANOSOMES

Detection of circulating trypanosomes in the early stages of *T. cruzi* infection is of great importance because at this stage effective treatment can be given which may prevent cardiac and gastrointestinal disease associated with chronic Chagas disease developing later in life.

The laboratory must therefore examine blood samples *carefully and for a sufficient length of time*.

Examination of a wet blood preparation

The trypanosomes of *T. cruzi* are fragile organisms easily lysed in thick blood films (as the blood dries) and easily damaged when spreading blood to make thin blood films.

The following technique is based on detecting motile trypanosomes.

Procedure

- 1 Apply a small drop of capillary blood or fresh anticoagulated blood to a slide and cover with a cover glass. Do not make the preparation too thick.

Caution: Wear protective gloves and handle the blood sample with care. Infection with *T. cruzi* can occur if parasites penetrate the skin, conjunctiva or mucous membranes.

- 2 Immediately examine the entire preparation microscopically using the 10× objective to focus the sample followed by the 40× objective to detect motile trypanosomes. Adjust the condenser iris to give good contrast or preferably use dark-field or phase contrast microscopy.

If no parasites are detected, use a concentration technique (see later text).

Differentiating *T. cruzi* from *T. rangeli*

T. rangeli is a non-pathogenic species transmitted by *Rhodnius* bugs. It can be found with *T. cruzi* mostly in Central America, Venezuela, Brazil and Columbia. In these areas all positive preparations should be checked to confirm that the trypanosomes seen are *T. cruzi* and not *T. rangeli*. This can be done by examining a Giemsa or Field stained thin blood film.

The appearance of *T. cruzi* and *T. rangeli* is shown in Plate 2.6.

T. cruzi trypanosomes

- Usually C-shaped, measuring 12–30 μm in length with a narrow membrane and free flagellum.
- Has a *large*, round to oval, dark-red staining kinetoplast at the posterior end.
- Nucleus is centrally placed and stains red-mauve.

Note: Occasionally slender forms of *T. cruzi* can be seen which have an elongated nucleus, subterminal kinetoplast and shorter free flagellum.

T. rangeli trypanosomes

Compared with *T. cruzi*, the trypanosomes of *T. rangeli* are longer and thinner, measuring 27–32 μm in length, have a long pointed posterior end and a much smaller kinetoplast which is situated a little way from the posterior end. The nucleus is in the anterior third of the body unlike in *T. cruzi* where it is central.

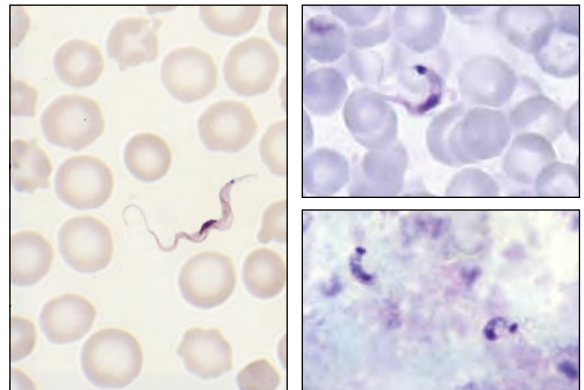


Plate 2.6 *Left:* *T. rangeli* in Field stained thin blood film. *Right upper:* *T. cruzi* in Giemsa stained thin blood film. *Right lower:* *T. cruzi* in Giemsa stained thick blood film. Right images, Photo credit CDC DPDx Image library

Important: To minimize damage to the parasites when spreading the thin blood film, hold the spreader at an angle of 60–70° and use as little pressure as possible when spreading the blood. Air-dry the film rapidly and immediately fix it with absolute methyl alcohol for 1–2 minutes. When dry stain the film by the Giemsa or Field technique for thin films as described in Section 2.1.

Concentration techniques to detect *T. cruzi*

The lysed blood concentration technique described

previously for trypanosomes that cause African trypanosomiasis can be used to concentrate *T. cruzi* parasites.

Note: As explained previously, in areas where *T. cruzi* occurs with *T. rangeli*, it is important to check any trypanosomes detected are *T. cruzi*.

The microhaematocrit concentration technique described previously for detecting trypanosomes that cause African trypanosomiasis can also be used for detecting *T. cruzi* parasites. Examining four capillary tubes increases the chances of detecting the trypanosomes.

Confirm that any trypanosomes detected are *T. cruzi* by examining a thin blood film (prepared from a lysed blood preparation).

SEROLOGICAL DIAGNOSIS OF CHAGAS DISEASE

Tests used in the serological diagnosis of Chagas disease and screening of donor blood include enzyme-linked immunosorbent assay (ELISA), indirect haemagglutination assay (IHA), indirect immunofluorescence assay (IFA), and rapid immunochromatographic tests. These tests become positive about 1 month following infection and usually remain positive after treatment.

Interpretation of test results can be difficult due to assays cross-reacting with other parasites such as *Leishmania* species and *T. rangeli*. To improve diagnostic accuracy the Pan American Health Organization (PAHO) suggests using two antibody assays based on different techniques.

More recently developed antibody tests use recombinant antigens, and/or synthetic peptides to increase sensitivity and specificity, making it possible to confirm or exclude a diagnosis of *T. cruzi* infection in those with chronic Chagas disease. Such assays include *BioELISA Chagas* (Biokit, Spain), *Chagatest ELISA* (Wiener Lab. Argentina) and *Chagas AB Rapid test* (Standard Diagnostics, Korea).⁷

ELISA techniques can be performed on finger-prick blood collected onto filter paper. Dried filter paper blood samples should be stored frozen in a sealed bag containing a desiccant (e.g. silica gel) until they can be tested.

Further information

Readers are referred to the 2019 WHO/PAHO publication: *Guidelines for the diagnosis and treatment of Chagas' disease*. Access the publication by entering the title in Google.

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2.3 Leishmaniasis

Leishmania parasites cause:

- **Visceral leishmaniasis** in which *Leishmania* organisms parasitize the reticuloendothelial system (liver, spleen, bone marrow, lymph nodes). If left untreated it can be fatal.
- **Cutaneous leishmaniasis**, in which *Leishmania* organisms parasitize the skin causing ulceration which may lead to life-long scars and disabilities. It can be localized, disseminated (diffuse), mucocutaneous (mucosal), or lupoid.

Leishmania species that cause visceral and cutaneous leishmaniasis include:

Disease	Species
VISCERAL	<i>L. donovani</i> , <i>L. chagasi</i> *
LEISHMANIASIS	<i>L. infantum</i> *

* *L. chagasi* and *L. infantum* are now recognized as the same organism.

CUTANEOUS LEISHMANIASIS

Cutaneous leishmaniasis (localized)
L. tropica, *L. major*
L. infantum, *L. aethiopica*
L. mexicana, *L. (V.) peruviana**
*L. (V.) guyanensis**
*L. (V.) panamensis**
*L. (V.) braziliensis**

Disseminated leishmaniasis
L. aethiopica, *L. amazonensis*
L. mexicana, *L. venezuelensis*

Mucocutaneous leishmaniasis
*L. (V.) braziliensis**
L. (V.) panamensis
*L. (V.) guyanensis**

Lupoid leishmaniasis
L. tropica

* Belong to the subgenus *Viannia*. Other species belong to the subgenus *Leishmania*. Treatment is different depending on species.

Distribution¹

Annually it is estimated that 70 000 – 1 million people become infected with leishmaniasis with 20 000 to 30 000 deaths. Visceral leishmaniasis is endemic in more than 80 countries. In 2020, more than 90% of new cases of visceral leishmaniasis were reported from Brazil, People's Republic of China, Ethiopia, Eritrea, India, Kenya, Somalia, South Sudan, Sudan and Yemen. In East Africa outbreaks occur frequently.

About 95% of cutaneous leishmaniasis occur in the Americas, Mediterranean basin, the Middle East and Central Asia. Over two thirds of new cases of cutaneous leishmaniasis occur in Afghanistan, Algeria, Brazil, Colombia, Islamic Republic of Iran, the Syrian Arab Republic, Pakistan and Peru.

It is estimated 95% of mucocutaneous leishmaniasis occur in Brazil, Peru and Ethiopia.

Transmission and life cycle

Leishmania species are transmitted by the bite of an infected female sandfly, belonging to the genus *Phlebotomus* in Africa, Asia and Europe and the genus *Lutzomyia* in the Americas. About 90 species of sandflies act as vectors, infecting humans and animal reservoir hosts.

Sandfly vectors

The feeding, breeding and flight habits of sandflies are species specific. Most sandflies feed mainly on plant juices, but female flies also require blood meals for egg development. Most species feed at night, dusk or dawn. Sandfly habitats include rotting leaves under trees, excreta, termite hills and rodent burrows. *Lutzomyia* sandflies inhabit forest areas.

Transmission of leishmaniasis is related to poverty, insanitary conditions, poor housing, living in close proximity to sandfly habitats and animal hosts, e.g. canine hosts and environmental changes such as deforestation, irrigation schemes and urbanization. Increases in transmission occur when non-immune people are brought into closer contact with sandfly vectors and reservoir hosts through work activities or population displacement caused by conflict, war, drought, flooding or other natural disaster. Climate changes affecting vectors and animal hosts can also influence transmission. Leishmaniasis can also be transmitted congenitally and through infected donor blood, transplantation of infected organs and through contaminated needles and syringes.

The life cycle of *Leishmania* species is summarized in Fig. 2.8. A person becomes infected when promastigotes are inoculated at the time a female sandfly takes a blood meal. The promastigotes are taken up by macrophages and develop into intracellular forms called amastigotes. They multiply, rupture from the macrophages and infect new cells.

In visceral leishmaniasis the amastigotes multiply in the macrophages of the spleen, liver, bone marrow, lymph glands, mucosa of the small intestine and other tissues of the reticuloendothelial system. Blood monocytes are also infected. In cutaneous leishmaniasis the parasites multiply in skin macrophages (histiocytes).

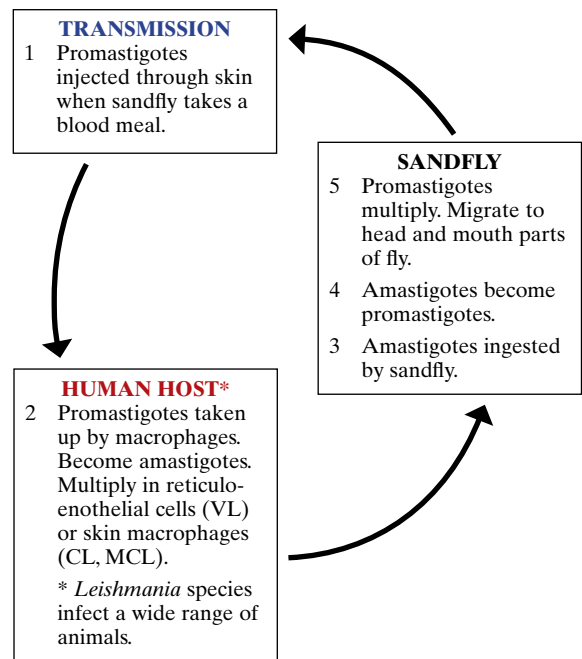


Fig 2.8 Transmission and life cycle of *Leishmania* parasites. VL: Visceral leishmaniasis, CL: Cutaneous leishmaniasis, MCL: Mucocutaneous leishmaniasis.

The life cycle is continued when intracellular and free amastigotes are ingested by a female sandfly. After about 72 hours, the amastigotes become flagellated promastigotes in the midgut of the sandfly. They multiply and fill the lumen of the gut. After 4–18 days (depending on species), the promastigotes move forward to the head and mouth-parts of the sandfly. The salivary glands are not parasitized.

Note: Species in the *Viannia* subgenus develop in the hindgut of the sandfly before migrating to the midgut and foregut.

Measures to prevent and control leishmaniasis

- Early detection and treatment of infected persons, especially in areas where humans are the only or important reservoirs of infection.
- Personal protection from sandfly bites by:
 - Using insect repellents and wearing clothing that covers exposed skin.
 - Using long-lasting insecticide impregnated bednets.
 - Avoiding sandfly habitats especially at times when the sandflies are most active.
- Vector control by the use of light traps, sticky paper traps, or residual insecticide spraying of houses and farm buildings where this is practical.
- Destruction of stray dogs in areas where dogs are the main reservoir hosts.
- Elimination and control of rodents in areas where these are sources of human infections.
- Whenever possible, siting human dwellings away from the habitats of animal reservoir hosts where sandflies are known to breed, e.g. rodent burrows or rocks where hydraxes live.
- Using fine-mesh screening on windows and doors.
- Community health education based on knowledge of local sandfly vectors, their breeding and biting patterns.
- Effective disease surveillance and rapid response during epidemics.

VISCERAL LEISHMANIASIS (VL)

This is the most severe form of leishmaniasis. It is caused by *L. donovani* and *L. infantum* (*L. chagasi*).

In endemic areas, the disease is more chronic with young adults and children being more commonly infected. About three times as many males are affected than females. In epidemics, all age groups are susceptible (except those with acquired immunity) and the disease is often acute. Without treatment, VL is usually fatal.

Symptoms in chronic VL include irregular fever, splenomegaly, hepatomegaly, and/or lymphadenopathy, loss of weight with wasting, diarrhoea, low white cell and platelet counts and anaemia. Skin changes are common. The local

Indian name for VL, *kala-azar* (meaning black sickness) is a reference to the greyish colour of the patient's skin. In acute VL there is splenomegaly, high undulating fever, chills, profuse sweating, rapid weight loss, fatigue, anaemia and leucopenia. Often there is epistaxis (nose bleed) and bleeding from the gums.

In the New World, VL is endemic or sporadic. Asymptomatic infections and subclinical forms of the disease are more common. Malnutrition and other infections increase the risk of developing symptomatic VL.

In active visceral leishmaniasis there is a poor cell-mediated immune response and therefore the parasites multiply rapidly. There is however a humoral response with large amounts of polyclonal non-specific immunoglobulin especially IgG being produced and also specific anti-leishmanial antibody. Patients who have recovered from visceral leishmaniasis are immune from reinfection but relapses can occur, particularly in those coinfecting with HIV.

Post kala-azar dermal leishmaniasis (PKDL)

In India and occasionally in East Africa, a cutaneous form of leishmaniasis can occur about 2 years after treatment and recovery from visceral leishmaniasis. This is referred to as post kala-azar dermal leishmaniasis and affects about 20% of patients in India. Hypopigmented and raised erythematous patches can be found on the face, trunk of the body and limbs. These may develop into nodules and resemble those of lepromatous leprosy, fungal infections or other skin disorders. Occasionally there is ulceration of the lips and tongue. Amastigotes are present in the papules and nodules.

VL HIV coinfection

In areas where both leishmaniasis and HIV infection occur, VL is being increasingly reported in those with immunosuppression caused by untreated HIV. As of 2021, *Leishmania*-HIV coinfection has been reported from 45 countries.

Parasites infect not just the reticuloendothelial system but also the lungs, central nervous system, skin and blood. Parasites have been found in phagocytic cells in the peripheral blood of up to 75% of coinfecting patients (98% in bone marrow aspirates).

Patients with VL/HIV coinfection with low CD4 counts do not respond well to treatment. VL is rapidly progressive in the absence of antiretroviral treatment with high parasite loads. Relapses are common and recurring. The results of different serological tests need to be interpreted with care as 50–75% of coinfecting patients have negative antibody responses.

In southern Europe between 25–70% of adult VL cases are estimated to be HIV-related. Intravenous

drug users are particularly at risk. In other parts of the world, eg. India, East Africa and Brazil the risk of *Leishmania*/HIV coinfection is increasing as VL is becoming more urbanized and HIV infection is becoming more common in rural areas. There are also reports of VL being caused by *Leishmania* strains normally of low virulence and also infections with species associated with CL and MCL.

LABORATORY DIAGNOSIS

Laboratory diagnosis of VL is required when VL is suspected clinically in a patient having fever for more than 2 weeks, enlarged spleen and usually weight loss and anaemia and lives in or has travelled to a VL endemic area.

The laboratory diagnosis of VL is by:

- Detection of amastigotes in:
 - material aspirated from the spleen, bone marrow or an enlarged lymph node,
 - nasal secretions,
 - peripheral blood monocytes and less commonly in neutrophils (buffy coat preparations).

The positivity rates for aspirates and peripheral blood (buffy coat) preparations are as follows:

	<i>% Positive</i>
Spleen aspirate	95–98%
Bone marrow aspirate	64–86%
Enlarged lymph node aspirate	55–64%
Buffy coat (India)	67–99%
Buffy coat (Africa)	About 50%

Note: In VL patients coinfecting with HIV, amastigotes are frequently found in blood monocytes and neutrophils in buffy coat preparations and also in aspirates from *enlarged* lymph nodes.

- Immunodiagnosis, including antibody tests of adequate sensitivity and specificity.

Other tests

- Malaria microscopy or RDT to exclude malaria.
- HIV test when the HIV status is not known in a person with a clinical diagnosis of VL.
- Haematological investigations including:
 - measurement of haemoglobin,
 - total and differential white cell (leucocyte) count,
 - platelet (thrombocyte) count.

In VL, the reticuloendothelial system becomes parasitized and the spleen greatly enlarged. Patients become anaemic, leukopenic and thrombocytopenic. During treatment, a rising haemoglobin and white cell count indicate a good response.

Other haematological findings usually include a positive direct antiglobulin test and often autoagglutination of red cells. Well washed red cells must be used when slide and tube blood grouping and an indirect antihuman globulin technique when crossmatching.

- Plasma albumin levels are greatly reduced (often below 2 g/L) and total protein raised in patients with VL.
- When available, measurement of serum or plasma alkaline phosphatase which is raised in about 40% of VL patients.

DETECTION OF AMASTIGOTES IN BUFFY COAT PREPARATIONS

In up to 50% or more of patients with VL, parasites can be detected in stained buffy coat smears prepared from EDTA (sequestrene) anticoagulated venous blood.

The same EDTA blood sample can be used to measure the haemoglobin and perform white cell and platelet counts.

Procedure

- 1 Collect 2.5 mL of venous blood, dispense into an EDTA container (see Reagent No. 17) and mix gently.
- 2 After performing a haemoglobin, white cell count, platelet count and making a thin blood film, centrifuge the EDTA blood in narrow bore tubes, e.g. *Eppendorf* plastic tubes or glass tubes 6 × 50 mm. Centrifuge for 15 minutes at medium to high speed, i.e. RCF about 1 000 × g to obtain the buffy coat (cream coloured layer of white cells and platelets above the red cells).

Filling narrow bore tubes: Using a long stemmed plastic bulb pipette or Pasteur pipette, aspirate the blood from the EDTA container. Insert the tip of the pipette to the bottom of the tube and fill the tube(s) with blood, withdrawing the pipette as the tube fills.
- 3 Using a plastic bulb pipette or Pasteur pipette, carefully remove and discard the plasma above the buffy coat. Transfer the buffy coat to a slide and mix. Place a drop on another slide and spread to make a thin smear. Air-dry the smear

and fix it with methanol for 1–2 minutes.

- 4 Stain the smear by Giemsa technique or Field rapid technique for thin films as described in Section 2.1.
- 5 When dry, spread a drop of immersion oil on the film and examine microscopically. Use the 40× objective to scan the smear for monocytes containing groups of amastigotes. Occasionally amastigotes can be detected in neutrophils. Use the 100× objective to identify the intracellular amastigotes. The small amastigotes may also be seen lying between cells.

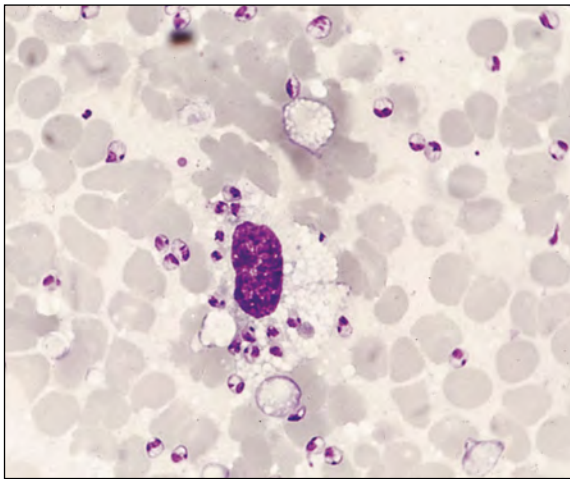


Plate 2.7 *Leishmania* amastigotes in and outside a monocyte in a Giemsa stained buffy coat preparation (100× objective).

DETECTION OF AMASTIGOTES IN ASPIRATES

Splenic aspiration

A splenic aspiration should not be performed when the patient is pregnant, has a soft spleen, is clinically jaundiced, there are signs of active bleeding, or the patient's blood pressure is low. Facilities for blood transfusion should be available should bleeding occur. Following a splenic aspiration the patient's blood pressure, pulse and respiratory rate should be monitored for up to 8 hours or longer if indicated.

Whenever possible, laboratory staff should assist the medical officer performing the aspiration to ensure films of the correct thickness are made, air dried rapidly and methanol fixed immediately. Before a splenic aspiration, perform the following tests:

- Platelet count and prothrombin time to check for abnormal bleeding. The aspiration should *not* be performed if the patient's platelet count is below

Amastigotes of Leishmania species

Structurally, the amastigotes of *Leishmania* species that cause VL, CL and MCL are similar. There are variations in size between species.

- *Small*, round to oval bodies measuring 2–4 μm.
- Can be seen in groups inside blood monocytes (less commonly in neutrophils), in macrophages in aspirates or skin smears, or lying free between cells.
- The nucleus and rod-shaped kinetoplast in each amastigote stain dark reddish-mauve.
- The cytoplasm stains palely.

40 × 10⁹/L (40 000/mm³) or the prothrombin time is 5 seconds or more longer than that of the control (see Further Reading).

Note: In VL field work when a platelet count and blood transfusion facilities are not available, a splenic aspirate should only be performed if the platelet to red blood cell ratio is more than 1:20 (assessed from an EDTA stained blood film) which is equivalent to a platelet count of 100 000/μL (Personal communication: Orgenes Lema).

- Haemoglobin (Hb) to check that the patient is not severely anaemic. The aspiration should not be carried out if the Hb is less than 55 g/L (5.5 g/dL).

Procedure for examining splenic, bone marrow, lymph node aspirates

- 1 Immediately after aspiration, make at least 2 thinly spread smears of the aspirate on *clean* slides. Only a small quantity of aspirate is required. Dilution with blood should be avoided.
- 2 Air-dry the smears as rapidly as possible. Fix by covering each smear with a few drops of absolute methanol or if more convenient, immerse the slides in a container of methanol. Fix for 1–2 minutes.
- 3 Stain the smears by the rapid Field technique for thin films or by the Giemsa staining technique as described in Section 2.1.
- 4 When dry, spread a drop of immersion oil on the smear and examine microscopically. Use the 40× objective to scan the smear for large mononuclear phagocytic cells containing groups of amastigotes. Use the 100× objective to identify the amastigotes.

Note: *L. donovani* amastigotes in splenic and bone marrow aspirates are shown in Plate 2.8.

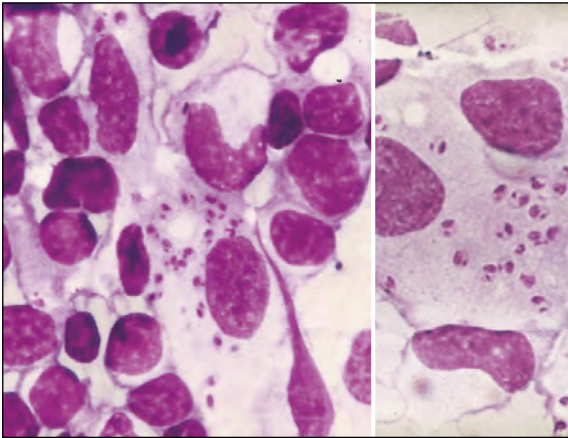


Plate 2.8 Giemsa stained amastigotes of *L. donovani*.
 Right: Bone marrow aspirate. Left: Splenic aspirate. As seen with the 100× objective.

Grading of aspirates for *Leishmania amastigotes**

When amastigotes are seen, systematically examine the smear using the 10× eyepiece and 100× objective, moving from field to field, grading the average parasite density as follows:

GRADE	NUMBER OF PARASITES	MICROSCOPE FIELDS
1+	1–10	1000
2+	1–10	100
3+	1–10	10
4+	1–10	1
5+	10–100	1
6+	> 100	1

* WHO Prevention, diagnosis and treatment of visceral leishmaniasis (Kala-Azar) in Kenya, 2017. National guidelines for health workers. Access from website: www.who.int/leishmaniasis/burden/Kala_Azar_Kenya_2017.pdf

Other findings in aspirates

Other parasites which may be found and should be reported in aspirates include malaria parasites and trypanosomes. If a smear contains pigment and eosinophils this may indicate *Schistosoma* infection. Eosinophils are absent or scanty in visceral leishmaniasis.

IMMUNODIAGNOSIS OF VL

In visceral leishmaniasis, specific antibody as well as non-specific polyclonal Ig G and Ig M are produced. Several serological techniques have been developed to detect and measure specific anti-leishmanial antibodies in patients' sera.

Antibody tests however are unable to distinguish active VL from subclinical or past treated infections. Antibody levels remain detectable up to several years after cure. VL relapse cannot be diagnosed using antibody tests. Detection of parasites is required. Patients should be asked if they have been previously diagnosed and treated for VL.

It has been reported that a significant proportion of healthy individuals living in endemic areas have reactive sera (>10% in low to moderate endemic areas, >30% in high transmission areas). Sera from patients with advanced HIV infection may be non-reactive. A negative test result cannot exclude a diagnosis of VL. In HIV-VL coinfection, antibody tests are negative in 50–75% of patients.

With antibody tests, false positive reactions can occur due to cross reactions with leprosy, tuberculosis, malaria, Chagas disease and schistosomiasis.

In district laboratories antibody tests used in the diagnosis of primary VL include:

- rK39 rapid test
- Direct agglutination test (DAT)

rK39 rapid antibody test

The rK39 test is an immunochromatographic rapid antibody test which is used when a patient is suspected clinically as having VL (fever for more than 2 weeks and splenomegaly or weight loss), has not been previously diagnosed with VL and from whom malaria has been excluded. The test is easy to perform and interpret, takes 10–20 minutes and tests can be stored at ambient temperature (below 30°C). Capillary blood is used and for some tests serum or plasma can also be used. Most tests are in strip format, packaged individually in moisture-proof pouches and all the items needed are provided in the test kit.

The rK39 enables VL testing to be decentralized and carried out at point-of-care providing a test is used that has been validated for the endemic area and those performing the tests are trained adequately in the correct use and interpretation of tests, safe handling of samples and supervisory support is provided technically and clinically.

An example of a rK39 test is the IT LEISH strip test manufactured by Bio-Rad Laboratories (previously DiaMed- IT LEISH, also known as Opti-Leish), shown in Plate 2.9 and represented diagrammatically in Fig. 2.9.

The performance of rK39 varies between endemic regions and test manufacturers. Studies have shown the test to have both high sensitivity and specificity when used to test immunocompetent persons in

the Indian subcontinent and Brazil. In East Africa a lower sensitivity has been reported which is thought to reflect the diversity of kinesin-related proteins found in East African samples. Sensitivity has improved in more recently developed rK28 tests which use additional antigens.² In East African endemic regions, the more sensitive DAT should be performed when a rK39 test is negative in a person suspected clinically as having VL.



Plate 2.9 Positive IT LEISH rK39 test. The following sensitivities and specificities have been reported for IT LEISH rK39 test in different endemic areas.³

	East Africa	Brazil	Indian subcontinent
Sensitivity (95% CI)	87.2%	92.0%	98.8%
Specificity (95% CI)	96.4%	95.6%	97.6%



Fig 2.9 IT LEISH rK39 test. Upper: Negative test showing only a colour band in the control (C) area.

Lower: Positive test showing colour bands in both the Test (L) and control (C) areas.

Courtesy of Bio-Rad Laboratories.

www.biorad.com/diagnostics

Direct agglutination antibody test (DAT)

The DAT is a semi-quantitative sensitive and specific antibody test. It is frequently used in field surveys and when a rK39 test is negative and VL is clinically suspected.

Unlike the rK39 test, the DAT is not a point-of-care test. It requires laboratory facilities and 2–3 days to complete. It can however be performed using samples eluted from dried blood spots (using Whatman No. 93 filter paper) collected in peripheral health units. Serum or plasma can also be used.

The test is performed in V-shaped microtitre plates. The sample is serially diluted from 1 in 100 to 1 in 51200 and antigen added. The antigen is freeze-dried and heat-stable. It consists of fixed promastigotes from *L. donovani* cultures stained with Coomassie brilliant blue. A negative control is used with each plate of tests.

Following incubation at room temperature for 12–18 hours the plates are examined for agglutination. A positive reaction is indicated by a hazy blue mat or cloudy appearance in the plate well. A negative reaction is seen as a dark blue dot. The titre is estimated by comparing the blue dot in the negative control with those of the samples. With a positive test, the reported titre is the last dilution that shows a hazy blue mat.

In endemic areas, the cut-off point for a positive DAT using freeze-dried antigen is 1 in 3200. This can change when using a liquid antigen. With borderline test results (1 in 400 to 1 in 1600), the DAT is usually repeated a few weeks later or parasitology tests are carried out.

The sensitivity of the DAT is reported as > 95% and specificity as > 85%. Cross-reactions are similar to those of the rK39 test and being an antibody test, the DAT cannot be used to diagnose VL relapse or monitor response to therapy.

New VL tests

Recently developed tests which are currently being evaluated include:

- rK28 rapid antibody test
- ELISA *Leishmania* antigen detection in urine
- Detection of *Leishmania* DNA using loop-mediated isothermal amplification (LAMP)

rK28 rapid antibody test

The rK28 test has been developed to improve the serological diagnosis of VL in East Africa. Recent evaluations in Sudan have shown the test to have a sensitivity of 92.5% and specificity of 100%.² The test is called *OnSite Leishmania rK39-Plus* and is being manufactured by CTK Biotech www.ctkbiotech.com

Detecting Leishmania antigen in urine

The ELISA which detects *Leishmania* antigen in urine has been developed to improve the diagnosis of VL in those coinfecting with HIV, assist in the diagnosis of PKDL and VL relapses and in treatment monitoring. The ELISA has been developed by the Foundation for Innovative New Diagnostics (FIND) www.finddx.org and Kalon Biological www.kalonbio.co.uk

Detecting Leishmania DNA using LAMP

The LAMP molecular test has been developed as a highly sensitive and specific diagnostic test for VL, replacing the need for invasive parasitology tests. The LAMP test is a ready-to-use kit using immobilized reagents that can be stored at ambient temperature and an incubator that incorporates an LED illumination unit to view test results in the reaction tubes. Negative and positive controls are included.

The *Loopamp Leishmania Detection Kit* has been developed by FIND in collaboration with Eiken Chemical Co, Japan www.eiken.co.jp/en/

CUTANEOUS LEISHMANIASIS (CL)

The clinical forms of CL vary according to the parasite species, region and response of the patient. The *Leishmania* species that cause cutaneous leishmaniasis (CL) and mucocutaneous leishmaniasis (MCL) are listed at the beginning of this Section.

MCL is the most severe and destructive form of cutaneous leishmaniasis in South America. Lesions are similar in development to those of oriental sore and the resulting ulcers may become very large and long-lasting. Early in the infection, parasites may migrate to tissues of the nasopharynx and palate and sometimes after many years when the first lesion has healed there begins a slow continuous erosion of these tissues. Disfiguration is often extreme with complete destruction of the nasal septum, perforation of the palate and damage to the tissues of the lips and larynx.

Patients with a history of inadequate treatment and prolonged scarring, tend to develop MCL. Mucosal lesions do not heal spontaneously and secondary and often severe bacterial infections can occur. A Sudanese form of MCL is referred to as oronasal leishmaniasis.

Disseminated cutaneous leishmaniasis (DCL)

DCL is usually caused by *L. aethiopica* (Old World) and *L. amazonensis* (New World). Skin lesions develop over a large area of the body. The lesions on the eyebrows, nose and ears resemble those of lepromatous leprosy. At first the lesions are smooth and firm. Later they become scaly and rough. They do not heal spontaneously. DCL relapses tend to occur after treatment.

COVID-19 and cutaneous leishmaniasis

Recent studies have shown that patients with a history of cutaneous leishmaniasis scarring show a reduced incidence of COVID-19 morbidity and mortality⁴. It is not known whether the protection is temporary or long-lasting.

LABORATORY DIAGNOSIS

The laboratory diagnosis of CL and MCL is by:

- Detecting amastigotes in smears taken from infected ulcers or nodules. In MCL, the parasites are scanty and difficult to find in smears.
- Culturing ulcer material and examining cultures for promastigotes.
- Where available, polymerase chain reaction (PCR).

Serological diagnosis of CL and MCL

Because of the poor antibody response in CL, serological tests are of little value in diagnosis. In MCL, antibodies can be found in the serum. District laboratories should contact their nearest Leishmaniasis Reference Laboratory for information on the most appropriate test to use.

COLLECTION AND EXAMINATION OF SLIT SKIN SMEARS FOR AMASTIGOTES

Material for examination should be taken from the inflamed *raised swollen edge* of an ulcer or nodule and also from the centre. Care should be taken to avoid contaminating the sample with blood.

Note: Secondary bacterial contamination makes it difficult to find parasites and therefore if bacterial infection is present, examination for *Leishmania* amastigotes is best delayed until antimicrobial treatment has been completed and the bacterial infection has cleared.

Procedure

- 1 Cleanse the area with a swab soaked in 70% v/v alcohol. Allow to dry completely.
- 2 Firmly squeeze the edge of the lesion between the finger and thumb to drain the area of blood (protective gloves should be worn).
- 3 Using a sterile scalpel blade, make a small cut into the dermis and blot away any blood. Scrape the cut surface in an outward direction to obtain tissue juice and cells.
- 4 Spread the material on a clean slide using a circular motion and working outwards to avoid damaging parasites in those parts of the smear that have started to dry.

The smear must be thinly spread and not left as a thick 'dab' smear. Parasites will be difficult to find in thick smears.

- 5 When dry, fix the smear by covering it with a few drops of absolute methanol. Fix for 1–2 minutes.

- 6 Stain the smear using the Giemsa technique or rapid Field technique for thin films as described in Section 2.1.
- 7 When the smear is dry, spread a drop of immersion oil on it and examine first with the 10× and 40× objectives to detect macrophages which may contain amastigotes (the parasites can also be found outside macrophage cells). Use the 100× oil immersion objective to identify the amastigotes, adding a further drop of oil if required.

Note: The features which identify *Leishmania* amastigotes are described in the previous text. *L. mexicana* amastigotes are larger than those of *L. (V.) braziliensis* and have a more centrally placed kinetoplast.

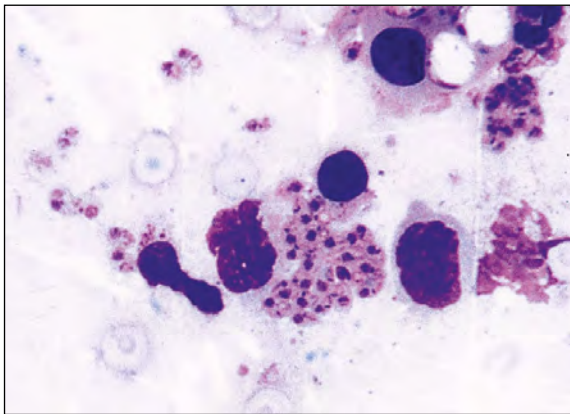


Plate 2.10 *Leishmania* amastigotes in Giemsa stained skin slit smear as seen with the 100× objective.

Culture of ulcer material

Culture is of value when cutaneous leishmaniasis is suspected and parasites cannot be found in smears. In leishmaniasis recidivans, culture is usually required to detect parasites.

Material for culture is best obtained by injecting and then aspirating a small quantity of sterile physiological saline in and out of the hardened margin of the ulcer. A few drops of the final aspirate is used to inoculate the culture medium. *L. tropica* grows rapidly in culture. *L. (V.) braziliensis* grows more slowly in culture than *L. mexicana* and the promastigotes are smaller.

Note: District laboratories should contact their nearest Leishmaniasis Reference Laboratory for advice on the culture medium to use, details of technique and how to minimize contamination of cultures.

Differentiation of *L. (V.) braziliensis* and *L. mexicana*

There are differences in the prognosis and treatment of diseases caused by parasites of the *L. (V.) braziliensis* and *L. mexicana* complexes. It is therefore important to know which organism is causing infection. Whenever possible, positive cultures or serum for serological testing should be sent to a Leishmaniasis Reference Laboratory for identification of the species.

PCR

More recently, polymerase chain reaction (PCR) is being used for *Leishmania* screening and subgenus differentiation (*Viannia* from *Leishmania*) using skin biopsy, blood or bone marrow.

REFERENCES

- 1 WHO *Leishmaniasis Fact Sheet*, <https://www.who.int/news-room/fact-sheets/detail/leishmaniasis>
- 2 Mukhtar M *et al.* Diagnostic accuracy of rK28-based immunochromatographic rapid diagnostic tests for visceral leishmaniasis: a prospective clinical cohort study in Sudan. *Trans R Soc Trop Med Hyg*, 2015 Sep; 109(9):594–600, doi: 10.1093/trstmh/trv060.
- 3 WHO, *TDR Visceral leishmaniasis rapid diagnostic test performance*. Diagnostic Evaluation Series No 4, 2011. Access from the WHO website www.who.int using the Search facility.
- 4 Bamorovat M *et al.* Prophylactic effect of cutaneous leishmaniasis against COVID-19: a case-control field assessment. *Int J Infect Dis*, 2022. doi:10.1016/j.ijid.2021.09.050

FURTHER READING

WHO Guideline for the treatment of visceral leishmaniasis in HIV co-infected patients in East Africa and South-East Asia, 2022. Access by entering the title in Google.

Interactive atlas of leishmaniasis in the Americas: Clinical aspects and differential diagnosis. PAHO, 2021. Access by entering the title in Google.

2.4 Lymphatic filariasis Loiasis Onchocerciasis

Lymphatic filariasis is caused by the filarial worms:

Wuchereria bancrofti, causing 90–95% of infections.

Brugia malayi
Brugia timori

The worms live in the lymphatic vessels and lymph nodes.

Loiasis, also known as Calabar swelling, is caused by the filarial worm:

Loa loa

L. loa lives in the subcutaneous tissues. It is often referred to as the 'African eye worm' because the adult worms can sometimes be seen migrating across the eyelid or conjunctiva.

Onchocerca volvulus

Onchocerca volvulus is a filarial worm that causes onchocerciasis. The disease is also known as river blindness because invasion of the eye can lead to visual impairment and permanent loss of vision.

LYMPHATIC FILARIASIS

Distribution

LYMPHATIC FILARIASIS

Lymphatic filariasis, also known as elephantiasis is a neglected tropical disease (see p. 49). WHO estimates there are 863 million people in 47 countries in the tropics and subtropics at risk of lymphatic filariasis¹. The WHO Programme to eliminate filariasis aims to stop transmission of infection with mass drug administration (MDA), and to alleviate suffering and prevent disability among those affected through an essential package of care. In 2020, COVID-19 restrictions interrupted MDA with possible increase in microfilarial load.

The filarial worms that cause lymphatic filariasis show what is called periodicity, i.e. the microfilariae (1st stage larvae) of the worms are present in the peripheral blood of those infected in greater numbers during certain hours which correspond to the peak biting times of their insect vectors. Distribution of the periodic variants of *W. bancrofti*, *B. malayi* and *B. timori* is as follows:

W. bancrofti

Nocturnal periodic *W. bancrofti* is endemic in tropical Africa, Melanesia, Micronesia, Indonesia, South Asia and South America. The diurnal subperiodic variant of *W. bancrofti* is found mainly in Polynesia. The nocturnal subperiodic variant is found in South and East Asia. *W. bancrofti* is found with *B. malayi* in parts of South East Asia and South India.

B. malayi

B. malayi is endemic in parts of South East Asia including many of the islands of the Malay Archipelago. It also occurs in south west India and Indonesia. Periodic *B. malayi* is commonly found in open swamps and the rice-growing areas of coastal regions. The subperiodic variant is found mostly in fresh water swamps in forests along major rivers.

B. timori

B. timori shows a nocturnal periodicity. It is found only in the Lesser Sunda Islands of Indonesia. The species takes its name from the island of Timor which forms part of the group. It is found in low lying riverine and coastal areas.

Transmission and life cycle

W. bancrofti, *B. malayi*, *B. timori* are transmitted by female mosquitoes belonging to the genera *Culex*, *Anopheles*, *Aedes*, and *Mansonia*.

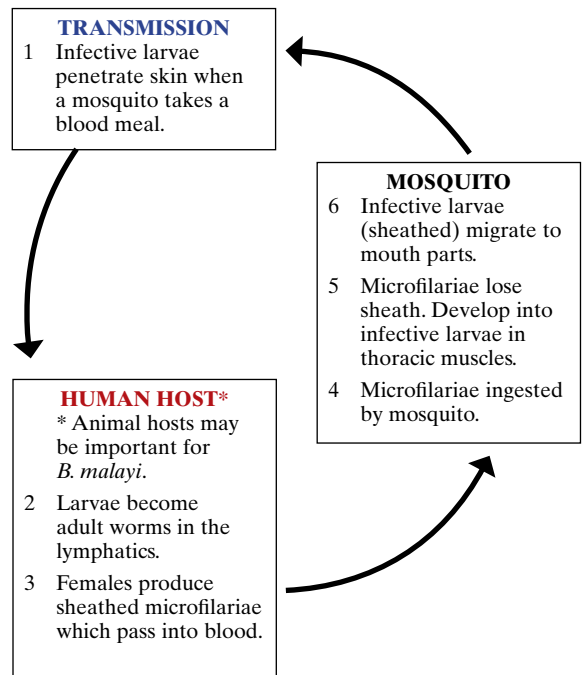


Fig 2.10 Transmission and life cycle of *W. bancrofti* and *Brugia* species.

Infection occurs when infective larvae are deposited on human skin when a mosquito vector takes a blood meal. The larvae penetrate the skin through the bite wound. Development of the larvae takes place in the lymphatics. Within 3–15 months, the

larvae become mature male and female worms. The females produce many sheathed microfilariae which can be found in the blood about 9 months after infection for *W. bancrofti* and after about 3 months for *Brugia* species.

The mature worms can live for many years in their host depending in part on the extent of the host's immune response. Their mean lifespan is 4–6 years but they can survive up to 15 years or more.

The microfilariae are taken up by a mosquito vector when it sucks blood (microfilariae which are not ingested die within 6–24 months). In the stomach of the mosquito the microfilariae lose their sheath and migrate from the midgut to the thorax of the vector where they develop into infective larvae. Development in the mosquito takes 1–2 weeks. Mature infective larvae migrate to the mouth-parts of the mosquito ready to be transmitted when the insect next takes a blood meal.

The life cycle of *W. bancrofti*, *B. malayi* and *B. timori* is summarized in Fig. 2.10.

Clinical features and pathology depend on the sites occupied by developing and mature worms, the number of worms present, length of infection and the immune responses of the host especially to damaged and dead worms. Infection is usually acquired in childhood.

Symptoms of infection differ from one endemic area to another. Infections can be asymptomatic, acute, or chronic. Adult filarial worms depend on endosymbiotic bacteria of the genus *Wolbachia* for their development, motility and fertility. *Wolbachia* also play an important role in the pathogenesis of filariasis.

Asymptomatic lymphatic filariasis

In endemic areas a proportion of infected persons develop no clinical symptoms although microfilariae can be found in their blood (microfilaraemia). Such persons can remain asymptomatic for several years or progress to acute and chronic filariasis. Asymptomatic infections can cause damage to the lymphatics, kidneys and immune system.

Acute lymphatic filariasis

In the acute form there are recurrent attacks of fever (filarial fever) with painful inflammation of the lymph nodes (lymphadenitis) and lymph ducts (lymphangitis). The lymphatics involved are those of the limbs, genital organs (especially spermatic cord) and breasts.

In bancroftian filariasis, the lymph glands in the groin and lymphatics of the male genitalia are

frequently affected. Inflammation of the spermatic cord and repeated attacks can lead to blockage of the spermatic lymph vessels, leading to accumulation of fluid in the scrotal sac which becomes distended (hydrocele). In brugian filariasis, the affected lymph nodes are mostly situated in the inguinal and axillary regions with inflammation of distal lymphatics.

Acute attacks can last for several days and are usually accompanied by a rash and eosinophilia. Secondary bacterial infection of the skin is common. Damage to the lymphatics leads to thickening and eventual blockage of lymphatic vessels with lymphoedema (tissue swelling). Infections with subperiodic *W. bancrofti* and *B. timori* are associated with ulcers which form along the inflamed lymph vessels.

Chronic lymphatic filariasis

This is characterized by hydrocele, lymphoedema and elephantiasis. Hydrocele is common in bancroftian filariasis. Microfilariae are rarely found in the blood of patients with hydrocele or elephantiasis but can be found occasionally in hydrocele fluid.

Elephantiasis is a complication of advanced lymphatic filariasis. It is seen as a coarse thickening, hardening and cracking of the skin overlying enlarged fibrosed tissues. The legs are more commonly affected than the arms and in *W. bancrofti* endemic areas, the thigh also is often involved. Grossly enlarged limbs make walking difficult. Secondary bacterial and fungal infections of the skin can occur. Elephantiasis is more commonly seen in filariasis endemic areas of Africa, China, India and the Pacific region.

In brugian filariasis, symptoms of infection develop more rapidly and children are often infected. Elephantiasis occurs less frequently and tends to involve only the lower limbs. In male adults, the scrotum and spermatic cord are not usually affected and hydrocele is rare.

Non-filarial elephantiasis

In tropical countries, causes of elephantiasis other than filarial worms include siliceous deposits. Endemic elephantiasis of the lower legs associated with siliceous deposits has been reported from the highlands of Kenya, Tanzania, Ethiopia, Ruanda, Burundi, western Sudan, Cape Verde Islands, Cameroon and Rajasthan in India. Damage to local lymphatics with obstruction occurs when silica from the soil is absorbed through bare feet, usually resulting in bilateral elephantiasis.

Chyluria

An uncommon complication of chronic bancroftian filariasis is chyluria. It occurs when the urogenital

lymphatic vessels which are linked to those that transport chyle from the intestine become blocked and rupture. Chyle and occasionally blood and microfilariae can be found in the urine especially in early morning samples. Microfilariae are usually present in the fibrin clots which form.

Occult filariasis and tropical pulmonary eosinophilia

The term occult filariasis refers to a rare condition which is caused by a hypersensitivity reaction to filarial antigens. The features of lymphatic filariasis are not present and microfilariae are not detected in the blood but may be found in tissues.

Tropical pulmonary eosinophilia is a form of occult filariasis in which there is a hypersensitive reaction to the destruction of microfilariae in pulmonary capillaries. It is found particularly in filariasis endemic areas of India and South East Asia. Males are more commonly affected than females. It interferes with breathing and can lead to chronic pulmonary fibrosis. Symptoms are worse at night.

There is a marked eosinophilia and high levels of filarial antibody including high titres of IgE. Eosinophils often appear vacuolated.

Measures to prevent and control lymphatic filariasis

- Preventive chemotherapy by mass drug administration (MDA) to entire at risk communities to reduce microfilariae in the blood aimed at preventing mosquitoes becoming infected.

Different treatment schedules are used in areas where lymphatic filariasis occurs with loiasis or onchocerciasis to avoid encephalopathy or a Mazzotti reaction. Full details can be found on the WHO website www.who.int/lymphatic_filariasis/en/
- Controlling mosquito vectors by:
 - Studying the ecology and behaviour of local vectors to reduce mosquito numbers and eradicate breeding sites. In stagnant and polluted water by improving the maintenance of pit latrines and septic tanks and covering the surface water with polystyrene beads. The use of selective weed killers has proved an effective measure against *Mansonia* mosquitoes that transmit *B. malayi*.
 - Using insecticides known to be effective against local vectors.
- Avoiding mosquito bites by wearing suitable clothing, using mosquito nets and as far as possible making houses mosquito-proof. The use of insecticide impregnated bed-netting is proving successful in many areas where the mosquito vector is a night feeder.
- Informing those living in endemic areas about the cause, early symptoms, detection and control of lymphatic filariasis.

LABORATORY DIAGNOSIS

Lymphatic filariasis is diagnosed by:

- Finding and identifying the microfilariae of *W. bancrofti* or *Brugia* species in blood samples.
- Occasionally detecting the microfilariae of *W. bancrofti* in hydrocele fluid or in the urine of patients with chyluria.
- Detection of *W. bancrofti* antigen in whole blood to diagnose bancroftian filariasis (cannot be used to diagnose brugian filariasis).

Blood samples require collection during the hours when the numbers of microfilariae circulating in the peripheral blood are at their highest, i.e. to coincide with their periodicity (see beginning of subunit).

SPECIES	COLLECTION TIME
<i>Wuchereria bancrofti</i>	
Periodic, nocturnal	22.00–04.00 h
Asia, Africa, Caribbean	Peak 24.00 h
South America, west Pacific	
Subperiodic, nocturnal	20.00–22.00 h
Thailand, Vietnam	Peak 21.00 h
Subperiodic, diurnal	14.00–18.00 h
South east Pacific	Peak 16.00 h
<i>Brugia malayi</i>	
Periodic, nocturnal	22.00–04.00 h
South and East Asia	Peak 24.00 h
Subperiodic, nocturnal	20.00–22.00 h
South East Asia	Peak 21.00 h
<i>Brugia timori</i>	
Nocturnal	22.00–04.00 h
	Peak 24.00 h

Mansonella microfilariae that can be found in blood

In areas where *W. bancrofti* and *L. loa* occur, patients may also be infected with microfilariae of *Mansonella* filarial worms. These parasites are mostly non-pathogenic or of low pathogenicity causing allergic reactions, fever and sometimes arthralgia and cutaneous swellings. Their microfilariae show no periodicity, i.e. they can be found in the blood both during day and night hours.

M. perstans is found in tropical Africa, Central America and South America. It therefore requires differentiation from *W. bancrofti* and *L. loa* (see later text).

M. ozzardi is found in the West Indies, Central America and South America. It therefore requires differentiation from *W. bancrofti* (see later text).

Even when samples are collected at the correct time, the numbers of microfilariae in the blood are often

few and therefore concentration techniques are frequently needed. Microfilariae are rarely found in the blood of patients with hydrocele or elephantiasis once the lymphatics become blocked.

Occasionally in endemic areas microfilariae can be present in the blood of asymptomatic persons. In *W. bancrofti* and *Brugia* infections, microfilariae numbers are higher in capillary blood than in venous blood.

Techniques to detect, identify and quantify microfilariae include:

- Thick stained blood technique using 60 µL (0.06 mL) of blood.
- Lyzed venous blood concentration technique using 10 mL of blood.
- Membrane filtration technique using 10 mL of blood.

Stained thick blood technique using 60 µL of blood

- 1 Collect three 20 µL blood samples, preferably using blood from the ear lobe. Using a clean slide, spread each 20 µL sample across the slide to make three lines of thick smears.

Ear lobe blood: More microfilariae can often be found in capillary blood collected from the ear lobe than from the finger.

- 2 Allow the smears to air-dry.
- 3 Stain the smears using Giemsa stain as described in Section 2.1.

Note: Smears should be stained within 48 hours of collecting the blood to avoid shrinkage and distortion of the microfilariae.

- 4 Wipe the back of the slide clean and allow the smears to air dry.
- 5 Examine the smears microscopically, first using the 10× objective to scan the smears. Use the 40× objective to identify the microfilariae, see later text.
- 6 Count the microfilariae (mf) in all three 20 µL smears (total of 0.06 mL of blood) Report the number of microfilariae/ mL:

$$\frac{\text{Number of mf counted}}{0.06} = \text{mf/mL}$$

Wet slide preparation to detect motile microfilariae

20 µL of capillary blood is mixed with 2 drops of water (to lyze the red cells). The preparation is covered with a cover glass and examined microscopically using the 10× objective for motile microfilariae. This technique is sometimes used as

a screening test but it is not as sensitive as examining a thick stained blood preparation using 60 µL of blood.

Lyzed venous blood concentration technique

Required

Saponin-saline solution to lyze the red cells.	Reagent No. 36
Methylene blue-saline or Field stain A	Reagent No. 28

Procedure

- 1 Collect 10 mL of venous blood and dispense it into 10 mL of saponin-saline lyzing solution.

Note: If saponin-saline is not available, dispense the blood into 10 mL of water.

- 2 Mix the blood gently in the lyzing solution and leave for 10–15 minutes to give time for the red cells to lyze.
- 3 Centrifuge the haemolysate for 10 minutes at slow speed, i.e. RCF 300–500× g.
- 4 Using a plastic bulb pipette or Pasteur pipette, immediately remove and discard the supernatant fluid. Transfer the sediment to a slide, add a *small* drop of methylene blue or Field stain A and cover with a cover glass. The stain will be taken up by the nuclei and show whether the microfilariae are sheathed.
- 5 Examine the entire preparation microscopically for motile microfilariae using the 10× objective with the condenser iris *closed sufficiently* to give good contrast.
- 6 Count the number of microfilariae in the entire preparation. Divide the number counted by 10 to give an approximate number of microfilariae per mL of blood (mf/mL).
- 7 If unable to identify the species, examine a fixed stained preparation with the 100× objective as follows:

- Remove the cover glass and add a *small* drop of plasma, serum, or albumin solution.* Mix and spread thinly. Allow the preparation to dry completely.

* The addition of albumin, plasma, or serum (known to be microfilaria-free) will help to prevent the preparation from being washed from the slide during staining.

- Fix with absolute methanol for 1–2 minutes.
- Stain using Field technique for thin films or Giemsa technique as described in Section 2.1.

Membrane filtration technique

10 mL of venous blood is collected into sodium citrate anticoagulant and the blood passed through a polycarbonate (clear) membrane filter of 5 µm porosity. The microfilariae are retained and the membrane is examined microscopically. The number of microfilariae counted divided by 10 gives the number of mf/mL. This is the most sensitive method for detecting small numbers of microfilariae but it is expensive for routine use.

Required

Nuclepore or other polycarbonate (e.g. *Isopore*) membrane filter, 25 mm diameter 5 µm pore size, available from Merck Millipore (www.merckmillipore.com), catalogue TMTF 02500 (pack of 100).

Syringe filter holder, 25 mm diameter available from Merck Millipore (www.merckmillipore.com), catalogue SX00 02500 (pack of 12).

Syringe, Luer, 10 mL capacity.

Sodium citrate anticoagulant Reagent No. 39

Methylene blue saline Reagent No. 28

Procedure

- 1 Collect 10 mL of venous blood and dispense it into 1 mL of sodium citrate anticoagulant. Mix well but do not shake.
- 2 Withdraw the plunger of a clean 10 mL Luer syringe.
- 3 Unscrew the filter holder and using blunt-ended forceps, *carefully* position the membrane filter (25 mm diameter, 5 µm pore size) on the filter support of the filter holder. Re-assemble the filter holder and attach it to the end of the syringe barrel.
- 4 Fill the syringe barrel with the anticoagulated blood, holding it over a beaker or other suitable container. Carefully replace the plunger of the syringe and slowly pass the citrated blood through the filter.
- 5 Remove the filter holder. Draw up about 10 mL of the methylene blue saline solution, re-attach the filter holder and pass the solution through the filter.
- 6 Remove the filter holder and draw air into the syringe. Re-attach the filter holder and pass the air through the filter. This will help the microfilariae to adhere to the filter.
- 7 Detach the filter holder, unscrew it and using blunt-ended forceps, carefully remove the filter and place it *face upwards* on a slide. Add a small drop of physiological saline and cover with a cover glass.
- 8 Examine the entire filter microscopically for motile microfilariae using the 10× objective with the condenser iris *closed sufficiently* to give good contrast.
Use the 40× objective to see whether the microfilariae are sheathed (seen as extensions at the head and tail end as shown in Plate a on page 95) and whether the blue stained nuclei extend into the tip of the tail.
- 9 Count the number of microfilariae in the entire preparation. Divide the number counted by 10 to give the approximate number of microfilariae per mL of blood (mf/mL).

EXAMINATION OF URINE FOR *W. BANCROFTI* MICROFILARIAE

In chronic bancroftian filariasis a condition called chyluria can occur, i.e. passing of chyle in urine. Chyle consists of lymph and particles of digested fat (soluble in ether). Urine containing chyle appears creamy white. When blood is also present, the urine appears pinkish-white. Microfilariae can often be found in the fibrin clots which form.

Samples: Collect 10–20 mL of early morning urine, i.e. first urine passed by the person after waking.

Procedure

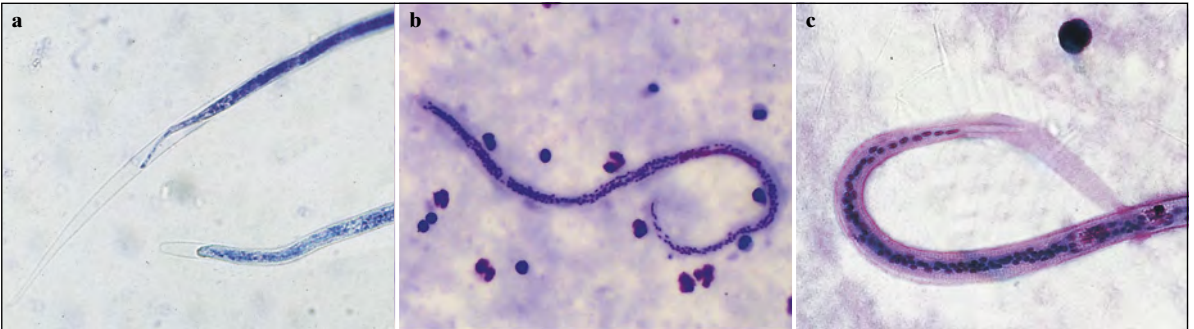
- 1 Report the appearance of the urine. Add about 2 mL of ether and shake to dissolve the chyle.
Caution: Ether is highly flammable, therefore use it well away from an open flame and ensure the room is well-ventilated.
- 2 Centrifuge the sample at slow speed, i.e. RCF 300–500× g (high speed centrifugation may cause the microfilariae to lose their sheath).
- 3 Remove and discard the supernatant fluid. If the sediment contains blood, lyse the red cells by adding an equal volume of saponin-saline solution (Reagent No. 36) or if unavailable add an equal volume of water. Mix and centrifuge again. Discard the supernatant fluid.
- 4 Transfer the sediment to a slide add a *small* drop of methylene blue-saline, or Field stain A and cover with a cover glass. Examine microscopically for motile microfilariae using the 10× objective with the condenser aperture closed sufficiently to give good contrast.

Note: *O. volvulus* microfilariae may also be found in urine in heavy infections and after treatment with diethylcarbamazine. Unlike *W. bancrofti*, the microfilariae of *O. volvulus* are unsheathed.

DIFFERENTIATION OF MICROFILARIAE THAT CAN BE FOUND IN BLOOD

Species	Sheath	Main Features	Notes
<i>W. bancrofti</i> Lymphatic filariasis See b, c	+	<ul style="list-style-type: none"> ● Large, measuring 275–300 × 8–10 µm. ● Body curves are few, nuclei are distinct. ● Sheath stains poorly with Giemsa and palely with haematoxylin.* <p><i>Tail:</i> There are no nuclei in the tip.</p>	<p>Test night blood (14–18 h: Pacific strain)</p> <p>Differentiated from <i>Brugia</i> species and <i>L. loa</i> by its tail features.</p> <p>Differentiated from <i>Mansonella</i> species by its larger size and sheath.</p>
<i>B. malayi</i> Lymphatic filariasis See e, f	+	<ul style="list-style-type: none"> ● Large, measuring 200–275 × 5–6 µm ● Body has many angular curves, nuclei are dense and stain darkly. ● Sheath stains dark pink with Giemsa and palely with haematoxylin (may be absent).* <p><i>Tail:</i> There are 2 nuclei in the end of the tail which tapers irregularly.</p>	<p>Test night blood.</p> <p>Differentiated from <i>W. bancrofti</i> by its darker stained sheath and nuclei, kinked body, and tail features.</p> <p>Differentiated from <i>B. timori</i> by its shorter length and darker stained sheath.</p>
<i>B. timori</i> Lymphatic filariasis See d	+	<p>The following features distinguish <i>B. timori</i> from <i>B. malayi</i>:</p> <ul style="list-style-type: none"> ● It is longer, measuring 290–325 × 5–6 µm. ● Sheath stains palely or not at all with Giemsa (often absent). ● Body nuclei are less dense and the space at the head end (cephalic space) is longer. <p><i>Tail:</i> Similar to <i>B. malayi</i>, i.e. has 2 nuclei in tail end.</p>	<p>Test night blood.</p> <p>Only found in Lesser Sunda Is.</p>
<i>L. loa</i> Loiasis (Calabar swelling) See a, g, h, i	+	<ul style="list-style-type: none"> ● Large, measuring 250–300 × 8–10 µm ● Body has several curves and kinks. Nuclei are not distinct. ● Sheath stains best with haematoxylin.* <p><i>Tail:</i> Nuclei extend to end of tail.</p>	<p>Test day blood.</p> <p>Differentiated from <i>W. bancrofti</i> by its tail features.</p> <p>Differentiated from <i>M. perstans</i> by its larger size and sheath.</p>
<i>M. perstans</i> Non-pathogenic or of low pathogenicity See i, j	–	<ul style="list-style-type: none"> ● Small and thin, measuring 190–240 × 4.5 µm ● Body nuclei are irregular. <p><i>Tail:</i> Nuclei extend to end of tail which is rounded. Large nucleus in tip.</p>	<p>In day and night blood.</p> <p>Differentiated from <i>L. loa</i> and <i>W. bancrofti</i> by its smaller size, absence of sheath, tail features, and non-periodicity.</p>
<i>M. ozzardi</i> Non-pathogenic or of low pathogenicity See k	–	<ul style="list-style-type: none"> ● Small and thin measuring 150–200 × 4.5 µm. ● Body nuclei are not distinct. Anterior nuclei are positioned side by side. <p><i>Tail:</i> No nuclei in the end of the tail which is long and pointed.</p>	<p>In day and night blood.</p> <p>Differentiated from <i>W. bancrofti</i> by its smaller size, absence of sheath, and non-periodicity.</p>

* **Delafield's rapid haematoxylin staining technique:** Can be found in *Part 1 District Laboratory Practice in Tropical Countries*, subunit 5.11, page 288. Access by entering the title in Google and clicking on entry <https://medicallabtechno.weebly.com>



a *L. loa* (head and tail) microfilaria in methylene blue preparation.

b *W. bancrofti* microfilaria. Giemsa stained (40× objective).

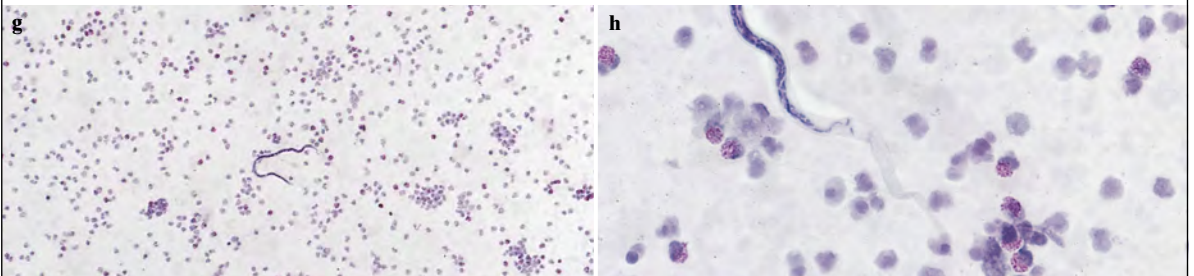
c Tail of *W. bancrofti*. (HE) No nuclei in tip (100× objective).



d *B. timori* microfilaria. Giemsa stained (40× objective).

e *B. malayi* microfilaria Giemsa stained (40× objective).

f Tail of *B. malayi*. Two nuclei in tip (100× objective).



g *L. loa* microfilaria. Haematoxylin and eosin (HE) stained (10× objective).

h Tail of *L. loa*. Nuclei extend to end of tail (100× objective).



i Left: *L. loa* microfilaria (HE). 40× objective.
Right: Unsheathed, smaller *M. perstans*.

j *M. perstans* microfilaria. (HE) stained (100× objective).

k *M. ozzardi* unsheathed microfilaria. Giemsa stained (100× objective).

Examination of aspirates for W. bancrofti microfilariae

The method described for examining urine (omitting the addition of ether) can also be used to examine aspirates of hydrocele fluid or lymph gland fluid for *W. bancrofti* microfilariae.

**FILARIASIS TEST STRIP TO DETECT
W. BANCROFTI ANTIGEN**

The Filariasis Test Strip is a rapid easy to perform immunochromatographic (ICT) qualitative test to detect circulating *W. bancrofti* antigen in capillary blood. Antigen is secreted by adult worms and therefore the antigen level is dependent on worm numbers.

Blood samples for the detection of *W. bancrofti* antigen can be collected at any time during the day unlike blood samples which need to be collected during night hours to detect nocturnal periodic and subperiodic microfilariae.

Compared to the ICT Filariasis Card test (*Binax NOW Filariasis*), the Filariasis Test Strip is more sensitive, requires less blood, can be stored at ambient temperature (2–37°C), has a longer shelf-life (16–24 months) and is less costly.

To perform the test, 75 µL of finger prick capillary blood or heparinized anticoagulated blood is collected using the micropipette provided in the test kit (EDTA anticoagulated blood should not be used). The blood is applied to the sample pad at the lower end of the test strip and the timer is set for 10 minutes.

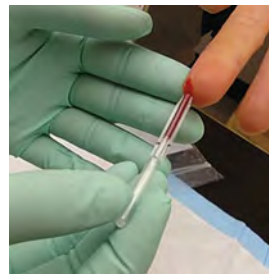
Antigen in the sample reacts with polyclonal antibody in the sample pad, the antibody antigen complex migrates along the nitrocellulose strip and is captured by specific monoclonal antibody, producing a pink band in the Test (T) area. A pink band is shown in the Control (C) area of the strip indicating correct migration of the sample. The test is invalid if there is no pink band in the Control area. Test results must be read in good light. A positive control should be used with each new test kit.

The Filariasis Test Strip has been reported as having an overall sensitivity of 93% and specificity of 99.3%. Evaluations of performance have emphasised the importance of following the procedure exactly when collecting the blood and applying it to the sample pad. When performing field surveys, the strip needs to be secured adequately to the plastic work tray supplied.

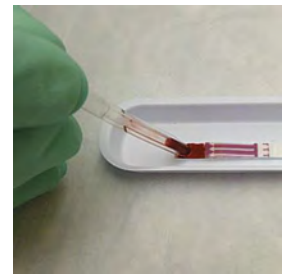
The Filariasis Test Strip is manufactured by Alere Inc, Scarborough, website: www.alere.com/en/home.html. Illustrated instructions showing how to use the Strip and a source of positive controls can be found on the following website: www.ntdsupport.org/resources/filariasis-test-strip-fts-bench-aid

Cross-reactivity of ICT Filariasis antigen test with L. loa.

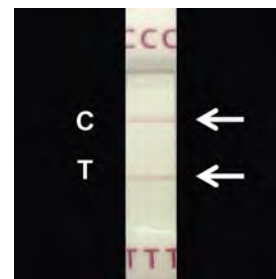
In Central Africa in areas of high loiasis prevalence, the ICT Filariasis Card Test has been reported as positive in patients with high *L. loa* microfilariae counts.



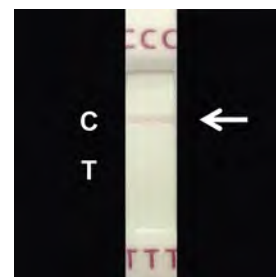
Using the micropipette to collect a 75 µL blood sample.



Applying the blood to the sample pad on the test strip.



Positive Filariasis Test Strip showing pink bands in the Test (T) and Control (C) areas.



Negative Filariasis Test Strip showing a pink band in the Control (C) area only
Courtesy of Alere Inc (now Abbott). www.alere.com

LOIASIS

L. loa has a diurnal periodicity. It is restricted to the equatorial rain forest areas of West and Central Africa, particularly the coastal plains of northern Angola, south eastern Benin, Central African Republic, Chad, Republic of Congo, Equatorial Guinea, Gabon, southern Nigeria, South Sudan, Democratic Republic of Congo and parts of Uganda. It is endemic in many areas where *W. bancrofti* also occurs.

L. loa is transmitted by blood-sucking daytime biting tabanid flies of the genus *Chrysops*. They are often referred to as horseflies. Infective larvae (often in large numbers) enter human skin through the deep wound made by an infected *Chrysops* fly when it sucks blood. The larvae penetrate subcutaneous connective tissues and within 6–12 months they develop into mature male and female worms. The viviparous females produce sheathed microfilariae which can be found in the blood during day hours. The mature worms may live for 4–12 years in their host. They wander in subcutaneous tissue and occasionally under the conjunctiva of the eye.

The microfilariae are taken up by a female *Chrysops* when sucking blood. In the stomach of the *Chrysops* the microfilariae lose their sheath. They pass through the stomach wall, penetrate thoracic muscles and develop into infective larvae. Development in the insect vector takes about 10 days. Mature infective larvae migrate to the mouth-parts of the insect ready to be transmitted when the *Chrysops* takes a blood meal.

Many people infected with *L. loa* do not develop clinical symptoms. The disease is characterized by the formation of swellings known as Calabar swellings which may last from a few days up to 3 weeks and measure from 3–10 cm in diameter. The arms are most frequently affected. The inflamed areas are an allergic response to adult *L. loa* worms migrating in the subcutaneous tissue. Worms are not usually present in the swellings but they can occasionally be seen migrating below the skin surface. In non-immune persons, infection with *L. loa* can cause severe allergic reactions.

Adult worms also migrate in subconjunctival tissues. They can be seen under the eyelids and occasionally slowly crossing the white of the eye. They can cause inflammation and irritation but not blindness. The microfilariae do not seem to cause any serious

symptoms although it has been reported that encephalitis can occur following treatment of heavy infections.

An eosinophilic leucocytosis and high titres of specific anti-filarial antibodies are found in patients with loiasis. Occasionally an immune complex related glomerulonephritis can occur with blood and protein being found in urine.

Measures to prevent and control loiasis

- Detecting and treating individuals, including using a rapid assessment procedure (RAP-LOA) based on clinical signs (history of eyeworm or Calabar swelling) to assess the endemicity of loiasis to help identify those at risk of severe adverse reactions to ivermectin treatment.
- Avoiding the bites of *Chrysops* flies (daytime feeders) by:
 - Wearing protective clothing, e.g. long trousers. Light-coloured clothing also gives some protection.
 - Siting settlements, including adequate water supplies, away from forest areas.
- Destroying *Chrysops* flies by:
 - Changing the character of breeding places wherever possible, e.g. clearing vegetation to allow in sunlight to dry out muddy areas which were previously heavily shaded.
 - Using insecticides as part of a control programme where this is feasible.

LABORATORY DIAGNOSIS

Loiasis is diagnosed by:

- Finding and identifying *L. loa* microfilariae* in blood samples.

* Diurnal: 10.00–15.00 h
Peak 13.00 h

* The presence of *L. loa* microfilariae in night blood has been reported from the Democratic Republic of Congo.

- Occasionally by finding *L. loa* microfilariae in joint fluid.

Note: Calabar swelling is accompanied by a marked eosinophilia.

Note: The techniques described previously to detect and identify microfilariae in lymphatic filariasis can be used to detect and identify *L. loa* microfilariae.

ONCHOCERCIASIS

Distribution

It is estimated that in 2017 there were 20.9 million people infected with *O. volvulus* with most infections (99%) occurring in 31 sub-Saharan African countries. Of those infected 14.6 million people had skin disease and 1.15 million had vision loss.²

Smaller onchocerciasis endemic areas occur in the Yemen Arab Republic, Brazil and the Bolivarian Republic of Venezuela.

Transmission and life cycle

O. volvulus is transmitted by *Simulium* blackflies. The flies breed in fast-running rivers and streams, in rain forests and savannah areas, frequently near human settlements and fertile agricultural land. The infective *O. volvulus* larvae enter through the bite wound after an infected female blackfly takes a blood meal. The larvae take several months to develop into mature worms.

The adult worms live in subcutaneous tissue and in lymph spaces, occurring singly or in tangled masses. In the later stages of infection a proportion of the worms become encapsulated in fibrous nodules. The worms can live up to 10 years or more in their host. The females produce many unsheathed microfilariae which can be found just

below the surface of the skin in the lymph spaces and in connective tissue. They can also be found in the fluid of nodules. Microfilariae are thought to be present in the skin from about 7 months onwards after infection. The microfilariae also migrate to the eye and other organs of the body.

The microfilariae are ingested by a blackfly as it feeds. After passing through the stomach wall of the fly, the microfilariae migrate to the thoracic muscles where they develop into infective larvae. Development in the blackfly vector takes about 10 days. The mature infective larvae pass to the mouthparts of the blackfly ready to be transmitted when the fly next takes a blood meal. The lifecycle of *O. volvulus* is summarized in Fig. 2.11.

Onchocerciasis is a major health and socioeconomic problem in endemic areas in Africa. The clinical features and pathology of onchocerciasis are caused mainly by the inflammatory reactions around damaged and dead microfilariae. The disease varies from one area of infection to another and within a particular population. The main clinical features are the formation of nodules, dermatitis and inflammatory reactions in the eye leading to blindness. Onchocerciasis is also thought to be a risk factor for epilepsy. Endosymbiotic bacteria of the genus *Wolbachia* also play a role in the pathogenesis of onchocerciasis.

Nodule formation

Nodules form under the skin when the adult worms become encapsulated in subcutaneous tissue. The nodules are called onchocercomas. They are firm, smooth and rubbery, round or elongated and measure from 5 mm across up to 50 mm when found in clusters. They may contain large numbers of microfilariae.

In many endemic areas of Africa, nodules are commonly found on the lower part of the body around the pelvis. In Central America and the savannah areas of Africa, nodules are often found on the upper part of the body. In young children (below 9 y), the nodules are found mainly on the head. In Yemen the lower limbs are mainly affected.

Skin disease

There is an inflammatory dermatitis which is usually accompanied by intense irritation, raised papules on the skin and subsequently alteration in the pigmentation of the skin. Dermatitis is more severe in those coinfecting with HIV. The term 'sowda' (black disease) is used to describe a severe allergic response usually affecting only one limb with darkening of the skin. The lymph nodes draining the limb become swollen and painful.

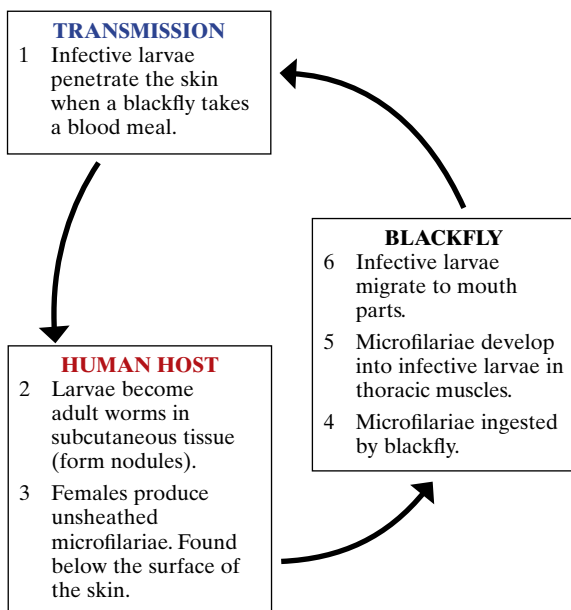


Fig 2.11 Transmission and life cycle of *Onchocerca volvulus*.

In chronic onchocerciasis, the skin loses its elasticity and becomes wrinkled which makes people look more aged than they are (known as 'elephant skin'). When the skin around the groin becomes affected 'hanging groin' develops. Spotted depigmentation of the skin is associated with chronic onchocerciasis.

Blindness

The most serious complication of onchocerciasis is when microfilariae in the skin of the face migrate into the eye. In early eye infections the microfilariae can be found in the cornea and in the anterior chamber. There is redness and irritation of the eye. Progressive changes caused by inflammatory reactions around damaged and dead microfilariae can cause sclerosing keratitis which can lead to blindness. Often the iris is also affected. Inflammation of the choroid and retina can also lead to blindness.

Nodding syndrome

In East Africa a serious epileptic disorder of unknown origin, referred to as Nodding Syndrome, has been reported in children infected with *O. volvulus*. Some studies suggest the condition may be a neuroinflammatory autoimmune disorder.³

Measures to prevent and control onchocerciasis

- By sustained community-directed treatment with ivermectin (CDTI). COVID-19 restrictions disrupted MDA in 2020. Careful monitoring and different treatment strategies are required in areas where *L. loa* occurs with onchocerciasis (Central African Republic, Cameroon, Nigeria, South Sudan, Democratic Republic of Congo) to avoid serious adverse treatment reactions.
- Interruption of transmission by the destruction of *Simulium* blackflies including: the selective use of insecticides, e.g. aerial spraying to destroy blackfly larvae in rivers and streams.
- Avoiding *Simulium* bites by covering as far as possible those parts of the body most at risk and siting human dwellings away from areas where blackflies breed. This often leads to the abandonment of fertile river valleys.

LABORATORY DIAGNOSIS

The laboratory diagnosis of onchocerciasis is by:

- Detecting and identifying *O. volvulus* microfilariae in skin snips.

Note: In heavy infections and following treatment, microfilariae can also be found in urine and most body fluids.

To assist in onchocerciasis population screening and surveillance:

- Antibody testing using the Ov 16 IgG4 antibody test.

COLLECTING AND EXAMINING SKIN SNIPS FOR *O. VOLVULUS* MICROFILARIAE

Skin snips should be taken from those sites most likely to be heavily infected. In Africa and South America, the highest number of microfilariae can usually be found in skin snips taken from the buttocks, iliac crests or calves of the legs, in Mexico and Guatemala from behind the shoulders or trunk and in Yemen from the lower limbs.

Important: A bloodless skin snip is required. It can be collected using a sterile needle and razor blade (or scalpel) see Plate 2.11 on the following page.

Procedure

A tube centrifugation-technique is recommended if a centrifuge is available. Alternatively, a slide technique can be used (skin snip immersed in saline on a slide and covered with a cover glass).

- 1 Cleanse the skin using a spirit swab. Allow the area to dry.
- 2 Insert a sterile fine needle almost horizontally into the skin. Raise the point of the needle, lifting with it a small piece of skin measuring about 2 mm in diameter.
- 3 Cut off the piece of skin with a sterile razor blade (or scalpel as shown in Plate 2.11).

Immerse the skin snip in a conical centrifuge tube containing about 1 mL of fresh physiological saline and leave it at room temperature for up to 4 hours. Do not tease (pull apart) the skin because this is not necessary and can damage the microfilariae.

Incubation time and the emergence of microfilariae

In some areas it has been shown that after 1 hour, up to 90% of microfilariae contained in a skin snip (saline preparation) will have emerged, whereas in other areas an incubation time of more than 4 hours is needed. Ivermectin reduces microfilaria motility.

- 4 Using forceps, remove the skin snip, place it on a slide and cover with a cover glass. Centrifuge the contents of the tube at medium to high speed, i.e. RCF \times 500–1000, for 5 minutes. Remove and discard the supernatant fluid. Transfer the entire sediment to a slide.
- 5 Examine both the skin snip and sediment microscopically for motile microfilariae using the

10× objective with the condenser iris closed sufficiently to give good contrast (see Plate 2.12). Report the number of microfilariae as scanty, few, moderate numbers, or many.

Note: If no microfilariae are seen, immerse the skin snip in a further 1 mL of saline and reincubate. If after overnight incubation no microfilariae are seen, report the preparation as 'Negative'.



Plate 2.11 Taking a skin snip for the detection of *O. volvulus* microfilariae.



Plate 2.12 Saline preparation showing *O. volvulus* microfilariae from a skin snip, using 10× objective.

If microfilariae are present identify them as *O. volvulus* as follows:

- Remove the cover glass and allow the preparation to dry *completely*.
- Fix the dried preparation with absolute methanol for 1–2 minutes.
- Stain with Giemsa as described in Section 2.1. Cover the preparation with a drop of immersion oil and examine it microscopically using the 40× and 100× objectives to identify the microfilariae (see Plate 2.13).

Differentiation of *O. volvulus* from *Mansonella* species

- In West Africa and Central Africa, *O. volvulus* requires differentiation from *M. streptocerca* (see Plate 2.13).
- In the West Indies, Central America and South America, *O. volvulus* requires differentiation from *M. ozzardi* (see page 101).

Mansonella species

M. streptocerca is a filarial worm that produces small unsheathed microfilariae that can be found in the skin. Most infections are asymptomatic or sometimes cause an itching dermatitis, hypopigmented macules and thickening of the skin. *M. streptocerca* is transmitted by *Culicoides* midges and is found only in the rain forests of Africa, especially in Ghana, Nigeria, Zaire and Cameroon.

M. ozzardi is a filarial worm that produces small unsheathed microfilariae that can be found both in the skin and blood (non-periodic). Most infections are asymptomatic or cause chronic arthritis, skin rashes and other symptoms. *M. ozzardi* is transmitted by *Culicoides* midges and *Simulium* blackflies. It is found in the West Indies, Surinam, Guyana, Colombia, Brazil, northern Argentina, Mexico and Panama.

Note: Other species of microfilariae may be found if the skin snip becomes contaminated with blood at the time it is collected.

Counting microfilariae in surveys

In onchocerciasis epidemiological surveys, the microfilariae from skin snips may need to be counted. To do this, a Walser corneoscleral punch can be used to collect skin snips weighing about 1.0 mg.

Each skin snip is immersed in 200 µL (0.2 mL) of physiological saline in the well of a microtitration plate or in a small tube and the plate or tube covered with *Parafilm* or cling film.

After overnight incubation, the contents of the well or tube are transferred to a slide, the microfilariae counted and the number reported per mg of skin.

Note: In low prevalence areas, the sensitivity of detecting microfilariae in skin snips is low.

Ov16 IgG4 ANTIBODY TEST

The Ov16 IgG4 antibody test has been designed to be used in population screening and surveillance in onchocerciasis elimination programmes. The test detects IgG4 antibodies in blood samples produced in response to *O. volvulus* Ov 16 antigen. It detects exposure to infection in present and past infections. *O. volvulus* antibodies can be found in the blood several years after infection.

The Ov16 IgG4 antibody test is available as an ELISA and a rapid ICT lateral flow test.

DIFFERENTIATION OF MICROFILARIAE THAT CAN BE FOUND IN SKIN

Species	Sheath	Main Features in Stained Preparations	Notes
<i>O. volvulus</i> Onchocerciasis See a	—	<ul style="list-style-type: none"> Large, measuring 240–360 × 5–9 μm. Head is slightly enlarged. Anterior nuclei are positioned side by side. <p><i>Tail:</i> No nuclei in the end of the tail which is long and pointed.</p>	<p>Differentiation from <i>M. streptocerca</i> (see below).</p> <p>Differentiation from <i>M. ozzardi</i> is mainly by its larger size and enlarged head.</p>
<i>M. streptocerca</i> Non-pathogenic or of low pathogenicity See b	—	<ul style="list-style-type: none"> Small and thin, measuring 180–240 × 4.5 μm. Anterior nuclei are positioned in single file. <p><i>Tail:</i> Nuclei extend to the end of the tail which is usually hooked. Tip is rounded or forked (West Africa).</p>	<p>Differentiation from <i>O. volvulus</i> is by its smaller size, single file anterior nuclei and tail features.</p> <p>Less motile than <i>O. volvulus</i> in wet preparations.</p>
<i>M. ozzardi</i> Non-pathogenic or of low pathogenicity See c	—	<ul style="list-style-type: none"> Small and thin, measuring 150–200 × 4.5 μm. Anterior nuclei are positioned side by side. <p><i>Tail:</i> No nuclei in the end of the tail which is long and pointed.</p>	<p>Differentiation from <i>O. volvulus</i> is mainly by its smaller size and different shaped head.</p> <p>Less motile than <i>O. volvulus</i> in wet preparations.</p>



Plate 2.13 **a** *O. volvulus* microfilaria. **b** *M. streptocerca* microfilaria. Smaller than *O. volvulus* usually with hooked tail. **c** *M. ozzardi* microfilaria. Smaller than *O. volvulus* with long pointed tail. Giemsa stained as seen with 40× objective.

Ov 16 IgG4 ELISA

The ELISA is performed as a quantitative laboratory test using whole blood or samples from dry blood spots. It has been used in onchocerciasis surveillance, mostly in Latin America.

Ov 16 IgG4 rapid ICT antibody test

The Ov 16 IgG4 rapid antibody test is a recently developed* qualitative test in cassette format which can be easily performed in field surveys.

* Developed by the Program for Appropriate Technology in Health (PATH) in collaboration with the National Institute for Health, Standard Diagnostics Inc, CDC and others. The test is manufactured by Standard Diagnostic Inc. Ordering details are available from PATH. E-mail dxinfo@path.org

All the items needed to perform the test are provided in the test kit. The test can be stored at ambient temperatures and has a shelf-life of up to 24 months. To perform the test, 10 µL of capillary blood from a finger prick is collected using the micropipette supplied (plasma, serum and dried blood spots can also be used). The blood is added to the circular well in the cassette. 4 drops of chase buffer are added to the square well. After 20 minutes the test result is read.

A positive test is shown by a pink colour band in the Test area and a pink band in the Control area indicating correct migration of the sample. A negative test is shown by a pink band in the Control area only.

Evaluations of the test are ongoing including evaluating the performance of the test in young children and post-treatment surveillance in different onchocerciasis endemic areas. For further information, readers are referred to the following NTD website:

www.ntdsupport.org/resources/Ov-16-meeting-notes

REFERENCES

- 1 *WHO Lymphatic filariasis Fact Sheet*, 2022. www.who.int/news-room/fact-sheets/detail/lymphatic-filariasis
- 2 *WHO Onchocerciasis Fact Sheet*, 2022. WHO website www.who.int/news-room/fact-sheets/detail/onchocerciasis
- 3 **Bovin M J**. New evidence for nodding disease as an autoimmune reaction to *Onchocerca volvulus*. *Ann Transl Med*, 2017, Dec;5(23):461. Doi 10.21037/atm.201709.06

2.5 Soil-transmitted helminth infections

Soil-transmitted helminth infections are caused by faecal pollution of the soil in areas where sanitation is poor. The infective forms of the parasites develop in the soil. The WHO estimates that there are approximately 1.5 billion people infected with soil-transmitted helminths worldwide with the greatest numbers occurring in tropical and subtropical areas. Those at greatest risk of infection are preschool and school-age children, women of childbearing age and adults in high-risk occupations such as tea-pickers and miners.¹

The main soil-transmitted helminths are:

- *Ascaris lumbricoides*, large intestinal roundworm.
- *Trichuris trichiura*, whipworm.
- *Necator americanus*, *Ancylostoma duodenale*, hookworms.
- *Strongyloides stercoralis*, dwarf threadworm.

ASCARIS LUMBRICOIDES

Transmission and life cycle

A person becomes infected by ingesting infective eggs in contaminated food or from hands that have become faecally contaminated.

The mature worms live free in the intestine. Fertilized female worms produce many eggs per day. The eggs can remain viable in soil and dust for several years. Heavy *Ascaris* infections can be found especially among children 3–8 years whose fingers become contaminated while playing on open ground. The worms can live 1–2 years in their host.

The direct life cycle of *A. lumbricoides* is summarized in Fig. 2.12. Eggs passed in the faeces are non-embryonated. They require about 30–40 days in the environment to mature to the infective stage. The larva does not hatch until the egg is ingested.

Ascariasis

During their migration, *Ascaris* larvae can cause inflammatory and hypersensitive reactions including pneumonia-like symptoms, attacks of coughing and wheeze. Eosinophilia is common and there is often urticaria. Signs of toxemia may develop and very occasionally neurological disorders.

Developing and mature worms in the intestine frequently cause abdominal pain, nausea, diarrhoea and vomiting. Intestinal muscle may become damaged and absorption impaired. *A. lumbricoides* infection in children is known to affect gastrointestinal function. Protein digestion or absorption is impaired, the absorption of fat decreases, and lactase activity in the small intestine is reduced. Infected children are often vitamin-A deficient and have low serum albumin levels. Frequent exposure to infection may result in impairment of physical and intellectual development.

Ascaris worms are large and in heavy infections, especially in children, worm masses can cause obstruction or perforation of the intestine and occasionally obstruction of the bile duct and pancreatic duct. Other complications include liver abscesses and appendicitis caused by migrating worms. Worms can pass through the anus or be vomited.

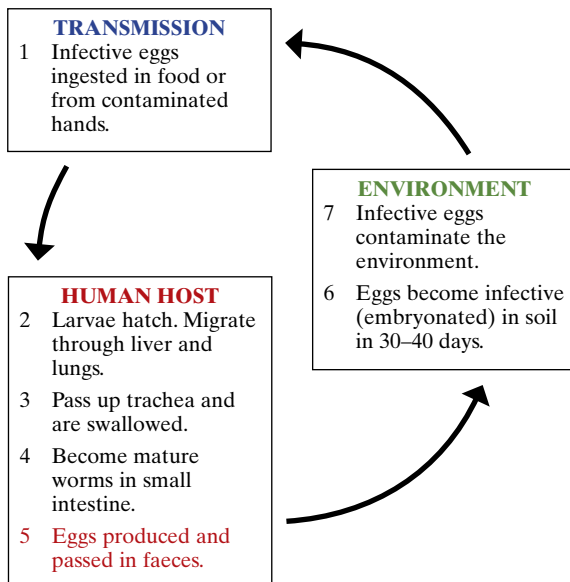


Fig 2.12 Transmission and life cycle of *Ascaris lumbricoides*.

Measures to prevent and control ascariasis

- Preventing soil becoming faecally polluted by:
 - Using latrines and avoiding the use of untreated human faeces as fertilizer.
 - Treating infected individuals as part of a control programme, especially children.
- Preventing infection by:
 - Washing hands before eating.
 - Avoiding eating uncooked vegetables, green salads and fruits which may be contaminated with faeces containing *Ascaris* eggs.

LABORATORY DIAGNOSIS

- Finding *A. lumbricoides* eggs in faeces. Concentration techniques are rarely required.

- Identifying *A. lumbricoides* worms expelled through the anus or mouth.

Eggs of *A. lumbricoides*

Usually fertilized eggs are found in faeces but occasionally infertile eggs are produced by unfertilized female worms.

FERTILIZED EGG (Plate 2.14 Right)

- Yellow-brown, oval or round, measuring 50–70 μm long by 30–50 μm wide.
- Shell is often covered by an uneven albuminous coat (mammilated).
- Contains a central granular mass which is the unsegmented fertilized ovum.

Decorticated egg: This term is used to describe an egg that has no albuminous coat. A decorticated egg has a smooth shell and appears pale yellow or colourless.

INFERTILE EGG (Plate 2.14 Left)

- It is darker in colour and has a thinner wall and more granular albuminous covering.
- More elongated than a fertilized egg, measuring about 90 \times 45 μm .
- Contains a central mass of large granules.

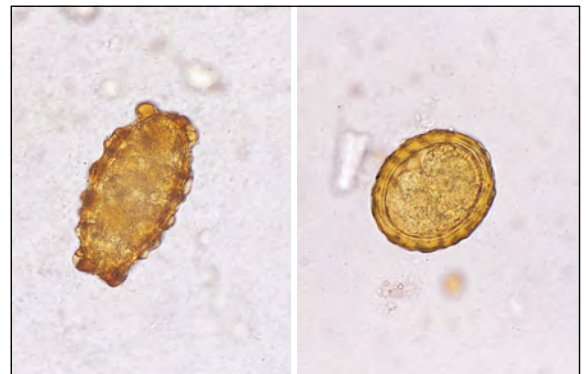


Plate 2.14 Left: Infertile egg of *A. lumbricoides*. Right: Fertile egg of *A. lumbricoides* in a saline preparation.

A. lumbricoides worms

Freshly expelled *Ascaris* worms are pinkish in colour with an appearance similar to earthworms. They measure 12–35 cm in length and taper at both ends. The tail of the male is curved. There is a small mouth surrounded by three lips.

Caution: Always use forceps to handle *Ascaris* worms. If storing *Ascaris* eggs in formol saline, e.g. for teaching purposes, first treat the faecal deposit with hot (70–80 °C) formol saline. This will prevent the eggs from developing to an infective stage.

TRICHURIS TRICHIURA

Transmission and life cycle

Infection is by ingesting infective eggs in contaminated food or from contaminated fingers. Children are more often infected than adults, due to playing on faecally contaminated ground. The direct life cycle of *T. trichiura* is summarized in Fig. 2.13. Many eggs are produced. They remain infective for several months in moist warm soil but they are unable to withstand drying.

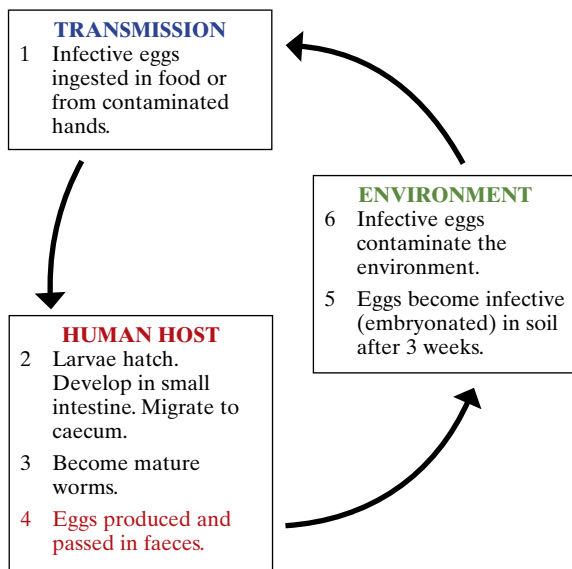


Fig 2.13 Transmission and life cycle of *Trichuris trichiura*.

Trichuriasis

Light infections produce few symptoms. In young children, severe infections can cause chronic diarrhoea, intestinal ulceration with blood and mucus being passed in the faeces, iron deficiency anaemia, failure to develop at the normal rate, weight loss and prolapse of the rectum. Massive infections can be fatal. Eosinophilia is common. Severe trichuriasis is thought to increase the risk of disease with *Entamoeba histolytica* and pathogenic enterobacteria such as *Shigella* species. Migrating worms occasionally cause appendicitis.

Measures to prevent and control trichuriasis are the same as those described for *A. lumbricoides*.

LABORATORY DIAGNOSIS

The laboratory diagnosis of *T. trichiura* infection is by finding *T. trichiura* eggs in faeces. Concentration techniques are rarely required to detect significant infections.

Note: Heavy infections can be diagnosed clinically by examining the rectum for worms using a proctoscope.

Egg of *T. trichiura*

- Yellow-brown and measures about 50 × 25 μm.
- Has a characteristic barrel shape with a colourless protruding mucoid plug at each end as shown in Plate 2.15.
- Contains a central granular mass which is the unsegmented ovum.



Plate 2.15 Egg of *T. trichiura* in a saline preparation.



Plate 2.16 Egg of *P. philippinensis*.

Differentiating the egg of Paracapillaria philippinensis from that of T. trichiura

The egg of *P. philippinensis* has a similar appearance to that of *T. trichiura*. In areas where both species occur, differentiation of the two species is required.

Paracapillaria philippinensis

P. philippinensis is a very small whipworm which is normally parasitic in fish-eating birds but can infect humans causing capillariasis. As its name suggests it is found in the Philippine Islands where it is fairly widely distributed in northern Luzon. It is also found in Thailand.

P. philippinensis is transmitted by the ingestion of infective embryonated eggs in raw, undercooked, or pickled fish. The larvae develop into mature worms in the small intestine. Immature eggs are passed in the faeces. Occasionally infective larvae develop in the intestine and cause autoinfection.

Capillariasis

Early and mild infections cause abdominal pain, intestinal 'gurgling', and chronic watery diarrhoea. Heavy infections can lead to muscle wasting and oedema caused by loss of protein and severe malabsorption of fats and sugars. Plasma potassium, sodium and calcium are reduced.

LABORATORY DIAGNOSIS OF CAPILLARIASIS

This is by finding the eggs in faeces (see Plate 2.16). They can be few and therefore if infection is suspected and the eggs are not found in direct preparations, a concentration technique should be used, such as the formol ether/ethyl acetate concentration method described in Appendix V.

Egg of *P. philippinensis* (see Plate 2.16)

Features used to identify the egg and differentiate it from *T. trichiura* are as follows:

- Smaller than *T. trichiura*, measuring about $45 \times 21 \mu\text{m}$.
- It is yellow-brown but less elliptical in shape than *T. trichiura*.
- The plugs (one at each end of the egg) are smaller and do not protrude like those of *T. trichiura*.

Eggs are normally excreted in the faeces 4–7 weeks after infection.

The first stage larva that hatches from the egg is called a rhabditiform larva. It feeds and in warm well-oxygenated soil, develops into the infective filariform larva. Infective non-feeding larvae can remain viable in damp warm soil for up to 2 years.

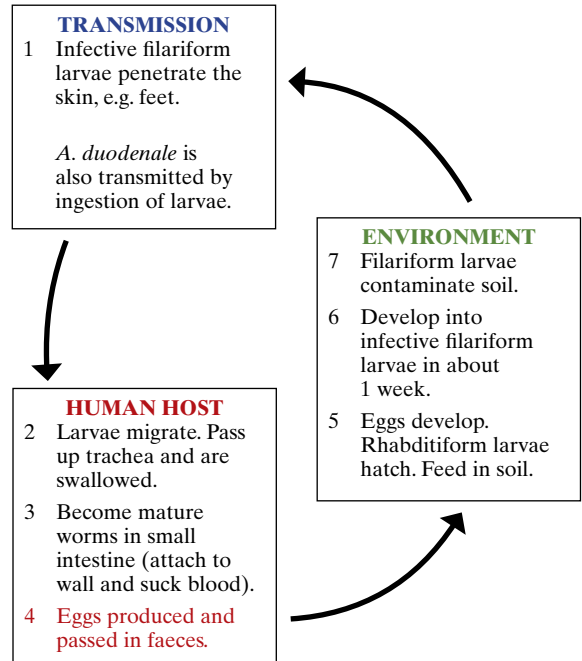
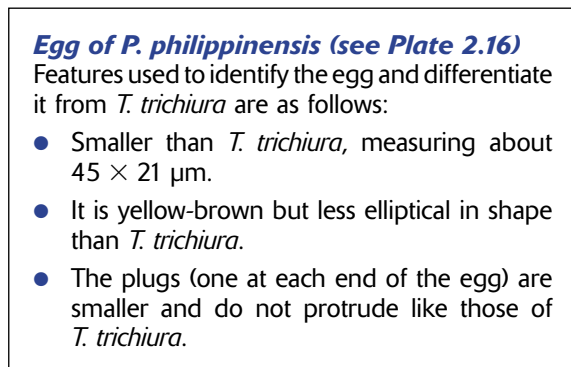


Fig 2.14 Transmission and life cycle of hookworms: *Ancylostoma duodenale* and *Necator americanus*.

HOOKWORMS

Hookworm infection is caused by :

Necator americanus
Ancylostoma duodenale

Hookworms are widespread in the tropics and subtropics.

Transmission and life cycle

Hookworm infection occurs when infective filariform larvae penetrate the skin, especially when a person is walking barefoot on infected ground. *A. duodenale* can also be transmitted by ingesting infective larvae. The direct life cycle of hookworms is summarized in Fig. 2.14.

Hookworm infection and hookworm anaemia

The first sign of hookworm infection is frequently a skin reaction at the site of larval penetration. This is known as 'ground itch' and is usually more intense in those previously infected. During migration of the larvae, mild respiratory symptoms may develop and also an eosinophilia.

Adult hookworms cause chronic blood loss. It has been estimated that a single *A. duodenale* worm ingests about 150 μL (0.15 mL) of blood per day and a *N. americanus* worm about 30 μL (0.03 mL). The test for occult blood in faeces is positive.

Iron deficiency anaemia: This usually develops with heavy prolonged infection, especially with *A. duodenale*. It may be severe and even fatal especially in those with inadequate iron stores and a low iron intake. Infected pregnant women are also at risk of becoming anaemic due to their increased need for iron. Loss of protein can lead to oedema.

Measures to prevent and control hookworm infection

- Preventing soil from becoming infected by improving environmental sanitation, particularly by the use of latrines and health education.

- Preventing infective larvae penetrating the feet by wearing adequate protective footwear. Open sandals are not effective barriers to infection.

LABORATORY DIAGNOSIS

The laboratory diagnosis of hookworm infection is by finding hookworm eggs in faeces less than 3 h old.* Morphologically, the eggs of *A. duodenale* and *N. americanus* cannot be differentiated. The direct examination of faeces is usually adequate to detect the eggs. If required the eggs can be concentrated by the formol ether/ethyl acetate concentration technique or the saturated salt flotation technique (see Appendix V).

Note: Hookworm infection is usually accompanied by a blood eosinophilia.

* A decrease of almost 50% in sensitivity for the detection of hookworm eggs has been reported when there is a delay of more than 3 h in the examination of faeces.

Egg of hookworm (*N. americanus* or *A. duodenale*)

In faecal samples less than 12 hours old, a hookworm egg has the following appearance:

- It is colourless with a thin shell which appears microscopically as a black line around the ovum.
- Oval in shape, measuring about $65 \times 40 \mu\text{m}$.
- Contains an ovum which appears segmented (usually 4–8 cell stage).

Note: If the sample is more than 12 hours old, a larva may be seen inside the egg. If the faeces is more than 24 hours old, the larva may hatch and must then be differentiated from a *Strongyloides* larva (see later text).

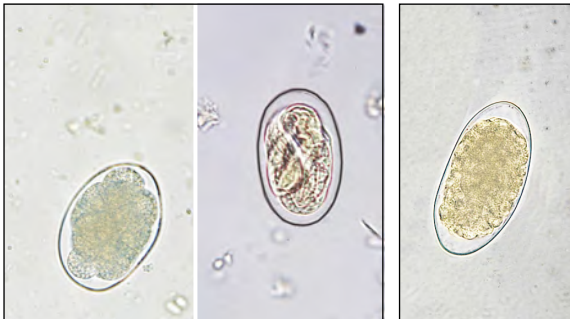


Plate 2.17 Hookworm eggs in saline preparations.

Left: Segmented. *Right:* Embryonated.

Plate 2.18 Egg of *Trichostrongylus*.

Eggs that can be mistaken for hookworm eggs

Hookworm eggs need to be distinguished from the eggs of *Trichostrongylus* species, *Ternidens deminutus*, *Oesophagostum* species and *Strongyloides fülleborni* (nematodes normally parasitic in animals which can infect humans), see following text. *S. fülleborni* is described after *Strongyloides stercoralis*.

Egg of *Trichostrongylus*

Compared with a hookworm egg, the egg of *Trichostrongylus* is:

- Longer and thinner than a hookworm egg, measuring 85–115 μm in length.
- More pointed at one or both ends as shown in Plate 2.18.
- Usually appears more segmented.

Egg of *Ternidens deminutus*

The egg of *Ternidens deminutus* has a similar structure to a hookworm egg but is much larger, measuring about 85 μm in length and contains more cells.

Egg of *S. fülleborni*

Often contains a larva. When at the non-embryonated segmented stage, it closely resembles a hookworm egg except that it is smaller, measuring about $50 \times 35 \mu\text{m}$ (see Plate 2.21 on page 108).

Egg of *Oesophagostum* species

It is about the same size as that of a hookworm but is passed in the faeces in an advanced stage of development.

Trichostrongylus species

Trichostrongylus nematodes, occasionally referred to as pseudo-hookworms, are mainly parasites of ruminants, equines and rodents but several species can infect humans.

Infections have been reported from parts of Africa, Egypt, Indonesia, Iran, Iraq, South East Asia, India, Japan and Chile.

A person becomes infected by ingesting third stage larvae in contaminated food or drink. The adult worms live in the small intestine with the head penetrating the mucosal wall. The head is without cutting teeth or plates. Eggs are produced which are passed in the faeces. They require differentiation from hookworm eggs (see previous text).

Like hookworms, *Trichostrongylus* worms also suck blood from their host. The clinical features of trichostrongyliasis, however, are less severe than those of hookworm infection and treatment is different.

Ternidens deminutus

T. deminutus is a nematode which resembles a hookworm. It is normally parasitic in monkeys and baboons but can infect humans. Infections have been reported mostly from South Africa and East Africa. Transmission is probably by ingestion of third stage larvae. The worms are found in the large intestine. Like hookworms, *T. deminutus* worms also

suck blood from their host and anaemia may develop in heavy infections. *T. deminutus* eggs can be found in faeces and require differentiation from hookworm eggs (see previous text).

Oesophagostom species

Human infections with *Oesophagostom* species have a high prevalence in West and East Africa. An infection rate of 30% has been reported from Togo and Ghana. Eggs resembling those of hookworm (see previous text) are passed in the faeces. In the soil the eggs hatch and the larvae develop into infective stages.

Infection is by the ingestion of infective larvae. The larvae develop in the large intestine where they form nodules and abscesses. When mature the worms leave the nodules and become attached to the intestinal wall.

STRONGYLOIDES STERCORALIS

Transmission and life cycle

Infection with *S. stercoralis* can occur:

- By infective filariform larvae penetrating the skin.
- By autoinfection (self-infection) with rhabditiform (first stage) larvae developing into infective filariform larvae in the intestine or on perianal skin followed by penetration of the intestinal wall or perianal skin. Autoinfection enables untreated infections to persist for many years.

The direct life cycle of *S. stercoralis* is summarized in Fig. 2.15. Unlike other soil-transmitted helminths, *S. stercoralis* reproduces in the soil (warm moist conditions). Adult worms which live in the small intestine are females and eggs are produced parthenogenetically from which rhabditiform larvae hatch. Larvae, not eggs are therefore excreted in the faeces. In the soil the larvae develop within a week into free-living male and female worms. The females produce a further generation of rhabditiform larvae. These develop into infective filariform larvae. The free-living cycle in the soil can be repeated several times.

Strongyloidiasis

When penetrating the skin, *S. stercoralis* larvae can cause an itchy dermatitis and rash. During migration of the larvae, allergic and respiratory symptoms may occur. Most infections are without serious symptoms. Heavy infections (especially common in children) can cause dysentery, malabsorption, steatorrhoea and dehydration with electrolyte disturbance. Abdominal pain is common and occasionally finger clubbing. There is usually an eosinophilia.

Hyperinfection: Autoinfection with *S. stercoralis* can become overwhelming and sometimes fatal when the body's normal immune responses are reduced, e.g. by drugs, steroids, malnutrition, pregnancy or puerperium, or when other diseases are present such as co-infection with HTLV-1 infection. In such infections, larvae can be found in most tissues and serous

cavities of the body. Ulcerative enterocolitis can occur with dysentery or rectal bleeding. It is therefore important to check for strongyloidiasis in those at risk of developing disseminated hyperinfection.

Prevention and control of *Strongyloides* infection are as described for hookworm infection.

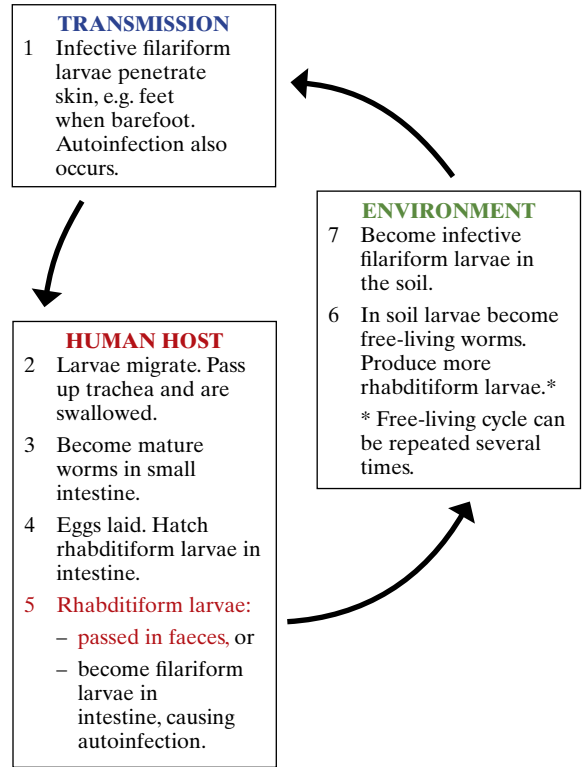


Fig 2.15 Transmission and life cycle of *Strongyloides stercoralis*.

LABORATORY DIAGNOSIS

The laboratory diagnosis of *S. stercoralis* infection is by finding motile *S. stercoralis* larvae in fresh faeces. The larvae can also be found in duodenal aspirates, but this method of diagnosis is not usually necessary. In disseminated infections, larvae can be found in most body fluids. Because *S. stercoralis* larvae tend to be excreted intermittently and their numbers can be few, the following technique is recommended if infection is suspected and the larvae are not detected by direct examination.

Water emergence technique for detecting *S. stercoralis* larvae in faeces

A fresh (not more than 2 hours old) formed

or semiformed faecal sample is required. The procedure is as follows:

- 1 Using a piece of stick, make a central deep depression in the sample. Fill the depression with warm water (not over 37 °C).
- 2 Incubate the sample in a 35–37 °C incubator or on a warm part of the bench for 1½–3 hours during which time the larvae will migrate out of the faeces into the warm water.
- 3 Using a plastic bulb pipette or Pasteur pipette, transfer some of the water to a slide and cover with a cover glass. Alternatively, transfer all the water to a conical tube, centrifuge and transfer the sediment to a slide.
- 4 Examine the preparation microscopically for motile larvae using the 10× objective with the condenser iris *closed sufficiently* to give good contrast.

Note: *S. stercoralis* larvae can also be concentrated by the formol ether/ethyl acetate technique described in Appendix V.

Rhabditiform larvae of *S. stercoralis*

- It is actively motile.*
* Following formol ether/ethyl acetate concentration, the larvae are immobilized.
- Large, measuring 200–250 µm × 16 µm, and unshathed.
- Shows a typical rhabditiform large bulbed oesophagus.
- It can be distinguished from a hookworm larva (sometimes seen in faeces more than 24 h old) by its shorter buccal cavity (mouth cavity) as shown in Plate 2.20.

Note: The buccal cavity can be more easily seen by running a drop of Dobell's iodine under the cover glass to immobilize the larva, and using the 40× objective to examine the depth of the buccal cavity.



Plate 2.19 Larva of *S. stercoralis* as seen with 10× objective.

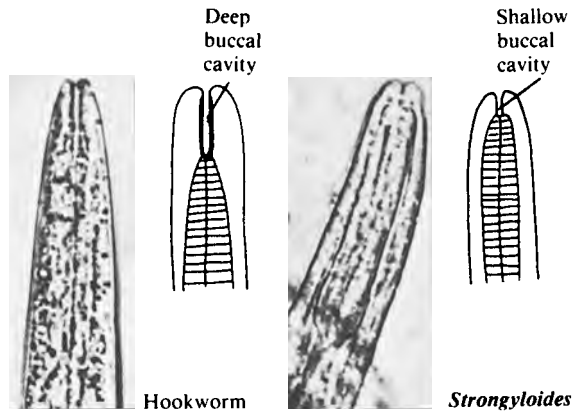


Plate 2.20 *Left:* Deep buccal cavity of hookworm. *Right:* Shallow buccal cavity of *S. stercoralis*.

Strongyloides füelleborni

S. füelleborni is a natural parasite of monkeys and dogs but it can also infect humans. The subspecies *S. füelleborni füelleborni* is found in tropical Africa, and the subspecies *S. füelleborni kellyi* is found in Papua New Guinea. Young children and infants, including babies as young as 2 months old, have been reported as being infected.

In Papua New Guinea, *S. füelleborni* infections are associated with an acute and often fatal infantile disease known as 'swollen belly illness'. *S. füelleborni* infection can be diagnosed by finding eggs in fresh faeces. Many eggs may be present and typically appear embryonated. Often the eggs are mistakenly reported as those of hookworm. Other laboratory findings include a low serum protein, moderate eosinophilia and sometimes anaemia.

Egg of *S. füelleborni*

- Colourless, oval in shape and measures about 50 × 35 µm, i.e. smaller than hookworm eggs which they can resemble.
- Often contains a partially developed larva.

Important: If there is a delay in examining the faeces, the larva will hatch.

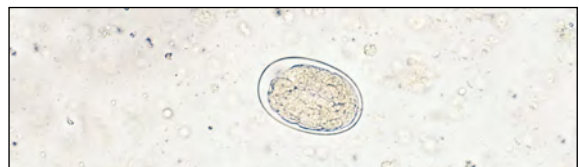


Plate 2.21 Egg of *S. füelleborni* in a saline preparation.

REFERENCES

- 1 *Soil-transmitted helminth infections*. World Health Organization Fact Sheet, 2022. <https://www.who.int/news-room/fact-sheets/detail/soil-transmitted-helminth-infections>
Fact Sheet includes the WHO targets for soil-transmitted helminth infections.

FURTHER READING

Trichuriasis, CDC DPDx, 2017 Update.

2.6 Schistosomiasis

The following species cause intestinal schistosomiasis:

Schistosoma mansoni
Schistosoma japonicum
Schistosoma intercalatum
Schistosoma guineensis
Schistosoma mekongi and related species

Urogenital schistosomiasis is caused by:

Schistosoma haematobium

INTESTINAL SCHISTOSOMIASIS

Distribution

Schistosomiasis is prevalent in the tropics and subtropics in areas without adequate sanitation and access to safe water supplies. Transmission has been reported from 78 countries.

In 2019, the WHO estimated the number of people requiring treatment for schistosomiasis (both intestinal and urogenital) was 236.6 million with at least 90% living in Africa.¹ Infection rates are highest among children (5–14 y).

S. mansoni is widespread in many African countries, Madagascar, and parts of the Middle East, South America (especially Brazil and Venezuela), Suriname and the Caribbean.

S. mansoni occurs with *S. haematobium* in African countries and the eastern Mediterranean. Water development projects for water conservation, irrigation, and hydroelectric power have contributed to the spread of *S. mansoni* and changes in its distribution.

S. japonicum is widely distributed in mainland China, the Philippines and western Indonesia.

S. mekongi is found in Laos People's Democratic Republic, and Cambodia in the lower Mekong River Basin and also in eastern Thailand. Prevalence rates are estimated at 15–50% with children (up to 15 y) being more commonly infected.

S. intercalatum has a high infectivity rate but is limited in its distribution mainly to West and Central Africa, i.e. Zaire, Gabon, and Cameroon. Infections have also been reported from the Republic of São Tomé (island off West Africa).

S. guineensis is found in the rain forests of central Africa and parts of West Africa.

Transmission and life cycle

Schistosoma species are transmitted by cercariae penetrating the skin when a person is bathing, washing clothes, fishing, or engaged in agricultural work or other activity involving contact with water that has been faecally contaminated and contains the snail hosts of the parasites. In its snail host the parasite multiplies and develops to its infective cercarial stage.

Hosts

Humans are the most significant definitive hosts of *S. mansoni*, *S. guineensis* and *S. intercalatum*. Intermediate hosts of *S. mansoni* are aquatic snails. They are found on vegetation in ponds, streams, rivers, lakes, dams, irrigation channels and rice paddies. *S. japonicum* infects a wide range of animals including water-buffaloes, dogs, cats, cattle, pigs, sheep, goats, and wild rodents. Dogs are important reservoir hosts in the transmission of *S. mekongi*. The snails that serve as intermediate hosts for *S. japonicum* and *S. mekongi* are water and land snails. They live mainly on vegetation along river banks and in rice paddies. Fresh water snails are the intermediate hosts for *S. guineensis*.

The indirect life cycle of *Schistosoma* species is summarized in Fig. 2.16 on page 110.

S. mansoni schistosomiasis

There may be irritation and a skin rash at the site of cercarial penetration ('swimmer's itch'). The majority of *S. mansoni* eggs penetrate through the intestinal wall and are excreted in the faeces sometimes with blood and mucus (estimated egg output is 100–300 eggs/day). Host reaction to eggs lodged in the intestinal mucosa leads to the formation of granulomata, ulceration, and thickening of the bowel wall. Large granulomata cause colonic and rectal polyps.

A proportion of the eggs reach the liver through the portal vein. In the liver, reaction to the eggs may eventually cause thickening of the portal vessels known as claypipe-stem fibrosis. Prolonged heavy infection can lead to a marked enlargement of the liver with fibrosis, portal hypertension,

and ascites. The spleen may also become enlarged. Death from haematemesis can occur from ruptured oesophageal varices.

Salmonella infections in patients with *S. mansoni* tend to become chronic and prolonged. Hepatitis B infections have been reported as more common and prolonged.

S. japonicum and *S. mekongi* schistosomiasis

About 2–60 days after infection a severe immune reaction to the products of young flukes and eggs may occur. It is known as Katayama reaction and takes the form of an acute illness with fever, muscular and abdominal pain, spleen enlargement, urticaria and eosinophilia. Although this can also occur with other schistosomiasis infections, it is more common with *S. japonicum*.

The clinical features and pathology of *S. japonicum* infection are similar to, but often more severe, than those of *S. mansoni* infection. The egg output of *S. japonicum* is higher (about 500–3 500 eggs/day). Enlargement of the liver and spleen is common in all age groups. Cerebral schistosomiasis and the depositing of eggs in other parts of the body also occur more frequently with *S. japonicum* infections.

S. mekongi symptoms are reported as being milder than those of *S. japonicum*. Portal hypertension is common.

S. intercalatum and *S. guineensis* schistosomiasis

The commonest clinical symptoms are dysentery and lower abdominal pain. Eggs trapped in the tissues appear to cause less host immune reaction and damage than the eggs of other schistosomes. Highest prevalence and intensity of infection occur in those aged 5–14 years.

Liver disease and portal hypertension have been reported with *S. guineensis* infection.

Measures to prevent and control schistosomiasis

- Avoiding contact with water known to contain cercariae by:
 - Providing safe water supplies in villages.
 - Constructing footbridges across infested rivers and streams.
 - Providing safe recreational bathing sites, especially for children.
- Preventing water becoming contaminated with eggs by:
 - Health education and providing sanitation facilities.
 - Treating infected persons.
 - Protecting water supplies from faecal pollution by animal reservoir hosts (*S. japonicum*, *S. mekongi*).
- Minimizing the risk of infection from new water conservation and irrigation schemes and hydroelectric developments by:
 - Treating workers when necessary.
 - Siting settlements away from canals, drains and irrigation channels and providing latrines and sufficient safe water for domestic use.
 - Lining canals with cement and keeping them free from silt and vegetation in which snails can breed.
 - Filling in formerly used irrigation ditches with clean soil to bury snail hosts.
 - Varying the water levels in the system.

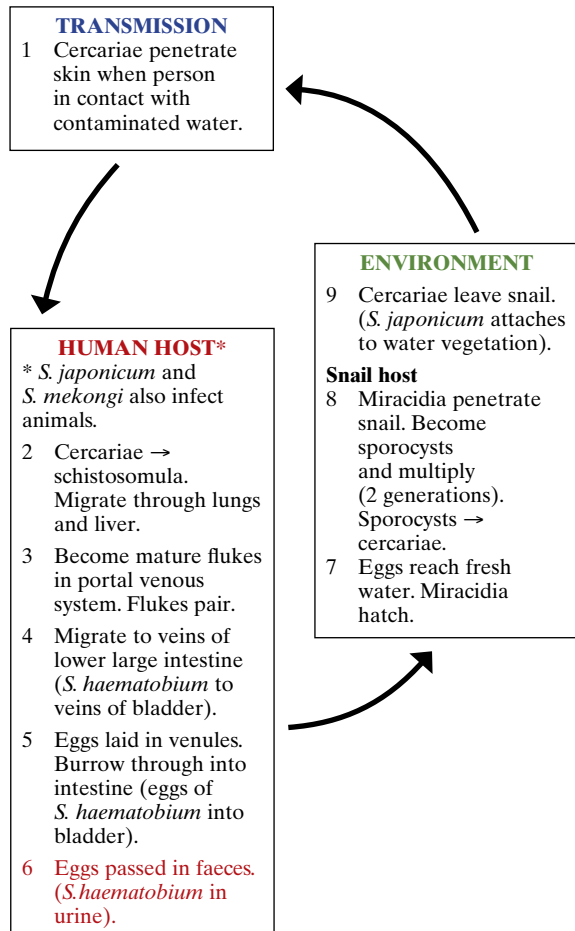


Fig 2.16 Transmission and life cycle of *Schistosoma* species.

- Destroying snail intermediate hosts, mainly by:
 - Using molluscicides where this is affordable and feasible and will not harm important animal and plant life.
 - Removing vegetation from locally used water places, draining swamps, and other measures to eradicate snail habitats.
 - Taking environmental measures to prevent seasonal flooding which results in an increase in snail numbers and transmission.
- Treating water supplies by:
 - Using a chlorine disinfectant where possible.
 - Storing water for 48 hours to allow time for any cercariae to die.
 - Using filter systems at water inputs to prevent cercariae from entering.
- Reducing disease through periodic large-scale treatment of at-risk population groups.

Updates: Readers are referred to the WHO schistosomiasis website www.who.int/schistosomiasis/en/

LABORATORY DIAGNOSIS

The laboratory diagnosis of intestinal schistosomiasis is by:

- Finding schistosome eggs in faeces by direct examination or more commonly by using a concentration technique. The sample will often contain blood and mucus.
- Examining a rectal biopsy for eggs when they cannot be found in faeces, especially after a patient has been partially treated.
- Detecting circulating cathodic antigen (CCA) in urine. CCA is a schistosomal antigen secreted by living adult schistosome flukes, indicating active infection.

Other findings

- There is usually a blood eosinophilia and patients are often anaemic.
- In patients with hepatic disease, serum total protein is raised due to raised globulin, serum albumin is often low, and serum alkaline phosphatase and aspartate aminotransferase (AST) activities are usually raised.

Examination of faeces for schistosome eggs

Schistosoma species that can be found in faeces

Depending on geographical area, eggs of the following *Schistosoma* species can be found in faeces:

S. mansoni
S. intercalatum
S. guineensis
S. japonicum
S. mekongi

Very occasionally, the eggs of *S. mattheei* (animal schistosome) are detected. *S. mattheei* is mainly found in parts of South Africa, Zaire and Zimbabwe.

Natural hybrids of *S. haematobium* and *S. mattheei* occur.

The preparation and examination of faeces by direct technique and the semi-quantitative reporting of egg numbers are described in Appendix V. Knowing the approximate number of eggs gives an indication of the intensity of infection. When eggs are not found in direct preparations a concentration method should be performed.

Concentration techniques used to detect schistosome eggs in faeces

Even in moderate to severe symptomatic infections, particularly those of *S. mansoni* and *S. intercalatum*, a concentration technique may be required to detect the eggs. Concentration techniques include:

- Formol ether/ethyl acetate concentration technique described in Appendix V.
- Mini-FLOTAC flotation technique described in Appendix V.
- Kato-Katz technique which is frequently used in field surveys to assess prevalence and intensity of infection. It is described in Appendix V.

Note: A comparison of the above techniques and emerging technologies for detecting schistosome eggs in faeces can be found in the paper of J. Utzinger *et al*: *New diagnostic tools in schistosomiasis*.²

Microscopical identification of *Schistosoma* eggs

S. mansoni

Distribution: Africa, South America, Caribbean, Middle East.

Egg of *S. mansoni*

- Pale yellow-brown, large, oval, measuring about $150 \times 60 \mu\text{m}$.
 - Has a characteristic side (lateral) spine.
- Note:* Sometimes the spine may appear terminal like that of an *S. haematobium* egg but if the egg is rolled over by pressing gently on the cover glass the spine will be seen to be lateral.
- Contains a fully developed miracidium.



Plate 2.22 Egg of *S. mansoni* with lateral spine in a saline preparation.

S. intercalatum

Distribution: West and Central Africa, island of São Tomé.

Egg of S. intercalatum

- Pale yellow-brown, large, elongate, measuring about $180 \times 60 \mu\text{m}$.
- Has a characteristic long spine at one end (terminal spine) which may appear bent.

Note: Because of its terminal spine, the egg of *S. intercalatum* can resemble that of *S. haematobium* but unlike *S. haematobium*, the egg of *S. intercalatum* is usually found in faeces, not in urine and it is also larger.

- Contains a fully developed miracidium.
- Unlike *S. haematobium*, the shell of *S. intercalatum* is usually acid fast by the ZN staining technique (stains red).



Plate 2.23 Egg of *S. intercalatum* with terminal spine.

S. guineensis

Distribution: West and Central Africa, island of São Tomé.

Egg of S. guineensis

Similar to the egg of *S. haematobium* (see page 115).

- Has a terminal spine which frequently appears slightly bent.
- Longer than the egg of *S. haematobium* measuring $170\text{--}190 \mu\text{m}$.
- Unlike *S. haematobium*, the shell is acid fast when stained by the ZN technique (stains red).

S. japonicum

Distribution: China, Philippines, Indonesia.

Egg of S. japonicum

- Colourless or pale yellow-brown, large and round to oval, measuring about $90 \times 65 \mu\text{m}$.
- A very small hook-like spine (rudimentary spine) can sometimes be seen projecting from the egg wall but often it is hidden by faecal debris and red cells.
- Contains a fully developed miracidium.

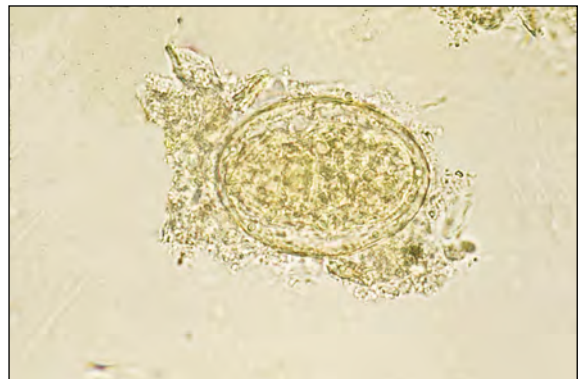


Plate 2.24 Egg of *S. japonicum*. Rudimentary spine is not visible.

S. mekongi

Distribution: Lao PDR and Cambodia.

Egg of S. mekongi

- It is similar to but smaller and rounder than the egg of *S. japonicum*, measuring about $56 \times 66 \mu\text{m}$.
- Has a small knob-like spine similar to the egg of *S. japonicum*.

Examination of a rectal biopsy for schistosome eggs

When schistosome eggs cannot be found in faeces they can sometimes be found in a rectal biopsy. The eggs are often non-viable and calcified.

A biopsy is examined as follows:

- 1 Immediately after removal, place the tissue in physiological saline and soak it for 30–60 mins.
- 2 Transfer the tissue to a slide and cover with a cover glass. With care, press on the cover glass

to spread out the tissue and make a sufficiently thin preparation.

- 3 Examine the entire preparation microscopically for eggs using the 10× objective with the condenser iris *closed sufficiently* to give good contrast. Constant focusing is necessary to detect the eggs.

Note: if the preparation is too thick to examine, add a drop of lactophenol solution (Reagent No 24) and wait a few minutes for the tissue to clear sufficiently.

- 4 Identify the eggs and estimate the number of uncalcified eggs in the biopsy and the proportion that are calcified (black). Uncalcified and calcified eggs of *S. mansoni* in tissue are shown in Plate 2.25.

Schistosome eggs found in rectal biopsies: A rectal biopsy, depending on geographical area, may contain the eggs of *S. mansoni*, *S. guineensis*, *S. intercalatum*, *S. japonicum*, *S. mekongi*, and occasionally the eggs of *S. haematobium*.

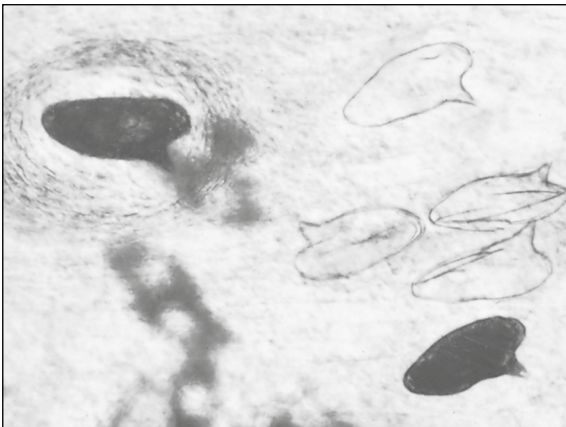


Plate 2.25 Uncalcified and calcified (black) eggs of *S. mansoni* in unstained rectal biopsy.

Detecting CCA in urine

Circulating cathodic antigen (CCA) can be detected qualitatively in urine using the *Schisto POC-CCA* test which is a lateral flow immunoassay. The test uses monoclonal antibody immobilized on the cassette membrane to bind CCA antigen in urine samples. Results are read after 20 minutes. A positive and negative test are shown opposite.

A positive test indicates active schistosomiasis. Sensitivity of the test depends on wormload. In early infections, antigen may be absent or produce a faint positive test result. Following successful treatment the concentration of CCA in urine decreases and is usually undetectable after 2–3 weeks.

CCA is produced by all *Schistosoma* species. The highest concentrations of CCA are detected in *S. mansoni* infections. The test is therefore particularly useful for diagnosing intestinal schistosomiasis.² In endemic areas, it has been shown to be more sensitive than the Kato-Katz technique. Levels in urogenital schistosomiasis are variable and appear to differ between regions. Most medium to high level *S. haematobium* infections can be diagnosed using the CCA test.

Urinary tract infections and haematuria can cause false positive tests. The test does not require urine to be collected at any particular time of day. Urine samples that have been stored at 4 °C or frozen at –20 °C can be used (warmed to room temperature before testing).



Positive CCA test showing a pink band in the Test (T) area and a pink band in the Control (C) area indicating the correct migration of the sample.

Courtesy of Rapid Medical Diagnostics.



Negative CCA test showing no pink band in the Test (T) area. A pink band in the Control (C) area indicates the correct migration of the sample.

Courtesy of Rapid Medical Diagnostics.

Availability of *Schisto POC-CCA* test

A 25 test kit is available and requires storage at constant 4–28 °C. Details of cost and shelf-life can be obtained from the manufacturer Rapid Medical Diagnostics: www.rapid-diagnostics.com

UROGENITAL SCHISTOSOMIASIS

S. haematobium causes urogenital schistosomiasis. The species contains several strains.

Distribution

S. haematobium is endemic in 54 countries, mainly in Africa, the Middle East, and Corsica. In some areas the distribution of *S. haematobium* overlaps with *S. mansoni* causing double infections.

The development of irrigation schemes and dams for hydroelectric power and flood control have greatly increased the prevalence of *S. haematobium* infections in several countries. The migration of refugees has contributed to an increase in distribution.

Transmission and life cycle

S. haematobium is transmitted by cercariae penetrating the skin when bathing, washing clothes, fishing, or engaged in agricultural work or other activity involving contact with contaminated water. In endemic areas a large proportion of children and teenagers become infected and reinfected.

S. haematobium has an indirect life cycle similar to that described for other *Schistosoma* species and summarized in Fig. 2.16.

It is the eggs of *S. haematobium* in the tissues not the adult flukes that stimulate host inflammatory responses that result in the clinical features and damage to the bladder and ureters that characterize urogenital schistosomiasis. The degree of illhealth and serious complications that develop later in life are related to the intensity and duration of infection.

Urogenital schistosomiasis

Within 24 hours of infection an intense irritation and skin rash, referred to as 'swimmer's itch', may occur at the site of cercarial penetration. Haematuria (blood in urine) caused by eggs penetrating through the wall of the bladder is a feature of urinary schistosomiasis. In endemic areas, up to 50–70% of infected persons (80% of infected children) have symptoms of urinary tract disease with haematuria, dysuria or frequency.

Eggs trapped in the wall of the bladder and surrounding tissues cause inflammatory reactions with the formation of granulomata. Many of the eggs die and become calcified eventually producing what are known as 'sandy patches' in the bladder. In heavy infections, eggs can be carried to other parts of the body. Following prolonged untreated infection and a marked cellular immune response, the ureters may become obstructed and the bladder wall thickened leading to abnormal bladder function, urinary tract infection, and eventually obstructive renal disease with kidney damage.

Complications can arise from genital schistosomiasis in both men and women. See the WHO website *

* www.who.int/schistosomiasis/genital_schistosomiasis/en/

The reproductive health of women can be particularly affected with the formation of granulomas in the uterus, fallopian tubes, and ovaries, leading to abdominal and pelvic pain, menstrual disorders, inflammation of the cervix, abortion, ectopic pregnancy and infertility. Urogenital schistosomiasis increases significantly the risk of being infected with HIV.

Chronic long-term infection with *S. haematobium* is associated with squamous cell bladder cancer. Infection is also linked to

an increase in *Salmomella* infections. Calculi (stones) in the bladder and urinary tract are also found with chronic urinary schistosomiasis. Anaemia is found particularly in those with low dietary iron intake, co-existing hookworm infection or malaria.

Prevention and control

The measures described for intestinal schistosomiasis also apply to the prevention and control of urogenital schistosomiasis.

Updates: Readers are referred to the WHO schistosomiasis website: www.who.int/schistosomiasis/en/

LABORATORY DIAGNOSIS

Laboratory diagnosis of *S. haematobium* infection is by:

- Finding the eggs or occasionally the miracidia of *S. haematobium* in urine. The report should be quantitative, i.e. number of eggs/10 mL of urine.
- Detecting eggs in a rectal biopsy or bladder mucosal biopsy.

Note: In mixed infections, the eggs of both *S. mansoni* and *S. haematobium* can sometimes be found in urine and occasionally *S. haematobium* eggs can be found in faeces.

Other findings

- Haematuria is a common finding.

Haematuria: In endemic areas, up to 80% of infected children have haematuria, and of those infected with more than 50 eggs/10 mL of urine, 98–100% have haematuria.

- Proteinuria is frequently present.
- Eosinophils can often be found in the urine. There is usually also a blood eosinophilia.

Identifying eosinophils in urine: Add a small drop of weak eosin solution (e.g. 1 in 10 dilution of Field stain B) to a urine sediment. The granules of eosinophils stain bright red whereas those of pus cells remain unstained or stain pale pink.

- Bacteriuria may accompany urogenital schistosomiasis.
- CCA can be detected in most medium to high level infections (see previous text).

Procedure for examining urine for schistosome eggs

The excretion of *S. haematobium* eggs in urine is highest between 10.00 h and 14.00 h, with a peak around midday. Even when persons are heavily infected, eggs may not be present in the urine. It may be necessary to examine several samples

collected on *different* days due to the irregular pattern of egg excretion. The routine examination of urine for schistosome eggs is as follows:

- 1 Collect 10–15 mL of urine (between 10.00 h and 14.00 h) in a clean dry container.

Note: Neither exercising before passing urine nor collecting terminal urine (last few drops), increase the number of eggs present in the sample. To avoid the miracidia hatching from the eggs, keep the sample in the dark if unable to examine it within 30 minutes.

- 2 Report the appearance of the urine. In moderate to heavy infections, the urine will usually contain blood and appear red or red-brown and cloudy.

When visible blood is present, add 2 drops of saponin solution (Reagent No. 36) to lyse the red cells. This will make it easier to detect the eggs.

If blood is not seen, test the sample chemically for blood and protein.

- 3 Transfer 10 mL of well mixed urine to a conical tube and centrifuge at RCF 500–1 000× g to sediment the schistosome eggs (avoid centrifuging at greater force because this can cause the eggs to hatch).

Note: If a centrifuge is not available, allow the eggs to sediment by gravity for 1 hour.

- 4 Discard the supernatant fluid. Transfer all the sediment to a slide, cover with a cover glass and examine the *entire* sediment microscopically using the 10× objective with the condenser iris closed sufficiently to give good contrast.

- 5 Count the number of eggs in the preparation and report the number/10 mL of urine. If more than 50 eggs are present, there is no need to continue counting. Report the count as 'More than 50 eggs/10 mL'. Such counts indicate a heavy infection.

In the early stages of urinary schistosomiasis, the egg count is an indicator of the severity of disease.

Other terminally spined Schistosome eggs

Occasionally the eggs of *S. guineensis* or *S. intercalatum* can be found in urine in areas where these species are found and when there is faecal contamination of the urine. They can be differentiated by Ziehl-Neelsen staining. The shell of *S. haematobium* is not acid fast whereas that of other terminally spined *Schistosoma* species is acid fast (stains red).

Egg of *S. haematobium*

- Pale yellow-brown, large and oval in shape, measuring about 145 × 55 µm.
- Has a characteristic small spine at one end (terminal spine).
- Contains a fully developed miracidium.

Note: Sometimes the miracidia hatch from the eggs and can be seen 'swimming' in the urine as shown in Plate 2.28.

- A living egg (in fresh unpreserved urine) shows what is called flame cell movement, i.e. flickering of the excretory flame cells (see Plate 2.26).

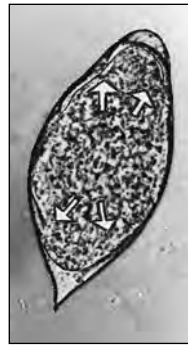


Plate 2.26 Position of flame cells.

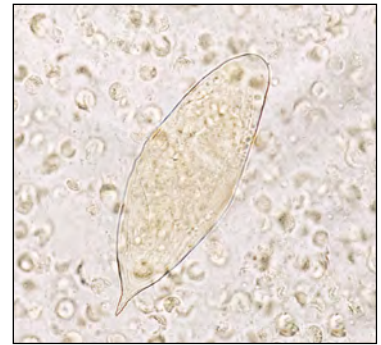


Plate 2.27 *S. haematobium* egg and red cells in urine, 40× objective.

Miracidia in urine: When the urine is dilute or has been left to stand for several hours in the light, the miracidia will hatch from the eggs. The ciliated miracidia are motile.

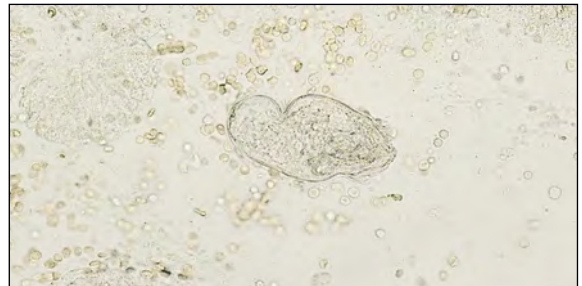


Plate 2.28 Ciliated miracidium of *S. haematobium* in urine.

Urine filtration technique

Filtration is the most sensitive, rapid, and reproducible technique for detecting and quantifying *S. haematobium* eggs in urine.

Required

- 10 mL Luer syringe.
- Syringe filter holder (Swinnex type), 13 mm diameter. This is suitable for holding filters of 12 mm or 13 mm diameter.
- Polycarbonate membrane filter of 13 mm diameter and 12–14 µm pore size. This is a *clear* filter. Other types of filters can also be used, e.g. *Nytre* woven filters.

Availability of syringe filter holders and membrane filters

A urine *S. haematobium* filtration test kit, SKU No 98230 is available from Sterlitech company:

www.sterlitech.com/schistosome-test-kit-98230.html

The kit contains:

- 500 polycarbonate 13 mm membrane filters, 12 µm pore size
- 5 plastic 13 mm Swinnex syringe filter holders
- 2 plastic 10 mL syringes

Procedure

- 1 Using blunt-ended (untoothed) forceps, carefully place a polycarbonate filter on the filter support of the filter holder. Re-assemble the filter holder and attach it to the end of a 10 mL Luer syringe (see Plate 2.29).
- 2 Remove the plunger from the syringe. Fill the syringe to the 10 mL mark with well-mixed urine, and replace the plunger. Holding the syringe over a beaker or other suitable container, slowly pass the urine through the filter.

Note: Filling the syringe is preferred to drawing up the urine into the syringe because it does not require tubing and the air which passes through the membrane after the urine, helps to stick the eggs to the filter.

Clogging of filters

If a membrane filter clogs, remove the filter holder and attach it to another syringe containing 3% v/v acetic acid. Pass the acid through the filter. This may help to clear it. If, however, the blockage does not clear, use another membrane to filter the remaining sample of urine. Both filters must be examined to give the number of eggs in the 10 mL urine sample.

- 3 Remove the filter holder and unscrew it. Using blunt-ended forceps, carefully remove the filter and transfer it face upwards (eggs on surface) to a slide. Add a drop of physiological saline, and cover with a cover glass.
- 4 Using the 10× objective with the condenser iris *closed sufficiently* to give good contrast, examine systematically the entire filter for *S. haematobium* eggs. Count the number of eggs and report the number per 10 mL of urine.

Note: If more than 50 eggs are present there is no need to continue counting (50 eggs or more/10 mL of urine is considered a heavy infection). Report the count as 'More than 50 eggs/10 mL'.

Differentiating non-viable from viable schistosome eggs on a filter

In assessing active infection or in judging whether treatment has been successful, it is helpful to know whether the schistosome eggs detected are viable or non-viable.

Although it is often possible to see flame cell movement in viable eggs (see Plate 2.26), a more reliable way of differentiating viable from non-viable schistosome eggs is to examine a preparation stained with 1% w/v trypan blue in physiological saline. A drop of stain is added and the preparation is left for 30 minutes at room temperature (in a damp chamber to prevent drying out).

Non-viable eggs	Stain blue
Viable eggs	Unstained

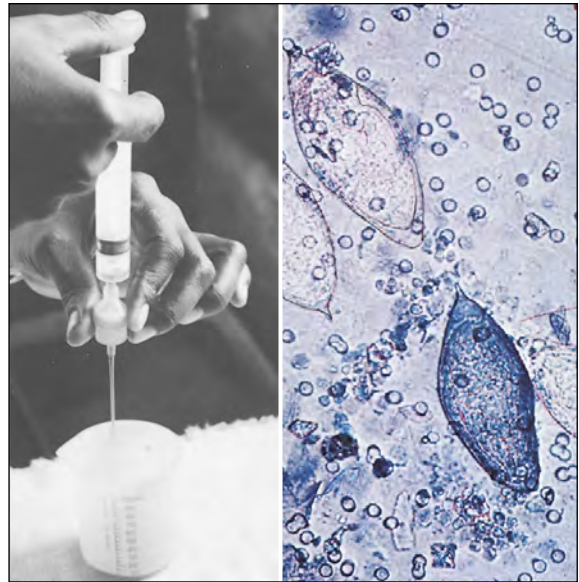


Plate 2.29 *Left:* Filtration concentration technique for detection of *S. haematobium* eggs. *Right:* Trypan blue preparation showing stained non-viable *S. haematobium* egg and unstained living eggs.

Examination of total volume of urine collected between 10.00 h and 14.00 h

In light infections to increase the possibility of finding *S. haematobium* eggs, the total volume of urine

excreted between 10.00 h and 14.00 h can be examined as follows:

- 1 Note the appearance of each sample of urine passed over the 4 hours. If blood is not visible test the sample chemically for protein and blood.
- 2 After testing for protein and blood, add about 0.1 mL of 10% v/v formol saline to each sample (50–100 mL) of urine to preserve the eggs. Red cells in the urine will be lysed.
- 3 Transfer the urine to a narrow cylinder, cover, and leave for about 2 hours for the eggs to sediment.
- 4 Discard all but the last approximate 15 mL of urine. Centrifuge to sediment the eggs.
- 5 Remove the supernatant, and transfer the sediment to a slide, cover with a cover glass and examine microscopically (using 10× objective) for schistosome eggs.

Examination of biopsies for *S. haematobium* eggs

The laboratory examination of rectal and bladder mucosal biopsies is as described for intestinal schistosomiasis. Non-viable calcified *S. haematobium* eggs appear black. Such eggs are frequently found in rectal biopsies.

REFERENCES

- 1 WHO Schistosomiasis Fact Sheet, 2022. <https://who.int/news-room/fact-sheets/detail/schistosomiasis>

FURTHER READING

Olumide A et al. Tools for detection of schistosomiasis in resource limited settings. *Medical Sciences*, 2018. Access by entering the title in Google.

2.7 Foodborne trematodiasis

The following are medically important foodborne trematode (flake) infections:

- Clonorchiasis caused by *Clonorchis sinensis*
- Opisthorchiasis caused by *Opisthorchis viverrini*
- Fascioliasis caused by *Fasciola hepatica* and *Fasciola gigantica*
- Fasciolopsiasis caused by *Fasciolopsis buski*
- Paragonimiasis caused by *Paragonimus* species

CLONORCHIASIS

Distribution

C. sinensis is endemic in south China, South Korea, Taiwan, northern Vietnam, the far east of Russia and Siberia.

High infection rates are found in areas where human untreated faeces and animal faeces pollute fish culture ponds or other fresh water which contain the snail and intermediate hosts.

Transmission and life cycle

C. sinensis is transmitted by eating raw, undercooked, smoked, or pickled fish or fish products containing infective metacercariae. Reservoir hosts include fish – eating cats, dogs, pigs and rodents.

The indirect life cycle of *C. sinensis* is summarized in Fig. 2.17. Following ingestion the metacercariae excyst in the duodenum, migrate to the intrahepatic bile ducts and gall bladder and occasionally to the pancreas where they become egg-producing flukes, about 4 weeks after infection. *C. sinensis* can live for many years in its host.

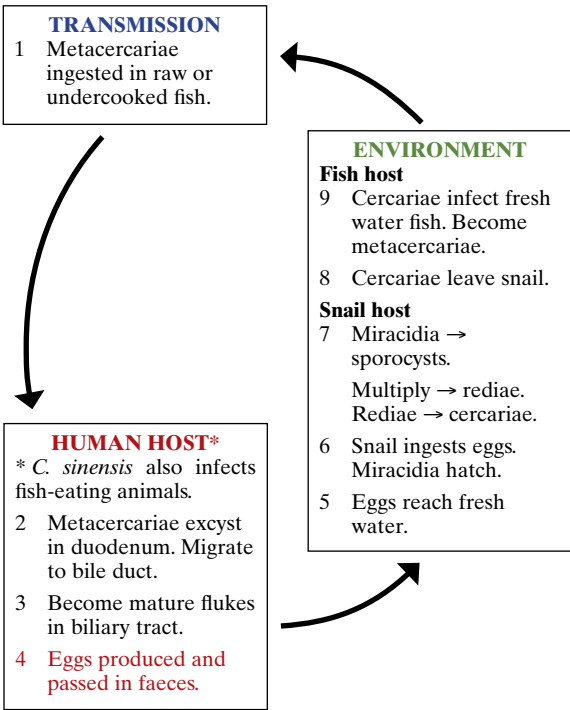


Fig 2.17 Transmission and life cycle of *Clonorchis sinensis*.

Clonorchiasis

Symptoms of infection with *C. sinensis* are rarely serious. In endemic areas, chronic and heavy infections can cause damage to the liver, and bile ducts with jaundice, hepatitis, cirrhosis, and biliary obstruction. The liver frequently becomes enlarged and in some cases cancer of the bile duct (cholangiocarcinoma) can develop. Chronic infection is associated with gall stones, recurrent attacks of cholangitis, and pancreatitis.

In heavy infections, symptoms include weakness, weight loss, abdominal fullness, diarrhoea, anaemia, and oedema. In advanced infections, portal hypertension, ascites, and upper gastrointestinal bleeding may occur.

Measures to prevent and control clonorchiasis

- Avoiding eating raw, pickled, smoked, or insufficiently cooked or processed fish or fish products which may contain metacercariae.
- Providing adequate latrines and health education to prevent faecal contamination of fish ponds and other fresh water that may contain snail hosts.
- Treating those infected.
- Using chemicals or other methods to eradicate snail hosts in areas where this is feasible.

LABORATORY DIAGNOSIS

The laboratory diagnosis of *C. sinensis* infection is by:

- Finding the eggs of *C. sinensis* in faeces.
- Detecting eggs in aspirates of duodenal fluid.

Note: Eosinophilia is common.

Examination of faeces for *C. sinensis* eggs

The eggs can be few and because they are small they can be easily missed.

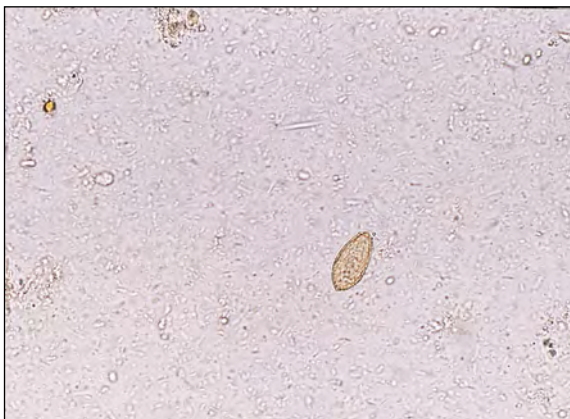


Plate 2.30 Egg of *C. sinensis* in a saline preparation.

Egg of *C. sinensis*

- Yellow-brown and small measuring $27\text{--}32 \times 15\text{--}18 \mu\text{m}$.
- Contains a ciliated miracidium but this is difficult to see through the surface of the egg.
- Has a clearly seen operculum (lid).
- Often described as having 'shoulders' (rim on which the operculum rests).
- A small projection can sometimes be seen at the other end of the egg.
- When examined with the high power objective, an indistinct outer covering of the shell can often be seen.

Note: The eggs of *C. sinensis* closely resemble those of *O. viverrini* and *O. felinus*.

A concentration technique should be used if *C. sinensis* infection is suspected and no eggs are found by direct examination. Substitution of formal water with a citric acid – Tween solution (Reagent No 14) in the formal ether/ethyl acetate technique is recommended for the best recovery of *Clonorchis* eggs.

Note: The 40× objective is required to identify *C. sinensis* eggs and the preparation must not be too thick otherwise the small eggs will be overlooked.

Examination of duodenal fluid to detect *C. sinensis* eggs

A filtration technique can be used to recover the eggs of *C. sinensis* from aspirates of duodenal fluid. A membrane filter of 8 μm pore size is required for filtering the eggs of *C. sinensis*. Staining the membrane with 1% w/v trypan blue in saline helps to show the eggs.

If unable to perform a filtration technique or if the sample is not suitable for filtration, transfer a drop of the aspirate (especially mucus) to a slide, cover with a cover glass and examine microscopically.

Make the preparation sufficiently thin otherwise the eggs will be missed. Use the 40× objective to identify the small eggs. If the aspirate is watery, centrifuge it first and examine a drop of the sediment.

Note: Further information on the epidemiology and pathogenesis of *C. sinensis* and the new technologies which are being developed to control and diagnose clonorchiasis can be found in the paper of Ze-Li Tang *et al.*¹

OPISTHORCHIASIS

Distribution

O. viverrini is endemic in Thailand, central Vietnam, Cambodia and Lao PDR, Kazakhstan, Ukraine and Russia. It is the commonest trematode infection in north east Thailand.

Transmission and life cycle

Transmission, hosts and life cycle are similar to those of *C. sinensis* described previously. Fish-eating animals especially cats and dogs serve as reservoir hosts. The mature flukes live in the biliary tract and pancreatic ducts.

Opisthorchiasis

Most of the symptoms associated with opisthorchiasis occur in persons with chronic and heavy infections. In Kampuchea and the Lao PDR, coinfection with *S. mekongi* can cause severe liver disease.

The flukes and deposited eggs cause inflammation and fibrosis around the bile ducts. Symptoms of chronic opisthorchiasis include diarrhoea, flatulence, upper abdominal pain, fatty food intolerance, fever, gall stones, progressive jaundice, enlarged liver, weakness and oedema.

Recurrent attacks of cholangitis and pancreatitis can occur. Cancer of the bile duct is associated with chronic infection. The measures described for *C. sinensis* apply also to the prevention and control of *O. viverrini* infections.

Readers are referred to the publication: *Toward intergrated opisthorchiasis control in northeast Thailand: The Lawaproject*. dx.doi.org/10.1016/j.actatropica.2014.07.017

LABORATORY DIAGNOSIS

The laboratory diagnosis of *O. viverrini* infection is the same as that described for *C. sinensis* (see previous text). Eggs can be found in faeces and aspirates of duodenal fluid.

Egg of *O. viverrini*

It closely resembles that of *C. sinensis* except that it is slightly smaller, measuring 19–29 × 12–17 µm.

Note: Information on advances in the diagnosis of human opisthorchiasis including development of *O. viverrini* antigen detection in urine can be found in the paper of C. Worasith *et al.*²

Opisthorchis felineus

O. felineus is a common parasite in cats, dogs, and fish-eating wild animals and also infects humans. It is mainly found in the Russian Federation, Kazakhstan, Ukraine, and Poland where it is widely distributed and a major public health problem.

Egg of *O. felineus*

It closely resembles that of *C. sinensis* except that it is slightly narrower, measuring 26–32 × 11–15 µm. Most of the eggs are asymmetrical, being slightly less convex on one side.

FASCIOLIASIS

F. hepatica and *F. gigantica* are important animal pathogens. They live in the liver and bile ducts of sheep, cattle, and other animals causing the serious disease liver rot. *F. gigantica* infections in cattle lead to considerable economic loss especially in some African countries. Very occasionally *Fasciola* flukes infect humans. Infection with *F. hepatica* is more common than infection with *F. gigantica* which is less adapted to humans.

Distribution

F. hepatica is found in sheep-raising areas in temperate countries and also in Egypt, parts of the Middle East, and South America, particularly Bolivia.

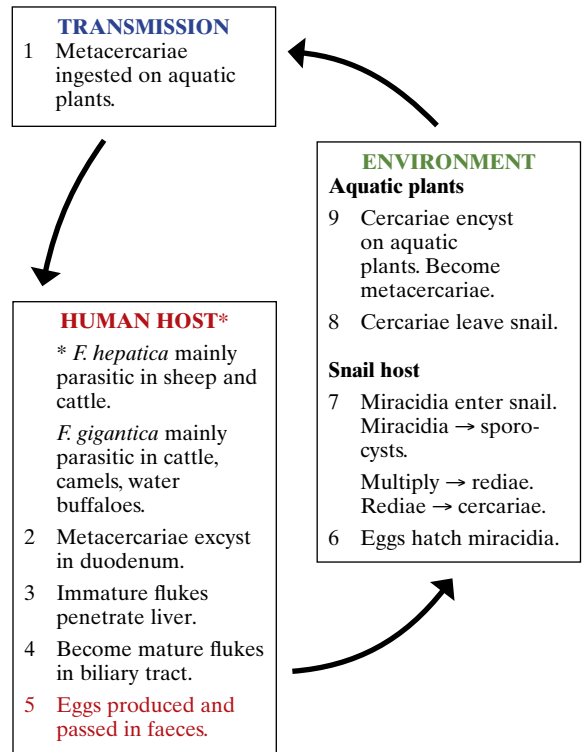


Fig 2.18 Transmission and life cycle of *Fasciola* species.

F. gigantica is found mainly in tropical Africa, South and South East Asia, and the Far East. Cattle, camels and water buffaloes are commonly infected. In some

areas, e.g. Egypt and the Islamic Republic of Iran, *F. hepatica* and *F. gigantica* occur together.

Transmission and life cycle

F. hepatica and *F. gigantica* are transmitted by ingesting metacercariae encysted on wild watercress or other aquatic plants grown in water contaminated with faeces from infected animals.

The indirect life cycle of *F. hepatica* and *F. gigantica* is summarized in Fig. 2.18. Following ingestion the metacercariae excyst in the duodenum and the young flukes migrate through the intestinal wall into the peritoneal cavity. They reach the bile ducts by penetrating through the liver capsule. About 4 months after infection the flukes become mature and produce eggs which are excreted in the faeces. The flukes can live for many years in their host.

Fascioliasis

Light infections are usually asymptomatic. Heavy infections, however, can cause acute disease with serious liver damage during the 4–6 weeks when the immature flukes migrate through the liver. Symptoms during this period include fever, sweating, abdominal pain, dizziness, cough, asthma, urticaria and a *marked* blood eosinophilia. In the bile ducts, the flukes cause inflammation. Occasionally, migrating flukes develop in other organs of the body.

With chronic infection there is thickening of the bile ducts due to a host cellular response. The liver becomes enlarged and blockage of the bile ducts can lead to obstructive jaundice. The gall bladder can also be infected. Anaemia is common in heavy infections.

Measures to prevent and control fascioliasis

- Avoiding eating watercress or other uncooked water plants which may contain infective metacercariae.
- Cultivating watercress in water free from faecal pollution.
- Reducing the infection rate in animals by fencing off grazing land known to be infected with metacercariae. Also treating animals to reduce egg output.
- Identifying and destroying snail habitats where this is feasible.

LABORATORY DIAGNOSIS

The laboratory diagnosis of fascioliasis is by:

- Finding eggs in faeces in chronic infections. *Fasciola* eggs can also be found in duodenal aspirates and in bile. Eosinophilia is common.

Note: Eggs will not be found in faeces in acute fascioliasis when the immature flukes are migrating through the liver and causing serious symptoms but not yet producing eggs. Diagnosis is best made serologically.

- Seriological diagnosis by testing serum for antibodies is particularly valuable in the early stages of infection when eggs are not present in the faeces. Cross-reactivity with other trematodes such as schistosomes can occur. In endemic areas where *Fasciola* infections are prevalent, district laboratories should consult with their nearest Public Health Laboratory regarding the availability of a suitable antibody test or faecal antigen test.

Examination of faeces for *Fasciola* eggs

A concentration technique such as the formol ether/ethyl acetate method described in Appendix V is recommended because the eggs are usually few. Several samples may need to be examined.

Note: If eggs are found in human faeces it must be confirmed that they are present due to a *Fasciola* infection and not from eating animal liver containing *Fasciola* eggs. Repeated finding of the eggs in faeces establishes parasitic infection.

Egg of *F. hepatica* or *F. gigantica*

- Yellow-brown, large and oval.
- *F. hepatica* eggs measure 130–145 × 70–90 μm.
- *F. gigantica* eggs are larger, measuring 156–197 × 90–104 μm.
- Has an operculum (lid).
- Contains an unsegmented ovum surrounded by many yolk cells.

Note: Morphologically *Fasciola* eggs resemble those of *Fasciolopsis buski* and *Echinostoma* species.

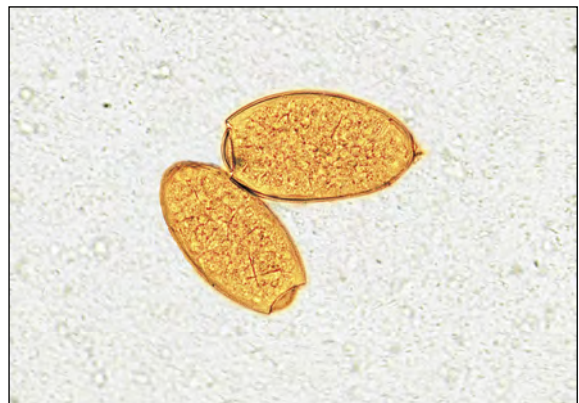


Plate 2.31 Eggs of *F. hepatica* in a saline preparation.

FASCIOLOPSIASIS

F. buski is the largest fluke that infects humans. It is known as the giant intestinal fluke. The pig and buffalo are the normal definitive hosts.

Distribution

Human infection with *F. buski* is widespread in South and East Asia including southern and central China, Taiwan, Vietnam and Thailand. It is also found in the Nile Delta and western South America. High prevalence rates occur in areas where water plants are cultivated in ponds that are fertilized by untreated infected pig or human faeces. Infection rates are particularly high in children.

Transmission and life cycle

Infection with *F. buski* is by ingesting metacercariae from water chestnuts, water caltrop, or other aquatic edible plants grown in water contaminated with faeces. The indirect life cycle of *F. buski* is summarized in Fig. 2.19.

Fasciolopsiasis

Most infections with *F. buski* are light and asymptomatic. Heavy infections can cause inflammation and ulceration of the intestinal wall with diarrhoea and abdominal pain. Toxins produced by the flukes can cause oedema of the face and

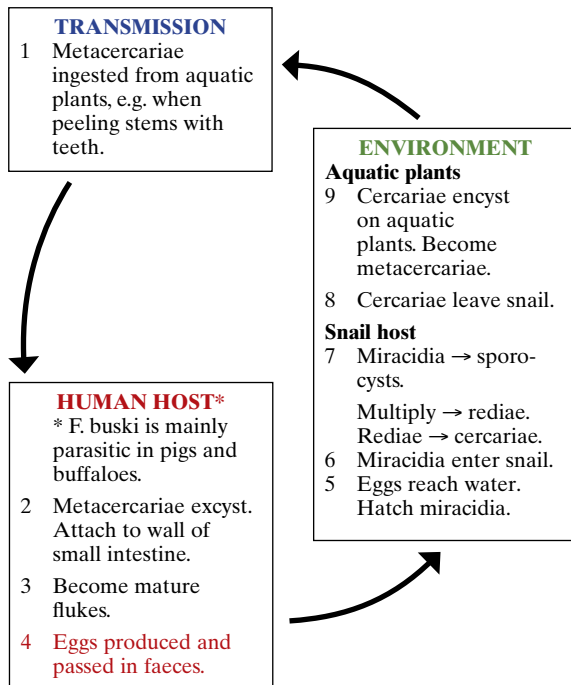


Fig 2.19 Transmission and life cycle of *Fasciolopsis buski*.

limbs, ascites, and other allergic reactions. Plasma albumin levels may fall due to protein loss. There is usually a blood eosinophilia and slight macrocytic anaemia. Toxic reactions in heavily infected children can be fatal.

Measures to prevent and control fasciolopsiasis

- Treating water plants which may be infected using boiling water or cooking them before eating or teeth-peeling.
- Providing latrines and health education to prevent eggs reaching the water (where humans are the main source of infection). Also, treating infected individuals (within a control programme).
- Avoiding the use of untreated human or pig faeces as fertilizer in cultivation ponds.
- Identifying and destroying snail hosts and their habitats where this is feasible.

LABORATORY DIAGNOSIS

The laboratory diagnosis of *F. buski* infection is by finding the eggs in faeces, examined by direct technique. Concentration techniques are rarely needed.

Egg of *F. buski*

- Yellow-brown, large and oval, measuring $130\text{--}154 \times 78\text{--}98 \mu\text{m}$.
- Has a small operculum (lid) which is usually difficult to see.
- Contains an unsegmented ovum surrounded by yolk cells.

Note: Morphologically the eggs of *F. buski* resemble those of *F. hepatica*, *F. gigantia* (similar but smaller), and *Echinostoma* species.



Plate 2.32 Egg of *F. buski* in a saline preparation.

PARAGONIMIASIS

Distribution

People are infected with *Paragonimus* species in areas where crabs and crayfish are eaten raw or undercooked. Species which cause paragonimiasis in tropical and subtropical countries include:

ASIA

- *P. westermani* (Oriental lung fluke) in the Far East and South East Asia (Korea, China, Philippines, Japan, Malaysia, Thailand, Indonesia, Vietnam), and also parts of India, and some Pacific Islands.
- *P. heterotremus*, also in Thailand, Lao Peoples Democratic Republic, and South China.
- *P. philippinensis*, in the Philippines.

AFRICA

- *P. uterobilateralis*, in the west and south of Africa. It is the main species in Nigeria. Also occurs in Burkina Faso, Central African Republic, Congo, Côte d'Ivoire, Equatorial Guinea, Gabon, Liberia, Sierra Leone, Zaire, Zambia.
- *P. africanus*, in Cameroon and also in Nigeria, and parts of central Africa.

CENTRAL AND SOUTH AMERICA

- *P. mexicanus*, in Central and South America, especially Ecuador and Peru. Also found in limited foci in Colombia, Costa Rica, Cuba, El Salvador, Guatemala, Honduras, and Mexico.

Transmission and life cycle

Infection with *Paragonimus* flukes is by ingesting the flesh or juice of raw, undercooked, or pickled crab or crayfish which contains metacercariae. In China, shrimps have also been found to be infected. Infection can also occur by ingesting metacercariae from fingers contaminated during food preparation.

The metacercariae of *Paragonimus* species that infect humans are also infective to crustacean-eating animals. In some areas animal reservoir hosts are important in transmission. The life-span of a *Paragonimus* fluke is usually 6–7 years but human infections of up to 20 years have been reported.

The life cycle of *Paragonimus* species is summarized in Fig. 2.20.

Paragonimiasis

Light to moderate *Paragonimus* infections are usually asymptomatic. Heavy infections, however, can cause pulmonary disease with inflammatory responses to the flukes and eggs. The flukes become encapsulated.

Symptoms of severe pulmonary paragonimiasis often resemble those of pulmonary tuberculosis with chest pain, cough, night sweats, pleural effusion, and haemoptysis (coughing up blood).

Paragonimus flukes in the intestine and liver cause liver disease, pain, diarrhoea, and vomiting. The most serious complications occur when *Paragonimus* flukes parasitize the central nervous system causing severe headache, cerebral haemorrhage, oedema, mental confusion, and sight impairment.

Measures to prevent and control paragonimiasis

- Not eating raw, undercooked, or pickled crabs or crayfish which may contain infective metacercariae.
- Not contaminating water with sputum or faeces.
- Detecting and treating infected persons in endemic areas.
- Identifying and destroying the snail hosts of local *Paragonimus* species where this is feasible.

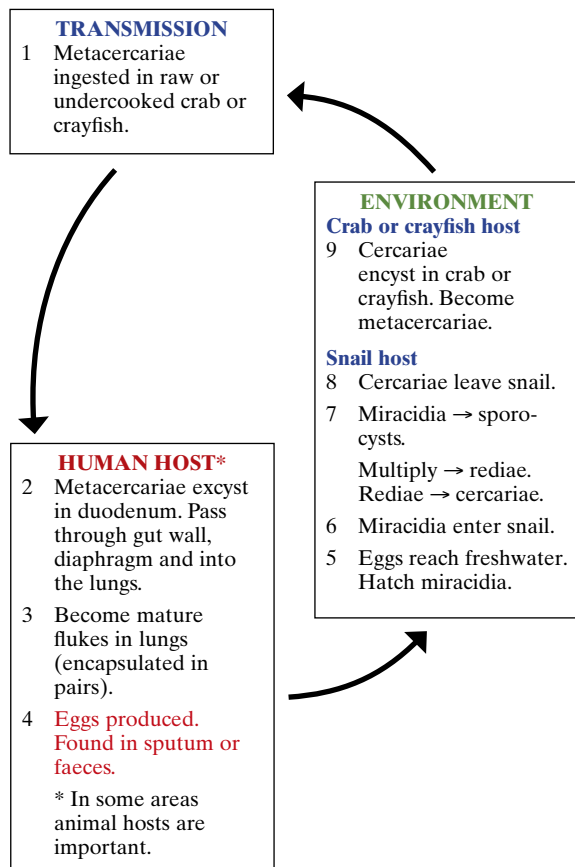


Fig 2.20 Transmission and life cycle of *Paragonimus* species.

LABORATORY DIAGNOSIS

The diagnosis of paragonimiasis is by:

- Finding *Paragonimus* eggs in sputum or occasionally in aspirates of pleural fluid.
- Examining faeces for *Paragonimus* eggs that have been swallowed in sputum.

Other findings

With heavy infections there is a moderate blood eosinophilia. In *Paragonimus* pericarditis many eosinophils can be found in aspirates of pleural fluid. Charcot Leyden crystals may also be seen in samples.

EXAMINATION OF SPUTUM FOR PARAGONIMUS EGGS

Sputum from patients with pulmonary paragonimiasis often contains blood, mucus, and stringy particles of rusty-brown gelatinous material in which masses of eggs can be found. Sputum from less heavily infected patients may contain very few eggs and a concentration technique may be necessary to detect the eggs.

Procedure

- 1 Report the appearance of the sputum, i.e. whether watery, mucoid, mucopurulent, or red and jelly-like, and whether it contains blood and rusty-brown particles.
- 2 If rusty-brown gelatinous particles are present, transfer a sample of this material to a slide and cover with a cover glass. Using a cloth or tissue, gently press on the cover glass to make a thin evenly spread preparation.

If no rusty-brown particles are present or if no eggs are found when the particles are examined, carry out a concentration technique (preferably using sputum collected over 24 h).

Concentration technique

- Add an equal volume of 30 g/L (3% w/v) sodium hydroxide solution to the sputum, shake, and leave for 15–30 minutes to allow time for the sodium hydroxide to dissolve the mucus.
- Shake well and centrifuge in a conical tube at slow to medium speed, i.e. not over RCF 500× g, for 5 minutes. Using a plastic bulb pipette or Pasteur pipette, remove and discard the supernatant fluid and transfer a drop of the sediment to a slide. Cover with a cover glass.

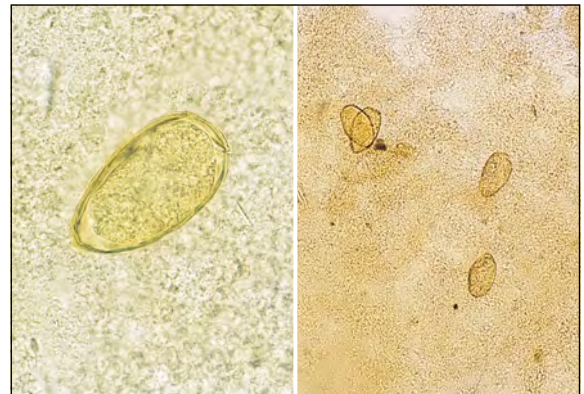


Plate 2.33 Eggs of *Paragonimus* species in unstained sputum. Right: As seen with the 10× objective. Left: As seen with the 40× objective.

- 3 Examine microscopically for eggs using the 10× objective with the condenser iris closed sufficiently to give good contrast. Use the 40× objective to identify the eggs.

Note: The eggs of *Paragonimus* species are identified from a knowledge of locally occurring species and by differences in egg size, shape and shell thickness.

FAR EAST

Egg of P. westermani

- Yellow-brown and usually asymmetrical in shape, being slightly flattened on one side. It measures 70–100 × 50–65 μm.
- Has a flat operculum (lid).
- Thickness of the shell varies. It is thicker at the end opposite the operculum.
- Contains an unsegmented ovum and mass of yolk cells.

AFRICA

Egg of P. uterobilateralis

Similar to the egg of *P. westermani* but smaller, measuring 50–98 × 35–52 μm.

Egg of P. africanus

This egg is larger than that of *P. uterobilateralis*, measuring 72–124 × 42–59 μm. It is similar to *P. westermani* but slightly thinner.

SOUTH AND CENTRAL AMERICA

Egg of P. mexicanus

Similar to that of *P. westermani* except that it has a thin irregularly undulating shell. It measures 73–92 × 47–53 μm.

Examination of pleural fluid for *Paragonimus* eggs

Aspirates of pleural fluid can be examined in the same way as described for sputum, except that there is no need to treat the fluid with sodium hydroxide before centrifuging. The fluid may contain many eosinophils.

Examination of faeces for *Paragonimus* eggs

A concentration technique should be used because only a few *Paragonimus* eggs are likely to be present in faeces. The formol ether/ethyl acetate concentration technique is recommended. Flotation techniques are not suitable. A *Paragonimus* egg is shown in Plate 2.33.

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- 2 **Worasith C et al.** Advances in the diagnosis of human opisthorchiasis: Development of *Opisthorchis viverrini* antigen detection in urine. *PLoS Negl Trop Dis*, 2015 9(10): e0004157, doi.org/10.1371/journal.pntd.0004157

FURTHER READING

Foodborne trematodiasis. World Health Organization Fact Sheet, 2021.
<https://www.who.int/news-room/fact-sheets/detail/foodborne-trematodiasis>

2.8 Taeniasis/cysticercosis Cystic echinococcosis

Taeniasis in humans is caused by:

Taenia solium (pork tapeworm)
Taenia saginata (beef tapeworm)
Taenia asiatica (Asian tapeworm)

Cysticercosis is caused by:

Ingesting the eggs of *Taenia solium*

In tropical and subtropical countries cystic echinococcosis (hydatid disease) is caused by:

Echinococcus granulosus

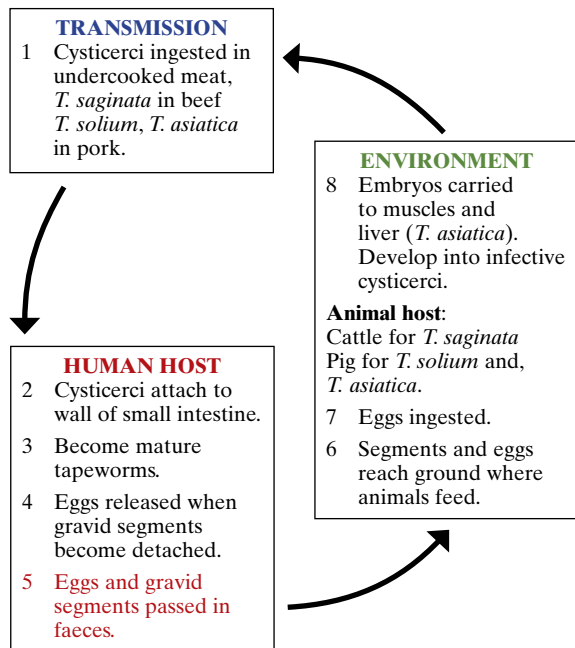
TAENIASIS CYSTICERCOSIS

Distribution

T. solium is mainly found in parts of Africa, China, India, Central America, South America, Papua New Guinea and non-Islamic South East Asia where raw or undercooked contaminated pork is consumed.

T. saginata has a worldwide distribution in countries where cattle are raised and contaminated beef is eaten raw or undercooked. In Egypt and Morocco the camel is the main source of infection.

T. asiatica is mainly found in China, Korea, Taiwan, Indonesia, Thailand, Vietnam, Philippines and Japan where contaminated pork, is eaten raw or undercooked.



Note: Infection with *T. solium* larvae can occur by ingesting eggs in food or from hands contaminated with faeces. Eggs develop into cysticerci causing cysticercosis and neurocysticercosis.

Fig 2.21 Transmission and life cycle of *Taenia solium*, *Taenia saginata* and *Taenia asiatica*.

Transmission and life cycle

T. solium is transmitted by ingesting raw or insufficiently cooked pork which contains cysticerci (tapeworm larval cysts) where sanitation and hygiene are inadequate and people live in close

contact with pigs. Infection with *T. solium* can also occur when viable eggs are accidentally ingested in contaminated water, food (particularly vegetables), or from contaminated fingers, resulting in serious disease (see *T. solium* cysticercosis).

T. saginata is transmitted by eating raw or undercooked beef which contains cysticerci where sewage disposal is inadequate. *T. asiatica* occurs when raw or undercooked pork, particularly pork liver, is eaten which contains cysticerci. Wild boar and occasionally cattle are also sources of infection.

Following ingestion, the cysts evaginate and become attached to the wall of the small intestine by suckers (*T. saginata*) or by suckers and hooklets (*T. asiatica*, *T. solium*). Segments (proglottids) are formed from the neck region. Within 2–4 months the larva grows into a long mature tapeworm.*

**T. solium* tapeworm measures 2–7 metres long and consists of 800–1000 segments. *T. saginata* measures 4–10 metres or longer with more than 1000 segments. *T. asiatica* measures about 350 cm long with 700–900 segments.

Fertilized eggs are produced in the mature segments at the end of the tapeworm. These gravid segments break off. Gravid segments containing eggs and eggs from ruptured segments are passed in faeces. Segments also migrate through the anus.

The life cycle of *Taenia* species is summarized in Fig. 2.21 on the previous page.

Taeniasis

Intestinal infection with *Taenia* species is rarely serious. There may be abdominal pain, nausea with intestinal disturbances and loss of appetite.

Measures to prevent and control taeniasis

- Avoid eating raw or undercooked beef or pork which may be contaminated. Cysticerci can be killed by heat for 5 minutes at 56 °C or by deep-freezing meat for a minimum of 3 weeks.
- Inspecting meat and condemning any found to contain cysticerci and improving sanitation in slaughter houses.
- Preventing animals having access to human faeces and not using untreated faeces as fertilizer.
- Providing latrines, health education, and guidance on food safety.
- Controlling the movement of pigs, improving pig husbandry and vaccinating pigs.
- Detecting carriers and treating infected persons.

T. solium cysticercosis

Following the ingestion of embryonated *T. solium* eggs, the larvae (cysticerci) cause cystic nodules in

subcutaneous tissues and muscles. When present in the brain they can cause epilepsy and other central nervous system disorders (neurocysticercosis). Dead and dying cysticerci may cause an inflammatory host response.

Prevention of cysticercosis: The prevention of cysticercosis caused by internal autoinfection is by diagnosing *T. solium* infection and treating it effectively.

Ingestion of eggs can be avoided by personal hygiene and by not eating food which may be contaminated with *T. solium* eggs such as raw vegetables grown on land fertilized with untreated human faeces.

LABORATORY DIAGNOSIS

The laboratory diagnosis of *Taenia* infection is by:

- Identifying gravid segments passed in faeces or recovered from clothing.

Note: Following treatment, the head and mature segments may be expelled and collected for identification.

- Detecting eggs in faeces. Morphologically the eggs of *T. solium*, *T. saginata* and *T. asiatica* are indistinguishable.

Cysticercosis: This is usually diagnosed serologically. When calcified, the cysts can be detected by X-ray. Cysts in the brain can also be detected by computed tomography (CT scan).

Identifying gravid segments

Caution: Because viable segments are actively motile, keep them in a closed container until they can be fixed and examined. *Always use forceps to handle samples and wear protective gloves. Ingestion of eggs can cause cysticercosis.*

Procedure for examining a segment

- 1 If recovered from faeces, wash the segment in clean water. If the segment is dry and shrivelled, soak it first in water before examining it.
- 2 Press the segment between two slides and hold the slides together with an elastic band or adhesive tape at each end.

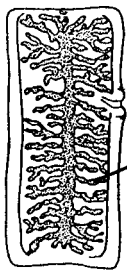
- 3 Firmly compressing the segment to give a thin preparation, hold it lengthways against the light and count the number of main branches arising from the central stem of the uterus (see Figs 2.22, 2.23). The branches are more easily counted with the help of a magnifying lens.

Note: If required, the uterine branches can be stained by injecting India ink through the opening (genital pore) on the side of the segment or by staining the segment with haematoxylin (Delafields or Harris'). Staining is not usually necessary to identify fresh segments.

Counting the number of main side branches from the uterine central stem can help to differentiate *Taenia* species.

	<i>T. saginata</i>	<i>T. solium</i>	<i>T. asiatica</i>
Gravid segment side branches:	14–32	7–16	11–32

T. saginata/T. asiatica



T. solium



Uterine side branches

Fig 2.22 Gravid segment *T. saginata/T. asiatica*

Fig 2.23 Gravid segment *T. solium*

Egg of *T. saginata*, *T. asiatica*, *T. solium*

- Round to oval, measuring 33–40 µm in diameter.
 - Embryo is surrounded by a thick, brown, radially striated wall (embryophore).
 - Hooklets are present in the embryo. Careful focusing is necessary to see the three pairs of hooklets.*
- *The term oncosphere is used to describe an embryo with six hooklets.
- Sometimes a clear membrane can be seen surrounding the egg but usually this is lost when the gravid segment disintegrates.

Note: The Ziehl-Neelsen staining technique is not sufficiently sensitive or specific to differentiate the eggs of *Taenia* species.



Plate 2.34 Egg of *Taenia* species showing small hooklets as seen in a saline preparation.

Examination of faeces for *Taenia* eggs

A concentration technique and the examination of several samples may be necessary to detect *Taenia* eggs in faeces. They are usually few because the eggs are not regularly discharged from the tapeworm in the intestine.

The eggs can be concentrated by the formol ether/ethyl acetate technique described in Appendix V.

Collection of a perianal sample for *Taenia* eggs

When required a clear adhesive tape technique can be used for the recovery of *Taenia* eggs from skin around the anus. A *Taenia* egg is shown in Plate 2.34.

Examining the scolex of *Taenia* species

Following successful treatment it is sometimes possible to recover the scolex (head) of a tapeworm (about 2 mm in diameter) for examination using a magnifying lens. Morphological features which can assist in differentiating *Taenia* species include whether a rostellum and hooklets are present as follows:

Scolex	<i>T. saginata</i>	<i>T. solium</i>	<i>T. asiatica</i>
Rostellum	Absent	Present	Present
Hooklets	Absent	Present	Absent

Note: Progress on the development of tests to diagnose *T. solium* infection and cysticercosis can be found in the WHO publication *Taenia solium taeniasis/cysticercosis diagnostic tools*.¹

CYSTIC ECHINOCOCCOSIS

Cystic echinococcosis, also known as hydatid disease is caused by ingestion of eggs of the tapeworm *Echinococcus granulosus* in food or water or from hands contaminated with dog faeces. Dogs and livestock are the definitive hosts of *E. granulosus* (harbour adult tapeworm). Following ingestion, the embryos are freed in the small intestine. They migrate through the intestinal wall into a blood vessel and are carried in the circulation to the liver, peritoneal cavity, lungs, kidneys, bone, and other sites in the body where they develop into hydatid cysts (larval form of *E. granulosus*).

Humans are not the natural intermediate hosts of *E. granulosus*. When a human becomes infected there is no transfer of the parasite to the definitive host. The normal intermediate hosts are sheep. Other animals including cattle, goats, pigs, camels and yaks may also become hosts. Different genotypes of *E. granulosus* have intermediate host preferences. Dogs become infected by eating tissue containing cysts from infected sheep or other intermediate animal host.

Distribution

Hydatid disease is known to occur in many parts of the world where sheep are in close association with dogs and humans or where dogs and wild carnivores are associated with livestock and wild herbivores. High infection rates occur in China, East Africa, North Africa, eastern Mediterranean, Central Asia, Chile, Uruguay, Peru and Argentina.

Hydatid disease

Infections can produce serious symptoms depending on the site and size of the hydatid cyst and host response. The cyst grows slowly but continuously. Patients may remain asymptomatic for several years. Obstruction and pressure on vital organs or rupture of the cyst with subsequent anaphylactic shock can be fatal. Some cysts may grow for only a short time and then die and calcify.

Up to 70% of hydatid cysts are found in the right lobe of the liver where they cause pain, fever and hepatomegaly. Up to 20% of cysts develop in the lungs where they cause pulmonary symptoms. Sputum may contain blood and cyst fluid. When hydatid cysts develop in bone there is no fibrous wall formed and therefore the cyst spreads causing pain

and bone fractures. Other sites include the brain, kidneys, muscles, eyes, or spleen but these organs are only rarely infected.

Prevention and control of hydatid disease include hand-washing and health education, food inspection, slaughterhouse hygiene, vaccination of lambs and deworming of dogs.

LABORATORY DIAGNOSIS

Hydatid disease (especially when the cysts are in the liver and abdomen) is usually diagnosed clinically with the assistance of ultrasound scanning and other imaging techniques.

Laboratory investigations include:

- To support a diagnosis, serological testing in Reference Laboratories using recombinant antigens (to improve sensitivity and specificity of tests).
- Examining cyst fluid for brood capsules and protoscolexes following the surgical removal of a cyst or fine needle aspiration. Protoscolexes can occasionally be found in sputum when a pulmonary cyst ruptures.
- Eosinophilia is a common finding.

Useful tests to differentiate liver hydatid disease from hepatoma

Serum glycoproteins, serum aminotransferases and other liver tests are usually normal with hydatid disease of the liver but abnormal with hepatoma.

Examination of cyst fluid for protoscolexes

Hydatid cyst: Structurally the cyst consists of an outer thick laminated cyst wall and an inner, thin, nucleated germinal layer. From the germinal layer, brood capsules are produced inside which small protoscolexes form (see Fig. 2.24).

The brood capsules may break off and sink down through the fluid which fills the cyst. Free brood capsules and individual protoscolexes released from the capsules, form what is called hydatid sand.

There is a marked cellular response by the host to the presence of the hydatid cyst which leads eventually to it being surrounded by a fibrous wall. Older cysts may become calcified.

Procedure

After centrifuging the cyst fluid (hydatid sand), transfer a drop of the sediment to a slide and cover with a cover glass. Examine the preparation microscopically for evaginated and invaginated

protoscolexes using the 10× objective. Use the 40× objective to identify the hooks of the scolex (double row). If the hydatid cyst is sterile, no protoscolexes will be found.

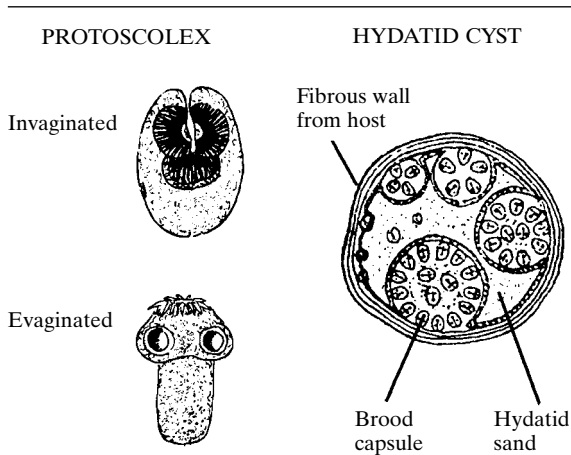


Fig. 2.24 Right: Morphology of hydatid cyst of *E. granulosus*. Left: An invaginated protoscolex (upper) and evaginated protoscolex (lower) of *E. granulosus*.

Protoscolex of *E. granulosus*

- Colourless, round to oval in shape, measuring about $140 \times 80 \mu\text{m}$. May be invaginated or evaginated (see Fig. 2.24).
- With focusing the hooks of the scolex can be easily seen.
- Viable protoscolexes do not stain with eosin.
- Non-viable protoscolexes stain red with eosin.

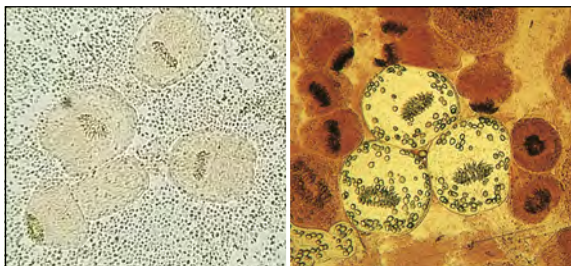


Plate 2.35 Left: Invaginated and evaginated protoscolexes of *E. granulosus* in hydatid cyst fluid. Right: Eosin preparation showing viable (unstained) protoscolexes and non-viable (red stained) protoscolexes.

Viability of protoscolexes

To test whether the protoscolexes are viable (living), prepare an eosin preparation (Reagent No. 18) of

the fluid. Viable protoscolexes remain unstained whereas dead (non-viable) protoscolexes have a damaged membrane and take up the eosin (see Plate 2.35).

Echinococcus multilocularis

E. multilocularis causes alveolar echinococcosis. Infections have been reported mainly from China, Russian Federation, Europe and North America. The disease usually occurs in a wildlife cycle between foxes, other carnivores and small mammals (mostly rodents). Domestic dogs and cats can also be infected. *E. multilocularis* is invasive and spreads like a malignant tumour causing cavities and necrosis. The liver is commonly infected and frequently other organs also become infected. The disease is usually fatal.

Multiceps species

The larval stage of these tapeworm species is a coenurus cyst and human infection with the larva is referred to as coenuriasis. Infection occurs following the ingestion of eggs in food or from hands contaminated with dog faeces. *Multiceps multiceps* is found in North and South Africa and the coenurus infects the brain. There is no treatment and infection is usually fatal.

Multiceps brauni is found in tropical Africa and causes a less serious disease because the coenurus does not infect the brain. The eyes and other parts of the body are infected. The definitive hosts of *Multiceps* species are dogs and jackals. The intermediate hosts of *M. multiceps* are sheep and the intermediate hosts of *M. brauni* are rodents.

REFERENCES

- 1 WHO *Taenia solium taeniasis/cysticercosis diagnostic tools*. WHO Report of stakeholder meeting, WHO, 2016. Access by entering the title in Google.
- 2 *Echinococcosis*. World Health Organization Fact Sheet, 2018. See below.

FURTHER READING

Taeniasis/cysticercosis. World Health Organization Fact Sheet, 2022.

<https://www.who.int/news-room/fact-sheets/detail/taeniasis-cysticercosis>

Echinococcosis. World Health Organization Fact Sheet, 2021. <https://www.who.int/news-room/fact-sheets/detail/echinococcosis>

WHO Guidelines on management of Taenia solium neurocysticercosis, 2021.

<https://apps.who.int/iris/handle/10665/344802>

2.9 Buruli ulcer Leprosy

Buruli ulcer is caused by *Mycobacterium ulcerans*
Leprosy is caused by *Mycobacterium leprae*

BURULI ULCER

Buruli ulcer is a chronic debilitating skin disease that can lead to permanent disfigurement and long-term disability. It has been reported in localized areas from 33 countries in tropical and subtropical Africa, South east Asia, Central and South America and the Western Pacific (including Australia). The majority of cases are reported from West and Central Africa including Benin, Cameroon, Côte d'Ivoire, Democratic Republic of the Congo and Ghana.¹

Those infected mainly live in rural low-lying areas near slow-moving or stagnant water. *M. ulcerans* is an environmental bacterium. Its mode of transmission is unknown. Strong evidence suggests the organism is transmitted through minor injuries possibly caused by spiky vegetation or aquatic biting insects. In Africa children under 15 years of age are most commonly affected with infections occurring on exposed parts of the body (usually the arms or legs).

Classification of Buruli ulcer

Infection with *M. ulcerans* begins as a subcutaneous nodule, plaque, or diffuse swelling which later develops into a painless ulcer with irregular borders and deeply undermined edges due to necrosis. Large areas of skin and sometimes bone are destroyed. The necrosis is caused by mycolactone toxin produced by the organism. The toxin causes local immunosuppression, increasing tissue damage. The mycolactones produced by African strains of *M. ulcerans* have been shown to be more potent in causing necrosis.² Following wound healing there can be extensive scarring leading to restricted movement of limbs and often permanent deformity and disability.

Classification of Buruli ulcer based on severity of disease¹

The WHO classifies Buruli ulcer into 3 categories:

- Category 1: Single small lesion (32%).
- Category 11: Non-ulcerative and ulcerative plaque and oedematous forms (35%).
- Category 111: Disseminated and mixed forms such as osteitis, osteomyelitis, joint involvement (33%).

The WHO estimates at least 70% of all persons with Buruli ulcer are diagnosed in the ulcerative stage. Medical treatment is the most effective in category 1 patients.

Depending on the patient's age, geographical area, location of lesions and pain caused, the following conditions require exclusion from a diagnosis of Buruli ulcer:¹

- Tropical ulcers with bacterial invasion, chronic lower leg ulcers due to arterial and venous insufficiency, diabetic ulcers, cutaneous leishmaniasis, ulcerative yaws, ulcers caused by *Haemophilus ducreyi*, leprosy, skin cancer.
- Boils, lipomas, ganglions, lymph node tuberculosis, onchocerciasis nodules, fungal subcutaneous infections.
- Cellulitis resembling oedema caused by *M. ulcerans*. Unlike Buruli ulcer, cellulitis lesions are painful and the patient has fever.

HIV co-infection

Buruli ulcer in patients co-infected with HIV is often more aggressive, has a poorer outcome and treatment is often less effective. Lesions are often larger and ulcerate more quickly. Multiple lesions are common. HIV also increases the risk of more widespread disease and bone involvement. A WHO Technical update is available on the *Management of Buruli ulcer HIV co-infection*.³

LABORATORY DIAGNOSIS

In endemic areas, Buruli ulcer is usually diagnosed clinically. Whenever possible the World Health Organization recommends that a diagnosis of Buruli ulcer be confirmed microbiologically:

- to confirm that the disease is Buruli ulcer,
- to determine the precise prevalence and incidence of Buruli ulcer in a given area,
- to confirm new foci,
- to manage appropriately the disease using antimycobacterial therapy with or without surgery,
- to confirm treatment failure, relapse, or reinfection after treatment.⁴

The following laboratory techniques are used to investigate Buruli ulcer:

- Direct microscopy
- Polymerase chain reaction (PCR)
- Culture
- Histopathology

Direct microscopy: This gives rapid results and can be performed at district level (see following text). It has been estimated to have a sensitivity of < 60%.

PCR: This technique has high sensitivity and specificity (>90%) for the diagnosis of Buruli ulcer. A laboratory is

required with PCR IS2404 facilities. WHO recommends that at least 70% of clinically suspected cases of Buruli ulcer be confirmed by a PCR positive result. The district laboratory should consult with the PCR referral laboratory regarding sample collection, transport requirements and patient information to accompany sample. PCR cannot be used for the follow-up of patients with recurrences.

Culture: Although not essential for the diagnosis of Buruli ulcer and the immediate management of patients, culture to detect viable bacteria may be required to identify treatment failures and recurrences of infection. Culture may also be required if drug-resistant strains emerge. A Buruli ulcer Reference Laboratory is required to culture *M. ulcerans*. Culturing takes 9–12 week (at 29–33 °C, low oxygen of 2.5–5%, pH range of 5.4–7.4). PCR is required for pathogen identification.

Histopathology: Requires a biopsy and the facilities of a Buruli ulcer Reference Laboratory. Histopathology is able to provide alternative diagnoses when the lesion is not Buruli ulcer.

Note: The advantages and disadvantages of each technique are summarised in the WHO *Laboratory diagnosis of Buruli ulcer: a manual for health care providers* (2014).⁴

Diagnosing Buruli ulcer in district laboratories

In most district laboratories in endemic areas, microscopy is used to assist in the diagnosis of Buruli ulcer. *M. ulcerans* is an acid fast bacillus (AFB). It can be stained using the same ZN and auramine O staining techniques described for *M. tuberculosis* in Section 2.11. *M. ulcerans* measures 1–3 × 0.5 µm and is often seen in dense clumps. Figs 2.25 and

2.26, taken from the WHO *Laboratory diagnosis of Buruli ulcer: manual for health care providers*⁴ describe the reporting of ZN and Auramine O stained smears for *M. ulcerans* AFB.

Collection of samples for microscopy

Samples should only be collected by an experienced clinician or health worker. For routine clinical management and case finding, samples are obtained using a swab or fine needle aspiration. A Buruli ulcer confirmation request form should accompany the sample.

Using a swab: Carefully clean the skin surrounding the ulcer (using an alcohol cotton wool swab). Take a sample from the undermined edges of a clinically diagnosed Buruli ulcer. Rotate the swab and swipe downward and clockwise around the tissues beneath the edges of the ulcer. The centre of the ulcer should not be swabbed (will not contain bacteria). Insert the swab into the sterile tube. Label the tube with the patient's name, age, gender and date.

Fine needle aspiration: This is mainly used to obtain a sample from a clinically diagnosed non-ulcerative lesion (nodule, plaque, oedema). To aspirate the bacteria, the needle must be advanced through the subcutaneous tissue to the fat tissue. This is where the bacteria will be found in non-ulcerative lesions.

Note: Full details of how to perform the technique can be found in the WHO *Laboratory diagnosis of Buruli ulcer: a manual for health care providers*.⁴

Fig 2.25 Reporting ZN stained smears for *M. ulcerans* AFB

Reproduced from *Laboratory diagnosis of Buruli ulcer* with permission of the World Health Organization.

No. of AFB seen on average	No. of fields to screen	Report
0/100 immersion fields	100	No AFB observed
1–9/100 immersion fields	100	Record exact figure
10–99/100 immersion fields	100	+
1–10/1 immersion field	50	++
>10/1 immersion field*	20	+++

AFB, acid-fast bacilli

* *Mycobacterium ulcerans* often forms clumps of densely packed AFB that are too numerous to be counted accurately. When large numbers of AFB are observed, the report should be “+++”.

Fig 2.26 Reporting Auramine O smears for *M. ulcerans* AFB

Reproduced from *Laboratory diagnosis of Buruli ulcer* with permission of the World Health Organization.

What you see (200x)	What you see (400x)	What to report
No AFB in one length	No AFB in one length	No AFB observed
1–4 AFB in one length	1–2 AFB in one length	Confirmation required*
5–49 AFB in one length	3–24 AFB in one length	Scanty
3–24 AFB in one field	1–6 AFB in one field	+
25–250 AFB in one field	7–60 AFB in one field	++
>250 AFB in one field	>60 AFB in one field	+++

*Confirmation required by another technician or preparation of another smear, to be stained and read.

Quality assurance

The use of control smears and other quality control procedures are essential to ensure the reliability of Buruli ulcer laboratory reports. On-site evaluations are required to ensure the accuracy of smear reporting. Details of internal and external quality control procedures can be found in the WHO *Laboratory diagnosis of Buruli ulcer: a manual for health care providers*.⁴

Diagnostic techniques in development

The WHO target for Buruli ulcer is that by 2030 the proportion of cases diagnosed in Category 111 will be reduced from 30% (base line) to less than 10%. To achieve this, rapid diagnostic tools for use at primary healthcare level need to be developed to ensure early diagnosis, reduction in morbidity and confirmation of cases.⁵

In 2018, WHO and FIND held a meeting to assess progress in the development of a rapid test to diagnose Buruli ulcer at the primary healthcare level and a test to assist in treatment monitoring and differential diagnosis at the district hospital or reference centre.⁶ The meeting also addressed the harmonization and standardization of sample collection, preparation and referral and the need for quality assurance in Buruli ulcer testing.

Currently mycolactone detection is being developed as a rapid diagnostic test using monoclonal antibodies and fluorescent thin-layer chromatography.⁶ It is being piloted in several countries. Mycolactone detection is also being considered as a test to monitor treatment and as a test of cure (levels of mycolactone in ulcerative lesions decrease with treatment).

LEPROSY

Mycobacterium leprae causes leprosy (Hansen's disease), a chronic infectious disease that mainly affects the skin, the peripheral nerves, mucosa of the upper respiratory tract and the eyes. Untreated leprosy can cause progressive and permanent damage to the skin, nerves, limbs and eyes. Loss of sensation and insensitivity to pain caused by nerve damage can lead to injuries from burns, ulcers and trauma, resulting in deformities particularly of the hands and feet. Early successful treatment with multidrug therapy (MDT) can prevent disability.

Leprosy is mainly transmitted via nasal secretions following close and prolonged contact with persons with untreated lepromatous leprosy. The incubation period is usually several years.

Clinical forms of leprosy

The clinical forms of leprosy and disease progression depend on a person's immune response to *M. leprae*. There are two distinct forms of leprosy: tuberculoid and lepromatous with several intermediate forms.

Tuberculoid: In this form of leprosy there is a high level of cell mediated immune (CMI) response. The term paucibacillary leprosy is used because few bacteria are present. There is usually only a single or very few well-defined skin lesions which are without feeling and perspiration and less pigmented than the surrounding skin. Bacteria are not present in the lesions. Loss of feeling is due to the invasion and destruction of nerve tissue by inflammatory cells. Affected nerves show marked thickening. Granulomas form containing giant cells.

Lepromatous: In this form of leprosy cellular immune responses are poor or absent. The term multibacillary leprosy is used because skin and mucous membrane lesions contain large numbers of bacteria. Patients show widespread areas of infection. Lesions are nodular, small and many with no loss of feeling. The bacteria multiply mainly in macrophage cells (histiocytes) in the skin dermis. Only CMI to *M. leprae* is affected (not to other organisms).

Figure 2.27 illustrates diagrammatically the cellular immune responses in tuberculoid and lepromatous leprosy and intermediate forms: BT (borderline tuberculoid), BB (mid-borderline) and BL (borderline lepromatous).

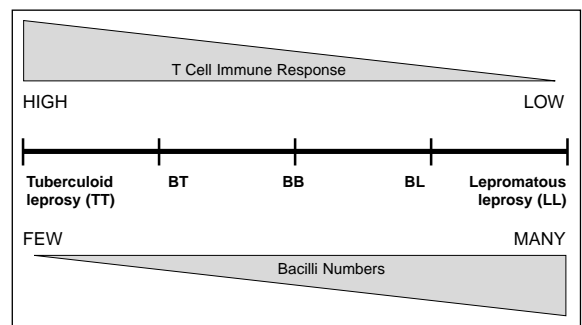


Fig. 2.27 Diagrammatic representation of cellular immune responses in the different forms of leprosy.

BT, BB, BL: Skin smears contain very few or no bacteria in the BT form and several to many in the BB and BL forms. Biopsies may in some cases be required to confirm the diagnosis and classification of patients with borderline leprosy especially those at, or near, the tuberculoid end.

M. leprae co-infection with HIV: Co-infection with HIV has not been shown to affect the clinical classification, progression and outcome of leprosy.

WHO Global Leprosy Strategy 2021–2030

Globally in 2020, 127 558 new leprosy cases were diagnosed (8 629 paediatric cases) with up to 79% reported from India, Brazil and Indonesia. In 2021 WHO published *Towards Zero Leprosy – Global Leprosy (Hansen’s disease) Strategy 2021–2030* with a long term vision of zero infection and disease, and zero disability, stigma and discrimination⁸.

Global targets for 2030⁸

- 120 countries reporting zero new autochthonous cases.
- 70% reduction in annual number of new cases detected.
- 90% reduction in rate/million population of new cases with grade-2 disability.
- 90% reduction in rate/million children of new child cases with leprosy.

Strategic pillars and key components⁸

- 1 Implement integrated, country-owned zero leprosy road maps in all endemic countries.
- 2 Scale up leprosy prevention alongside integrated active case detection.
- 3 Manage leprosy and its complications and prevent new disability.
- 4 Combat stigma and ensure human rights are respected.

The COVID-19 pandemic disrupted the implementation of leprosy programmes and resulted in a 37% reduction in new cases detected in 2020 compared with 2019⁷.

LABORATORY DIAGNOSIS

Leprosy is usually diagnosed and classified by clinical examination. The WHO advises that only in rare instances is there a need to use laboratory investigations to confirm a diagnosis of leprosy.⁹ Examination of a skin smear for the presence of *M. leprae* may occasionally be needed to confirm a clinically difficult diagnosis of multibacillary leprosy. A smear should be collected from only one or two sites. PCR is useful for drug resistance surveillance.

Morphology

M. leprae is a non-motile, non-spore, straight or slightly curved rod measuring 0.2–0.5 × 5–8 µm. Bacteria with pointed or enlarged ends are sometimes seen. The organisms can be found singly, in clusters, and in large groups within macrophage cells. Organisms in large groups are called globi. Single *M. leprae* organisms and globi are shown in colour Plate 2.36.

M. leprae is acid fast when stained by the Ziehl-Neelsen technique (see following text). It is not as acid fast as the tubercle bacillus and therefore a weaker acid solution, i.e. 1% acid alcohol is used to decolorize smears. *M. leprae* is also less heat-resistant than tubercle organisms and therefore smears must be gently heat-fixed for just a few seconds.

Method of collecting and examining a skin smear for *M. leprae*

A smear for the examination of *M. leprae* must be collected by a *trained and experienced* observer using an aseptic and safe technique. The site sampled should be the edge of a leprosy lesion.

- 1 Explain the procedure to the patient (or parent if the patient is a child).
- 2 Fit a new scalpel blade in its scalpel holder. Sterilize the blade by wiping it carefully with a piece of absorbent cotton wool soaked in 70% v/v ethanol (alcohol) and flaming it for 2–3 seconds in the flame of a spirit lamp. Allow the blade to cool, making sure it is not touching any unsterile surface.
- 3 Wearing protective rubber gloves, cleanse the area from where the smear is to be taken, using a cotton wool swab moistened with 70% v/v ethanol (alcohol). Allow the area to dry.
- 4 Pinch the skin tightly between the thumb and index finger until it becomes pale due to loss of blood.
Important: The area must be kept bloodless while the smear is collected because a smear which contains red cells will be difficult to examine and report.
- 5 Using the sterile blade, make a small cut through the skin surface, about 5 mm long and deep enough into the dermis (2–3 mm) where the bacteria will be found. Continue to hold the skin tightly.
- 6 Using a dry piece of cotton wool, blot away any blood which appears at the site of the cut.
Note: Providing pressure is maintained between the thumb and index finger, little or no bleeding should occur.
- 7 Turn the scalpel blade until it is at a right angle to the cut. Using the *blunt* edge of the blade, scrape firmly two or three times along the edges and bottom of the cut to collect a sample of tissue juice and cells.

- 8 Transfer the sample to a slide. Make a small circular smear, covering *evenly* an area measuring 5–7 mm in diameter.
- 9 Cover the cut with a small dressing. Instruct the patient to remove the dressing as soon as the cut has healed.
- 10 Ensure the slide is clearly labelled with the patient's name and identification number.
- 11 When the smear has dried, *gently* heat-fix it by holding it, smear uppermost, over the flame of a spirit lamp or the pilot flame of a Bunsen burner for a *few* seconds. *Do not* over-heat because this will interfere with the staining of *M. leprae*.

Ziehl-Neelsen technique for staining

M. leprae

Required

- | | |
|--------------------------------------|----------------|
| – Carbol fuchsin stain (filtered) | Reagent No. 12 |
| – 1% v/v acid alcohol | Reagent No. 3 |
| – Malachite green, 5 g/L (0.5% w/v)* | Reagent No. 27 |

*If preferred, methylene blue, 5 g/L may be used instead of malachite green.

Method

- 1 Cover the smear with filtered carbol fuchsin stain. Heat the stain until vapour just begins to rise (i.e. about 60 °C). *Do not overheat*. Allow the heated stain to remain on the slide for 10–15 minutes (ensure the stain does not dry on the smear).
- 2 Wash off the stain with clean water. When the tap water is not clean, wash the smear with filtered tap water or clean boiled filtered rain water.
- 3 Decolorize the smear rapidly (about 5 seconds) by rinsing it with 1% v/v acid alcohol.
Caution: Acid alcohol is flammable, therefore use it with care well away from an open flame.
- 4 Wash well with clean water. Cover the smear with malachite green stain (or methylene blue) for 1–2 minutes.
- 5 Wash off the stain with clean water. Wipe the back of the slide clean and place it in a draining rack for the smear to air-dry (do *not* blot-dry). Protect it from direct sunlight.

- 6 Examine the smear microscopically, first with the 40× objective to see the distribution of material and then with the oil immersion lens to look for acid fast bacilli.

Results

M. leprae Red solid bacilli or beaded forms, occurring singly or in masses (globi)
 Macrophage cells Green*
 Background material Green*

*Blue if methylene blue counterstain has been used.

Note: The appearance of *M. leprae* in a Ziehl-Neelsen stained smear is shown in colour Plate 2.36.

Reporting *M. leprae* smear

Report the smear as 'Positive' if *M. leprae* bacteria are seen or 'Negative' if no bacteria are seen after examining the entire smear or at least 100 high power microscope fields.

B1 and M1: Reporting the bacterial index (B1) and morphological index (M1) is no longer required for routine purposes.

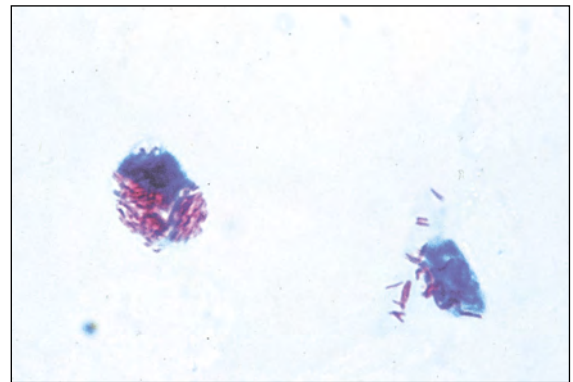


Plate 2.36 *Mycobacterium leprae* in Ziehl-Neelsen stained skin smear as seen with the 100× objective.

Quality control (QC) of skin smears

To collect and report smears for *M. leprae* reliably, laboratory staff must receive adequate training and supervision. QC must include the laboratory supervisor spot-checking smear reporting (same day as smears are stained, to prevent discrepancies due to fading of the stain). A control known positive smear must be used to check the staining reaction of every newly prepared batch of carbol fuchsin. Fixed smears for control purposes should be stored in an airtight light-proof box.

Biopsies

Examination of a biopsy does not form part of the routine day to day work of leprosy control but may occasionally be required to diagnose a patient with tuberculoid leprosy or borderline-tuberculoid leprosy. A pathologist or other person with experience in examining leprosy biopsy sections (in the Central Hospital or Centre with specialist facilities), will be able to interpret a cellular response and invasion of nerve tissue by inflammatory cells.

Mycobacterium lepromatosis

M. lepromatosis also causes leprosy. It was identified by genetic analysis in 2008. It was first reported as causing diffuse lepromatous leprosy and since then has been identified as also causing other forms of multibacillary leprosy⁹. It is endemic in Brazil, Myanmar and the Philippines and has also been reported from Mexico, Malaysia and USA. Only small numbers of infections have been identified but it is probable that infections may have been under-diagnosed. *M. leprae* and *M. lepromatosis* dual infection has been reported in endemic countries.

Microbiologically and clinically *M. lepromatosis* is similar to *M. leprae*. It is acid fast, cannot be cultured in the laboratory and responds to the same treatment as used for *M. leprae*⁹. Studies are ongoing to determine whether viable *M. lepromatosis* occurs in non-human sources and whether zoonotic transmission is occurring.

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2.10 Dengue

Dengue is caused by dengue virus (DENV), an RNA flavivirus. There are four distinct but closely related dengue virus serotypes: DENV-1, DENV-2, DENV-3 and DENV-4. Dengue virus is transmitted by female *Aedes* mosquitoes, mainly *Ae. Aegypti* (day-biting mosquito found in urban areas, preferring to feed on humans and breeding in man-made containers) and less commonly by *Ae. Albopictus* (able to survive in cooler temperatures). The disease is found in tropical and subtropical urban and semi-urban areas, particularly in South east Asia, Indian subcontinent, Western Pacific, Central and South America. Dengue prevalence is increasing in Africa. In 2019, 5.2 million cases of dengue were reported³.

Dengue is a major and escalating global health problem with an estimated 50% of the world at risk of infection.¹ An important cause of increased dengue incidence and serious dengue outbreaks is climate change with associated global warming. Unseasonal heavy rainfall with flooding lead to increases in mosquito numbers, lengthening of transmission seasons and the spread of dengue geographically to less tropical and subtropical countries (including Nepal, Afghanistan, parts of Europe and North America). Greater air-travel, increased urbanization and inadequate mosquito control are also contributing to the rapid spread of dengue.

Dengue fever and severe dengue

Many infections with DENV are asymptomatic and self-limiting. Dengue fever should be suspected when a person has lived or travelled in a dengue endemic area in the last 14 days and has a sudden high fever (40 °C/104 °F) typically of 2–7 days duration accompanied by two or more of the following symptoms: severe headache, pain behind the eyes, muscle and joint pains, swollen glands, nausea/vomiting, rash, petechiae, progressive leucopenia.² Cases also include any child coming

from or living in an area with dengue transmission, with acute febrile illness, usually of 2–7 days and no apparent focus.

Warning signs of severe dengue

Warning signs of severe dengue developing (3–7 days after the first symptoms in conjunction with a fall in temperature to below 38 °C/100 °F) include: severe abdominal pain, persistent vomiting, fluid accumulation (ascites, pleural effusion, pericardial effusion), mucosal bleeding, lethargy/restlessness, postural hypotension, liver enlargement, progressive increase in haematocrit.²

Severe dengue

Severe dengue is classified when one or more of the following are present: shock or respiratory distress due to plasma leakage, severe bleeding, organ impairment.² Severe dengue is a serious life-threatening condition, frequently affecting children and those who have been previously infected with one serotype of dengue virus. Recovery from infection by one serotype provides lifelong immunity against that particular serotype, however subsequent infections by other serotypes increase the risk of developing severe dengue. Diabetes mellitus increases the risk of severe dengue.

Severe dengue is characterized by a rising haematocrit (due to plasma leakage) together with a reduced platelet count, enlarged liver and haemorrhagic symptoms. In some patients, severe dengue can progress to circulatory collapse. Laboratory tests can help to monitor the response of patients to treatment and avoid fatal complications. (see Laboratory Diagnosis).

Immunization

The first dengue vaccine, *Dengvaxia* (CYD-TDV) developed by Sanofi Pasteur was licensed in December 2015. In clinical trials the live attenuated dengue vaccine, CYD-TDV, has been shown to be efficacious and safe in persons who have had a previous dengue virus infection (seropositive individuals) but carries an increased risk of severe dengue in those who experience their first natural dengue infection after vaccination (seronegative individuals).³ Pre-vaccination screening is therefore recommended (based on an antibody test or documented laboratory confirmed dengue infection in the past).

Implementing dengue vaccination as part of dengue prevention and control requires consideration of the sensitivity, specificity and cost of locally available tests, local priorities, dengue epidemiology, hospitalization rates and affordability of the vaccine and screening tests. The main methods of controlling and preventing dengue transmission are reducing mosquito numbers, using mosquito protective measures personally and in the home and by active monitoring and surveillance of vectors.

LABORATORY DIAGNOSIS

Laboratory investigations for dengue include:

- Tests to diagnose dengue.
- Tests to monitor the treatment of patients with dengue fever and severe dengue.

Tests to diagnose dengue

In most district laboratories the following tests are used to diagnose dengue:

- NS1 (non-structural protein – 1) rapid antigen test
- Serological tests: IgM and IgG antibody tests

Molecular testing: PT-PCR is the most sensitive and specific test for the diagnosis of dengue in the acute stage of infection (1–5 days after the onset of symptoms). Most PT-PCR tests also serotype the virus. The facilities of a Dengue Reference Laboratory are required for PCR testing.

Sample:

- For rapid NS1 testing: 60–100 µL of anticoagulated (EDTA) blood, plasma or serum (depending on test manufacturer) are required. Samples can be stored for up to 48 hours at 4–6 °C.
- For rapid IgM or IgG antibody testing: 10–20 µL of anticoagulated (EDTA) blood, serum or plasma (depending on test manufacturer) are required. Stability is as for NS1 testing.
- For IgM and IgG antibody titre testing: The volume of serum required will depend on the test procedure. Samples can be stored for up to 48 hours at 4–6 °C or for up to 1 week when stored frozen at 10–20 °C.

NS1 antigen test

NS1 protein is produced as the dengue virus replicates. It can be detected in plasma and serum in the early stages of acute dengue infection before the development of IgM antibodies.² It persists for about 9 days. Levels of NS1 fluctuate during the course of the disease.

A positive NS1 test is indicative of active dengue. The test is highly specific. A negative test however, cannot rule out a diagnosis of dengue. Sensitivity of the test is variable being reported as 46.8–79.8%.^{4,5} Sensitivity is influenced by virus serotype, whether the infection is primary or secondary (less sensitive in secondary infections), the stage of infection and the antibodies used in the test.²

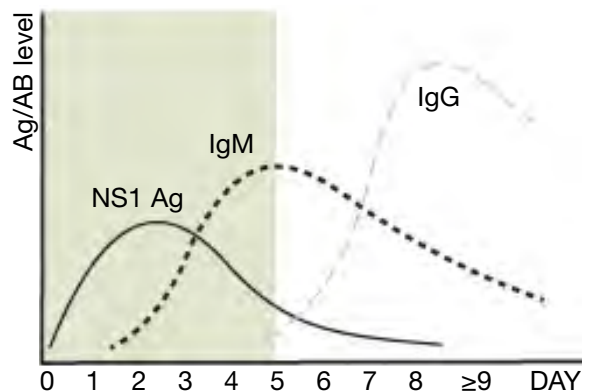


Fig 2.28 NS1 antigen response in dengue acute phase samples (day 1–5). Courtesy of Access Bio Inc.

Several ELISA and rapid immunochromatographic (IC) cassette and strip tests have been developed to detect NS1 antigen. Most rapid tests can be used with whole blood, serum, or plasma, take 15–20 minutes to perform, can be stored at 1–30 °C, have a shelf-life of 24 months from date of manufacture and are available in pack sizes of 25.

Rapid tests are available that combine NS1 testing with the detection of IgM and/or IgG. An example of a combined (Combo/Duo) test is shown in Fig. 2.30. The NS1, IgG and IgM tests are not quantitative. More than 80% of samples can be diagnosed as dengue when both NS1 and IgM tests are performed.

Note: Manufacturers of rapid NS1 tests and Combo/Duo NS1, IgM, IgG tests include: Wells Bio Inc, AccessBio, Abbott Diagnostics, InBois International, Standard Diagnostics, Panbio Diagnostics.

Quality assurance of rapid tests

To ensure the reliability of rapid dengue tests it is important to ensure samples and tests are stored correctly and the test procedure as instructed by the manufacturer is followed exactly. Each test has a built-in control (C) to demonstrate satisfactory migration of the sample. Tests should be read in a good light and reported as indicated by the manufacturer. Any test limitations should be noted.

IgM and IgG antibody tests

The antibody response in primary and secondary dengue infection is as follows:

Primary infection

IgM antibodies are detectable 5–6 days after the onset of fever, peaking at 14–15 days and declining after 30–60 days.² They may remain detectable for up to 6 months. IgG antibodies become detectable

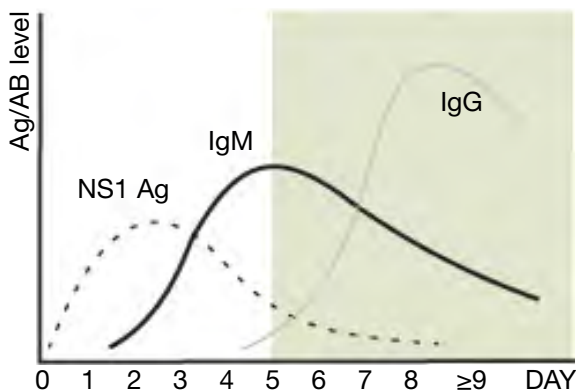


Fig. 2.29 Antibody response as seen in dengue convalescence phase samples (after day 3–5). Courtesy Access Bio Inc.

NS1 Test (cassette left)

- 1 80 μ L whole blood added to sample well.
Can also use serum or plasma (60 μ L).



IgG/IgM Tests (cassette right)

- 2 20 μ L whole blood added to sample well.
Can also use serum or plasma (10 μ L).



- 3 1 drop of assay buffer added to IgG/IgM sample well only.

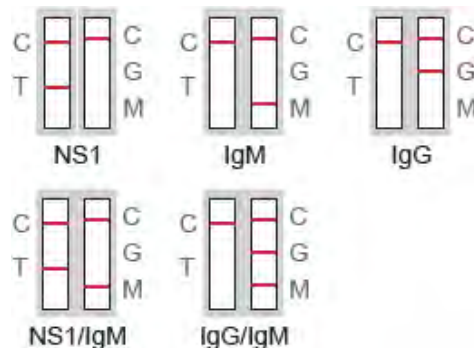


- 4 Test results read after 15–20 minutes.



Interpretation of test results T: NS1 G: IgG M: IgM

Positive



Negative



Note: Validity of each test is shown by a positive Control Line (C).

Fig. 2.30 Wells Bio CareUS (also known as CareStart) Dengue Combo NS1, IgG/IgM test. An evaluation of the test in children with acute dengue in Myanmar reported a test specificity of 100% and sensitivity of 79.82%.⁴ Artwork courtesy of Access Bio Inc CareSmart www.accessbio.net/

10–14 days after the onset of symptoms and persist at a lower level for life.

Secondary infection

In a secondary dengue infection, there is a much weaker and shorter IgM response (antibodies may be undetectable). The IgG response is rapid (2–3 days following fever) and of high titre. The antibodies persist for 30–40 days at high level before falling to levels found in primary infection. Secondary infection is associated with more severe disease and higher mortality. Determining the IgM, IgG ratio can help to differentiate primary from secondary infections in acute disease.

Accuracy of antibody tests

The accuracy of antibody tests in diagnosing dengue is dependent on test manufacturer and also whether the infection is primary or secondary, the stage of infection and dengue serotype.

Cross-reactions may occur with other flaviviruses (e.g. yellow fever virus, West Nile virus, Zika virus), Chikungunya virus (togavirus, transmitted by same *Aedes* mosquitoes that transmit dengue virus) and other conditions (e.g. malaria, scrub typhus, leptospirosis, cytomegalovirus, Epstein-Barr infection, rheumatoid factor). Cross-reactions with other flaviviruses are uncommon in primary dengue infection.

Confirmation of dengue can be made serologically by:

- demonstrating a serological conversion of IgG from negative to positive accompanied by a positive IgM test.
- demonstrating a fourfold increase in IgG antibody titre in a second serum sample collected 14–21 days after the first sample.

Important: In dengue endemic areas, district laboratories should consult their nearest Dengue Reference Laboratory regarding which dengue tests are the most appropriate to use and how to interpret test results. The Reference Laboratory should also provide information on indications for RNA RT-PCR testing and sample requirements.

Tests used to monitor treatment

The following tests are helpful in monitoring the treatment and progress of dengue patients:

- **Measurement of haematocrit** (see Section 3.1): In severe dengue there is a significant rise in haematocrit (often by 20% or more), indicating haemoconcentration due to increased vascular permeability and plasma leakage. Haematocrit levels will be affected by fluid replacement and bleeding.

- **Platelet count** (see Section 3.1): This is greatly reduced in severe dengue. Monitoring platelet numbers can warn of approaching severe dengue.
- **White blood cell count** (see Section 3.1): Leucopenia is common and often marked, accompanied by a low lymphocyte count. Blood films may show reactive lymphocytes.
- **Coagulation tests:** These may be required to monitor patients with haemorrhagic symptoms. When available, they include bleeding and clotting times, prothrombin time, partial thromboplastin time and fibrinogen.
- **Clinical chemistry tests** (see Sections 3.3, 3.4): In severe dengue, liver enzymes (ALT, AST) are raised and frequently also creatinine levels. Serum sodium and albumin are reduced. Albuminuria is usually present and often haematuria. When available, measurement of electrolytes is helpful in monitoring treatment.
- **Measurement of C-reactive protein** (see Section 2.19): This test is useful in areas where Chikungunya and Zika viruses are found with dengue. CRP levels are normal in dengue fever and non-severe dengue but raised in Chikungunya and Zika virus infections. In severe dengue, CRP levels become significantly raised.

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2.11 Tuberculosis

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis*.

***Mycobacterium tuberculosis* complex (MBTC)**

M. tuberculosis is a complex of genetically closely related variants.

- *M. tuberculosis*: This variant is the principal cause of human TB.
- *M. bovis*: This is the main cause of TB in cattle and occasionally in other animals. Humans can become infected by close contact with infected animals and ingesting the organisms in raw untreated milk.
- *M. africanum*: This variant is midway between *M. bovis* and *M. tuberculosis*. It causes human TB in equatorial Africa. Type 1 (more closely related to *M. bovis*) is found mainly in west Africa and type 2 (more closely related to *M. tuberculosis*) is found in east Africa.

Distribution

TB is an infectious disease of global public health importance with high prevalence and mortality rates in tropical and low resource countries. Contributing factors include poverty, co-infection with HIV (major risk), overcrowded living conditions, under-nutrition, diabetes mellitus, tobacco use, conflict situations disrupting health care services, and the emergence of drug-resistant tuberculosis. Inadequate facilities to diagnose TB and test for drug resistance delay effective treatment.

In 2020 there were an estimated 10 million people with TB worldwide (1.1 million children). There were 1.5 million deaths¹. Only about 1 in 3 people with drug resistant TB were able to access treatment. Two thirds of new cases of TB were reported from India, China, Indonesia, the Philippines, Pakistan, Bangladesh and South Africa.

COVID-19 restrictions disrupted TB case-finding, diagnosis and treatment, leading to greater mortality and morbidity.

United Nations Meeting on TB¹

The Meeting reaffirmed:

- The SDG 3.3 target (see p.1) of ending TB by 2030 with a 90% reduction in the number of TB deaths and 80% reduction in TB incidence rate compared with levels in 2015.
- The WHO End TB target of ending TB by 2035 with a 95% reduction in the number of TB deaths and 90% reduction in TB incidence rate compared with levels in 2015.

Information on the six WHO core functions to achieve these targets can be found in the 2021 WHO Tuberculosis Fact Sheet¹.

Stop TB Partnership Global Plan to end TB 2023-2030

Launched in July 2022 the *Global Plan to end TB 2023–2030*² details the actions and financial resources needed to end TB as a global health threat by 2030. The Plan lays out the investment required to save millions of lives by the early diagnosis and treatment of 50 million people with TB, the development, approval and distribution of a new TB vaccine and the development of new TB medicines, treatment regimens and diagnostics.

Transmission

Most infections with *M. tuberculosis* are caused by inhaling tubercle bacilli in respiratory droplets from a person with active pulmonary TB (coughing, sneezing) or inhaling the organisms in dust particles. The bacilli become lodged in the lung and are taken up by macrophages. Activated macrophages form a granuloma around the site of primary infection which usually limits it. Only about 5–10% of infected persons develop active TB at the time of infection or at a later date due to poor health, under-nutrition or defective immune responses. Disease can occur several months or years later when dormant viable tubercle bacilli become reactivated.

Pulmonary TB: This can occur when the primary infection does not heal and there is continued multiplication or reactivation of the tubercle bacilli at a later stage. An inflammatory reaction leads to liquefied destruction of lung tissue with caseation. Erosion through the wall of a bronchus leads to the discharge of liquefied tissue and the formation of a cavity. Bacilli multiply in the wall of the cavity and can be found in sputum (“open” infectious stage). Patients with advanced infections have difficulty in breathing due to cavities in their lungs.

The main symptoms of pulmonary TB in adults are chronic cough, production of mucopurulent sputum which may contain blood (haemoptysis), loss of weight, fever, night sweats, fatigue, chest pain, pericarditis and occasionally lung collapse.

In children, pulmonary TB is more difficult to diagnose because there is rarely a cough with sputum production. There is weight loss and failure to thrive. Enlargement of lymph glands may cause bronchial obstruction and occasionally a gland may rupture into a bronchus causing acute infection of the affected lung. Infection may progress to tuberculous meningitis.

Tuberculous meningitis: Occurs more frequently in non-immune infants and young children as a complication of primary TB but it can occur at any age. The condition is often fatal unless treated at an early stage, but acid fast bacilli (AFB) are difficult to find in cerebrospinal fluid. Lymphocytes are usually found (neutrophils in the early stages).

Miliary tuberculosis: Many small granulomata are formed which on a chest X-ray look like millet seeds (hence the name miliary tuberculosis). Patients are often acutely ill with fever but a chronic form of the disease can also occur. The liver, spleen and lymph glands may be enlarged and the meninges may also become infected.

Renal and urogenital TB: Renal infection is often suspected when repeated urine samples are found to contain pus cells but no organisms are isolated by routine culture. There

may be frequency in passing urine, haematuria and usually recurring fever. TB of the genital tract (epididymitis in males, endometrial TB in females) can cause infertility and pelvic inflammatory disease.

Bone and joint TB: A commonly infected site is the spine which may lead to the collapse of vertebrae and the formation of a “cold” abscess in the groin. This form of the disease is rare.

Tuberculosis and HIV

Infection with HIV greatly increases the risk of developing active TB and drug-resistant TB. WHO estimates that people living with HIV are 15–22 times more likely to develop active TB than people without HIV¹. Progression to advanced HIV disease is also accelerated.

In 2020, 214 000 people died of HIV-associated TB, 85% of whom were living in Africa¹. 88% of TB patients living with HIV were on antiretroviral therapy. Extrapulmonary TB (without lung cavitation) is common in TB patients with HIV. Sensitive molecular tests are required to diagnose TB in those co-infected with HIV.

Tuberculosis and diabetes

Persons with diabetes mellitus are three times more likely to develop active TB. Diabetes reduces immune responses. In 2020 an estimated 370 000 new cases of TB were attributable to diabetes². Patients respond less well to TB treatment, remaining infectious for a longer period than TB patients without diabetes. The risk of TB relapse and reinfection is increased and diabetes may also increase the risk of developing multidrug-resistant TB. In diabetic patients with active TB, control of hyperglycaemia may be more difficult. TB is associated with hyperglycaemia and glucose intolerance.

The global increase in diabetes mellitus is increasing the prevalence of TB in low-and middle-income countries (see Section 3.2). WHO recommends that all those with TB should be systematically screened for diabetes and screening for TB in those with diabetes should be considered in settings with high TB prevalence.³

Management of Diabetes mellitus-Tuberculosis. A guide to the essential practice

Published in 2019 by the The World Diabetes Foundation and International Union Against TB and Lung Disease (IUATLD) this practical guide has been produced to help front-line health workers responsible for the diagnosis, management and care of those with tuberculosis and diabetes.⁴ It includes information on the clinical aspects of TB and diabetes mellitus, those at higher risk of developing TB, drug-resistant TB, laboratory tests used to screen and diagnosis TB in those with diabetes and tests to diagnose diabetes in those with TB.

LABORATORY DIAGNOSIS

At district level, depending on resources, infrastructure, and trained staff, TB is diagnosed by:

- Examining stained sputum smears microscopically for tubercle bacilli which has high specificity but low sensitivity.
- Using nucleic acid amplification testing (NAAT) which has high diagnostic accuracy.
- Testing urine for lipoarabinomannan (LAM) to assist in the diagnosis of TB in HIV-seropositive patients.

Note: Culture of *M. tuberculosis*, testing for multidrug resistance and the investigation of treatment failures are carried out in Regional Tuberculosis Reference Laboratories.

SPUTUM SMEAR MICROSCOPY

Sputum smear microscopy is used to monitor TB treatment and when facilities for molecular testing are not available, to assist in the diagnosis of TB. Smears are examined for acid fast bacilli (AFB).*

***AFB:** This refers to the acid fastness of the tubercle bacillus. When stained with heated carbol fuchsin or auram-ine-phenol, the *M. tuberculosis* bacillus retains its red colour when an acid decolourizer is applied.

For AFB to be detected microscopically, sputum smears need to contain 5 000–10 000 AFB/mL which may be found in patients with cavity lesions. AFB may not be detected in patients without cavity lesions, e.g. those co-infected with HIV*, young children and the elderly.

*In AIDS patients it is sometimes possible to detect AFB in buffy coat smears prepared from EDTA anticoagulated blood.

While the specificity of detecting AFB in sputum smears is high, sensitivity is low. Sensitivity can be increased by:

- Improving the quality of sputum samples.
- Using fluorescence microscopy.

Improving the quality of sputum samples

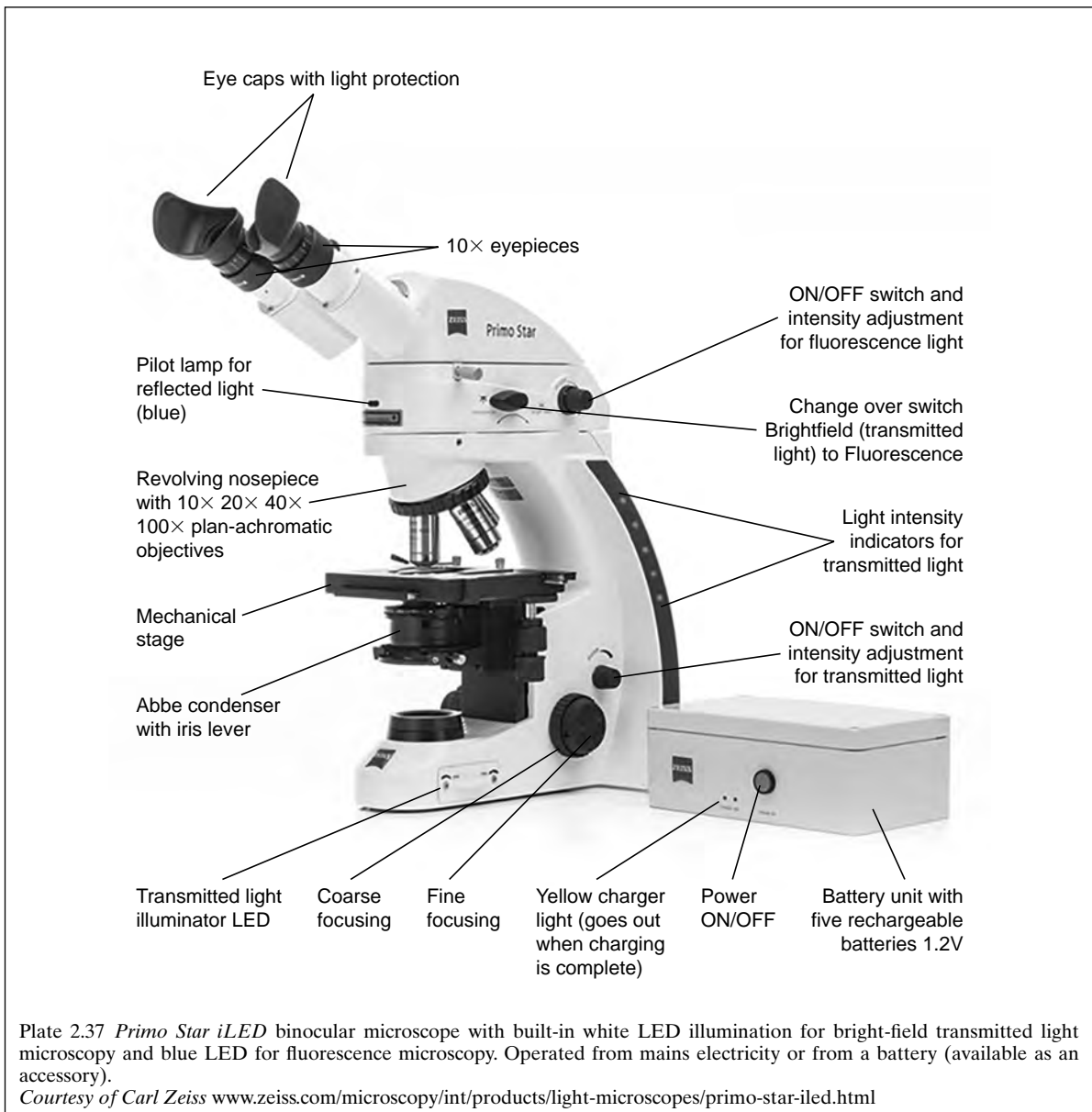
The successful detection of AFB in sputum is dependant on examining good quality sputum samples. Examining at least two sputum samples is recommended. Depending on national guidelines, the samples may be collected on separate days (one spot sample at the time of first consultation

and a second sample as an early morning sputum the following day), or both samples collected on the same day of consultation, i.e. same-day-diagnosis.

Several studies have shown that examining an early morning sample does not increase the number of patients diagnosed with pulmonary TB compared with examining two samples on the same day (at least 1 hour apart). The majority (95–98%) of smear-positive patients are identified by quality assured same-day-diagnosis.^{5,6,7} This lowers costs and enables smear positive patients to be treated the same day.

Collecting a good quality sputum sample

- 1 Advise the patient why a sputum sample is needed.
- 2 Request the patient to produce a sample well away from others, in an open area (not in the laboratory, patient waiting area, latrine or other closed-in area).
- 3 Give the patient a wide-mouthed disposable container (not a snap-closing one which can create aerosols).



- 4 To ensure the sample is sputum and not saliva, mucus, or nasal secretions, instruct the patient to inhale and exhale several times and then cough deeply to provide 3–5 mL of sputum, particularly a mucopurulent sample. It should not contain food particles.
- 5 Label the container with the patient's name, age, hospital/health centre number, time and date of collection. Complete a request form.
- 6 Test the sample and issue the report with the minimum of delay.

Using fluorescence microscopy

Compared with Ziehl-Neelsen (ZN) transmitted light microscopy, auramine O (AO) fluorescence is more sensitive (by at least 10%), efficient and cost-effective at detecting AFB in sputum smears.^{8,9} It reduces the time spent examining smears by 25–30%. A lower power magnification is used, enabling more of the smear to be examined in a shorter time.

Primo Star iLED microscope

An example of an LED fluorescence microscope is the *Primo Star iLED* microscope developed by the Foundation for Innovative New Diagnostics (FIND) and Carl Zeiss to improve the diagnosis of TB in resource-limited countries. It is available at reduced price to countries with high TB burden.

The *Primo Star iLED* binocular microscope model 415500 – 0040 with built-in white LED microscope is affordable, robust and easy to operate. It uses a white LED for transmitted light microscopy and a blue LED for reflected light fluorescence as shown in Plate 2.37. The LEDs have a long life, i.e. 10 000 hour light years, equivalent to a life-span of 10 years and consume very little power, enabling the microscope to be operated from a battery unit (available accessory) where power supplies are intermittent. No warm-up period for the light source is required, there are no harmful emissions and a dark room is not needed.

AURAMINE O FLUORESCENCE STAINING*

*Fluorescence staining

In fluorescence microscopy, ultra-violet light which has a short wavelength and is not visible to the eye (or just visible deep blue light) is used to illuminate organisms which have been previously stained with a fluorescence dye (fluorochrome) such as auramine O. The fluorochrome transforms the short wavelength light (or just visible deep blue light) into longer wavelength visible light. The fluorescent stained organisms are seen glowing against a dark background.

In the auramine O (AO) sputum staining procedure, auramine O stain containing phenol binds to the mycolic acids in the cell wall of tubercle bacilli and is not removed by acid-alcohol. The smear is washed with potassium permanganate or methylene blue to darken the background. The acid-fast tubercle bacilli fluoresce bright yellow against the dark background.

Preparing and fixing a sputum smear

- 1 Record the appearance of the sputum sample, i.e. whether salivary, mucoid, mucopurulent or blood stained.
- 2 Select a purulent or mucopurulent part of the sputum, or if present, yellow caseous material and spread this on a slide using a wooden stick, covering an area about 2 × 3 cm. Avoid making the smear too thick (it should be possible to read newsprint through it).
- 3 Allow the smear to air-dry in a safe place out of direct sunlight and protected from dust and insects.

Caution: *M. tuberculosis* is a highly infectious pathogen, therefore handle samples with care away from the face and near to the flame of a spirit lamp or Bunsen burner. Spread smears slowly to avoid creating aerosols and avoid using springy wire loops.

Fixing a sputum smear

When *completely* dry, fix the smear with 70% v/v alcohol for 2 minutes (or until the alcohol evaporates). This method of fixation is recommended in preference to heat-fixation because it kills *M. tuberculosis*. Heat-fixation does not kill the organisms.

Heat-fixation can be used when the sputum has been pretreated with bleach as sodium hypochlorite also kills tubercle organisms.

AO staining procedure¹⁰

Prepare an AO staining SOP, including principle of fluorescence staining (see previous text), reagents and other materials required, staining procedure, reporting smears, quality assurance (see later text) and procedure reference.

Reagents required

- 0.1% Auramine Reagent No. 8
- 0.5% Acid-alcohol Reagent No. 2
- 0.5% Potassium permanganate* Reagent No. 35

* 0.3% methylene blue (Reagent No. 30) can also be used. It provides a lighter coloured background which reduces contrast slightly but can make focusing easier.

Procedure

- 1 Place the slides, smear upwards, on a staining rack about 1cm apart to avoid carrying over material from one slide to another.

Note: Include positive and negative control smears with each batch of smears.

- 2 Cover the slides with filtered 0.1% auramine for 20 minutes. Do not allow the stain to dry.
- 3 Wash off the stain with water, preferably distilled or deionized.

Note: When not available use tap water if clean and not chlorinated (chlorine may affect fluorescence), or boiled filtered water.

- 4 Cover the slides with 0.5% acid-alcohol for 2 minutes. Wash with water.
- 5 Cover the slides with 0.5% potassium permanganate* or 0.3% methylene blue for 1 minute, followed by several rinses with water.

* Timing is critical to avoid over-quenching the fluorescence. When using 0.3% methylene blue, timing is less critical.

- 6 Wipe the back of each slide clean and place in a draining rack for the smear to air-dry. *Do not* blot dry.

Important: To prevent fading of the fluorescence, protect the stained smears from sunlight and bright light and examine within 24 hours.

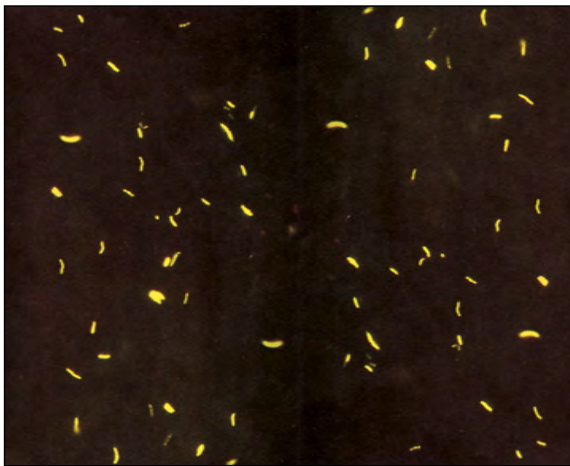


Plate 2.38 Appearance of acid-fast tubercle bacilli (AFB) in an auramine O fluorescence stained sputum smear using a 40× objective 10× eyepiece.

Reporting a fluorescence stained smear

Examine the smear systematically using a fluorescence microscope. First use the 10× objective to focus the smear and the 40× objective with 10× eyepiece to detect and report the AFB.

Look for small straight or slightly curved yellow rods occurring singly or in groups against a dark background. The appearance of AFB in a fluorescence stained sputum smear is shown in Plate 2.38.

Check the positive control smear for correct AFB fluorescence and the negative control for acid-fast contaminants.

The following commonly used system of reporting AFB in fluorescence stained smears is based on the WHO/IUATLD grading system:

Number of AFB seen Report

No AFB in 100 fields	No AFB seen
1–19 AFB in 100 fields	Report actual AFB number
20–199 AFB in 100 fields	AFB 1+
5–50 AFB per field	AFB 2+
>50 AFB per field	AFB 3+

ZIEHL – NEELSEN STAINING

In the Ziehl-Neelsen (ZN) sputum staining technique, heated carbol fuchsin stain binds to the mycolic acid in the cell wall of tubercle bacilli. Following the application of an acid decolourizer, the stain is not removed from the bacilli. They are acid-fast (AFB). The smear is counter-stained with methylene blue or malachite green which stains the background material providing a contrast colour against which the red AFB can be seen.

Note: The preparation and fixing of sputum smears are as described previously for fluorescence staining.

Ziehl-Neelsen staining procedure¹⁰

Prepare a ZN staining SOP, including staining principle, reagents and other materials required, staining procedure, reporting smears, quality assurance (see later text) and procedure reference.

Reagents required

– 1% carbol fuchsin	Reagent No. 12
– 3% acid-alcohol or	Reagent No. 4
25% sulphuric acid	Reagent No. 41
– 0.1% methylene blue*	Reagent No. 28
*malachite green counter-stain can also be used.	

Procedure

- 1 Place the slides, smear upwards on a staining rack, about 1cm apart to avoid carrying over material from one slide to another.

Note: include positive and negative control smears with each batch of smears.

- Cover the slides with filtered 1% carbol fuchsin and heat the stain until vapour just begins to rise. *Do not* allow the stain to boil or dry. Stain for 10 minutes.

Caution: Apply only a *small* flame under the slides. Keep the flame well away from flammable reagents.

- Wash off the stain with water, preferably distilled or deionized.*

*When not available and tap water is not suitable, use boiled filtered water.

- Cover the slides with 3% acid-alcohol or 25% sulphuric acid for 3 minutes. Wash with water.
- Counter-stain with 0.1% methylene blue (or malachite green) for 1 minute. Wash with water.
- Wipe the back of each slide clean and place in a draining rack for the smear to air-dry. *Do not* blot dry.

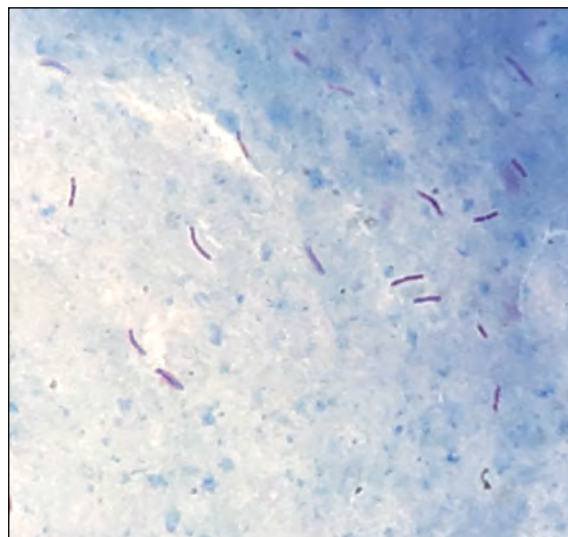


Plate 2.39 Appearance of acid-fast tubercle bacilli (AFB) in a Ziehl-Neelsen stained sputum smear counter-stained with methylene blue using a 100× objective and a 10× eyepiece.

Reporting a ZN stained smear

Examine the smear systematically using the 10× objective to focus and the 100× objective to detect and report AFB.

Look for red straight or slightly curved rods or beaded rods occurring singly or in groups. The appearance of AFB in a ZN stained sputum smear is shown in Plate 2.39.

The following commonly used system of reporting AFB in ZN stained smears is based on the WHO/IUATLD grading system.

Number of AFB seen	Report
No AFB in 100 fields	No AFB seen
1–9 AFB in 100 fields	Report actual AFB number
10–99 AFB in 100 fields	AFB 1+
1–10 AFB per field	AFB 2+
>10 AFB per field	AFB 3+

Quality assurance for sputum smear microscopy

- Follow the SOP exactly. Use positive and negative control smears to check the quality of new batches of reagents, staining reactions and presence of acid-fast contaminants.
- Re-check randomly smears during a supervisory visit or sooner by a senior member of staff to avoid discrepancies in reporting AO smears due to fluorescence fading.

- Ensure the microscope is in good working condition. Check the eyepiece and objective lenses are clean and illumination is adequate.
- Check reagents are being stored correctly and not used beyond their expiry date.
- Monitor the quality of sputum samples, labelling of samples, registration process, accompanying documentation and preparation of smears.
- Check whether samples are being examined and reported during a patient's hospital/health centre visit to avoid delays in patients being treated.
- Ensure biosafety procedures are being followed including the handling, storage and safe disposal of samples and infectious material, correct use of disinfectants and daily cleaning and disinfecting of the work area.
- Whenever possible participate in an external quality assessment scheme which includes sputum smear microscopy.

NAAT TO DIAGNOSE TUBERCULOSIS

In nucleic acid amplification testing (NAAT), *M. tuberculosis* genetic material is amplified (multiplied) and detected with high sensitivity and specificity. TB can be diagnosed rapidly and more effectively than by smear microscopy (40–50% more sensitive).

The following NAATs have been endorsed by WHO and recommended as initial diagnostic tests for TB in preference to sputum smear microscopy:

- *GeneXpert MTB/RIF* and *GeneXpert MTB/RIF Ultra*, manufactured by Cepheid Inc.
- *Truenat MTB*, *MTB Plus*, and *MTB-RIF*, manufactured by Molbio Diagnostics Ltd.

Both molecular tests can be used to diagnose pulmonary and extrapulmonary tuberculosis in children and adults at point-of-patient care and to detect rifampicin resistance. Recommendations on the use of the two tests can be found in the *WHO consolidated guidelines on tuberculosis. Module 3: Diagnosis – Rapid diagnostics for tuberculosis detection, 2021 July Update*¹¹. The tests cannot be used to monitor treatment, microscopy is required (molecular tests cannot distinguish between living and dead bacilli). The biosafety requirements for performing both tests are similar to those for sputum smear microscopy.

Equipment used in both systems can be used to diagnose other bacterial and parasitic infections of importance in tropical countries and also to diagnose COVID-19.

GeneXpert MTB/RIF, MTB/RIF Ultra

M. tuberculosis DNA is detected using real-time polymerase chain reaction (PCR) and reverse transcriptase PCR. Rifampicin resistance is detected by identifying mutations in the *rpoB* gene. *GeneXpert MTB/RIF* and *MTB/RIF Ultra* are fully automated assays. An advantage of the GeneXpert system is that a single use cartridge contains all the reagents required to carry out sample processing, nucleic acid amplification and detection of target sequences. Test results are reported as MTB not detected or MTB detected, and for rifampicin resistance, RIF not detected or RIF detected.

Sensitivity of *MTB/RIF* has been reported as 88% and specificity as 99% when compared with liquid culture. The *Ultra* cartridge is more sensitive, detecting as few as 16 colony-forming units/mL compared to 131/mL for *MTB/RIF*. Test time for the *Ultra* is 90 minutes (2 hours for *MTB/RIF*). Shelf-life of *Ultra* cartridges is 16 months from date of manufacture (24 months for *MTB/RIF*).

The *GeneXpert* system requires a *GeneXpert* instrument (see Plate 2.40), an *MTB/RIF* or *Ultra* cartridge, a computer to run the *GeneXpert Dx* system software and barcode scanner. *Ultra* cartridges run from software version 4.7b or later (Cepheid provides a free of charge CD to update software). An uninterrupted electricity supply is required, an

operating temperature below 30 °C and minimal interference from dust (a dust filter is available as an optional accessory). In places where electricity supplies are intermittent, the use of an uninterrupted power supply on-line unit and back-up battery pack are recommended (available from Stop TB Partnership).

Principle of test

Following sample preparation which involves lysing the sample at room temperature for 15 minutes (reagent and pipettes supplied), the sample is added to the cartridge and inserted in the *GeneXpert* instrument. The PCR process is fully automatic. Test results are available in under 2 hours, reported as MTB not detected or MTB detected and for rifampicin resistance, RIF not detected or RIF detected.

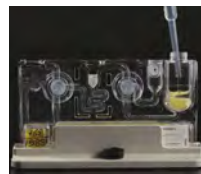
Quality control of the *GeneXpert MTB/RIF* system includes a sample processing control which checks for correct sampling and the presence of sample inhibitors in the PCR reaction. A probe check control measures the fluorescent signal from the probes, reaction-tube filling, probe integrity and dye stability. The system requires annual calibration.

Cartridges and reagents require storage at 2–28 °C. The number of samples that can be processed at one time depends on the capacity of the *GeneXpert* instrument (1, 2, 4 or 16 module capacity), see Plate 2.40.



GeneXpert MTB/RIF cartridge with equipment module for testing four samples.

Courtesy of Cepheid Inc.



a Truenat reagent cartridge



b Sample Prep device



c MTB micro chip on chip tray



d Truenat PCR analyzer (2 chip port)

Truenat system for diagnosing TB.

Courtesy of Stop TB Partnership. Images reproduced from *Practical Guide to implementation of Truenat Tests*.

Plate 2.40 Molecular tests to diagnose TB and detect rifampicin resistance.

Truenat MTB, MTB plus, MTB RIF

Truenat MTB and *MTB Plus* are chip-based micro real-time polymerase chain reaction assays that detect *M. tuberculosis* in sputum. *Truenat MTB RIF* is used to detect rifampicin resistance in positive samples.

Truenat assays were endorsed by WHO in 2020 following a multicenter prospective clinical evaluation study conducted in 19 clinical sites and 7 reference laboratories in Peru, India, Ethiopia and Papua New Guinea. The study found the diagnostic accuracy of *Truenat* assays to be comparable to that of *GeneXpert MTB/RIF* and *MTB/RIF Ultra*¹¹.

Principle of assay

A sputum sample is first liquefied and lysed using the *Truenat Auto MTB Sample Pre-treatment Pack* (supplied with the test kit). *M. tuberculosis* DNA is extracted from the sample using the *Truenat Sample Prep Device* and *Cartridge Based Sample Prep Kit* reagents. PCR reagents, the *Truenat MTB* micro chip and *Truenat* micro analyzer are used for PCR amplification and fluorescent probe-based detection of MTB. The PCR test results are displayed on the analyzer screen and if required can be printed using the *Truenat* PCR printer. Positive and negative controls are used to ensure the analyzer is working accurately.

Truenat equipment is both mains and battery operated (rechargeable from mains or a solar panel), enabling *Truenat* tests to be used in areas of unstable electricity. The tests can also be used in operating temperatures up to 40 °C. A portable field testing kit is available (contains single chip port analyzer).

Unlike the *GeneXpert* system, the *Truenat* system requires a separate piece of equipment (*Cartridge Based Sample Prep Device*) and cartridge-based *Sample Prep Kit* reagents (see Plate 2.40 a,b) to extract DNA from the sputum sample.

The *Truenat MTB* or *MTB Plus* kit contains a microtube containing freeze-dried PCR reagents and the *Truenat MTB* or *MTB/Plus* micro chip (in sealed pouches). Positive and negative controls are available to ensure the test is performing correctly.

A *Truenat* micro PCR analyzer and MTB micro chip (see Plate 2.40,c,d) are required for PCR amplification and fluorescent probe-based target detection. The analyzer is available with 1, 2, or 4 chip ports enabling 10-48 samples to be tested at one time. The PCR analyzer displays test results, showing whether MTB is detected. For *MTB Plus*, positive test results are displayed as “high, medium, low or very low”. The detection limit is about 30 CFU/mL of sputum sample. For *Truenat MTB*, the estimated number of bacteria are shown as CFU/mL with a

detection limit of about 100 CFU/mL of sputum sample. For each assay the validity of the test is also shown. Test results can be transferred to a laboratory computer or remote computer via a Wifi network. They can be printed using the *Truenat* micro PCR printer.

Detecting rifampicin resistance

When MTB is detected in a sample the same DNA eluate can be used to test for rifampicin resistance using a *Truenat MTB-RIF Dx* chip. Major mutations that are known to cause resistance to rifampicin are detected. An additional 1 hour is added to the test giving a total running time of 2 hours.

Note: Further information on the use of *Truenat* tests, including a suggested SOP, can be found in the Stop TB Partnership 2021 *Practical Guide to Implementation of Truenat Tests for the detection of TB and rifampicin resistance*. Access by entering the title in Google.

Availability of *Truenat* supplies: The 3 different reagent packs and 3 items of equipment required to perform *Truenat* tests can be obtained from Stop TB Partnership (see end of subunit).

TB LIPOARABINOMANNAN (LAM) URINE TEST

TB-LAM Ag is a lateral flow (LF) immunochromatographic strip test that detects LAM antigen qualitatively in urine. The antigen is a component of the bacterial cell wall of *Mycobacterium* species. It is released by active and degenerating bacilli. The test therefore is not specific for *M. tuberculosis* and has a low sensitivity. WHO recommends using TB-LAM to assist in the diagnosis of active TB in HIV-seropositive persons who are seriously ill or have a low CD4 cell count (< 200 cells/mm³ for inpatients, < 100 cells/mm³ for outpatients), irrespective of symptoms of TB. The test should not be used for HIV-seropositive outpatients who have not been assessed for TB or are without TB symptoms and with an unknown CD4 cell count or CD4 count > 100 cells/mm³. The test should not be used as a general screening test for TB.

Determine TB LAM Ag

The test is manufactured by Abbott (previously Alere). It is available in a card containing 10 strips and has a shelf-life from manufacture of 15 months when stored at 2–30 °C. The test requires 60 µL of fresh urine and takes 25 minutes to perform.

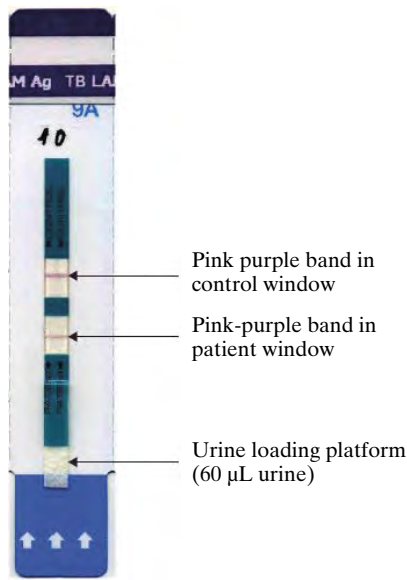


Plate 2.41 Positive *Determine TB LAM Ag* test. Band colour intensity is interpreted using a Reference scale card provided with the test.

Courtesy of Alere.

A positive test is indicated by a pink-purple band in the control window of the strip (indicating a valid test) and a pink-purple band in the patient window as shown in Plate 2.41. A Reference scale is provided to assist in reading the colour intensity of the test band. A negative test (pink-purple band in control area only) cannot be used to exclude TB.

Where facilities are available and patients are able to produce samples for testing, a molecular test should be used to diagnose pulmonary and extra-pulmonary TB in patients with HIV disease.

Note: The value of C-reactive protein (CRP) testing to improve the specificity of the WHO-recommended four-symptom screen for TB in HIV-positive patients is described on p.173.

AVAILABILITY OF LABORATORY TB DIAGNOSTICS

A supplier of laboratory TB diagnostics is the organization Stop TB Partnership Global Drug facility. It supplies quality assured TB diagnostic products that are WHO approved, technically evaluated and validated for use at lowest possible prices.

The May 2022 *Diagnostics, Medical devices and other health products Catalogue* lists products with prices for ZN and AO staining supplies, *GeneXpert MTB/RIF & Ultra* tests, UPS and auxiliary battery pack, *Determine TB-LAM* urine test strips, *Truenat assays*, *Primo Star iLED* microscope and other items relevant to laboratories performing TB investigations.

The Catalogue can be downloaded by entering the Catalogue title in Google.

REFERENCES

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- 2 *Global Tuberculosis Report 2021*. WHO 2022. Access by entering the title in Google.
- 3 *Tuberculosis and diabetes. The dual epidemic of TB and diabetes*. World Health Organization, 2016. https://www.who.int/tb/publications/diabetes_tb.pdf
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- 5 **Davis J L.** Bringing patient-centered tuberculosis diagnosis into the light of day. *BMC Med*, 2017 15:219. DOI 10.1186/s12916-017-0992-4
- 6 **Murphy M E *et al.*** Spot sputum samples are at least as good as early morning samples for identifying *Mycobacterium tuberculosis*. *BMC Med*, 2017;15:192
- 7 **Datta S *et al.*** Comparison of sputum collection methods for tuberculosis diagnosis: a systematic review and pairwise and network meta-analysis. *Lancet Glob Health*, 2017; 5(8);e760-71
- 8 **Goel S *et al.*** Impact of introducing light-emitting diode fluorescence microscopy services for diagnosis of pulmonary tuberculosis under Revised National Tuberculosis Control Program India. *Lung India*, 2018 Jul-Aug;35(4):307–311. DOI: 10.4103/lungindia.lungindia_475_17
- 9 **Shariq Ahmed *et al.*** Light-emitting diode-fluorescence microscopy: determining its sensitivity and specificity in diagnosis of pulmonary tuberculosis in a high-burden tuberculosis region and resource-limited country like India. *Chrimed J Health Res*, 2019, Vol 6, Issue 1, Page 44–51. DOI: 10.4103/cjhr_46_18
- 10 *Laboratory diagnosis of tuberculosis by sputum microscopy Handbook*, 2013. Global Laboratory Initiative, Working Group of the Stop TB Partnership. Access by entering the title in Google.
- 11 *WHO consolidated guidelines on tuberculosis. Module 3: Diagnosis – Rapid diagnostics for tuberculosis detection 2021 update*. <https://who.int/publications/i/item/9789240029415>

FURTHER READING

WHO Consolidated Guidelines on Tuberculosis (TB). Consisting of:
Module 1 Prevention: Tuberculosis preventive treatment, 2020.

Module 2 Screening: Systematic screening for tuberculosis disease, 2020.

Module 3 Diagnosis: Rapid diagnostics for tuberculosis detection, update 2021.

Module 4 Treatment: tuberculosis care and support, 2022. Complemented by: *Operational Handbook on Tuberculosis: Drug-susceptible TB treatment*.

Module 5 Management of tuberculosis in children and adolescents, 2022.

Tuberculosis Work Safe Laboratory Safety. Global Laboratory Initiative, 2019.

Stop TB Partnership.

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Lin Y et al. *Management of Diabetes Mellitus-Tuberculosis. A Guide to the Essential Practice*, 1st ed 2019. International Union Against Tuberculosis and Lung Disease and World Diabetes Foundation.

https://www.theunion.org/what-we-do/publications/technical/english/theUnion_DMTB_Guide.pdf

Harries A D et al. What can National TB Control Programmes in low-and middle-income countries do to end tuberculosis by 2030. *F100Res*, 2018 Jul 5.

doi:10.12688/f1000research.14821.1

McKenna L. The tuberculosis diagnosis pipeline for children. *Treatment Action Group*, 2018.

<http://www.pipelinerreport.org/2018/tb-peds-diagnosis-pipeline>

2.12 Meningitis

Meningitis is an inflammatory infectious disease of the meninges, the membranes that cover the brain and spinal cord. It is mainly caused by bacteria or viruses and occasionally by fungi and parasites. Pathogens reach the meninges in the blood stream or occasionally by spreading from nearby sites such as the middle ear or nasal sinuses. Person to person transmission can occur through respiratory droplets and throat secretions. The risk of infection increases in overcrowded conditions and in the absence of immunization.

Common symptoms of meningitis are high fever, headache, neck stiffness, intolerance to light, confusion and vomiting. Early diagnosis and effective treatment are essential to reduce loss of life and impairments following meningitis. At least one third of patients experience impairments which may include seizures, limb loss, memory and behaviour changes, cognitive impairments, vision loss, neuromotor disability and hearing loss.¹

Bacterial meningitis

The main causes of acute bacterial (pyogenic) meningitis are *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenzae* type b and *Streptococcus agalactiae* group B. Other causes of bacterial meningitis include *Escherichia coli* and other enterobacteria, *Mycobacterium tuberculosis*, *Salmonella* serovars, *Listeria monocytogenes* and *Staphylococcus aureus*.

***Neisseria meningitidis*:** This can cause severe brain damage and septicaemia. It is fatal in 50% of cases with children under 5 years of age and young adults being most at risk.¹ Following infection, 20% or more of patients suffer impairments.

N. meningitidis serogroups A, B, C, W, X, Y can cause meningitis epidemics particularly in sub-Saharan Africa. Outbreaks occur in the dry season in the “meningitis belt” which extends from Senegal to Ethiopia (26 countries). Nasopharyngeal mucosal damage from dust and respiratory infections leads to increased susceptibility to infection. Carriers of *N. meningitidis* are particularly important in the transmission of meningococcal meningitis. A hyper-invasive strain of serogroup C is spreading in sub-Saharan Africa with the potential to cause major epidemics in the absence of adequate supplies of effective meningitis C vaccine.²

Polysaccharide vaccines used in prevention and outbreaks are available for *N. meningitidis* serogroups A, C, Y and W. They offer 3 year protection after 2 years of age.¹ There is no universal vaccine against meningococcal meningitis. Conjugate vaccines (used in prevention) confer

longer-lasting immunity i.e. 5 years or more and can be administered after 1 year of age.¹ In recent years mass vaccination of people aged 1–29 with meningococcal A conjugate vaccine (*MenAfriVac*) has greatly reduced the incidence of meningitis serogroup A epidemics in sub-Saharan Africa. Protein-based vaccines for use in outbreaks against serogroup B are available.² They protect against meningitis but not transmission.

***Streptococcus pneumoniae*:** This pathogen causes endemic pneumococcal meningitis with high fatality rates, particularly in young children. Epidemic pneumococcal meningitis is rare. Following infection patients frequently experience long-term disability. Pneumococcal conjugate vaccination is reducing the incidence of pneumococcal meningitis (effective after 2 years of age).

***Haemophilus influenzae* type b:** In recent years the availability and widespread use of *H. influenzae* type b conjugate vaccine has reduced the occurrence of *Haemophilus* type b meningitis in young children in tropical countries. A combined vaccine, *H. influenzae*/meningococcus serogroup C is also available.

***Streptococcus agalactiae* group B:** This species is a common cause of meningitis and sepsis in neonates. A vaccine to immunize against *S. agalactiae* is being developed but not yet available.

Note: Further information on conjugate and polysaccharide vaccines used in the prevention and treatment of bacterial meningitis can be found in the WHO 2021 Meningitis Fact Sheet (see Further Reading).

Tuberculous meningitis: This form of tuberculosis mainly affects young children and those infected with HIV (see Section 2.11).

Syphilitic meningitis: This may occur as a complication of late syphilis.

Viral meningitis

The term aseptic meningitis is frequently used to describe viral meningitis and other forms of non-bacterial meningitis.

The main causes of viral meningitis particularly in young children are enteroviruses such as Coxsackie virus and echovirus. Other causes include Herpes simplex virus types 1 and 2, some arboviruses, human parechoviruses, cytomegalovirus, lymphocytic choriomeningitis virus, measles virus and HIV. Primary infection and reactivation of varicella-zoster virus can also cause meningitis.

Fungal meningitis

The commonest cause of fungal meningitis in tropical countries is the yeast fungus, *Cryptococcus neoformans*. It can cause meningitis in HIV infected persons with advanced immunodeficiency. It is more common in adults than children and is thought to be a reactivation of latent infection. Raised intracranial

pressure is a frequent serious complication of cryptococcal meningitis.

Less common causes of fungal meningitis include the dimorphic fungi, *Coccidioides* and *Histoplasma* which also cause meningitis in persons with reduced immune responses.

Parasitic meningoencephalitis

In meningoencephalitis both the brain and meninges are infected. *Naegleria fowleri*, a free-living amoeba causes acute meningoencephalitis in tropical countries and elsewhere. The condition is referred to as primary amoebic meningoencephalitis. Most infections can be traced to bathing in stagnant freshwater lakes, pools contaminated with sewage or in under-chlorinated swimming pools. Unless treated at an early stage the infection is fatal within a few days of the onset of symptoms.

The free-living amoeba *Acanthamoeba castellanii* can also cause meningoencephalitis but unlike *Naegleria*, most *Acanthamoeba* infections occur in immunocompromised persons.

Note: Encephalitis occurring in African trypanosomiasis is described in Section 2.2.

WHO Global roadmap Defeating meningitis by 2030

In “looking towards a world free of meningitis”, the global roadmap *Defeating meningitis by 2030* sets out three goals:

- Elimination of bacterial meningitis epidemics.
- Reduction of cases of vaccine-preventable bacterial meningitis by 50% and deaths by 70%.
- Reduction of disability and improvement of quality of life after meningitis due to any cause.

Actions required to achieve the goals of the roadmap

- Prevention and epidemic control focused on the development of new affordable vaccines, high immunization coverage, improvement of prevention strategies and response to epidemics.
- Diagnosis and treatment, focused on speedy confirmation of meningitis and optimal patient care.
- Disease surveillance to guide meningitis prevention and control.
- Care and support of those affected by meningitis, focusing on early recognition and improved access to care and support for complications from meningitis.
- Advocacy and engagement, to ensure high awareness of meningitis, to promote country engagement and to affirm the right to prevention, care, and after-care services.

LABORATORY DIAGNOSIS

In district laboratories meningitis is investigated by examining cerebrospinal fluid (CSF) for the causes of bacterial and aseptic meningitis using:

- Microscopy, including Gram staining, counting and differentiating white cells in CSF and examining wet preparations.
- Rapid diagnostic tests.
- Biochemical tests, including measuring protein and glucose.

Note: Few district laboratories have facilities for CSF culture, pathogen identification and antimicrobial susceptibility testing.

Collection of CSF

CSF *must* be collected by an experienced medical officer or health worker. It must be collected *aseptically* to prevent organisms being introduced into the central nervous system. The CSF is usually collected from the arachnoid space between the fourth and fifth lumbar vertebrae.

When possible the CSF opening pressure should be measured. Normal CSF opening pressure is < 20cm H₂O for adults and < 10cm for children below age 8. A raised opening pressure indicates increased intracranial pressure from cerebral oedema which can occur in bacterial and fungal meningitis. The opening pressure is usually normal in viral meningitis.

Before performing a lumbar puncture the laboratory should be advised so that staff are prepared to receive and examine the CSF. A delay in examining CSF may result in a lower cell count due to white cells being lysed and a falsely low glucose value due to glycolysis. It is usual to collect the CSF in two sterile screw-cap containers about 1 mL in container labelled No.1 and about 2–3 mL in container No.2.

Reporting CSF appearance

Normal CSF appears clear and colourless. In meningitis, the CSF may appear purulent, cloudy, slightly turbid or clear. It may contain clots and blood.

Purulent or cloudy CSF: This indicates the presence of polymorphonuclear neutrophils (pus cells), suggestive of acute bacterial meningitis. CSF begins to appear cloudy when it contains about 200 WBC × 10⁶/L.

Slightly turbid or clear CSF: This may indicate tuberculous meningitis, viral meningitis or fungal meningitis.

Blood in CSF: This may be due to haemorrhage in the central nervous system. When due to a traumatic lumbar puncture, CSF sample No.2 will usually contain less blood than sample No.1. Following a subarachnoid haemorrhage, the fluid may appear xanthochromic, i.e. yellow-red (seen after centrifuging).

Clots in CSF: These indicate a high protein concentration with increased fibrinogen which may occur in acute bacterial meningitis or when there is spinal constriction. Occasionally in tuberculous meningitis a spider-web clot may form.

ACUTE BACTERIAL MENINGITIS

When the CSF appears purulent or cloudy, examine a Gram stained CSF smear for bacteria, particularly organisms suggestive of meningitis caused by *N. meningitidis*, *S. pneumoniae*, *Haemophilus* type b or *S. agalactiae* group B.

GRAM STAINING

Preparing a CSF smear for Gram staining

- 1 Centrifuge the CSF at RCF 1 000 × g for 10 minutes. Transfer the supernatant fluid to another tube (to be used for glucose and protein tests should these be required). Mix the sediment and transfer one or two drops to a slide. Spread to make a smear which is not too thick or too thin. Allow to air-dry.

Note: Do not centrifuge a purulent sample. Prepare a smear from the uncentrifuged CSF.

- 2 When completely dry, fix the smear with 1–2 drops of absolute methanol. Allow the alcohol to evaporate.

Note: Avoid heat-fixing CSF smears because this can damage cells and organisms.

Gram staining procedure⁴

Principle of Gram staining

Based on their cell wall structure, most bacteria can be classified as Gram positive or Gram negative. Gram positive organisms have thick peptidoglycan cell walls. They stain dark purple with crystal violet and are not decolorized by acetone-alcohol. Gram negative organisms have thinner cell walls. Following

crystal violet staining, they are decolorized by acetone-alcohol. They stain pink-red following the application of a red counterstain. Retention of crystal violet by Gram positive bacteria may also be due in part to their more acidic protoplasm which binds to the basic crystal violet dye helped by iodine which acts as a mordant.

Gram stain SOP: This should include principle and purpose of the test, sample details, reagents and other materials required, staining procedure, reporting of smears, quality assurance and procedure reference.

Reagents required

- | | |
|--------------------------------|----------------|
| – Crystal violet stain | Reagent No. 15 |
| – Lugol's iodine | Reagent No. 26 |
| – Acetone-alcohol decolorizer | Reagent No. 1 |
| – Neutral red 1g/L w/v (0.1%)* | Reagent No. 31 |

* Neutral red is recommended because it stains *Neisseria* organisms particularly well. 0.1% safranin and dilute carbol fuchsin (1 in 10) can also be used.

Procedure

- Cover the smear with crystal violet stain for 30 seconds. Wash off with clean water.*
*If the tap water is not clean, use filtered water or clean boiled rainwater.
- Cover the smear with Lugol's iodine for 30 seconds. Wash off with clean water.
- Decolorize rapidly (few seconds) with acetone-alcohol. Wash off with clean water.
- Cover the smear for 2 minutes with 0.1% neutral red, 1 minute with 0.1% safranin, or 30 seconds with dilute carbol fuchsin.
- Wipe the back of the slide clean and place in a draining rack for the smear to air-dry.
- Examine the smear microscopically, first with the 40× objective to check the staining and distribution of material. Use the 100× objective to report the smear.

Results

Gram positive bacteria dark purple
Gram negative bacteria pink-red
Nuclei of pus cells (neutrophils) pink-red

Reporting CSF Gram smears

Report the presence of pus cells and the Gram reaction, morphology and number of bacteria (few, moderate or many). Look particularly for:

- Gram negative intracellular and extracellular diplococci, suggestive of *N. meningitidis*, see Plate 2.42.

- Gram positive diplococci surrounded by a capsule (clear or pale pink area surrounding the diplococci), suggestive of *S. pneumoniae*, see Plate 2.43.
- Gram positive cocci in chains (streptococci), suggestive of *S. agalactiae* group B, see Plate 2.44.
- Small Gram negative rods and coccobacilli (often staining palely) and occasionally filamentous forms, suggestive of *H. influenzae* type b, see Plate 2.45.

Important: Issue the Gram stain report as soon as possible.

Quality assurance

Prepare control smears of Gram positive organisms e.g. *S. aureus* and Gram negative organisms, e.g. *E. coli* and use these smears to check newly prepared Gram stains and reagents. At weekly intervals include a control smear with routine Gram staining.

Variations in Gram reaction

Gram positive organisms may lose their ability to retain crystal violet and stain Gram negatively for the following reasons:

- Cell wall damage due to antibiotic treatment or when a smear is heat-fixed.
- Over-decolorizing a smear.
- Using an iodine solution past its expiry date or when it appears pale yellow instead of brown.
- Preparing a smear from an old culture (in laboratories performing culture).

Gram negative organisms may not be fully decolorized and appear as Gram positive when a smear is too thick.

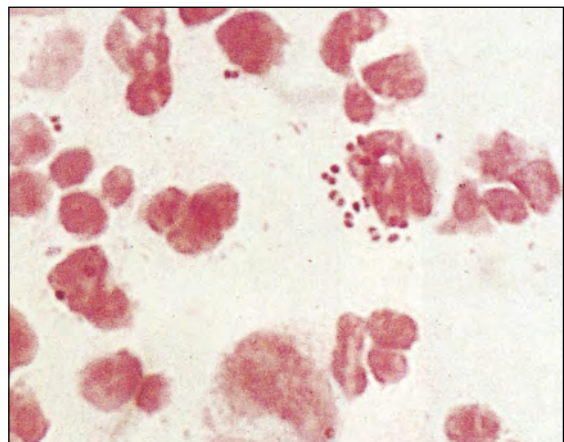


Plate 2.42 *Neisseria meningitidis* in Gram stained CSF. Note the pus cells and intracellular Gram negative diplococci. As seen with the 100× objective (neutral red counterstain).

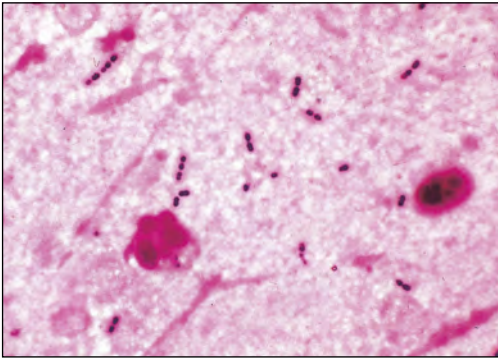


Plate 2.43 *Streptococcus pneumoniae* in Gram stained CSF. Note the Gram positive diplococci surrounded by a capsule (seen as a pink stained area around the diplococci). As seen with the 100× objective (dilute carbol fuchsia counterstain).

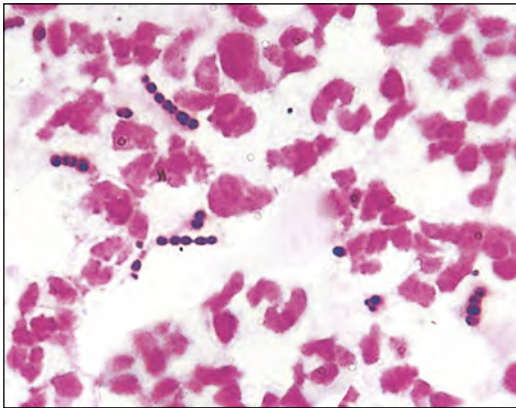


Plate 2.44 *Streptococcus agalactiae* group B in Gram stained CSF. Note the pus cells and Gram positive streptococci. As seen with the 100× objective (dilute carbol fuchsia counterstain).

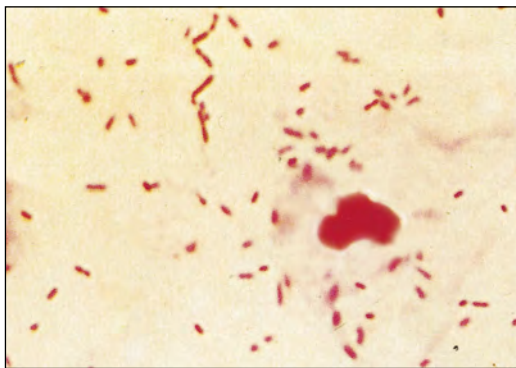


Plate 2.45 *Haemophilus influenzae* type b in Gram stained CSF. Note the pus cell and small Gram negative rods and coccobacilli. As seen with the 100× objective (neutral red counterstain).

RAPID MENINGITIS TESTS

Rapid CSF tests have been developed to diagnose the causes of acute bacterial meningitis and to identify the serogroups causing meningococcal meningitis to guide vaccination response in the control of epidemics. Currently available tests include:

- Latex agglutination (LA) tests
- Lateral flow assays (LFA)

LA tests to detect bacterial meningitis

LA tests use specific antibody coated latex particles to detect specific bacterial antigen by visual agglutination. They require storage at 2–8 °C, have a short shelf-life and CSF samples need to be heated to 100 °C and centrifuged prior to testing.

An example of an LA test is the *Pastorex Meningitis* test manufactured by Bio-Rad laboratories Inc. The test kit contains reagents to identify antigens to:

- *N. meningitidis* serogroups A, B (also *E. coli* strain K1) and W/Y (does not differentiate between W and Y serogroups). It cannot detect serogroup X.
- *H. influenzae* type b
- *S. pneumoniae*
- *S. agalactiae* (group B *Streptococcus*)

Each test kit contains reagents for 25 tests, positive and negative controls, agglutination cards and dispensing pipettes. Once opened the reagents can be used for up to 1 month. In the test procedure, a minimum of 0.5 mL of CSF is heated at 100 °C for 3 minutes. Following cooling to room temperature, the CSF is centrifuged for 5 minutes at RCF 3 000 × g. For each test, 50 µL of sample is mixed with 1 drop of latex reagent and the card rotated at 120 rpm for 10 minutes. A positive test is shown by agglutination.

Note: Information on the performance of *Pastorex Meningitis* can be found in the paper, *Systematic review of rapid diagnostic tests for the diagnosis of meningitis in outbreak response in sub-Saharan Africa*.⁴

LFAs to diagnose meningococcal meningitis

LFAs are immunochromatographic (IC) tests. They do not require refrigeration for transport or refrigeration and have a longer shelf-life than LA tests. They are also easier to use, not requiring pre-heating or centrifuging of CSF. Some IC tests have been validated for testing both CSF and urine.

An example of a recently developed LFA is the three cassette test *MeningoSpeed*, manufactured by

BioSpeedia Company (see Plate 2.46). It is available as a 20 test pack. *MeningoSpeed* detects *N. meningitidis* serogroup X as well as serogroups A, C, W, Y. Serogroup X has been reported recently as causing epidemics in sub-Saharan Africa.

MeningoSpeed is stable for up to 2 years from manufacture. It does not require refrigeration (recommended storage is 4–30 °C). The test is performed by adding 1 drop (30 µL) of CSF or urine to the Sample well in the cassette and adding 3 drops of diluent. The test result is read after 15 minutes. A positive test is shown by a pink band appearing in the Control (CL) area of the viewing window and a pink band in the Test area of the viewing window, depending on the serogroup detected. Positive tests for serogroups A, W, Y, C and X are shown in Fig. 2.31.

MeningoSpeed is reported by Biospeedia as having a sensitivity for CSF of 95.6% (versus PCR) and specificity of 93.8% and for urine a sensitivity of 93% and specificity of 96%. Field evaluations are awaited.



Plate 2.46 *MeningoSpeed* three cassette IC test to detect *N. meningitidis* serogroups: A, W, Y, C, X

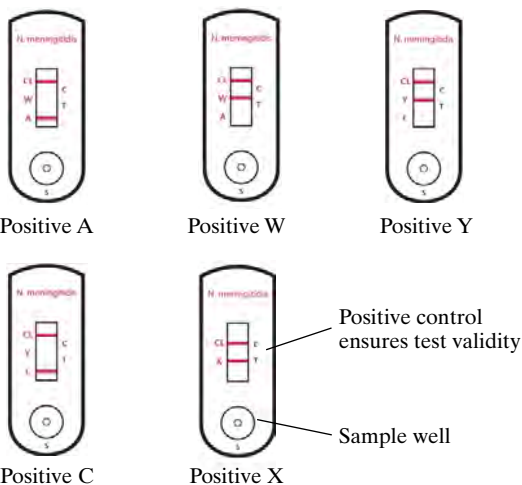


Fig. 2.31 Positive *MeningoSpeed* IC cassette tests for serogroups A, W, Y, C and X
Courtesy of Biospeedia Company
<http://www.biospeedia.com/index.php/en/products-en/39-meningospeed>

Note: Also available is a duplex IC dipstick which detects *N. meningitidis* serogroups A and W135/Y (RDT 1) and serogroups C and Y (RDT 2). It is manufactured by the Pasteur Institute in France and Centre de Recherche Médicales et Sanitaire (CERME) in Niger. Details of the performance of the test can be found in paper Reference.⁴ A dipstick to detect serogroup X is under development.

Rapid tests to diagnose pneumococcal meningitis

These include:

- *PneumoSpeed* cassette IC test
- *BinaxNow Streptococcus pneumoniae* card antigen test

PneumoSpeed cassette IC test is available from Biospeedia Company. It has a similar shelf-life, stability and easy test procedure as described previously for *MeningoSpeed*. It detects *S. pneumoniae* in CSF or urine.

Alere *BinaxNow Streptococcus pneumoniae* antigen card test is a rapid IC test for detecting *S. pneumoniae* in CSF or urine. The test is manufactured by Abbott (previously Alere). It has a shelf-life of 1 year from manufacture when stored at 2–30 °C.

To perform the test, the card is opened, the CSF or urine is collected on a *BinaxNow* swab which is then inserted in a hole in the card. Citrate phosphate buffer is added and the card is closed. After 15 minutes the test result is read through a viewing window on the front of the card. A positive test is shown by a pink band in the Control area of the viewing window and a pink band in the Sample area. The test result can also be read using an Alere test reader (available as an optional accessory). Positive and negative controls are provided with the test. Sensitivity of the test has been reported as 88–100% and specificity as 96–100%.⁴

TUBERCULOUS MENINGITIS VIRAL MENINGITIS, FUNGAL MENINGITIS

In district laboratories the following tests are used to differentiate acute bacterial meningitis from tuberculous, viral and fungal meningitis:

- Counting and differentiating WBCs in CSF.
- Measuring CSF total protein.
- Measuring CSF glucose.
- Tests to investigate tuberculous meningitis: ZN, molecular testing.
- Tests to investigate cryptococcal meningitis: Antigen test and India ink preparation.

Note: A summary of the results of laboratory tests in the different forms of meningitis and other central nervous system disorders can be found at the end of this Section.

COUNTING & DIFFERENTIATING WBCS IN CSF

A CSF WBC count with an indication whether the cells are pus cells (polymorphonuclear neutrophils) or lymphocytes, is required when the CSF appears slightly cloudy or clear or when the Gram smear does not indicate acute bacterial meningitis.

Note: Samples that are heavily blood stained or contain dots are unsuitable for cell counting. Make a Gram smear and report as previously described.

To identify whether WBCs in the CSF are neutrophils or lymphocytes, dilute the CSF in a fluid which stains the cells. Isotonic 0.1% toluidine blue is recommended because it stains lymphocytes and the nuclei of neutrophils blue. *C. neoformans* yeast cells stain pink. Red cells remain unstained. When toluidine blue is unavailable, isotonic methylene blue can be used. If preferred, WBCs can be differentiated by examining a Leishman, Giemsa or rapid Field stained smear (sediment from centrifuged CSF).

Procedure

- 1 Mix the uncentrifuged CSF. Dilute the fluid 1 in 2, i.e. 1 drop of CSF with 1 drop of toluidine blue diluting fluid (Reagent No. 43)*

* The drops must be of equal volume, therefore use Pasteur pipettes of the same bore size, and hold the pipettes vertically when dispensing the drops.

- 2 Assemble a modified Fuchs-Rosenthal ruled counting chamber,* making sure the chamber and cover glass are completely clean.

*When unavailable, an improved Neubauer (preferably *Bright-Line*) chamber can be used. A Fuchs-Rosenthal chamber is recommended because it has twice the depth (0.2mm) which is more suitable for counting WBCs in CSF.

- 3 Using a fine bore Pasteur pipette or capillary tube, carefully fill the counting chamber with the *well-mixed* diluted CSF. The fluid must not overflow into the channels on each side of the chamber.
- 4 Wait about 2 minutes for the cells to settle. Count the cells microscopically.
- 5 Focus the cells and rulings using the 10× objective with the condenser iris *closed sufficiently*

to give good contrast. Before starting the count, use the 40× objective to check that the cells are white cells and not red cells (unstained smaller cells without a nucleus) and note whether the WBCs are mainly polymorphonuclear neutrophils (with lobed nucleus) or lymphocytes. If a mixture of both, estimate approximately the percentage of each type of cell.

When yeast cells are seen, examine an India ink preparation (see later text).

Note: When red cells are seen, mention this in the report. When many red cells are present, the CSF is unsuitable for WBC cell counting.

- 6 Count the cells in 5 of the large squares as shown in Fig. 2.32.

Note: When the cells are too many to count, dilute the CSF 1 in 10 (1 drop CSF mixed with 9 drops of diluting fluid), refill the chamber and count the cells. See later text for calculation factor to use.

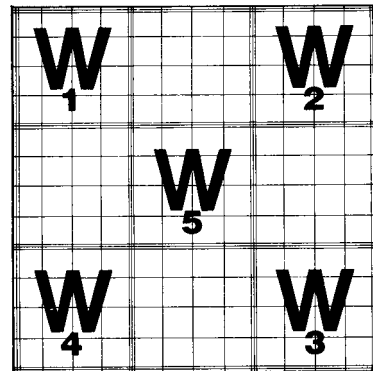


Fig 2.32 Modified Fuchs-Rosenthal ruled chamber. Cells are counted in the squares marked W1, W2, W3, W4, W5.

Multiply the cells counted by 2. Report the number of cells per litre (L) of CSF.

Example (Using 1 in 2 CSF dilution and Fuchs-Rosenthal chamber)

If 240 cells are counted in 5 squares:

$$240 \times 2 = 480$$

Report as 480×10^6 cells/L

When using an Improved Neubauer chamber: Count the cells in 4 of the large squares as shown in Fig. 2.33. Multiply the cells counted by 5.

Example

If 64 cells are counted in 4 squares:

$$64 \times 5 = 320$$

Report as 320×10^6 cells/L.

Calculation factors when using 1 in 10 CSF dilution

- **Fuchs-Rosenthal chamber:** Multiply cells counted in 5 squares by 10. Report number of cells per litre of CSF (see above text).

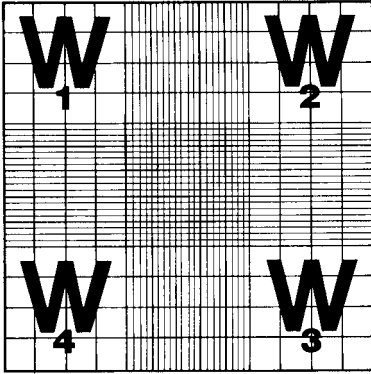


Fig 2.33 Improved Neubauer ruled chamber (can be used when a Fuchs-Rosenthal chamber is not available). Cells are counted in the squares marked W1, W2, W3, W4.

- **Improved Neubauer chamber:** Multiply cells counted in 4 squares by 25. Report number of cells per litre of CSF (see previous text).

Normal CSF: Contains up to 5×10^6 cells/litre (up to 15 in neonates).

When no WBCs are seen, report the count as: Below $5 \text{ cells} \times 10^6/\text{L}$.

MEASURING CSF TOTAL PROTEIN

CSF total protein is measured to assist in the diagnosis of non-acute bacterial meningitis and other disorders of the central nervous system. If the CSF contains blood it is unsuitable for measuring protein.

In district hospital laboratories, CSF total protein is usually measured using an analyzer and a CSF protein reagent test kit. In peripheral laboratories a visual comparative method can be used to measure total protein semi-quantitatively. When this is not possible, Pandy's test can be helpful in screening for globulin in CSF.

Measuring CSF total protein using a reagent test kit

Most CSF protein test kits measure total protein

using the pyrogallol red method or a turbidometric technique.

Pyrogallol red method

In an acidic medium, CSF protein combines with pyrogallol red and sodium molybdate to form a blue-purple coloured complex, the absorbance of which is read at 600 nm. The colour is stable for at least 30 minutes.

The reagent and protein standard are supplied ready for use and are stable when stored in tightly closed containers at 2–8 °C, protected from light and contamination. Linearity of the test depends on the test kit used. Sample dilution and retesting are required when the test result is beyond the linearity of the test kit.

Turbidometric technique

At 37 °C in the presence of sulphosalicylic acid and sodium sulphate, CSF protein produces a turbidity, the absorbance of which is read at 530 nm and is directly proportional to the concentration of protein in the CSF. The test requires reading within 30 minutes.

The reagent and standard are supplied ready for use and are stable when stored tightly stoppered at 2–8 °C. Sample dilution and retesting are required when the CSF concentration is beyond the linearity of the test kit.

Visual comparative CSF protein method

Trichloroacetic acid is added to the CSF. The resulting turbidity is compared visually with a set of protein standard solutions, providing a semi-quantitative measurement of CSF protein.

Required

- Trichloroacetic acid, 50 g/L Reagent No. 44 (5% w/v)
- Protein standards containing 0.44 g/L (44 mg/dL), 0.88 g/L (88 mg/dL), and 1.10 g/L (110 mg/dL) protein

Preparation of protein standard solutions

The standards may be easily prepared from a 22% albumin solution, as follows:

- 1 Take three screw-cap bottles of 50 mL capacity and label them 0.44 g/L, 0.88 g/L, and 1.10 g/L.

Pipette into each bottle as follows:

Bottle	22% albumin solution*
0.44 g/L	0.1 mL
0.88 g/L	0.2 mL
1.10 g/L	0.25 mL

* If only a 30% albumin solution is available, obtain a 22% solution by mixing 1.6 mL of distilled water with 4.4 mL of the 30% solution.

- Add 50 mL of distilled water (sterile if possible) to each bottle, stopper, and mix well.
Store the solutions at 2–8 °C. Renew every month or before they become contaminated.

Procedure

The test requires 0.5 mL of CSF.

- Take four tubes and label them 1, 2, 3, and P (Patient).
- Pipette into each tube as follows:

Tube:	1	2	3	P
mL of trichloroacetic acid, 50 g/L	1.5	1.5	1.5	1.5
mL of 0.44 g/L standard:	0.5	–	–	–
mL of 0.88 g/L standard:	–	0.5	–	–
mL of 1.10 g/L standard:	–	–	0.5	–
mL of CSF:	–	–	–	0.5

- Mix the contents of each tube, and leave for 5 minutes.
- Remix the solutions in each tube, and make an estimate of the approximate amount of protein in the CSF by comparing the cloudiness in the patient’s tube (P) with that in each of the three standard tubes.

Report the approximate protein concentration in grams per litre (g/L).

Note: It is easier to match the turbidity in the patient’s tube with that in the standard tubes if a printed card is held behind the tubes.

Values greater than 1.10 g/L

If the turbidity in the patient’s tube is greater than 1.10 g/L, repeat the test after diluting the CSF 1 in 5 in physiological saline (0.1 mL of CSF added to 0.4 mL of saline will give a 1 in 5 dilution). Multiply the result by 5.

Pandy’s globulin test

This test is only of value when it is not possible to measure CSF total protein. It detects globulin.

Procedure

- Pipette about 1 mL of saturated phenol solution (Reagent No. 33) into a small tube.
Caution: Phenol is a highly corrosive and harmful chemical, therefore handle it with care.

- Using a glass dropper or plastic bulb pipette and holding the tube at eye level against a dark background, add 1 large drop of CSF. *Do not mix.*
- Look for an immediate cloudiness around the drop of CSF, indicating the presence of excess globulin.

Test result

Immediate cloudiness Pandy’s test positive
No cloudiness Pandy’s test negative

The cloudiness may disappear after a few minutes..

Test control

Add 1 drop of water to 1 mL of phenol reagent. No cloudiness should form.

Interpreting CSF protein results

The reference interval for CSF protein in an adult is 0.15–0.40 g/L (15–40 mg/dL). The range for ventricular fluid is slightly lower. Values up to 1.0 g/L are normal for newborn infants. Only traces of globulin are found in CSF, insufficient to give a positive Pandy’s test.

Increase in total protein and positive Pandy’s test

- All forms of meningitis
- Trypanosomiasis and amoebic meningoencephalitis
- Cerebral malaria
- Brain tumours
- Cerebral injury
- Spinal cord compression
- Poliomyelitis
- Guillain-Barré syndrome
- Polyneuritis

Increases in CSF total protein also occur in diseases which cause changes in plasma proteins such as myeloma.

When the total protein exceeds 2.0 g/L, the fibrinogen level is usually increased sufficiently to cause the CSF to clot. This may occur in acute bacterial meningitis, spinal block or following haemorrhage.

MEASURING CSF GLUCOSE

CSF glucose with plasma glucose are measured when the results of CSF microscopy, cell count and protein concentration are inconclusive. It may also help to exclude a diagnosis of bacterial meningitis when it is not possible to culture CSF.

Note: If the CSF contains blood it is unsuitable for measuring glucose.

Blood sample

When testing for CSF glucose, collect a blood sample at the same time to calculate the CSF/plasma glucose ratio. CSF glucose is about 70% of plasma glucose or a ratio of CSF to plasma glucose of 0.6.*

* Calculate as follows:

$$\text{Ratio of plasma glucose} = \frac{\text{CSF glucose}}{\text{plasma glucose}}$$

In acute bacterial meningitis, tuberculous and fungal meningitis the CSF/plasma glucose ratio is reduced to 0.4–0.5. In viral meningitis, the CSF/plasma glucose ratio is frequently normal or > 0.6.

Glucose oxidase method for measuring CSF glucose

This is the same method as described for measuring plasma glucose in Section 3.2 except four times the amount of CSF is used (test result is divided by four). A greater quantity of CSF is used because less glucose is found in CSF and markedly reduced glucose levels may occur in meningitis.

Use of blood glucose meters for measuring CSF glucose

Several studies have shown that a POCT glucose meter can detect abnormal CSF/plasma glucose ratios with sufficient accuracy (when testing CSF immediately after lumbar puncture), enabling bacterial meningitis to be diagnosed and managed rapidly.⁶

Note: Urine reagent test strips for measuring CSF glucose should not be used because although specific, sensitivity is low. Likewise the use of urine test strips for measuring CSF protein is not recommended because they have poor specificity for CSF protein.

Interpreting CSF glucose results

The reference range for adult CSF glucose is 2.5–4.4 mmol/L (45–80 mg/dL) with a CSF/plasma glucose ratio of 0.6.

Low CSF glucose

Low CSF glucose levels and low CSF/plasma glucose ratio are found in most forms of meningitis, particularly acute bacterial meningitis when glucose may even be absent.

In viral meningitis, the CSF glucose concentration is usually normal. Occasionally the CSF/plasma glucose ratio may be > 6.0. In fungal meningitis the CSF/plasma glucose ratio may be normal or low.

Increased CSF glucose

A raised CSF glucose is found when the plasma glucose is raised and sometimes with encephalitis or following damage to cerebral capillaries.

An increased or normal CSF glucose may be found in diabetic patients with meningitis.

TUBERCULOUS MENINGITIS

In tuberculous meningitis the CSF opening pressure is increased, the CSF contains lymphocytes, the glucose concentration is low and total protein is raised. A diagnosis of tuberculous meningitis can be made using a molecular technique to test the CSF for *M. tuberculosis* DNA.

When molecular testing is not possible, examine a stained smear microscopically for AFB. The sensitivity of finding AFB in CSF is low. The following technique increases the chance of finding the bacilli:

- 1 Centrifuge the CSF at RCF 3 000 × g for 20 minutes. Remove the supernatant fluid and mix the sediment. Transfer several drops of the sediment to a slide, allowing each drop to dry before adding the next. When completely dry, fix the smear with absolute methanol. Allow the alcohol to evaporate.
- 2 Stain the smear using the auramine O staining technique and examine for AFB using fluorescence microscopy as described in Section 2.11.

When fluorescence microscopy is not available, stain the smear using the ZN technique and examine for AFB as described in Section 2.11.

CRYPTOCOCCAL MENINGITIS

Cryptococcal meningitis is a life-threatening condition unless diagnosed and treated early. In cryptococcal meningitis the CSF opening pressure is increased, the fluid contains lymphocytes, CSF protein is raised and glucose concentration is reduced.

WHO recommendations for diagnosing and screening for cryptococcal meningitis⁷

In diagnosing cryptococcal meningitis WHO recommends testing CSF for cryptococcal antigen. When no rapid antigen test is available, examination of a CSF India ink preparation is recommended (see later text). When no lumbar puncture is possible or is contraindicated, testing serum, plasma, or whole

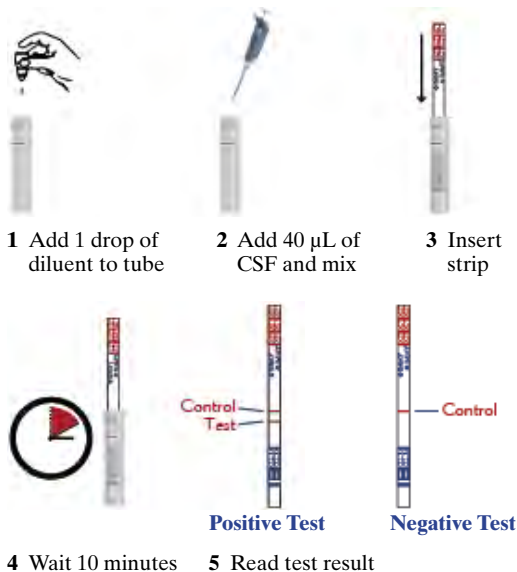
blood for cryptococcal antigen is the preferred diagnostic approach.

In screening HIV-positive persons for cryptococcal infection (to identify those at risk of developing cryptococcal meningitis), WHO recommends testing those with a CD4 cell count below 100 cells/mm³. Testing may also be considered in those with a count below 200 cells/mm³.

Cryptococcal antigen test

An example of a rapid cryptococcal antigen test is *CrAg LFA*, manufactured by IMMY Corporation. *CrAg LFA* is a rapid IC dipstick detecting antigen to all four *Cryptococcus* serotypes including *C. gatti*. The test has a shelf-life of 2 years from manufacture and can be stored at room temperature. It is available as a 50 test kit. No pre-treatment of the sample is required. The test takes 10 minutes to perform. The procedure is shown in Fig. 2.34. The reagent and all the items needed to perform the test are included in the test kit.

In diagnosing cryptococcal meningitis, *CrAg LFA* has been shown to perform equally well when testing CSF, serum, plasma or whole blood.⁸ When used to screen HIV-infected persons for cryptococcal infection, the accuracy of the test was shown to be highest when testing serum.⁹



Note: Extremely high antigen levels can result in weak or negative reactions (prozone effect). If suspected, titrate the CSF as detailed in manufacturer's test insert sheet.

Fig. 2.34 Procedure for performing *CrAg LFA* rapid cryptococcal antigen test. *Courtesy of IMMY Corporation.* www.immy.com/products/lateral-flow-assays/crag-ifa/

India ink preparation

India ink is a negative staining technique. Unstained encapsulated cryptococcal yeasts are seen against a dark background. The test has a low sensitivity.

Procedure

- 1 Centrifuge the CSF for 5–10 minutes at RCF 1 000 × g. Remove the supernatant fluid and mix the sediment.
- 2 Transfer a drop of the sediment to a slide, add a drop of India ink (Pelikan black drawing ink is suitable*) and cover with a cover glass.

*When not available, use nigrosin stain (Reagent No. 32)

Note: Do not make the preparation too thick otherwise the encapsulated yeasts will not be seen.

- 2 Examine the preparation using the 40× objective. Look for oval or round cells, some showing budding, irregular in size, measuring 2–10 µm in diameter and surrounded by a large unstained capsule as shown in Plate 2.47.



Plate 2.47 India ink preparation showing single and budding encapsulated *Cryptococcus neoformans* yeast cells as seen with the 40× objective.

Dark-field microscopy to detect cryptococci in CSF

Using dark-field microscopy is an alternative technique to the India ink preparation to demonstrate encapsulated cryptococci in CSF. When using 10× and 40× objectives, adequate dark-field microscopy can be obtained by using a black patch stop below the condenser. Further details can be found in subunit 4.3, pages 122–123 in *Part 1 District Laboratory Practice in Tropical Countries*.*

* Can be accessed online by entering the book title in Google and viewing: <https://medicallabtechno.weebly.com>

CSF Test Results in meningitis and other diseases of the central nervous system

	Appearance	WBCs	Protein	Glucose	Microscopy/ Other tests
Normal CSF	Clear Colourless	< 5 × 10 ⁶ /L ^a Lymphs	0.15–0.40 g/L (15–40 mg%) <i>Pandy's</i> : Negative	2.5–4.0 mol/L (45–72 mg%) 0.6 plasma/ glucose ratio	–
Acute bacterial meningitis	Purulent or cloudy May contain clots	> 200 × 10 ⁶ /L Neutrophils ^b	High > 1 g/L <i>Pandy's</i> : Positive	Very low 0.4–0.5 ratio	Gram: Bacteria, e.g. <i>N. meningitidis</i> <i>S. pneumoniae</i> <i>H. influenzae</i> type b <i>S. agalactiae</i> Group B enterobacteria
Viral meningitis	Clear or slightly turbid	> 10 × 10 ⁶ /L Lymphs ^c	Normal or increased <i>Pandy's</i> : Negative or positive	Normal or > 0.6 ratio	–
Tuberculous meningitis	Clear or slightly turbid	25–500 × 10 ⁶ /L Lymphs ^c	High > 1.5 g/L <i>Pandy's</i> : Positive	Low 0.4–0.5 ratio	Molecular testing AO or ZN : To detect AFB
Cryptococcal meningitis	Clear or slightly turbid	> 10 × 10 ⁶ /L Lymphs	Usually increased <i>Pandy's</i> : Positive	Normal or low 0.4–0.5 ratio	Antigen test: Positive India ink: Encapsulated yeast cells
Primary amoebic meningoencephalitis	Cloudy	> 200 × 10 ⁶ /L Neutrophils	Increased > 1 g/L <i>Pandy's</i> : Positive	Low	Wet preparation: Motile amoebae
Trypanosomiasis meningoencephalitis	Clear or slightly turbid	> 10 × 10 ⁶ /L Lymphs	High > 1 g/L <i>Pandy's</i> : Positive	Normal or low	Wet preparation: Motile trypanosomes Giemsa: Morula cells
Neurosyphilis	Usually clear	> 20 × 10 ⁶ /L Lymphs	Normal or increased	Usually normal	CSF VDRL: Reactive in about 50% of patients. A non-reactive TPHA excludes neurosyphilis.

^a *Values in infants 1–12 months*: Cell count 0–15 cells × 10⁶/L, Glucose: 1.94 – 5.0 mmol/L, Protein: (4–8 weeks) 0.5–0.9 g/L, (2m – 18y) 0.05–0.35 g/L.

^b *In partially treated acute bacterial infection*: Lymphocytes may be more common than neutrophils.

^c *In viral and tuberculous meningitis*: Neutrophils are often found early in infection.

NOTES

Opening CSF pressure: This is increased in most forms of meningitis except viral meningitis when the pressure is usually normal or slightly raised. Normal CSF opening pressure is < 20 cm H₂O for adults and < 10 cm H₂O for children below age 8 years.

Guillain-Barré syndrome: CSF total protein is raised in the absence of WBCs.

C-reactive protein (CRP): In acute bacterial meningitis, CSF and serum CRP are significantly raised whereas in viral meningitis CRP is usually absent or low. Measurement of serum CRP is described in Section 2.19.

C. neoformans in Giemsa stained CSF

As shown in Plate 2.48, cryptococci in CSF can also be detected in a Giemsa stained preparation. Make a smear using sediment from centrifuged CSF, fix and stain the smear using the Giemsa technique described in Section 2.2. Single and budding cryptococci stain mauve surrounded by an unstained capsule.

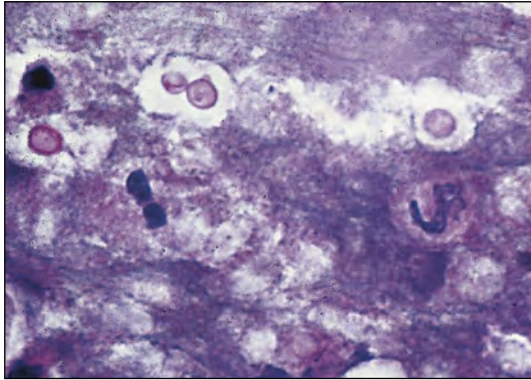


Plate 2.48 Single and budding encapsulated *C. neoformans* in Giemsa stained CSF sediment as seen with the 100× objective.

PRIMARY AMOEBIC MENINGOENCEPHALITIS

Primary amoebic meningoencephalitis caused by *Naegleria fowleri* is a rare life-threatening condition which unless diagnosed and treated at an early stage, is fatal within a few days of the onset of symptoms. The CSF is purulent with a neutrophil count similar to that found in acute bacterial infection. Total protein is increased and glucose concentration is low. The CSF may also contain red cells and eosinophils.

Examine the CSF microscopically for motile *Naegleria* trophozoites (amoebae) as follows:

- 1 Transfer a drop of uncentrifuged purulent CSF, or a drop of centrifuged cloudy CSF, to a slide and cover with a cover glass.
- 2 Examine the preparation using the 10× objective with the condenser iris closed sufficiently to give good contrast.
Look for small, clear motile elongated amoebae among the pus cells.
- 3 Use the 40× objective to identify the rapidly moving amoebae as described in the following text (even if not detected first with the 10× objective, always examine the preparation with the 40× objective).

Important: When amoebae are found in CSF, notify immediately the medical officer attending the patient.

Naegleria amoeba

- Elongate in form, measuring 10–22 × 7 μm.
- Rapidly motile (more than 2 body lengths per minute). Pseudopodia are lobulate and show characteristic explosive protrusion.
- When viewed with the 40× objective, vacuoles can often be seen in the cytoplasm (the small nucleus with its nucleolus is not usually visible in an unstained preparation).
- Does not contain ingested red cells.

Note: *Naegleria* amoebae will remain motile for several hours at room temperature and up to 24 hours at 35–37 °C.

The amoebae can be stained with Giemsa stain, but they do not stain well by the Gram technique. They can also be seen in acridine orange stained preparations examined by fluorescence microscopy (cytoplasm appears red and foamy).

In distilled water, the amoebae develop flagella after 2–4 hours.

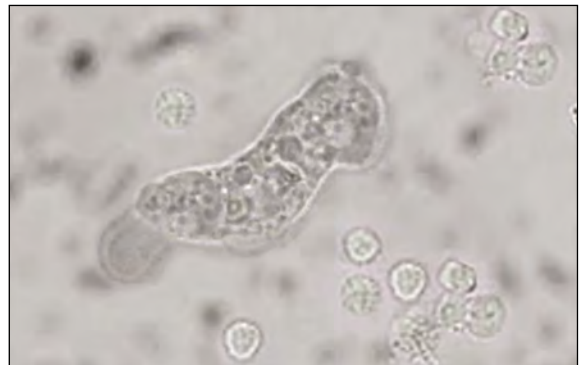


Plate 2.49 Trophozoite of *Naegleria* in CSF with pus cells. Unstained preparation as seen with the 40× objective.

Note: The cysts of *Naegleria* are free-living and not found in humans.

Acanthamoeba species

Other free-living amoebae belonging to the genus *Acanthamoeba* have been reported as causing chronic granulomatous amoebic encephalitis and skin abscesses in

immunocompromised persons. The brain is probably infected via the blood-stream, possibly from infected skin, eye, or lung. *Acanthamoeba* species can also cause chronic amoebic keratitis in healthy people.

Acanthamoeba cysts as well as the amoebae are thought to be infective. The cysts can survive for several years in dust. They are angular in shape with a double wall. The amoebae are slow moving with spiky projections.

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FURTHER READING

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Mazzola L, Kelly-Cirino C. Meningitis Diagnostics Use Cases. World Health Organization, 2018. <https://www.who.int/emergencies/diseases/meningitis/meningitis-diagnostics-use-cases.pdf>

2.13 Sexually transmitted infections

The following sexually transmitted infections (STIs) can be diagnosed in district laboratories using point-of-care tests (POCTs):

- HIV infection (described in Section 2.14)
- Syphilis
- Trichomoniasis
- Gonorrhoea
- Urogenital chlamydia
- Hepatitis B infection (described in Section 2.15)

Bacterial vaginosis, vaginal candidiasis and donovanosis can also be diagnosed in district laboratories.

Note: The increased risk of persons with STIs becoming infected with HIV is described on page 190.

Laboratory diagnosis of STIs^{1,2}

When using POCTs to diagnose STIs:

- Patients can be treated specifically with the minimum of delay, often in a single clinic visit.
- Sexual partners can be treated appropriately to prevent re-infection.
- There is less risk of developing complications from untreated STIs.
- Overuse of antibiotics associated with syndromic treatment can be avoided.
- The risk of acquiring HIV is reduced following a reduction in the incidence of STIs.
- The information provided from diagnostic testing can help in STI surveillance, development of treatment guidelines and the planning of STI screening, prevention and control programmes.

Reliable laboratory diagnosis of STIs requires the use of tests of proven performance in the settings in which they will be used. Laboratory staff are essential in monitoring POCT by health care workers, including providing training in STI testing and quality assurance.

Note: Information on the performance of currently available POCTs for diagnosing STIs and diagnostic technologies under development can be found in the 2018 paper *The Point-of-Care Diagnostic landscape for Sexually Transmitted Infections (STIs)*.³

WHO targets for ending sexually transmitted infection epidemics as major health concerns by 2030⁴

- 90% reduction of *T. pallidum* incidence globally (2018 global baseline).
- 90% reduction of *N. gonorrhoeae* incidence globally (2018 global baseline).
- 50 or fewer cases of congenital syphilis per 100 000 live births in 80% of countries.
- Sustain 90% national coverage and at least 80% in every district (or equivalent administrative unit) in countries with the human papillomavirus vaccine in their national immunization programmes.

SYPHILIS

Syphilis is caused by *Treponema pallidum*, a spirochaete bacterium. It is transmitted through sexual contact with infected skin or mucous membrane lesions, transplacentally from an infected mother to her foetus or through blood transfusion.

Sexually acquired syphilis

Sexually acquired syphilis has an early infectious stage, occurring within the first 2 years of infection and a late non-infectious stage.

Early stage: Includes primary syphilis, secondary syphilis and early latent syphilis.

Late stage: Includes late stage latent syphilis, late stage tertiary syphilis, cardiovascular syphilis and neurosyphilis. Sexual transmission occurs only during primary, secondary and early latent stage infection.

Primary syphilis

Within 3–10 weeks of infection an ulcer, known as a chancre develops at the site of infection (vagina, penis or anus). About 10% of ulcers are extragenital. The ulcer is shallow, well-defined with undurated edges and a smooth red surface that exudes serum. It is usually painless. Treponemes can be found in the chancre fluid. The chancre heals after 4–6 weeks.

In those coinfecting with HIV, healing may be delayed. Plasma

HIV viral load is increased and the CD4 cell count is reduced in untreated HIV coinfection.

Secondary syphilis

If untreated, secondary syphilis develops 4–8 weeks after the primary infection. A widespread non-itchy maculopapular rash appears including on the palms and soles. The secondary skin lesions contain treponemes and are highly infectious. Treponemes are also present in the blood. Mucous membranes are infected and mouth ulcers are common. Lymphadenopathy, fever, malaise and joint pains are common.

In those coinfecting with HIV, the syphilitic rash may be severe. In most patients the secondary lesions heal although relapses can occur.

Latent and late stage syphilis

During the latent stage there are no clinical symptoms of syphilis but infection can be detected serologically. About 25% of patients with untreated latent syphilis progress to late stage tertiary syphilis, a slowly progressive inflammatory stage occurring up to 30 years or more after infection. Gummatous lesions develop in skin, bones, liver and other organs. Cardiovascular disease develops and degenerative changes occur in the central nervous system causing neurosyphilis and general paralysis, stroke, cranial nerve dysfunction and dementia.

Treponemes are not present in late stage syphilitic lesions. In those coinfecting with HIV, late stage syphilis may progress more rapidly.

Congenital syphilis

In congenital syphilis, a mother with infectious syphilis infects her newborn infant. Treponemes in the blood pass through the placenta. Syphilis in pregnancy can lead to abortion, premature delivery, stillbirth, perinatal death and the birth of infants with congenital syphilis.

Infants born with congenital syphilis, depending on the severity of infection, have a rash, skin and mucous membrane lesions, fail to gain weight, often have bone defects, joint swellings and hepatosplenomegaly. Difficulties in hearing and sight problems may develop at a later stage. Treponemes may be found in samples from the umbilical cord, the placenta, nasal discharge or skin lesions.

Transmission of syphilis by blood transfusion

The risk of transmitting *T. pallidum* in donated blood or blood products from donors with active syphilis is low. When donated blood is stored at 2–8 °C for 72–120 hours, *T. pallidum* is inactivated. In most blood transfusion centres, donated blood is screened serologically for *T. pallidum* and blood is usually collected from low risk donors (based on a questionnaire). In emergency situations where blood is collected from a family member for immediate transfusion, there is usually sufficient time to screen the blood for *T. pallidum* antibody using a rapid IC test (see later text).

LABORATORY DIAGNOSIS

Syphilis is diagnosed by:

- Detecting motile treponemes in serous fluid from chancres and moist secondary skin lesions in early syphilis or from mucocutaneous lesions in newborns with congenital syphilis.
- Testing serum serologically using non-specific cardiolipin antibody tests. They are 70–80% sensitive in primary syphilis and 70–100% in latent syphilis.
- Testing serum serologically using specific treponemal antibody tests. They are 70–90% sensitive in primary syphilis and 90–100% in latent syphilis.

DETECTING MOTILE TREPONEMES

A diagnosis of active syphilis can be made in about 50% of patients with primary or secondary syphilis by detecting motile treponemes in serous fluid using dark-field microscopy. Treponemes may be found providing the preparation is examined within 15 minutes of collecting the sample and the patient has not been treated with antibiotics.

Collecting a serous sample

- 1 Wearing protective gloves, cleanse the area around the ulcer using a swab moistened with physiological saline. Remove any scab which may be present.

Caution: *T. pallidum* treponemes are highly infectious.

- 2 Gently squeeze the lesion to obtain serous fluid. Collect a drop on a cover glass and invert it on a microscope slide.

Note: The cover glass and slide *must be completely clean*.

- 3 Immediately deliver the preparation to the laboratory for examination by dark-field microscopy.

Detecting treponemes by dark-field microscopy

A good light is essential and a thin preparation is required. Examine the preparation using the 10× and 40× objectives.

Dark-field microscopy

Adequate dark-field microscopy can be obtained when using the 10× and 40× objectives by inserting a dark-field stop in the filter holder below the condenser. A special dark-field condenser is not required.

T. pallidum treponemes : Look for brightly illuminated, thin, delicate, tightly wound spirochaetes, measuring 6–10 μm long with 8–14 evenly sized coils as shown in Plate 2.50. They have a bending and slowly rotating motility and may be seen lengthening and shortening.

The spirochaetes of *T. pallidum* require differentiating from saprophytic genital spirochaetes. These are of variable size, thicker than *T. pallidum*, have fewer coils and a different motility.

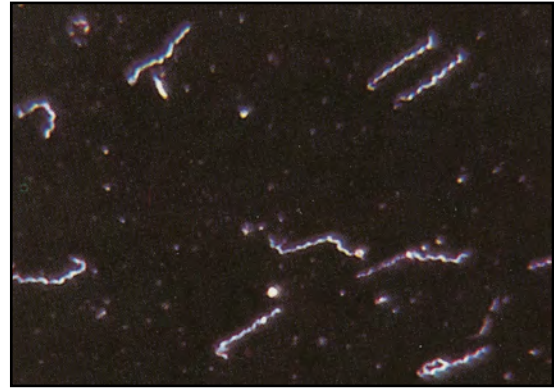


Plate 2.50 *Treponema pallidum* in chancre fluid as seen by dark-field microscopy using the 40× objective.

SEROLOGICAL DIAGNOSIS OF SYPHILIS

A person infected with *T. pallidum* produces two types of antibody:

- Non-specific antibody that reacts with cardiolipin antigen.

Cardiolipin

This is a phospholipid substance extracted from beef heart tissue, it is complexed with cholesterol and lecithin to make cardiolipin antigen. A similar substance is released from treponemes and damaged cells during active syphilis infection. This substance (often referred to as reagin) stimulates the production of anti-cardiolipin Ig G and Ig M antibodies which can be detected in the serum of patients.

- Specific treponemal antibody that reacts with treponemal antigen.

Antibody reactions in HIV coinfection

Immunosuppression can result in a poor antibody response in syphilis with tests being weakly reactive or non-reactive. Occasionally infection with HIV can produce high antibody titres and prozone reactions (see later text) due to B cell activation. Coinfection with HIV is also associated with the failure of antibody titres to fall following treatment.

Non-specific antibody tests

Non-specific tests include:

- VDRL (Venereal Diseases Research Laboratory) test which is read microscopically.
- RPR (Rapid plasma reagin) test which is read macroscopically.

VDRL

In the VDRL test, serum is heat-inactivated to destroy complement, reacted with freshly prepared cardiolipin-cholesterol-lecithin antigen and the resulting flocculation (suspended antigen-antibody complex) is read microscopically using a 10× objective and 10× eyepiece. Reactive tests are quantitated to obtain the antibody titre (using a double dilution technique).

RPR

In the RPR test, the cardiolipin-cholesterol-lecithin antigen has choline chloride added to it which removes the need for heat-inactivation of samples and enables plasma as well as serum to be used in the test.

The antigen is supplied in a ready-to-use stabilized form which can be kept for up to 6 months when stored at 4–10 °C. Carbon is also added to the antigen enabling test reactions to be read macroscopically. The patient's serum or plasma is spread within a circular area on a plastic coated card, antigen is added and the mixture rotated at 100 rpm for 8 minutes using a rotator. Reactive tests are quantitated to obtain the antibody titre.

Non-specific anti-cardiolipin antibodies are produced about 3–5 weeks following infection (about 2 weeks after the chancre forms). Large amounts of antibody are produced in secondary syphilis. In latent and late syphilis, anti-cardiolipin antibodies decline (even without treatment) and tests often become non-reactive or weakly reactive. Following successful treatment, anti-cardiolipin antibody titres fall, becoming negative after 6 months in primary syphilis and after 12–18 months in secondary syphilis.

A positive non-specific antibody test indicates possible active syphilis but is not a definitive diagnosis. A positive test can also occur (usually in low titres) in other infections, immune disorders and narcotic drug abuse, resulting in biological false positive (BFP) tests.

Biological false positive tests

Transient (usually less than 6 months) BFPs can occur with infective hepatitis, virus pneumonia, chickenpox, measles, lymphogranuloma venereum, acute malaria, trypanosomiasis, following immunization and during pregnancy. Long term BFPs can occur in systemic lupus erythematosus, rheumatoid arthritis, lepromatous leprosy, tuberculosis, malignancy and narcotic addiction.

When a non-specific test is reactive, a specific treponemal test should be performed. When the specific test is non-reactive and the antibody titre of the non-specific test is 1 in 8 or below, a BFP is indicated. When the titre is greater than 1 in 8, both the non-specific and specific test should be repeated at a later stage to look for a rising titre.

Prozone reaction

A prozone reaction can occur when the anti-cardiolipin antibody titre is very high, e.g. in secondary syphilis. Excess antibody prevents normal antibody-antigen reactivity which may result in a test being falsely reported as "non-reactive". With experience most prozone reactions can be recognised by their "roughness" or abnormal grainy appearance.

Quality assurance

Known positive and negative controls must be included with each batch of tests. Manufacturers' instructions must be followed exactly. Samples and reagents must be at the correct temperature for testing and samples must not be haemolyzed or lipaemic.

Specific treponemal antibody tests

Specific treponemal tests are able to diagnose syphilis presumptively but are unable to differentiate between active infection and past treated infection. *T. pallidum* IgG antibody can persist for a patient's lifetime even following successful treatment. Unlike non-specific antibody tests, specific treponemal tests do not produce BFPs. A specific antibody test is required to confirm a positive non-specific test and in late syphilis when a cardiolipin test may be non-reactive.

Specific treponemal tests do not differentiate between infections caused by *T. pallidum* and the non-venereal *Treponema* species causing endemic treponematoses. These include *T. carateum* causing pinta and the *T. pallidum* subspecies, *T. p.* subsp. *pertenue* (causing yaws) and *T. p.* subsp. *endemicum* (causing bejel).

Differentiation is based on the clinical appearance of lesions and areas of the body infected, differences in transmission and geographical distribution and the age of the patient.

Further information can be found in the 2014 paper: *The Endemic Treponematoses, Clinical Microbiology Reviews*.⁵

Specific treponemal antibody tests include:

- TPHA (*T. pallidum* haemagglutination assay)
- TPPA (*T. pallidum* particle agglutination assay)
- FTA-ABS (Fluorescent treponemal absorption) test
- Rapid IC treponemal antibody tests

TPHA

In the TPHA, patient's serum is mixed with red cells coated (sensitized) with *T. pallidum* antigen. If antibody is present the sensitized cells are agglutinated and they settle in a characteristic mat pattern in the bottom of the well of the microtitration plate. In a negative test, unagglutinated cells form a button or smooth ring at the bottom of the well. Cross-reacting antibodies which may be in the patient's serum due to the presence of commensal treponemes are removed by an extract of a non-pathogenic treponeme contained in the diluent. The TPHA is less sensitive than the FTA-ABS in primary syphilis but gives similar results to the FTA-ABS in secondary and late syphilis. False positive reactions can occur in connective tissue disorders and lepromatous leprosy.

TPPA

The TPPA test is similar to the TPHA test except that gelatin particles instead of red cells are sensitized with *T. pallidum* antigen, making the reagent more stable. A positive test is indicated by clumps of particles.

FTA-ABS

The fluorescent antibody test is the first serological test to become positive following infection. It is an expensive test mainly performed in reference public health laboratories.

Rapid IC treponemal antibody tests

Rapid IC treponemal antibody tests are of particular value in antenatal care. WHO recommends screening all pregnant women for syphilis during their first antenatal care visit.⁶ When laboratory facilities are unavailable, rapid IC tests enable on-site same day testing. When blood samples need to be sent for off-site laboratory testing, mothers may not return for their test results leading to those with syphilis not being treated.

Rapid IC tests can be used with serum, plasma or whole blood, take 5–20 minutes to perform and do not require refrigerated transport or storage. The tests detect IgG, IgM and IgA treponemal antibodies. When compared to high performance laboratory-based treponemal antibody assays with 100% sensitivity, rapid IC tests are less sensitive, particularly when testing whole blood. The positive predictive value of a rapid test will also depend on the prevalence of syphilis in the area.

An example of a rapid IC lateral flow treponemal antibody test that performs well as a syphilis screening test is *SD Bioline Syphilis 3.0*. When testing serum, the test has been shown to have a sensitivity of 87.06% and specificity of 95.85% and when testing whole blood, a sensitivity of 84.50% and specificity of 97.95%.³ The test uses 20 μ L of whole blood or 10 μ L of plasma or serum and has a shelf-life of 24 months from date of manufacture when stored at 2–30 °C. The test procedure is shown in Plate 2.51.

It is important that those performing IC rapid treponemal tests are well trained, follow standardized

procedures for testing samples, reporting and interpreting test results and participate in external quality assessment. Other considerations include the safe handling and disposal of samples, management of a reliable supply chain and stock control and ensuring test kits are stored as instructed by the manufacturer.



Add 20 μ L whole blood or 10 μ L of serum or plasma to sample (S) well.

Add 4 drops of assay diluent. Read test result after 5–20 minutes.



Positive test
Pink Test (T) line
Pink Control (C) line



Negative test
Pink Control (C) line

Plate 2.51 *SD Bioline Syphilis 3.0* test procedure and test results.

Courtesy of Standard Diagnostics Inc. www.standardia.com

Combined non-specific and specific rapid test

A rapid IC test that combines both non-specific and specific antibody testing has been developed recently by Chembio Diagnostic Systems. Called *DPP Syphilis Screen and Confirm Assay*, the test requires 10 μ L of whole blood or 5 μ L of serum or plasma. Test time is 15–20 minutes. When performed in laboratories using serum, it was concluded that the DPP test would identify more than 93% of active syphilis infections.³ It was noted however that the sensitivity of the treponemal antibody line was lower than when using uncombined single treponemal antibody tests. The results of field evaluations are awaited.

Combined HIV/Syphilis rapid tests

Several combined rapid anti-HIV/treponemal antibody tests have been developed for use in point-of-care, particularly for HIV and syphilis screening during pregnancy. WHO recommends testing pregnant women for HIV and syphilis at least once, preferably in the first trimester of pregnancy.⁷

An example of a rapid HIV/syphilis combined test prequalified by WHO in 2015 is the IC lateral flow *SD Bioline HIV/Syphilis Duo* cassette test. It detects

Main differences between non-specific and specific syphilis antibody tests

Non-specific cardiolipin tests

Examples: VDRL, RPR

- Screening tests. Cannot confirm or exclude syphilis.
- Tests detect IgM and IgG antibodies against cardiolipin.
- Indicate possible active disease.
- Antibody titre falls with effective treatment.
- Tests may become non-reactive in late syphilis.
- Biological false positives occur (titre usually 1 in 8 or below).
- Prozone reactions can cause reactive sera to be missed.
- Rotator required for RPR test (100 rpm for 8 minutes).

Specific treponemal tests

Examples: TPHA, TPPA, FTA-ABS, rapid immunochromatographic tests

- Positive test indicates present or past infection.
- Higher specificity than cardiolipin tests.
- Used to check positive cardiolipin test reaction.
- Not suitable for assessing response to treatment.
- Tests often remain positive for many years due to persistence of IgG.
- More complex tests can be used to differentiate IgM and IgG.
- TPPA test can be modified to use whole blood collected on card.

Syphilis serology diagnosis table

Test	STAGE OF SYPHILIS					
	Primary	Secondary	Latent	Late (Tertiary)	Congenital	On Treatment
Dark-field microscopy	+	+	n/a	n/a	+	n/a
Non-specific cardiolipin antibody tests: VDRL, RPR	+	+	+/-	+/-	+	-ve
Treponemal antibody tests: TPHA, TPPA, FTA-ABS, Rapid IC	+	+	+	+	n/a	+

Neonate level should be >4× maternal level

n/a: Test not appropriate

Useful reference: Ratnam S. The laboratory diagnosis of syphilis. *Can J Infect Dis Med Microbiol* 2005;16(1):45-51.

Table: Courtesy of Dr Paul Shears.

Pregnancy: Whenever possible, a rapid immunochromatographic syphilis test should be used.

To evaluate treatment: Use quantitative cardiolipin tests (3 monthly intervals). When there is relapse (or re-infection) there is usually a rise in titre (also seen in TPHA test).

Note: In HIV coinfection, serological responses may be altered (see text).

antibody to HIV-1 including subtype 0, HIV-2 and *T. pallidum* antibody from whole blood (venous or finger stick), serum or plasma. It is available as a 25 test kit and has a shelf-life of 2 years from date of manufacture when stored at 1–30 °C. The test is performed by dispensing 20 µL of whole blood or 10 µL of serum or plasma into the sample well of the cassette, adding 4 drops of assay diluent and reading the results after 15–20 minutes. Test results are shown in Plate 2.52.

The WHO prequalification evaluation of *SD Bioline HIV/Syphilis Duo* gave a final sensitivity for HIV antibodies of 100% and specificity of 99.5% and for *T. pallidum* antibodies a final sensitivity of 87% and specificity of 99.5% when compared with reference assays.⁷

Interpretation of test results

- **Positive treponemal antibody:** Indicates an active or past treated syphilis infection. A non-specific antibody test e.g. RPR is required to differentiate between active and treated syphilis in high syphilis prevalence areas (> 5%).
- **Positive HIV antibody:** Indicates possible HIV infection but a reactive test result *must be confirmed* as described in Section 2.14.

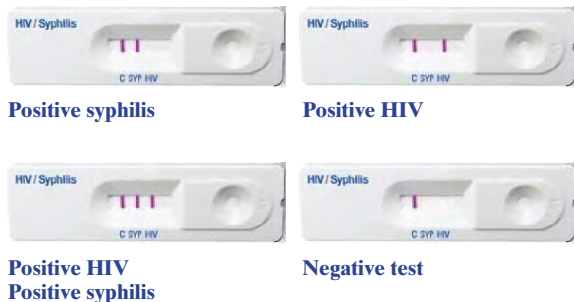


Plate 2.52 *SD Bioline HIV/Syphilis Duo* test results showing positive HIV, positive syphilis, positive HIV and syphilis and negative test result.

Courtesy of Standard Diagnostics. www.standardia.com

Laboratory diagnosis of congenital syphilis

In infants born with congenital syphilis it may be possible to detect motile treponemes (by dark-field microscopy) in fluid from skin lesions or nasal discharge. A presumptive diagnosis of congenital syphilis can be made if an infant is born with symptoms of infection (see previous text), the mother has untreated syphilis at the time of delivery and the infant's serum gives a reactive specific treponemal antibody test at 3 months.

Most of the antibody found in an infant's serum at birth will be maternal IgG. In an uninfected infant this will disappear in 2–3 months. Testing both infant and maternal sera can be helpful. Infection in the infant is indicated when the infant's RPR antibody titre is higher (\geq 4-fold) than that of the mother and the titre is shown to rise. Testing for IgM treponemal antibody is not sufficiently reliable to diagnose congenital syphilis.

Laboratory investigation of neurosyphilis

Although syphilitic meningitis may develop during secondary syphilis, neurosyphilis usually occurs as a complication of late syphilis. A person with neurosyphilis will have a reactive specific treponemal serological test and therefore serum should always be tested before collecting cerebrospinal fluid. Serum cardiolipin tests may be reactive or non-reactive.

Note: Contamination of CSF with even a small amount of blood can give misleading test results.

The following CSF tests are helpful in diagnosing neurosyphilis:

- VDRL test using unheated CSF: The CSF VDRL test is reactive in about 50% of patients with neurosyphilis. When the CSF VDRL test is non-reactive, perform a CSF TPHA. If this test is non-reactive, neurosyphilis can be excluded. A reactive TPHA, in the absence of any red cells in the CSF along with a raised cell count and raised protein, is strongly suggestive of neurosyphilis.
- Cell count (see Section 2.12): More than 10 cells \times 10⁶/L (lymphocytes) will be found.
- Total protein: The total CSF protein may be normal or increased.

Note: Following successful treatment the CSF cell count should return to normal within 6 months. The CSF VDRL will usually show a gradual decrease in reactivity.

TRICHOMONIASIS

Trichomoniasis is caused by *Trichomonas vaginalis*, a flagellated protozoan parasite. Globally it is the commonest cause of non-viral STI.

Symptomatic infections in women cause vaginitis with a purulent vaginal discharge of pH > 5 and vulvo-vaginal irritation. Symptoms are often worse during menstruation. In pregnancy, *T. vaginalis* infection is associated with preterm delivery and

low birth weight. Trichomoniasis is less frequent in men than women and often transient. Infection can cause a non-purulent urethral discharge. Most *T. vaginalis* infections in men are asymptomatic and also asymptomatic in a high percentage of women. Inflammation caused by trichomoniasis increases significantly the risk of acquiring and transmitting other STIs particularly HIV.

LABORATORY DIAGNOSIS

T. vaginalis infection can be diagnosed by:

- Detecting *T. vaginalis* microscopically in wet preparations and stained smears.
- Detecting *T. vaginalis* from a vaginal swab using a rapid point-of-care IC antigen test.
- Culturing *T. vaginalis* from a vaginal swab, male urethral swab or urine samples (usually in a referral laboratory) to assist in the diagnosis of trichomoniasis, genotyping, or antimicrobial susceptibility testing.
- Molecular testing using nucleic acid amplification tests (NAATs) to diagnose trichomoniasis from genital and urine samples. The tests have high sensitivity and specificity. They are expensive.

Note: Vaginitis can also be caused by vaginal candidiasis and bacterial vaginosis (see later text).

DETECTING *T. VAGINALIS* MICROSCOPICALLY

Motile *T. vaginalis* can be detected occasionally in vaginal discharge (rarely in urethral exudate) and when examining urine samples routinely. Examining samples microscopically is the least sensitive technique for diagnosing trichomoniasis (36–75%).⁸

Vaginal discharge and urethral exudate must be examined *as soon as possible* after collection. The preparation must not be too thick (add a drop of physiological saline if indicated). Examine the sample using the 10× and 40× objectives with the condenser iris diaphragm closed sufficiently to give good contrast.

T. vaginalis in wet preparations

T. vaginalis trichomonads are a little larger than pus cells measuring 10–20 μm. They are round or oval in shape and move by means of an undulating membrane and 4 anterior flagella. An axostyle protrudes from the end of the organism. A careful search is required to detect the flagellates among

pus cells. Movement is often slight (on the spot) and not progression. *T. vaginalis* trichomonads are shown in Plate 2.53.

Note: When more than 10 minutes have passed since collection of the sample, motility can often be increased by incubating the preparation at 35–37 °C for a few minutes (in a petri dish containing a damp piece of cotton wool).

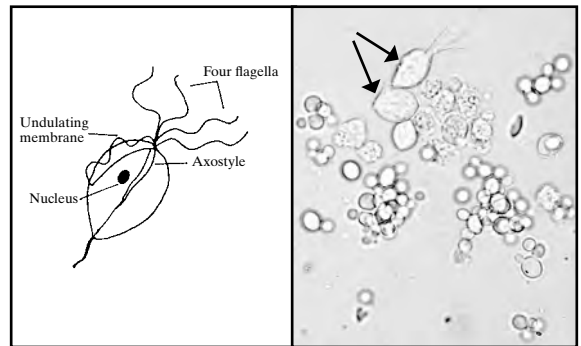


Plate 2.53 *Left:* Morphology of *T. vaginalis*. *Right:* Wet preparation showing *T. vaginalis* trichomonads, pus cells and yeast cells in a vaginal sample as seen with the 40× objective.

Staining *T. vaginalis*

When *T. vaginalis* is not detected in a wet preparation remove the cover glass, allow the sample to air-dry, fix with methanol and stain using Giemsa or Field technique described in Section 2.2. When facilities for fluorescence microscopy are available, examination of an acridine orange stained smear is recommended. *T. vaginalis* trichomonads are shown in Plate 2.54.

Note: For the microscopical diagnosis of vaginal candidiasis and bacterial vaginosis, see later text.

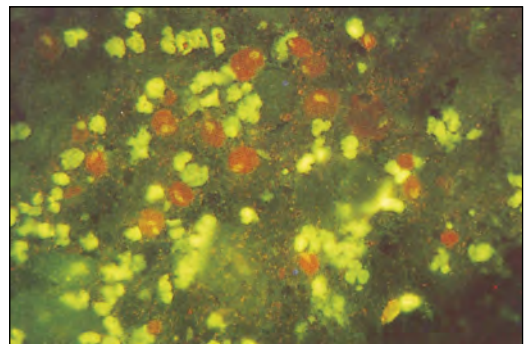
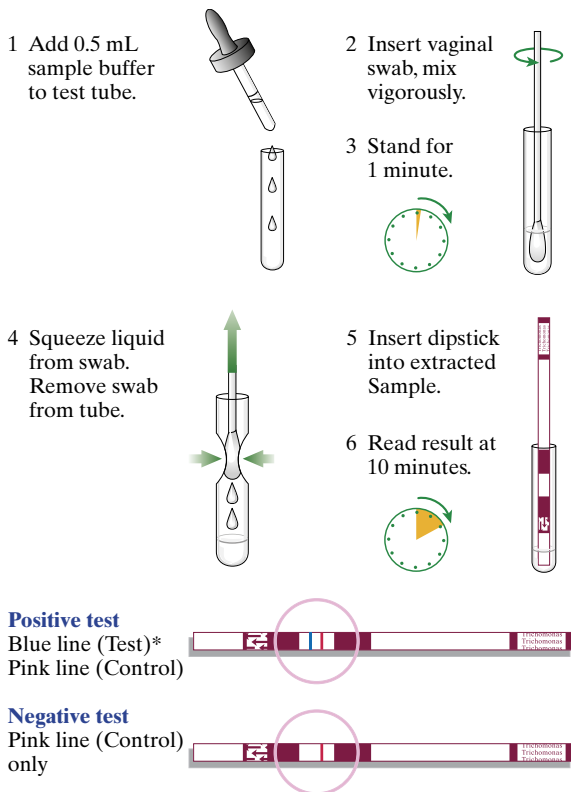


Plate 2.54 *T. vaginalis* trichomonads (red-brown) in an acridine orange preparation as seen by fluorescence microscopy using the 40× objective.

RAPID *T. VAGINALIS* ANTIGEN TEST

A rapid *T. vaginalis* antigen test is recommended in preference to microscopy for the diagnosis of vaginal trichomoniasis. An example is the *OSOM Trichomonas Rapid Test* manufactured by Sekisui Diagnostics. It is an enzyme IC immunoassay dipstick which detects qualitatively solubilized *T. vaginalis* proteins extracted from a vaginal swab. The test time is 11 minutes.

OSOM Trichomonas Rapid Test can be stored at room temperature (15–30 °C) and has a shelf-life of 16 months from date of manufacture. All the items needed to perform 25 tests, including sterile collection swabs, test tubes and sample buffer are included in the test kit. Positive and negative control swabs are also provided to check the performance of new test batches. The test has been shown to have both high sensitivity (83.3%) and specificity (98.8%) in symptomatic women when compared with NAATs.⁸ The performance of *OSOM Trichomonas Rapid Test* is shown in Fig. 2.35.



* Unlike most other IC tests, the IC Test line is blue because the primary antibody is conjugated to blue coloured particles.

Fig. 2.35 *OSOM Trichomonas Rapid Test* procedure. Courtesy Sekisui

VULVOVAGINAL CANDIDIASIS

Vulvovaginal candidiasis is caused by *Candida albicans* fungi and occasionally by other *Candida* species. *Candida* is normally present in the genital tract of up to 25% of healthy child-bearing women. Most symptomatic infections occur endogenously as a result of antibiotic treatment, reduced immune responses, diabetes mellitus, or changes in reproductive hormone levels. Symptoms include a white odourless vaginal discharge with pH < 5.0, vulvovaginal irritation and dysuria.

Vulvovaginal candidiasis is often diagnosed from clinical symptoms. Narrow range pH papers can be used to confirm the pH is < 5.0 (in trichomoniasis and bacterial vaginosis, the pH is > 5.0).

LABORATORY DIAGNOSIS

Laboratory confirmation of vulvovaginal candidiasis is by:

- Examining wet preparations of vaginal discharge and urine sediment for budding yeast cells as shown in Plate 2.55 (also in Plate 2.53).
- Examining a Gram stained smear of vaginal discharge and urine sediment for Gram positive yeast cells and pseudohyphae as shown in Plate 2.56. The Gram staining technique is described in Section 2.12.

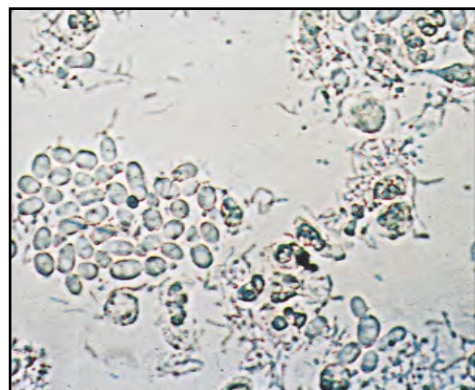


Plate 2.55 *Candida* yeast cells as seen in a wet preparation, showing single and budding yeasts. They can be differentiated from red cells by their small oval shape and some yeasts will show budding, as seen with the 40× objective.



Plate 2.56 Gram stained smear showing Gram positive *Candida* yeast cells and pseudohyphae as seen with the 100× objective.

BACTERIAL VAGINOSIS

Bacterial vaginosis (non-specific vaginitis) occurs in women of child-bearing age when there is a change in the normal acidic microbial flora of the vagina from lactobacilli to a mixed anaerobic microbial flora. *Gardnerella vaginalis* may also be involved. Bacterial vaginosis can cause vaginitis with an offensive-smelling grey-white watery vaginal discharge. With bacterial vaginosis there is an increased risk of acquiring HIV.

LABORATORY DIAGNOSIS

Laboratory confirmation of bacterial vaginosis is by:

- Examining a Gram stained smear for clue cells which are epithelial cells with adhering short Gram negative rods, coccobacilli and Gram variable rods as shown in Plate 2.57. In bacterial vaginosis more than 20% of epithelial are clue

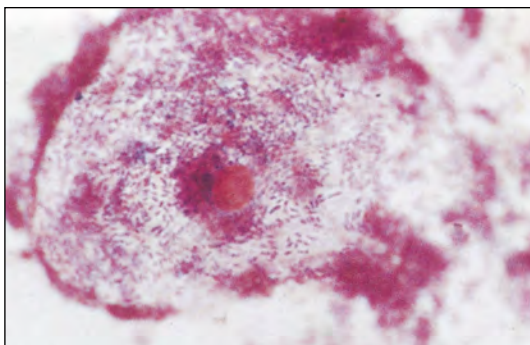


Plate 2.57 Clue cell in Gram stained vaginal smear showing adhering short Gram negative rods, coccobacilli and Gram variable rods, suggestive of bacterial vaginosis, as seen with the 100× objective.

cells. Report the smear as “Clue cells seen, suggestive of bacterial vaginosis.”

- Testing the pH of the vaginal discharge using narrow range pH papers. The normal vagina has a pH of 4.0. In bacterial vaginosis the pH is > 5.0.
- Performing an amine odour slide test. A positive test for bacterial vaginosis is indicated when a characteristic fishy amine odour is released after adding a drop of 10% potassium hydroxide to a vaginal sample on a slide.

Note: Information on the Ison-Hay criteria for grading Gram stained smears in bacterial vaginosis can be found in the WHO Manual: *Laboratory diagnosis of sexually transmitted infections, including human immuno-deficiency virus.*⁹

GONORRHOEA

Gonorrhoea is caused by *Neisseria gonorrhoeae*. In men infection can cause urethritis with urethral discharge and dysuria (painful urination). Untreated and repeated infections can lead to infertility, epididymitis and urethral stricture. In women *N. gonorrhoeae* can cause cervicitis, vaginal discharge and lower abdominal pain. Many infections are asymptomatic. Complications of untreated gonococcal infection in women include pelvic inflammatory disease, ectopic pregnancy, miscarriage and infertility. Pregnant women with gonorrhoea can pass infection to their newborn infant at birth causing neonatal conjunctivitis with purulent ocular discharge and swollen eyelids. If untreated it can lead to blindness.

In HIV-coinfection, it has been shown that untreated gonococcal infection increases genital HIV shedding and mucosal transmission of HIV. Treatment of gonorrhoea has become a major problem worldwide due to increased resistance to most antibiotics previously used to treat gonococcal infections.

LABORATORY DIAGNOSIS

In district laboratories gonorrhoea can be diagnosed by:

- Examining a Gram stained smear of urethral discharge and urine sediment for intracellular Gram negative diplococci.
- Performing a point-of-care nucleic acid amplification test (NAAT).

Gram staining

In symptomatic men up to 95% of gonococcal urethritis can be diagnosed presumptively by Gram staining urethral discharge. In women a presumptive diagnosis of gonorrhoea from Gram staining an endocervical swab sample has both low sensitivity and low specificity. Culture is required (see later text).

Methanol fixed smears of urethral discharge or sediment from centrifuged urine can be Gram stained following the procedure described in Section 2.10.

Reporting Gram stained urethral smears

Look for pus cells containing Gram negative diplococci as shown in Plate 2.58. Only the nuclei of pus cells stain. When pus cells have been damaged, the organisms may be seen lying outside the pus cells (extracellular). When organisms are seen, report the smear as "Pus cells with Gram negative diplococci, suggestive of gonorrhoea" and refer a sample for culture and antimicrobial susceptibility testing (see later text).

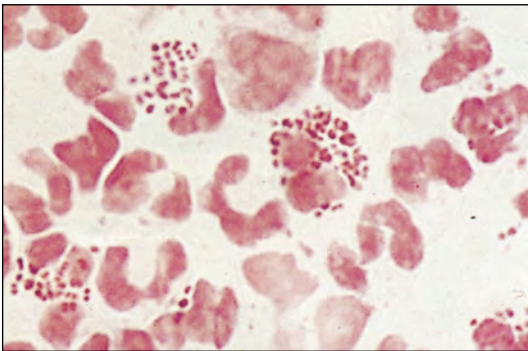


Plate 2.58 Gram stained urethral smear showing pus cells and intracellular Gram negative diplococci, indicative of gonorrhoea, as seen with the 100× objective (neutral red counterstain).

Diagnosis of gonorrhoea by point-of-care NAAT

An example of a point-of-care automated NAAT which can be used to screen and diagnose gonorrhoea in asymptomatic and symptomatic patients is *GeneXpert CT/NG* manufactured by Cepheid Inc. It is a combined *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) NAAT, detecting and differentiating *C. trachomatis* and *N. gonorrhoeae* DNA.

The test takes appropriately 90 minutes to perform. Samples that can be tested include urine (male and female), endocervical and vaginal swab samples (previously collected in Cepheid Transport Reagent).

Quality control of *Xpert CT/NG*

For each test this includes:

- Sample processing control which ensures correct processing of the sample.
- Sample adequacy control which ensures the sample contains human cells and the cells are lysed adequately to extract nucleic acids.
- Probe check control which measures the fluorescence signal from the probes to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability.

GeneXpert CT/NG has been shown to perform accurately, diagnosing gonorrhoea and chlamydia with high sensitivity and specificity.³ The same Cepheid *GeneXpert* equipment can be used as described for diagnosing tuberculosis in Section 2.11. The *Xpert CT/NG* cartridge has a shelf-life of 12 months from date of manufacture when stored at 2–28 °C.

Rapid lateral flow *N. gonorrhoeae* IC tests

Several rapid POC lateral flow antigen tests have been developed for diagnosing gonorrhoea. Evaluations have shown the tests to perform poorly often with sensitivities below 50%.³ New rapid point-of-care tests are in the pipe-line.

Culture and antimicrobial susceptibility testing

To provide effective treatment and assist in monitoring *N. gonorrhoeae* antibiotic resistance, presumptive gonorrhoea samples and *N. gonorrhoeae* NAAT positive samples require referral to a laboratory with facilities for *N. gonorrhoeae* culture, identification and antimicrobial susceptibility testing.

To ensure the viability of *N. gonorrhoeae* microorganisms, urethral and endocervical swab samples require transporting in Amies or Stuart transport medium. The samples should be transported in a cool box to reach the referral laboratory as soon as possible and within 48 hours at the latest.

BD ESwab

This consists of a sterile nylon flocked swab available with a regular size swab tip, minitip or flexible minitip and tube containing 1 mL of modified liquid Amies medium. The sample elutes rapidly from the swab.

The referral laboratory performing *N. gonorrhoeae* culture and susceptibility testing should advise district laboratories on sample collection procedures, transportation requirements and provide sterile transport medium.

Note: Details of sample collection procedures, culture and identification of *N. gonorrhoeae* and antimicrobial susceptibility can be found in the WHO Manual: *Laboratory diagnosis of sexually transmitted infections including human immunodeficiency virus*.⁹

UROGENITAL CHLAMYDIA

Urogenital chlamydia is caused by *Chlamydia trachomatis*.

C. trachomatis

Chlamydiae are obligate intracellular bacteria, existing as intracellular replicating bodies and spore-like infectious elementary bodies.

C. trachomatis serovars D-K infect epithelial cells of the genital tract causing urogenital chlamydia. Serovars A-C infect epithelial cells of the eye causing trachoma, a major cause of preventable infectious blindness in tropical countries.

C. trachomatis L1-L3 serovars replicate in lymphatic tissue causing lymphogranuloma venereum (LGV), a systemic infection. A small ulcer forms at the site of infection, followed by enlarged painful lymph glands in the groin and surrounding tissues (buboes). LGV is endemic in parts of the tropics and subtropics.

In men *C. trachomatis* is a common cause (up to 50%) of non-gonococcal urethritis with dysuria and a watery non-purulent urethral discharge. Untreated infections can progress to epididymitis and prostatitis.

In women *C. trachomatis* infects the epithelial cells of the endocervix. It can cause cervicitis with a mucopurulent cervical discharge and dysuria. Most infected women are asymptomatic. When untreated, chlamydia infection can spread to the upper reproductive tract causing pelvic inflammatory disease and increased risk of ectopic pregnancy. Infertility can result following repeated infections. Untreated cervical chlamydia infection is associated with cervical squamous cell carcinoma. Infants of mothers with chlamydia can be infected at delivery resulting in neonatal mucopurulent conjunctivitis.

LABORATORY DIAGNOSIS

In district laboratories tests used to diagnose and screen for urogenital chlamydia include:

- Diagnosing urogenital chlamydia presumptively in men by demonstrating non-gonococcal urethritis.
- Using a point-of-care NAAT to screen and diagnose urogenital chlamydia.

Demonstrating non-gonococcal urethritis

A presumptive diagnosis of urogenital chlamydia in men can be made by examining a Gram stained preparation for pus cells without Gram negative diplococci (chlamydiae cannot be visualized in a Gram stained preparation).

Point-of-Care NAAT to detect *C. trachomatis*

An example of a point-of-care NAAT to diagnose and screen for symptomatic and asymptomatic chlamydia is the *GeneXpert CT/NG* combined test, previously described for diagnosing gonorrhoea. The test detects with high sensitivity and specificity *C. trachomatis* serovars D-K that cause urogenital chlamydia and serovars L1-L3 that cause LGV.

Rapid lateral flow Chlamydia antigen tests

Compared with NAATs, currently available lateral flow *Chlamydia* antigen tests, lack sensitivity to screen and diagnose urogenital chlamydia infections.¹⁰ New tests are being developed.

DONOVANOSIS

Donovanosis, also called granuloma inguinale, is caused by *Klebsiella granulomatis*. It is a sexually transmitted genital ulcerative condition occurring mainly in tropical and subtropical areas, including Papua New Guinea, South Africa, South India, Brazil and the Caribbean.

Ulceration of the genitalia and surrounding skin can be extensive with bleeding from lesions but unlike LGV, the lymph glands are less involved. Secondary bacterial infection can occur and also coinfection with other STIs. The risk of HIV transmission is increased.

Laboratory diagnosis

In district laboratories, donovanosis is usually diagnosed by finding intracellular Donovan bodies in Giemsa stained smears from infected tissue. Methanol fixing of smears and Giemsa staining are described in Section 2.1.

Appearance of *K. granulomatis*

In Giemsa stained smears, *K. granulomatis* organisms appear as encapsulated coccobacilli showing bipolar

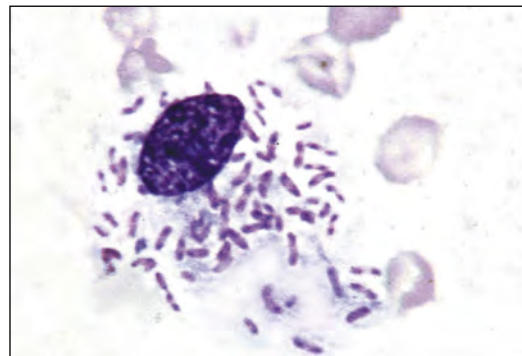


Plate 2.59 Giemsa stained smear showing *K. granulomatis* encapsulated bipolar stained coccobacilli (Donovan bodies) in the cytoplasm of a macrophage cell as seen with the 100× objective.

staining. They are found in the cytoplasm of macrophage cells and are known as Donovan bodies. A Giemsa stained preparation is shown in Plate 2.59.

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2.14 HIV Infection

The human immunodeficiency virus (HIV) weakens the immune system by infecting and destroying directly and indirectly cells bearing the CD4 antigen receptor, the most important being T helper lymphocytes. In untreated HIV infection, the depletion of CD4 cells causes bacterial and fungal opportunistic infections and cancers. The gradual loss of CD4 cells over time can lead to advanced HIV disease and the acquired immunodeficiency syndrome (AIDS)*.

* AIDS is defined by the development of certain cancers, infections or other severe long-term manifestations (WHO 2022 HIV Fact Sheet¹).

HIV retrovirus

HIV is an enveloped RNA virus belonging to the lentivirus subgroup of retroviruses. By possessing the enzyme reverse transcriptase, retroviruses are able to reverse-transcribe RNA to DNA. The DNA genome produced (provirus) becomes integrated in the DNA of the infected cell ensuring permanent infection and replication of the virus.

Structurally HIV consists of:

- An inner core containing two copies of single stranded RNA, viral enzymes and reverse transcriptase, surrounded by a nucleocapsid composed of p24 protein (group specific core antigen which does not vary).

- Double layered lipid envelope derived from the membrane of the host cell. The envelope contains virus specific glycoproteins: gp 120 (protrudes from the surface) and gp 41 (embedded in the envelope). These enable the virus to attach to and infect host cells. The gene that encodes gp 120 mutates rapidly producing many antigenic variants.

There are two genetically distinct types of the virus, HIV-1 which causes about 95% of HIV infections worldwide and HIV-2 which is found mainly in West Africa and has also been reported from Brazil, India, Europe and USA. HIV-2 is less infectious than HIV-1, progresses more slowly and does not cause diseases

such as Kaposi's sarcoma. Not all antiretroviral drugs are active against HIV-2.

HIV-1 is divided into three groups, M, O and N with more than 90% of HIV-1 infections belonging to Group M. Group O is found in west and central Africa. Group N is rare (has been reported from Cameroon). Within HIV-1 Group M there are at least nine genetically distinct subgroups which differ in their geographical distribution and transmission preferences.

Distribution of HIV-1 subgroups

HIV-1 is classified into subgroups A, B, C, D, F, G, H, J, K, L and several circulating recombinant forms. Subgroup C is the predominant subgroup in Africa and parts of Asia, subgroup F in South America, central Africa and Europe and recombinant CRFOI-AE (formally subgroup E) in Thailand. Subgroup B is mostly found in western countries, Australasia, North America and North Africa. K and L are found in the Democratic Republic of Congo.

Transmission of HIV

HIV is present in semen, vaginal/cervical secretions, blood and breast milk and these are the main vehicles by which the virus is transmitted. The risk of transmitting the virus is highest in the early stage (seroconversion) and late stages of HIV infection when viral load is highest.

In tropical countries, HIV is mainly transmitted by:

- Unprotected heterosexual intercourse (85–95% transmission). HIV tends to be more easily transmitted from males to females. Male to female transmission risk is highest in immature girls aged 16 years or less. Frequent changes in sexual partners increase the risk of transmission. Sex workers and their clients are particularly at risk.
- Sexual practice involving anal intercourse. The risk of acquiring HIV is particularly high among men who have sex with men.
- Mother-to-child transmission during pregnancy, labour and delivery (commonest mode of transmission and carries 15–30% risk) or breast-feeding (10–15% risk). Antiretroviral therapy (ART) during pregnancy greatly reduces the risk of transmission. Early infant diagnosis can be made by detecting HIV nucleic acid in the blood of infected infants (see later text).
- Transfusion of infected blood or blood products where HIV screening of donor blood is not performed or is inadequate.
- Use of contaminated syringes, needles and drug solutions by intravenous drug users (carries a high risk).

- Use of inadequately sterilised needles in clinics and hospitals and the use of non-sterile instruments in tribal ceremonies.
- Accidental needle-stick injuries by healthcare staff. Well documented studies have shown there is no risk to laboratory staff of acquiring HIV when applying good laboratory practice.

There is no epidemiological evidence that HIV is transmitted through air, water or food, sharing eating utensils, coughing or sneezing, insect bites, shaking hands or other casual contact.

Distribution of HIV infections¹

At the end of 2021 WHO estimated there were globally 33.9–43.8 million people living with HIV, two-thirds of whom were living in Africa. The global distribution of HIV is shown in Fig. 2.37. In 2021 1.1–2.0 million people became infected with HIV and there were 510 000–860 000 deaths from HIV-related causes. Approximately 28.7 million people were receiving antiretroviral treatment (ART). Only 52% of children (0–14 years) were receiving ART. HIV-seropositive people do not transmit the virus when regularly receiving ART and are virally suppressed.

During the time of COVID-19 restrictions, fewer people were tested for HIV and fewer HIV-seropositive patients were tested for opportunistic infections. Clinic visits, viral load assessment, CD4 cell counting services and treatment schedules were also disrupted in some areas.

Clinical features

The early stages of HIV infection may be asymptomatic or flu-like symptoms may develop including fever, headache, muscle aches and joint pains, rash, and sore throat. Further symptoms may develop as immune responses weaken. These include weight loss, fatigue, swollen lymph glands, diarrhoea, cough and painful mouth sores. Untreated infection can progress to advanced HIV disease and the development of cancers such as Kaposi's sarcoma, lymphomas and cervical cancer.

Advanced HIV disease

WHO defines advanced HIV disease for adults, adolescents and children older than 5 years as having a CD4 count < 200 cells/mm³ or WHO clinical stage 3 or 4 disease^{2,*}

* One of WHO HIV strategy targets for late stage HIV disease is to reduce the percentage of people starting ART with a CD4 count < 200 cells/mm³ from 30% in 2020 to 10% by 2030³.

All children younger than 5 years living with HIV are considered to have advanced HIV disease.






	People living with HIV in 2021	People acquiring HIV in 2021	People dying from HIV-related causes in 2021
 Total	38.4 million [33.9–43.8 million]	1.5 million [1.1–2.0 million]	650 000 [510 000–860 000]
 Adults (15+ years)	36.7 million [32.3–41.9 million]	1.3 million [990 000–1.8 million]	560 000 [430 000–740 000]
 Women (15+ years)	19.7 million [17.6–22.4 million]	640 000 [480 000–870 000]	240 000 [180 000–320 000]
 Men (15+ years)	16.9 million [14.6–19.7 million]	680 000 [500 000–920 000]	320 000 [250 000–430 000]
 Children (<15 years)	1.7 million [1.3–2.1 million]	160 000 [110 000–230 000]	98 000 [67 000–140 000]

Fig. 2.36 Summary of the global HIV epidemic in 2021. Source UNAIDS/WHO estimates. Updated: July 2022.

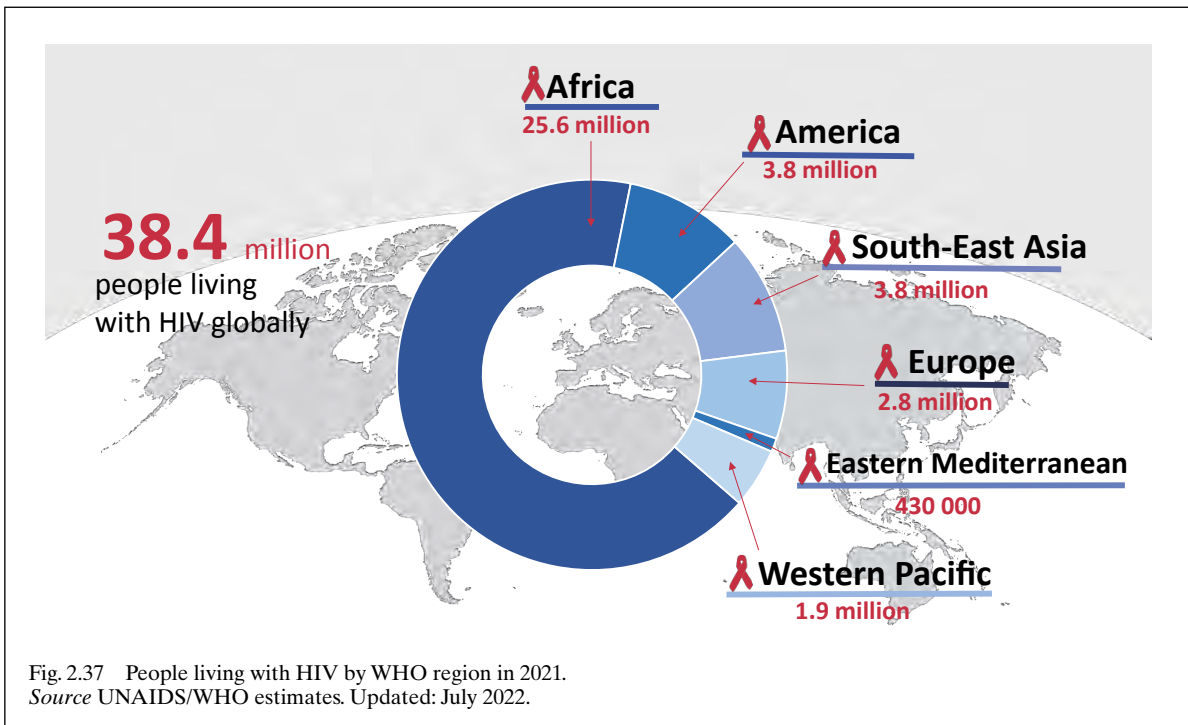


Fig. 2.37 People living with HIV by WHO region in 2021. Source UNAIDS/WHO estimates. Updated: July 2022.

Chart 2.14.1 WHO clinical staging 3 and 4 of HIV disease in adults, adolescents and children

Adults and adolescents	Children (< 15 years)
Clinical stage 3	
<p>Unexplained severe weight loss (>10% of presumed or measured body weight)</p> <p>Unexplained chronic diarrhoea for longer than 1 month</p> <p>Unexplained persistent fever (intermittent or constant for longer than 1 month)</p> <p>Persistent oral candidiasis</p> <p>Oral hairy leukoplakia</p> <p>Pulmonary tuberculosis</p> <p>Severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia)</p> <p>Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis</p> <p>Unexplained anaemia (<8 g/dl), neutropaenia (<0.5 x 10⁹/l) and/or chronic thrombocytopaenia (<50 x 10⁹/l)</p>	<p>Unexplained moderate malnutrition^b not adequately responding to standard therapy</p> <p>Unexplained persistent diarrhoea (14 days or more)</p> <p>Unexplained persistent fever (above 37.5°C, intermittent or constant, for longer than one 1 month)</p> <p>Persistent oral candidiasis (after first 6 weeks of life)</p> <p>Oral hairy leukoplakia</p> <p>Lymph node tuberculosis</p> <p>Pulmonary tuberculosis</p> <p>Severe recurrent bacterial pneumonia</p> <p>Acute necrotizing ulcerative gingivitis or periodontitis</p> <p>Unexplained anaemia (<8 g/dl), neutropaenia (<0.5 x 10⁹/l) or chronic thrombocytopaenia (<50 x 10⁹/l)</p>
Clinical stage 4	
<p>HIV wasting syndrome</p> <p><i>Pneumocystis (jirovecii) pneumonia</i></p> <p>Recurrent severe bacterial pneumonia</p> <p>Chronic herpes simplex infection (orolabial, genital or ano-rectal of more than 1 month's duration or visceral at any site)</p> <p>Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)</p> <p>Extrapulmonary tuberculosis</p> <p>Kaposi sarcoma</p> <p>Cytomegalovirus infection (retinitis or infection of other organs)</p> <p>Central nervous system toxoplasmosis</p> <p>HIV encephalopathy</p> <p>Extrapulmonary cryptococcosis, including meningitis</p> <p>Disseminated nontuberculous mycobacterial infection</p> <p>Progressive multifocal leukoencephalopathy</p> <p>Chronic cryptosporidiosis</p> <p>Chronic isosporiasis</p>	<p>Unexplained severe wasting, stunting or severe malnutrition^d not responding to standard therapy</p> <p><i>Pneumocystis (jirovecii) pneumonia</i></p> <p>Recurrent severe bacterial infections (such as empyema, pyomyositis, bone or joint infection, meningitis, but excluding pneumonia)</p> <p>Chronic herpes simplex infection (orolabial or cutaneous of more than 1 month's duration or visceral at any site)</p> <p>Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs)</p> <p>Extrapulmonary tuberculosis</p> <p>Kaposi sarcoma</p> <p>Cytomegalovirus infection (retinitis or infection of other organs with onset at age more than 1 month)</p> <p>Central nervous system toxoplasmosis (after the neonatal period)</p> <p>HIV encephalopathy</p> <p>Extrapulmonary cryptococcosis, including meningitis</p> <p>Disseminated nontuberculous mycobacterial infection</p> <p>Progressive multifocal leukoencephalopathy</p>

Adults and adolescents	Children (< 15 years)
Disseminated mycosis (extrapulmonary histoplasmosis, coccidioidomycosis)	Chronic cryptosporidiosis (with diarrhoea)
Lymphoma (cerebral or B-cell non-Hodgkin)	Chronic isosporiasis
Symptomatic HIV-associated nephropathy or cardiomyopathy	Disseminated endemic mycosis (extrapulmonary histoplasmosis, coccidioidomycosis, penicilliosis)
Recurrent septicaemia (including nontyphoidal <i>Salmonella</i>)	Cerebral or B-cell non-Hodgkin lymphoma
Invasive cervical carcinoma	HIV-associated nephropathy or cardiomyopathy
Atypical disseminated leishmaniasis	

Reproduced with WHO permission from *Consolidated guidelines on the use of antiretroviral drugs for treating and preventing HIV infection*, 2nd edition, 2016.

People with interrupted ART or treatment failure are at risk of developing advanced HIV disease.

The leading causes of opportunistic infections and mortality among adults with advanced HIV disease include:

- tuberculosis,
- severe bacterial infections such as pneumonia, bacteraemia, meningitis, gastrointestinal infections, cryptococcal meningitis,
- histoplasmosis,
- toxoplasmosis,
- *Pneumocystis jirovecii* pneumonia,
- talaromycosis in South-East Asia.

The laboratory diagnosis of HIV-related conditions and opportunistic infections is described at the end of the subunit (see p. 188–190).

WHO HIV Global Health Sector Strategy³

Targets for the HIV 2022-2030 Strategy include:

Reducing:

- Number of people newly infected with HIV yearly from 1.5 million in 2020 to 335 000 by 2030.
- Number of people dying from HIV related causes yearly from 680 000 in 2020 to < 240 000 by 2030.

Increasing:

- Percentage of people who know their HIV status from 84% in 2020 to 95% by 2030.
- Percentage of HIV-seropositive people accessing ART from 87% in 2020 to 95% by 2030.

LABORATORY DIAGNOSIS

HIV testing services.

HIV testing services should include pre-test information and post-test counselling, information on laboratory tests, HIV prevention and long-term treatment and care. It is important that those being offered HIV testing are assured that testing will be voluntary, confidential, conducted in a private non-stigmatizing welcoming environment with informed consent and their test results will be correct.

Reasons for HIV testing include:

- To confirm HIV infection in patients with clinical symptoms of infection or possible advanced HIV disease-defining condition.
- To screen pregnant women to prevent mother-to-child transmission of HIV.
- To test newborns and children for HIV infection.
- To diagnose HIV infection in patients with tuberculosis or a sexually transmitted infection.
- To screen patients for HIV infection prior to surgery or treatment with immunosuppressive drugs.
- To provide couples with their HIV status as part of family planning services.
- To screen donor blood to prevent transmission of the virus in whole blood or blood products.
- To reduce virus transmission, HIV illness and mortality.

HIV testing services need to ensure tests are available where e.g. health facility, community-based, or home, and when they are needed based on health needs, peoples' preferences and cost-effectiveness². Test results need to be timely.

HIV testing should be integrated with other clinical services such as those for TB, maternal and child health, sexual and reproductive health, diabetes and programmes for intravenous drug users. Whenever possible, HIV testing should be available in work places, schools and recreational establishments using when appropriate mobile testing units. The benefits of HIV testing should be promoted.

HIV self-testing

WHO recommends that HIV self-testing (HIVST) be offered as an additional approach to HIV testing services.² HIVST enables more people with HIV to be reached, helping to meet the United Nations target of 95% of all people with HIV knowing their status by 2030. A reactive self-test requires confirmatory testing and self-testers require the support and treatment provided by an HIV testing unit. HIV self-testing is described on pages 180–182.

Laboratory investigations

HIV testing services include the following laboratory investigations:

- Tests to diagnose HIV infection.
- Tests to monitor treatment.
- Tests to diagnose coinfections, opportunistic infections and HIV-related health conditions.

TESTS TO DIAGNOSE HIV INFECTION

Antibody tests are used to diagnose HIV infection except for infants below 18 months when HIV nucleic acid tests are required (see later text).

A *single* reactive HIV test cannot be used to diagnose HIV infection with certainty due to differences in circulating HIV subtypes, variations in the performance of tests and possible cross-reactions with other pathogens.

A *definitive diagnosis of HIV* can only be made by using a validated HIV testing strategy that uses 2 and preferably 3 different consecutive reactive tests. Three tests are indicated when the number of people testing positive for HIV and prevalence of HIV among those untreated fall below 5%². Before commencing antiretroviral therapy, WHO recommends re-testing to confirm an HIV-positive

diagnosis. Also recommended is the periodic retesting of persons using ART prevention (PrEp).

HIV tests should not be used to confirm whether a person diagnosed with HIV receiving ART is still HIV-seropositive.

Window period

HIV antibodies can be detected in blood or other body fluids 4-6 weeks following infection and in nearly all infected persons within 3 months of exposure. The seroconversion period before markers of HIV can be detected is referred to as the window period. The length of the window period is determined by the HIV antibody test used, virus characteristics, and a person's immune response.

Generations of HIV antibody tests

Second, third and fourth generation of HIV rapid tests are available based on how soon following exposure they can potentially detect HIV infection.

Most rapid antibody tests used in HIV testing strategies are second and third generation tests.

- 2nd generation tests detect potentially HIV-1/2 antibodies (IgG only) about 28 days following exposure.
- 3rd generation tests detect potentially HIV-1/2 antibodies (IgG and IgM) about 21 days following exposure.
- 4th generation tests detect both HIV-1/2 antibodies (IgG and IgM) and p24 antigen about 14 days following exposure.

WHO prequalified HIV RDTs

The 2022 list of WHO prequalified 2nd and 3rd generation HIV RDTs can be found on the WHO website. Enter in Google *WHO list of prequalified in vitro diagnosis products*.

WHO evaluations are awaited on 4th generation HIV RDTs.

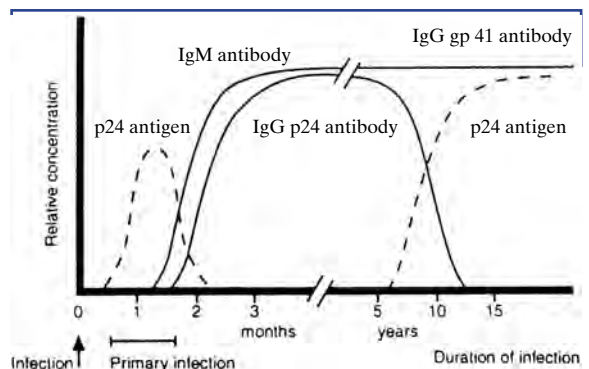


Fig. 2.38 HIV p24 antigen and HIV antibody responses in HIV infection.

HIV TESTING STRATEGY

An HIV testing strategy should achieve a positive predictive value of at least 99%, indicating the probability that an HIV-positive diagnosis is correct². As mentioned previously, using 3 separate tests is recommended as shown in Fig. 2.39.

The first test used in the strategy should be the most sensitive test available to detect all true reactive samples, i.e. sensitivity $\geq 99\%$. The 2nd and 3rd tests used in the testing strategy should be high specificity tests to rule out false reactivity, i.e. specificity $\geq 98\%$.

HIV TESTING ALGORITHM

The HIV testing algorithm refers to the HIV tests selected for use in the testing strategy based on:

- Performance characteristics of tests, including sensitivities, specificities, antigens used and test formats (tests with different technologies should be selected).

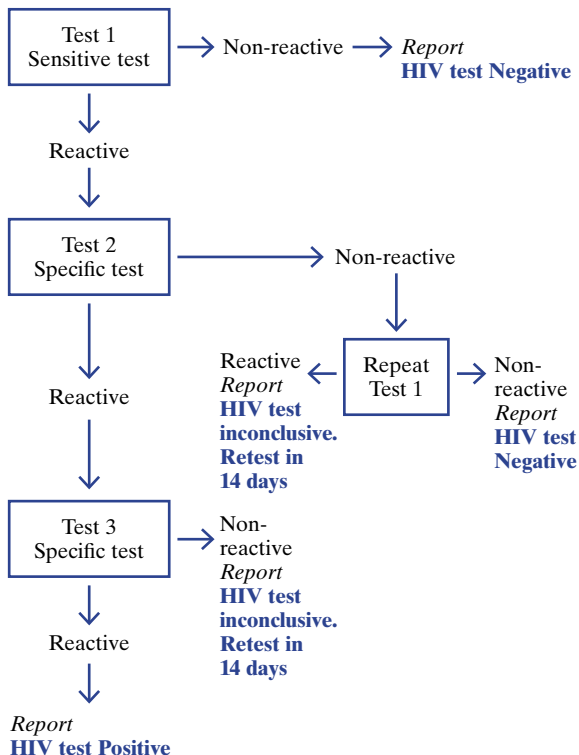


Fig. 2.39 WHO recommended HIV testing strategy using 3 tests.²

- Lowest inter-reader variability (< 5% is the WHO recommendation).
- Lowest invalid test rate.
- Availability, cost of tests and reliability of supply.
- Shelf-life of tests and test kit sizes.
- Training required and quality control.
- How well the tests can be performed at point-of-care by non-laboratory personnel.

More than one testing algorithm should be developed in the event of tests becoming unavailable. Testing algorithms should be reviewed every 3–5 years and amended as new improved performance HIV RDTs become available and national HIV policies change.

Examples of rapid HIV tests used in HIV testing strategies include:

- *Determine HIV-1/2 strip test* which is frequently used as a first test in HIV testing strategies, having a sensitivity of 99.1-100%.
- *HIV1/2 STAT-PAK dipstick* and *Wondfo One Step HIV1/2* which have high specificities and are therefore often used as second or third tests.

Other WHO prequalified rapid HIV tests that are used in HIV testing strategies can be found by entering in Google: *WHO list of prequalified in vitro diagnostic products*.

Interpretation of 3 line rapid HIV antibody tests

Most recently developed rapid HIV antibody tests are 3 line tests i.e. incorporating test bands to detect HIV-1 (T1), HIV-2 (T2) and the test Control band. Because of a 30-70% cross-reactivity between HIV-1 and HIV-2, HIV-1 positive samples, particularly high antibody titre samples often show reactivity (weak pink bands) also in the HIV-2 test band. HIV-2 positive samples frequently show reactivity also in the HIV-1 band, often of similar intensity. For some rapid HIV antibody tests, confusion in interpreting test results has led to increased reader variability.

Differentiation between HIV-1 and HIV-2

In West Africa and other areas where HIV-2 occurs it is important to select a test that detects reliably HIV-2 because HIV-2 is resistant to some drugs used in the treatment of HIV-1 infection (see Further Reading **Honge et al.**).

Determine HIV-1/2

Determine HIV-1/2 is an immunochromatographic (IC) lateral flow strip test manufactured by Abbott. It detects antibodies to HIV-1 and HIV-2 in serum, plasma or capillary/venous blood. The test has a shelf-life of 18 months (24 months for Chase buffer) from date of manufacture and is available as a 20 and 100 test pack. It requires storage at 2–30 °C

out of direct sunlight. The Chase buffer and EDTA capillary tubes required to test capillary blood need to be ordered separately.

Determine HIV-1/2 is a sensitive HIV test with a WHO prequalification final evaluation sensitivity of 99.1–100%, specificity of 97.8–99.6%, inter-reader variability of 1.4% and invalid rate of 0.3%.⁴ Antigens used are recombinant HIV-1 (gp120 and gp41) and recombinant HIV-2 (gp36).

Test procedure using capillary blood

- 1 Collect 50 µL of blood using the EDTA capillary tube and apply to the application pad of the test strip.
- 2 Wait for the blood to be absorbed into the pad.
- 3 Add 1 drop of Chase buffer.
- 4 Read the test result after 15 minutes (can be read up to 60 minutes).

Test results

A reactive test is shown by a pink band in both the Control and Test areas.

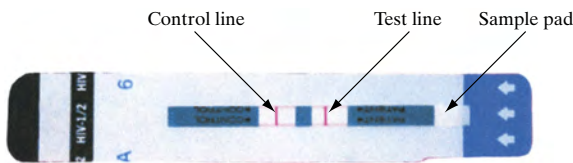
A non-reactive test is shown by a pink band in the Control area only.

The test is invalid if there is no pink Control band.

Reactive and non-reactive test results are shown in Plate 2.60.

Note: the test is reactive even if the colour of the sample band appears lighter or darker than the Control band. Weak coloured bands should be reported as reactive tests. The possible causes of false test results are summarized under *Quality control* (see later text).

Reactive test



Non-reactive test

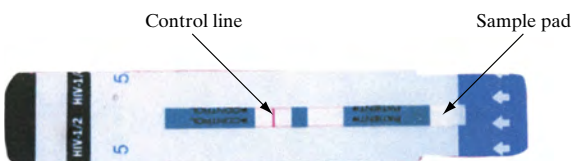


Plate 2.60 *Determine HIV-1/2* test results.
Courtesy of Abbott

HIV 1/2 STAT-PAK Dipstick

HIV 1/2 STAT-PAK Dipstick is an IC lateral flow strip test manufactured by Chembio Diagnostic Systems Inc. It detects antibodies to HIV-1 and HIV-2 in serum, plasma or capillary/venous blood. The test has a shelf-life of 24 months from date of manufacture and is available as a 30 test pack.. It requires storage at 8–30 °C out of direct sunlight. The kit contains all the items needed to perform the test including the Running buffer, sample loops and backing cards.

In the WHO prequalification final evaluation, the test gave a of specificity of 98.97–99.9%, sensitivity of 99.1–100%, inter- reader variability of 0.1% and 0% invalid test rate.⁵ A combination of HIV-1 and HIV-2 antigens are used.

Test procedure for finger-stick blood sample

- 1 Remove a test strip from the container. Peel the liner from the adhesive strip on the back of the strip. Attach the strip to the backing card provided, label and lay the card on a flat surface.
- 2 Using the 5 µL sample loop provided, collect 5 µL of capillary blood. Holding the loop vertically, touch it to the sample pad of the test strip.
- 3 Holding the Running buffer bottle vertically, slowly add 3 drops of buffer allowing each drop to be absorbed before adding the next.
- 4 Read the test result between 15 and 20 minutes (*do not read after 20 minutes*).

Note: If preferred an alternative dipstick tube procedure can be used (see manufacturer's test insert sheet).

Test results

A reactive test is shown by a pink band in both the Control (C) and Test (T) areas.

A non-reactive test is shown by a pink band in the Control (C) area only.

The test is invalid if there is no pink Control band.

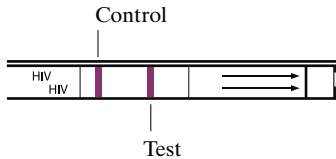
HIV 1/2 *STAT-PAK* test results are shown in Plate 2.61 on page 180.

Note: the intensity of colour in the Test area will vary with the concentration of antibody from barely visible to a dark colour. Even a weak (faint line) in the Test area indicates a reactive test. In between use, keep the vial containing the test strips tightly closed.

The possible causes of false test results are summarized under *Quality Control* (see later text).



Reactive test



Non-reactive test

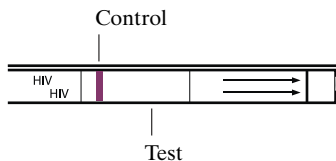


Plate 2.61 HIV1/2 STAT-PAK Dipstick test results.
Courtesy of Chembio Diagnostic Systems Inc.

Quality control

In diagnosing HIV infection it is particularly important that HIV testing is accurate and reliable. This can be achieved by:

- Following the testing strategy previously described and ensuring the testing algorithm is validated.
- Using tests of different technologies and tests of proven performance, e.g. WHO prequalified HIV tests.
- Following carefully the manufacturer's test procedure and instructions for the transport and storage of test kits.
- Checking new batches of test kits using quality control samples and not using kits that have expired.
- Reading test reactions in a good light.
- Confirming an HIV diagnosis by re-testing before commencing ART.
- Participating in an external quality assessment scheme which includes HIV testing and sending reactive samples for checking to a Reference Laboratory.

Possible causes of false reactive tests

- Cross reacting antigens from other pathogens.
- Contaminating proteins in sample.

Possible causes of false non-reactive tests

- Antibody below the detection limit of the test (e.g. pre-seroconversion samples).
- Not using a sufficiently sensitive test.
- Infection with an HIV variant not detected by the test.
- Re-testing a patient being treated with antiretroviral drugs.

HIV SELF-TESTING

WHO recommends HIV Self-Testing (HIVST) as a means to reach first-time testers, people with undiagnosed HIV infection, or those at ongoing risk who are in need of frequent retesting.

HIVST is becoming more widespread following lower prices for self-tests and surveys that have shown self-testing to increase the uptake of HIV testing and enable more HIV infected people to become aware of their HIV status⁶. HIVST has been shown to be highly acceptable in various settings and among different groups of users, leading to early HIV diagnosis, treatment and prevention. It should never be coercive.

Self-testers have been shown to perform HIV self-tests, using capillary blood or oral fluid samples, as well as trained healthcare workers. Tests using blood are seen by some people as more accurate while oral fluid testing is preferred by others. It is *important* for self-testers to be aware of the window period and that it may take up to 3 months for antibody levels to reach a level sufficient to give a positive test.

When a test is positive a trained HIV test provider is required to confirm the test result, provide guidance and a link to treatment and care. When a test is negative and there is an ongoing risk or recent exposure, retesting is indicated. Self-testing should not be used by people receiving ART to check infectivity. It is not a replacement for regular HIV testing for people receiving pre-exposure prophylaxis. It should not be used by parents to test an infant. (see later text). It should not be used by people with a blood disorder.

Wondfo HIV Self-Test

Wondfo HIV Self-Test is a WHO prequalified self-test. It is widely used as it requires only a small

drop (10µL) of capillary blood, is easy to perform and is inexpensive (recent discount initiative). It is manufactured by Guangzhou Wondfo Biotech Co Ltd. The *Wondfo HIV Self-Test* is a lateral flow cassette-based qualitative immunoassay that detects HIV-1 and HIV-2 antibodies in a measured volume of capillary blood. It has a shelf-life of 24 months from date of manufacture and is available as a single test, 20 and 100 test packs. The test requires storage at 2–30 °C. All the items needed to perform the test are provided and also well-illustrated test instructions, information on HIV disease and the actions to take following test results.

Based on WHO prequalification of *Wondfo One Step HIV1/2 Whole blood/Serum/Plasma test* (PQDx 0357-004-00), the sensitivity of the test is reported as 99.2–100.0% and specificity as 99.5–100%. The test is easy to interpret with a 0% reader variability.

Test procedure

- 1 Collect 10µL of capillary blood as detailed in the test instructions.
- 2 Add the blood to the well of the Test cassette.
- 3 Add 4 drops of buffer.
- 4 Read the test results after 15 minutes but not after 30 minutes.

A positive test is shown by a pink band in the Test line and Control line as shown in Plate 2.62. The intensity of colour is not an indication of the concentration of antibody in the sample.

A negative test is shown by a pink band in the Control line only.

The test is invalid if there is a band in the Test line but no band in the Control line.

Important

When a test is positive, an HIV test provider is required to confirm the test result, provide support and a link to treatment and care.

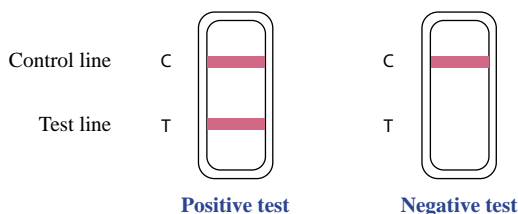


Plate 2.62 test results for *Wondfo HIV Self Test*.
Courtesy of Guangzhou Wondfo Biotech Co Ltd.

OraQuick HIV Self-Test

Prequalified by WHO in 2017 (PQD 0159-055-01), *OraQuick HIV Self-Test* is a widely used lateral flow qualitative immunoassay that detects HIV-1/2 antibody in oral fluid. It is manufactured by OraSure Technologies Inc.

OraQuick HIV Self-Test has a shelf-life of 30 months from date of manufacture and is available as a 25 or 100 test pack. It requires storage at 2–30 °C. In a field study in South Africa the test was shown to have a sensitivity of 93.88–99.95% and a specificity of 99.62–100%.⁷ Other evaluations have also shown the test to have high sensitivity and specificity.

Test procedure

Preparation: Do not eat, drink or chew gum 15 minutes before testing and do not use mouth-cleaning products 30 minutes before testing. Test at between 15–37 °C.

- 1 Slide the tube containing the developing solution into the stand provided.
- 2 Collect an oral sample by swabbing the outer upper gum and outer lower gum with the flat pad of the testing device provided. Do not touch the flat pad with the fingers.
- 3 Insert the testing device into the tube until the flat pad touches the bottom of the tube.
- 4 Read the test result not before 20 minutes and not after 40 minutes.

Test results

A positive test is shown by a pink band in both the Test (T) area and Control (C) area. The intensity of colour of the Test band does not necessarily correlate with the amount of antibody in the sample. A negative test is shown by a pink band in the Control (C) area only.

The test is invalid if there is a pink band in the Test area but no pink Control (C) band.

Positive and negative tests for *OraQuick HIV Self-Test* are shown in Plate 2.63.

Important: Self-testers with a reactive test result require further testing from a trained provider using a validated testing strategy and the support of an HIV-testing programme for counselling, treatment and care. It is important for self-testers to be aware of the window period.

Limitations of *OraQuick HIV Self-Test* are listed under the WHO Prequalification Report Version 2.⁸

**Positive OraQuick
HIV Self-Test**



**Negative OraQuick
HIV Self-Test**



Plate 2.63 *OraQuick HIV Self-Test*, positive and negative test results.

Courtesy of OraSure Technologies Inc. www.orasure.com/

DIAGNOSING HIV INFECTION IN NEWBORN INFANTS

Mother-to-child transmission of HIV infection can occur in pregnancy, labour or during delivery. Following birth the virus can also be transmitted in breast milk. Effective treatment of an HIV infected mother greatly reduces the risk of mother-to-child transmission. Early diagnosis and treatment of HIV infected infants are essential to prevent infant mortality in the first few months of life, particularly when infants are infected during pregnancy.

HIV antibody tests cannot be used to diagnose HIV infection in infants up to 18 months of age due to the placental transfer of maternal antibodies to the infant and the possible transmission of antibodies during breast feeding. Tests are required that detect HIV viral nucleic acid in whole blood or from dried blood spot samples using polymerase chain reaction (PCR).

When HIV infection occurs in utero, HIV nucleic acid can be detected in blood samples within 48 hours of birth. When infants are infected at or around the time of delivery, HIV nucleic acid can usually be detected at 1–2 weeks. Early HIV testing may be affected by antiretroviral drugs taken by the mother resulting in false negative tests. When a nucleic acid test is negative at birth further testing is required at 4–6 weeks. Most tests to diagnose infant HIV infection are performed during a mother's first postnatal visit (at 4–6 weeks). National guidelines should be followed. A positive nucleic acid test requires confirmation by testing a second sample.

Note: Further information on HIV diagnosis among infants and children, infant prophylaxis, and advanced HIV disease among children and adolescents can be found in the 2021 WHO *Consolidated guidelines in HIV prevention, testing, treatment, service delivery and monitoring*². The publication can be accessed by entering the title in Google.

Point-of-care nucleic acid testing

Recently developed technologies are now making it possible for district laboratories to perform on-site HIV nucleic acid testing or send dry blood spot (DBS) samples to a regional laboratory with facilities for rapid nucleic acid testing and connectivity for the rapid return of test results.

Preparation of dry blood spots

- 1 Label a Munktell or Whatman No. 903 perforated filter paper collection card (see Fig. 2.40) with the infant's name, identity number and date of collection. Do not use ordinary filter paper.



Fig. 2.40 Whatman No 903 blood collection filter paper card.

- 2 Fill each circle of the card with free-flowing blood from a heel prick if the infant is under 4 months of age or from the big toe if older. Each circle holds about 70 μL of blood. Ensure the blood fills and fully saturates at least 3 circles.
- 3 Place the card upwards in a draining rack for the blood spots to air-dry, protected from sunlight, insects and dust. Overnight drying is recommended. Do not stack cards together and do not touch the blood spots.
- 4 When the blood spots are *completely dry*, place each card individually in a sealable plastic envelope with 3 desiccant packs.
- 5 Place 5–10 envelopes in a plastic ziplock bag. Add a DBS Humidity Card. Enclose the request forms in the bag. Gently squeeze air from the bag before inserting it in a despatch envelope (preferably bubble-wrap envelope) for transporting to the Referral Laboratory.

Note: When kept dry, DBS samples can be kept at ambient temperature (18–28 $^{\circ}\text{C}$) for up to 14 days or at 4 $^{\circ}\text{C}$ for up to 3 months. If required, they can be stored for up to 60 days in a -20°C freezer or for longer at -70°C .

Two automated point-of-care technologies have been prequalified by WHO for early infant HIV diagnosis:

- *Xpert HIV-1 Qual Assay*⁸
- *m-PIMA HIV-1/2 Detect*⁹

Xpert HIV-1 Qual Assay

Xpert HIV-1 Qual Assay is manufactured By Cepheid (recently acquired by Danaher Corporation). It detects qualitatively HIV-1 RNA and proviral DNA in DBS samples, EDTA capillary and venous blood, using real-time reverse transcriptase PCR (RT-PCR). HIV-2 is not detected. Several tests can be processed at one time (depending on the capacity of the GeneXpert instrument). Test running time is 90 minutes.

The *GeneXpert* instrument is the same as that used for diagnosing tuberculosis, described in Section 2.11. The *Xpert HIV-1 Qual* cartridge requires storage at 2–28 °C and has a shelf-life of 8 months from date of manufacture. Cartridges are available as a 10 test pack.



Plate 2.64 Upper: *Xpert HIV-1 Qual* instrument with inserted cartridge. Lower: *Xpert HIV-1 Qual* cartridge. Courtesy of Cepheid. www.cephheidinternational.com

Items provided in the test kit include the test cartridge (containing the RT-PCR reagents), sample reaction vial containing lysis reagent, 1 mL transfer pipettes and 100 µL transfer micropipettes. Not provided are the *GeneXpert* instrument, computer, *GeneXpert* DX software, barcode scanner and equipment for processing DBS samples, i.e. *Eppendorf ThermoMixer* and *Eppendorf SmartBlock* (heat block which fits in the *ThermoMixer*).

Test procedure for processing DBS samples

- 1 Turn on the *ThermoMixer* and heat to 56 °C.
- 2 Label the Sample Reagent vial. Remove one 12 mm diameter dry blood spot disc from the filter paper card and place it in the Sample Reagent vial, ensuring the disc is fully submerged in the buffer solution.
- 3 Incubate at 56 °C in the *ThermoMixer* for 15 minutes while rotating at 500 rpm.
- 4 Using the 1 mL transfer pipette provided, transfer the lysed DBS sample into the cartridge sample chamber.
- 5 Close the cartridge lid, insert the cartridge in the *GeneXpert* instrument (see Plate 2.64) and start the test.
- 6 Test results are available after 90 minutes, reported as HIV-1 Detected or HIV-1 Not detected.

Procedure for processing EDTA blood samples

- 1 Using the 1 mL transfer pipette provided, transfer 750 µL of reagent from the Sample Reagent vial into the cartridge sample chamber.
- 2 Using the micropipette provided, add 100 µL of well-mixed EDTA sample to the cartridge sample chamber.

Note: EDTA samples may be stored at 15–30 °C for up to 24 h and at 2–8 °C for up to 72 hours.

- 3 Close the cartridge lid, insert the cartridge in the *GeneXpert* instrument and start the test.
- 4 Test results are available after 90 minutes, reported as HIV-1 Detected or HIV-1 Not detected.

Quality control

For *Xpert HIV-1 QUAL*, the WHO prequalification evaluation reported final sensitivities and specificities as:

- For DBS, sensitivity 99.34%, specificity 100%.
- For infant whole blood (< 72h old), sensitivity 98.86%, specificity 100%.

The system incorporates internal controls to ensure adequate processing of the nucleic acids and monitoring of inhibitors in the RT and PCR reactions. A probe check control verifies reagent rehydration, PCR tube filling, probe integrity and dye stability. External quality control includes sending previously tested samples to a Reference laboratory for rechecking.

Power back-up for *GeneXpert* instrument

To avoid tests not being completed due to a power failure, a back-up battery pack is recommended for use with the *GeneXpert* instrument as described in Section 2.11.

m-PIMA HIV-1/2 Detect

m-PIMA HIV-1/2 Detect is manufactured by Alere Technologies GmbH. It is a qualitative nucleic acid amplification test for the detection of HIV-1 groups M/N and O and HIV-2 RNA in fresh capillary blood, EDTA whole blood and plasma samples using RT-PCR. It cannot be used for dried blood spots (DBS). Samples are processed automatically in the *m-PIMA* analyzer with all the reagents contained in the *HIV-1/2 Detect* cartridge (see Plate 2.65).

The *m-PIMA* analyzer is portable (weighing 7.8 Kg), has a touch screen and a built-in battery providing 8 hours running time in the event of a power failure. The cartridge has a shelf-life of 9 months from date of manufacture. It requires storage at 4–30 °C. The test kit includes the analyzer and test cartridges. Not included but available are a printer, power drum, connectivity pack and plastic EDTA capillaries.

Test procedure

- 1 Flip open the HIV-1/2 cartridge cap to expose the sample capillary.
- 2 When using blood from a heel/toe prick apply the sample directly to the cartridge. Sufficient blood is applied when the control window appears full (requires 25 µL of blood).
- 3 When using EDTA capillary or venous blood or plasma, transfer 25 µL of well mixed sample to the cartridge.

Note: EDTA samples can be stored at ambient temperature (18–28 °C) for up to 24 hours following collection (or for longer when frozen at –80 °C).

- 4 Close the cartridge cap. Press Run Test on the analyzer and immediately insert the cartridge. The test starts automatically.

Test results are displayed on the analyzer screen after 52 minutes, reported as HIV-1 M, N and O Detected or Undetected and HIV-2 Detected or Undetected (see Fig. 2.41).

Quality control

For *m-PIMA HIV-1/2 Detect*, the WHO prequalification evaluation (PQDx 0226-032-00) reported a final sensitivity of 99.33% and specificity of 100% for infant samples. Assay process controls include positive and negative hybridization controls and internal process controls for HIV-1 and HIV-2.

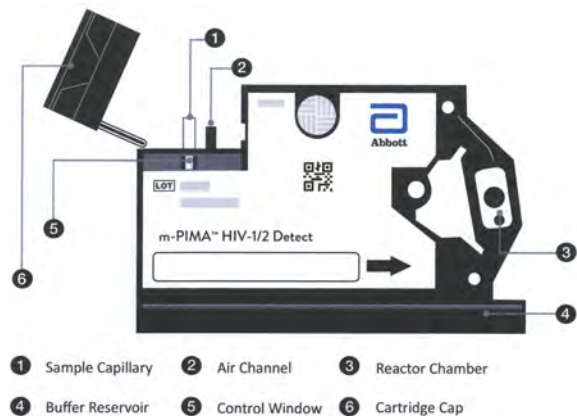


Plate 2.65 Upper: *m-PIMA HIV-1/2 Detect* analyzer. Lower: *m-PIMA HIV-1/2 Detect* cartridge. Courtesy of Abbott

Abbott Test Report

m-PIMA™ HIV-1/2 Detect

Sample ID
23-03-2018-ABC

HIV detected (positive)

HIV-1 M/N	Detected
HIV-1 O	Not detected
HIV-2	Not detected

Result No. 107

Date / Time 2019-07-23 15:50

Cartridge ID 0123456789

Operator SAM MILLER

Device Serial NAT-04000935

Software 0.26.3

QC

Sample Detection	Pass
Device	Pass
HIV-1 Positive Control	Pass
HIV-2 Positive Control	Pass
Negative Control	Pass
Analysis	Pass

Signature

Fig. 2.41 Example of *m-PIMA HIV-1/2 Detect* HIV positive Report.

- Age-appropriate TB symptom screening (see Section 2.11).
- Cryptococcal antigen test for adults and adolescents if the CD4 cell count is at or below 200 cells/mm³.

The following tests are desirable (if feasible):

- Hepatitis B screening (HbsAg, see Section 2.15).
- Hepatitis C screening (HCV antibody test, HCV RNA test, see Section 2.15).
- Screening for sexually transmitted infections for adults (see Section 2.13).
- Assessment for major noncommunicable chronic diseases and comorbidities such as renal disease, liver disease, diabetes mellitus, and opportunistic infections (see end of Section).

Note: Such conditions, and patient medications should be considered before starting ART to assess possible drug interactions, contraindications or dose adjustment.

- Measuring haemoglobin if starting AZT (see pages 232–237).
- Measuring serum creatinine and estimating glomerular filtration rate (eGFR) before starting TDF (see Section 3.3).

Note: Guidance on helping people prepare for ART, dosages for ART drugs, key drug interactions, monitoring ART drug toxicity, drug resistance, and pre-and post prophylaxis can be found in the 2021 WHO *Consolidated guidelines on HIV prevention, testing, treatment, service delivery and monitoring*².

TESTS TO MONITOR ART

WHO recommends lifelong antiretroviral therapy (ART) for all those living with HIV regardless of clinical stage or CD4 cell count. Retesting prior to starting treatment is also recommended to confirm an HIV diagnosis.² Benefits of early effective ART include:

- less severe illness and opportunistic infections caused by disease progression,
- better immune response,
- lower viral load resulting in reduced HIV transmission.

HIV monitoring tests

The following test are recommended by WHO²:

- CD4 cell count to identify advanced HIV disease. ART should not be delayed while waiting for the CD4 test result.

Monitoring ART

Monitoring ART is essential to assess whether:

- treatment is successful,
- patients are adhering to their treatment regime,
- treatment is failing and change to a second-line treatment regimen is indicated,
- drug resistance is developing.

Laboratory monitoring of ART is by:

- Viral load testing which measures quantitatively HIV virus (as viral RNA) in plasma, expressed as the number of virus RNA copies/mL. It is the recommended technology for monitoring ART response and treatment failure.
- CD4 counting which monitors ART immunologically. It has a low sensitivity and low positive predictive value in monitoring treatment failure and should therefore only be used when viral load testing is not possible.

VIRAL LOAD TESTING

Viral load (VL) testing is the most effective way of determining whether ART is suppressing HIV viral replication. It detects treatment failure accurately and at an early stage. VL testing is performed 6 months following the start of ART, at 12 months and then every 12 months if the patient is stable on ART. Treatment failure is defined by a detectable viral load exceeding 1 000 copies/mL in two consecutive viral load measurements within a 3 month interval after at least 6 months of starting a new ART regime (with adherence support).

Point-of-care WHO prequalified technologies include:

- *Xpert HIV-1 Viral load*
- *m-PIMA HIV-1/2 VL*

Xpert HIV-1 Viral load

Xpert HIV-1 Viral load, prequalified by WHO in 2017,¹⁰ is manufactured by Cepheid AB. It measures quantitatively HIV-1 RNA automatically by RT-PCR in plasma using an *Xpert HIV-1 Viral load* cartridge (of similar format to that shown in Plate 2.64 for diagnosing HIV infection in newborn infants) and the same *GeneXpert* instrument. HIV-2 is not detected and DBS samples cannot be used. The shelf-life of the cartridge is 12 months from date of manufacture. It requires storage at 2–28 °C. It is available as a 10 test pack. Several tests can be run together depending on the capacity of the *GeneXpert* instrument.

Test procedure

- 1 Using the 1 mL transfer pipette provided, pipette 1 mL of plasma directly into the test cartridge (contains all the reagents needed for RNA extraction, amplification and detection).

Plasma separated from EDTA whole blood (within 48 h) can be kept at 15–30 °C for up to 24 hours, at 2–8 °C for up to 6 days or frozen (≤ -18 °C and ≤ 70 °C) for up to 6 weeks. Ensure refrigerated plasma samples and thawed frozen samples are allowed to warm to room temperature and mixed prior to testing.

- 2 Insert the cartridge into the *GeneXpert* instrument and start the test.

Test results are available after 92 minutes, reported as HIV-1 RNA Detected copies/mL or HIV-1 RNA Not Detected.

The test quantifies HIV-1 RNA over the range 40–10 000 000 copies/mL.

Quality control

Each *Xpert HIV-1 Viral load* test includes a sample volume adequacy control, an internal quantitative control (high and low) which is also a sample processing control and a probe check control. External quality control includes testing known samples and sending previously tested samples for confirmation of test results to a Reference Laboratory.

m-PIMA HIV-1/2 VL

m-PIMA HIV-1/2 VL, prequalified by WHO in 2019¹¹ is manufactured by Alere Technologies GmbH. It measures quantitatively HIV-1 (groups M/N and O) RNA and HIV-2 RNA automatically in plasma by RT-PCR using an *m-PIMA HIV-1/2 VL* cartridge (of similar format to that shown in Plate 2.65 for diagnosing HIV in newborn infants) and the same *m-PIMA* analyzer. DBS samples cannot be used. The shelf-life of the cartridge is 9 months from date of manufacture. It requires storage at 4–30 °C. It is available as 50 test pack. Tests are run singly.

Test procedure

- 1 Using the sample transfer tool provided, transfer 50 μ L of plasma into the sample capillary of the *m-PIMA HIV-1/2 VL* cartridge. When the correct volume of blood has been added, this is shown in the Sample control window.

Venous EDTA whole blood samples can be held at 18–28 °C for up to 48h. Plasma samples can be kept up to 6 hours at 18–28 °C or up to 26 hours at 2–8 °C. If required, plasma samples can be stored frozen at –80 °C.

The cartridge contains all the reagents needed for sample precessing, amplification and detection.

- 2 Close the cartridge cap. Press Run Test and insert the cartridge.

Test results are available within 70 minutes. Quantitative test results (i.e. copies/mL) are provided separately for HIV-1 group MN, HIV-1 group O and HIV-2.

The test quantifies HIV, RNA over the range 800–1 000 000 copies/mL.

Quality control

Quality control for the *m-PIMA HIV-1/2 VL* includes a sample detection control, internal process controls for HIV-1 and HIV-2 and positive and negative

hybridization controls. External quality control includes testing known samples and sending previously tested samples for confirmation of test results to a Reference Laboratory.

DBS samples for viral load testing

When facilities for VL testing using plasma samples are not available, DBS samples can be collected (as described previously for early infant HIV diagnosis) and sent to a Referral Laboratory for VL testing providing the laboratory is using an analyzer that has a validated protocol for testing DBS samples. VL testing using DBS samples is less sensitive than VL testing using plasma which may result in a false negative test result when an HIV viral load is low.

CD4 CELL COUNTING

When viral load testing is not available, CD4 cell counting and clinical monitoring are recommended for monitoring ART and diagnosing immunological failure. In adults and adolescents, immunological failure is indicated when the CD4 cell count is ≤ 250 cells/mm³ following clinical failure or when CD4 levels are persistently < 100 cells/mm³. For children under 5 y, persistent CD4 levels < 200 cells/mm³, indicate immunological failure (for children > 5 y, below 100 cells/mm³)².

A CD4 cell count is performed at the start of ART to assess immune responses and identify those with advanced HIV disease at risk of opportunistic infections.

When VL testing is available for ART monitoring, WHO suggests CD4 cell counting can be discontinued in patients who are stable on ART and virally suppressed.

An example of a point-of-care cell counter that has been designed for use in tropical countries in places without stable electricity supplies is the *BD FACSPresto Near-Patient CD4 Counter*.

BD FACSPresto Near Patient CD4 Counter

The *BD FACSPresto Counter* is manufactured by Becton, Dickinson and Company,. It was prequalified by WHO in 2014.¹² It is an automated system combining CD4 cell counting (absolute and percentage CD4 cell counts) and measurement of haemoglobin using capillary and EDTA blood samples. The system uses fluorescence imaging and absorbance reading technology to classify and count CD4 T lymphocytes and measure haemoglobin. Test time is approximately 23 minutes with through-put of 10 samples/hour.

The *BD FACSPresto* CD4 cell counter is shown in Plate 2.66. It is a portable instrument (weighing 7 Kg) with a touch screen and a built-in battery which can be recharged from mains electricity, a car battery or from a solar power unit (available as an accessory). The *BD FACSPresto* cartridge (shown in Plate 2.66) contains fluorochrome-conjugated antibody reagents and an integral control in dried form enabling the cartridge to be stored at 4–31 °C. It has a shelf-life from manufacture of 23 months (recently lengthened) and is available as a 100 test pack.

Test procedure

- 1 Add a drop of capillary blood or EDTA venous blood sample into the cartridge inlet port and close the cartridge cap. A minimum of 25 μ L of blood is required.

EDTA venous blood samples can be kept at 20–25 °C for up to 24 hours before testing. Samples refrigerated before staining can give incorrect test results. Do not test haemolyzed samples.

- 2 Leave the cartridge in the workstation on the bench at room temperature (10–40 °C) for 18 minutes. The workstation holds 10 slots individually linked to a timer on the instrument. During this time the sample flows through the reagent disc and into the channel where the fluorescence antibody reagent stains the white cells.
- 3 At the end of 18 minutes, remove the channel protector strip and insert the cartridge in the *BD FACSPresto* instrument. Imaging and analysis take 4 minutes.

Test results can be read from the analyzer screen and printed using the integral printer. The instrument can store up to 12 000 test results.

BD FACSPresto validated range

Absolute CD4 count: 50–4000 cells/ μ L

% CD4 count: 5–60%

Haemoglobin concentration: 2.0–20.0 g/dL

Reference intervals

Reference intervals provided by Becton Dickinson for absolute CD4 cells counts, % CD4 cell counts and haemoglobin using *BD FACSPresto* analyzer are as follows:

		Reference interval
Absolute CD4 cells	Male	462 – 1306 cells/ μ L
	Female	440 – 1602 cells/ μ L
% CD4 cells	Male	29–54%
	Female	32–55%
Haemoglobin	Male	13.5–18.0 g/dL
	Female	12.0–16.0 g/dL

Note: Reference intervals should be established locally.

Quality control

For *BD FACSPresto*, WHO reported a precision of < 4% for CD4 absolute counts in both venous and capillary whole blood.¹² The system performs a power-on-self test to verify system parameters, checking counting accuracy for normal and low counts and reagent performance.

Whenever possible district laboratories should participate in an external quality assessment scheme which includes CD4 counting.

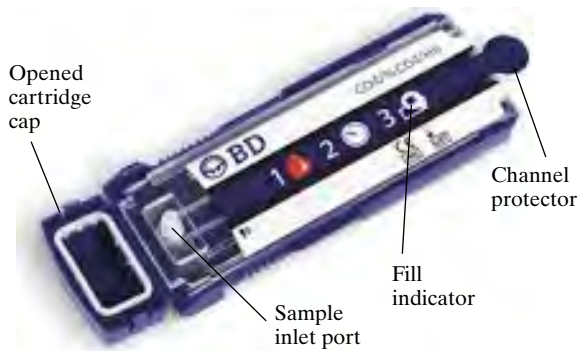


Plate 2.66 Upper: *BD FACSPresto* CD4 analyzer for counting absolute and % CD4 cells and measuring haemoglobin. Lower: *BD FACSPresto* cartridge. Courtesy of Becton Dickinson.

HIV-ASSOCIATED DISEASES OPPORTUNISTIC INFECTIONS

The range of pathogens and diseases associated with HIV infection include those which:

- Increase the risk of acquiring HIV infection, increase viral load, reduce immune responses, cause severe HIV disease, affect ART regimens and treatment outcomes.
- Cause opportunistic infections in those with a low CD4 cell count.

The disease characteristics of opportunistic pathogens may also be affected.

HIV associated diseases and opportunistic infections that can be investigated in district laboratories at point-of-patient-care include:

- Tuberculosis
- Sexually transmitted infections
- Hepatitis B and C
- Cryptococcal meningitis
- Disseminated histoplasmosis
- Leishmaniasis
- *Talaromyces* infection
- Malaria
- Buruli ulcer
- *Cryptosporidium* infection and isosporiasis
- Diabetes mellitus

Tuberculosis

It has been estimated that people living with HIV are 15–22 times more likely to develop active TB than people without HIV. Extrapulmonary TB is common. TB is the leading cause of death among people living with HIV. In 2021 WHO recommended that people living with HIV should be systematically screened for TB at each visit to a health facility². In 2020 a meta-analysis was carried out to review the accuracy of the WHO-recommended four-symptom screen for TB (cough, fever, night sweats and weight loss). It was shown that a C-reactive protein test with a cut-off of >5mg/L among outpatients living with HIV, not yet receiving ART, was as sensitive as the four-symptom screen for TB but significantly more specific (C-reactive protein testing is described on p. 223–224).

The *TB_{LAM} Ag* test can be used in advanced HIV disease to assist in the diagnosis of TB in HIV-positive

patients with a low CD4 cell count (see p. 145–146). An accurate diagnosis of TB in those co-infected with TB requires molecular testing (see p. 144–146).

Sexually transmitted infections

The risk of becoming infected with HIV and transmitting the virus is greatly increased in persons with sexually transmitted infections (STIs), particularly those that cause discharge and genital ulceration such as primary syphilis, genital herpes (HSV-2) and chancroid. A significant risk is also associated with gonorrhoea, chlamydia, bacterial vaginosis and trichomoniasis.

HIV increases the infectiousness and severity of STIs. HIV viral load in genital secretions can be high even when viral load in the blood is low or undetectable. For people infected with HIV, STIs may take longer to treat.

Note: The laboratory diagnosis of syphilis, gonorrhoea, trichomoniasis, urogenital chlamydia, bacterial vaginosis, vaginal candidiasis and donovanosis is described in Section 2.13.

Hepatitis B and C

In those living with HIV, coinfection with hepatitis B (HBV) and hepatitis C (HCV) can be life-threatening. Coinfection is common because the routes of transmission are common for HBV, HCV and HIV.

In HIV/HBV coinfection there is a more rapid progression to cirrhosis and hepatocellular carcinoma particularly in those with a low CD4 cell count. Mortality is increased and treatment response is reduced compared with people who do not have HIV. A hepatitis B vaccine is available.

In HIV/HCV coinfection, HCV-related liver disease progresses more rapidly and treatment responses are often poor. Treatment is also more complicated due to possible interactions between drugs used to treat HCV and HIV and drug toxicities. No vaccine has yet been developed for HCV.

Note: Laboratory tests used to diagnose HBV and HCV are described in Section 2.15. Early testing should be performed in those infected with HIV to delay the progression of liver disease particularly in areas of high HBV and HCV transmission and when there is a high risk of infection.

Further information on testing for chronic HBV and HCV infection, managing coinfections including treatment regimens, and preventing mother to child transmission of HBV, can be found in the 2021 WHO *Consolidated guidelines on HIV*

*prevention, testing, treatment, service delivery and monitoring*².

Cryptococcal meningitis

Cryptococcal meningitis is caused by *Cryptococcus neoformans*. It is a major cause of mortality in advanced HIV disease when the CD4 cell count is below 100 cells/mm³. Early diagnosis and treatment are essential.

Note: The laboratory diagnosis of cryptococcal meningitis is described in Section 2.12.

Additional information on the diagnosis, prevention and management of cryptococcal disease can be found in the 2018 WHO *Guidelines for the diagnosis, prevention and management of cryptococcal disease in HIV-infected adults, adolescents and children*¹³.

Visceral leishmaniasis

Visceral leishmaniasis (VL) is being increasingly reported in those living with HIV with low CD4 cell counts. HIV increases greatly the risk of developing active severe visceral leishmaniasis and atypical infections. Parasites infect not just the reticuloendothelial system but also the lungs, gastrointestinal tract, central nervous system, skin and blood. Both HIV disease and VL cause immunosuppression.

VL/HIV coinfection increases HIV replication and disease progression. Patients do not respond well to VL treatment. Relapses are common. VL infections have been reported as being caused by *Leishmania* strains normally of low virulence. Cases of VL have been described in association with immune reconstitution inflammatory syndrome (see later text) among people living with HIV with latent *Leishmania* infection or among people already treated for VL receiving ART².

Note: The laboratory diagnosis of VL is described in Section 2.3. The results of serological tests need to be interpreted with care as 50-70% of HIV/VI coinfecting patients have negative antibody responses. The specificity of tests is also affected.

Further information on VL/HIV coinfection can be found in the 2022 WHO *Guideline for the treatment of visceral leishmaniasis in HIV co-infected patients in East Africa and South-East Asia*¹⁴.

Malaria

Falciparum malaria is a common coinfection with HIV in tropical countries. In untreated HIV infection,¹⁷ susceptibility to malaria is increased, malaria episodes are more frequent and the progression of HIV infection is accelerated. HIV viral load increases

significantly during a malaria attack. Antimalarial drugs are less effective in HIV coinfection.

During pregnancy HIV immunosuppression in HIV/malaria coinfection, contributes to more frequent and severe anaemia, low birth weight, premature delivery and poor infant survival. The risk of mother-to-child transmission of HIV increases due to increased viral load. In young children, untreated HIV infection increases the frequency of malaria fever, anaemia, severe falciparum malaria and coma.

Note: The microscopical diagnosis of malaria and the use of malaria rapid diagnostic tests are described in Section 2.1.

Further information on HIV/malaria coinfection can be found in the Kwenti T E 2018 paper: *Malaria and HIV coinfection in sub-Saharan Africa: prevalence, impact and treatment strategies*¹⁵.

Buruli ulcer

HIV is common in sub-Saharan Africa where Buruli ulcer is endemic and may increase the risk of Buruli ulcer disease². In patients coinfecting with HIV, Buruli ulcer is often more aggressive, has a poorer outcome, and treatment is often less effective. Lesions tend to be larger and ulcerate more quickly. Multiple lesions are common. HIV also increases the risk of more widespread disease and bone involvement.

Note: Laboratory tests to screen for Buruli ulcer disease and confirm a diagnosis are described in Section 2.9.

Further information on Buruli ulcer HIV coinfection can be found in the 2020 WHO Technical update *Management of Buruli ulcer – HIV coinfection*¹⁶.

Cryptosporidiosis

Cryptosporidium can cause severe, prolonged, life-threatening diarrhoea in persons with untreated HIV infection and a low CD4 cell count (<200 cells/mm³). *Cryptosporidium* coinfection is a major cause of mortality among those with advanced HIV disease. Contributing factors include, unsafe drinking water supplies and poor personal hygiene.

Note: Laboratory tests used to diagnose cryptosporidiosis can be found on pages 214, 215–216.

Further information on HIV/*Cryptosporidium* coinfection can be found in the 2020 paper of Ahmadpour E *et al*: *Cryptosporidiosis in HIV-positive patients and related risk factors: A systematic review and meta-analysis*¹⁸.

Diabetes mellitus

People with HIV infection have been shown to be at increased risk of developing diabetes and lipid abnormalities linked to cardiovascular disease. Older people living with HIV are at greater risk due to complications associated with long-term HIV infection and ART combined with dietary and life-style factors that contribute to the development of diabetes type-2 diabetes and raised LDH cholesterol.

Note: The laboratory diagnosis of diabetes mellitus, measurement of total cholesterol and LDH cholesterol are described in Section 3.2.

Information on HIV, diabetes and high cholesterol can be found in the 2018 paper of Alcorn K: *High cholesterol and diabetes common among people on HIV treatment in Malawi*¹⁸.

Talaromycosis

Talaromyces marneffei is a fungal pathogen found in South-East Asia, causing systemic infection. It is transmitted by inhaling *T. marneffei* spores in the environment. Agricultural workers in rural areas are mostly infected, particularly in the rainy season during times of high humidity.

T. marneffei is an important HIV associated opportunistic pathogen with a high fatality rate in advanced HIV disease with most deaths occurring in patients with a CD4 cell count < 200 cells/mm. Most opportunistic infections cause disseminated disease involving the lungs, gastrointestinal tract, liver, spleen, blood, skin and bone marrow.

Laboratory diagnosis

Occasionally talaromycosis can be diagnosed microscopically by examining Giemsa stained touch

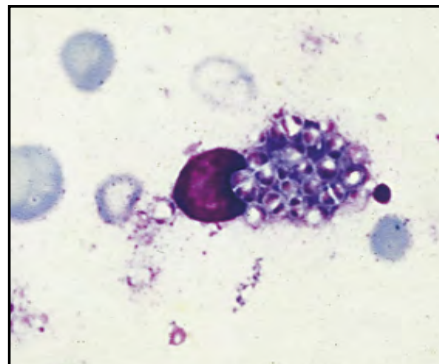


Plate 2.67 *Talaromyces marneffei* yeast cells in a macrophage cell in a Giemsa stained smear. The yeast cells are oval-elliptical with a characteristic dividing line (septation). As seen with 100× objective.

smears of skin, lymph node biopsies or bone marrow for yeast cells in mononuclear cells as shown in Fig. 2.67.

Further information on talaromycosis and HIV can be found in the 2021 paper of Narayanasamy S *et al*: *A global call for talaromycosis to be recognised as a neglected tropical disease* (see Further Reading).

Histoplasmosis

Histoplasmosis is caused by *Histoplasma capsulatum*, a dimorphic fungus with yeast forms in tissues. The disease is endemic in parts of North, Central and South America and is also found in some countries in Africa and Asia. Infection is by inhaling *Histoplasma* spores from damp soil contaminated with excreta from birds and bats, usually when the soil is disturbed.

In people living with HIV, disseminated histoplasmosis occurs in untreated infections when the CD4 cell count falls below 150 cells/mm³. The disease is often difficult to diagnose as the symptoms are non-specific and can resemble other infectious diseases such as disseminated TB. Unless diagnosed and treated early, disseminated histoplasmosis in advanced HIV disease is usually fatal.

Laboratory diagnosis

The recommended method of diagnosing disseminated histoplasmosis in advanced HIV disease is by detecting circulating *Histoplasma* antigen using an enzyme immunoassay. Rapid lateral flow tests are under development which will make it easier to make a diagnosis in district laboratories. Cross reactions with other fungal diseases have been reported. Antibody tests are not useful.

Occasionally disseminated histoplasmosis can be diagnosed by examining Giemsa stained smears of infected tissue for yeast cells in mononuclear or endothelial cells or yeast cells in blood smears as shown in Plate 2.68.

Note: Further information on disseminated histoplasmosis in people living with HIV can be found in the 2020 WHO publication *Guidelines for diagnosing and managing disseminated histoplasmosis among people living with HIV*¹⁹.

Note: The laboratory diagnosis of other opportunistic infections occurring in late stage HIV infection require the facilities of a Reference Laboratory e.g. CNS toxoplasmosis, non-tuberculosis mycobacterial infection, chronic herpes simplex infection, cytomegalovirus infection, lymphoma and Kaposi's sarcoma.

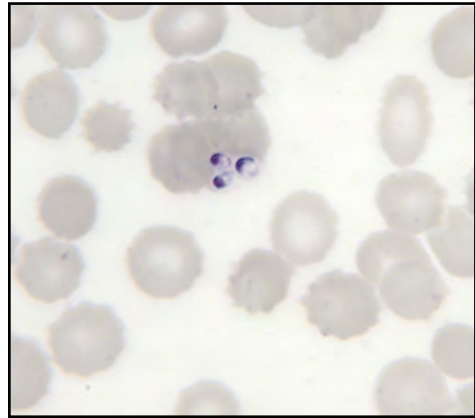


Plate 2.68 *Histoplasma* yeast cells in a Giemsa stained blood film. The yeast cells can be differentiated from *Leishmania* amastigotes by being encapsulated and having no kinetoplast. As seen with the 100× objective.

Immune reconstitution inflammatory syndrome (IRIS)

IRIS is the overwhelming inflammatory immune response to a pathogen which sometimes occurs when a person's immune system begins to recover following treatment with antiretroviral drugs. The condition may be life-threatening. The term paradoxical IRIS refers to the worsening of a previously successful treated infection after ART is started. The term unmasking IRIS refers to the inflammatory response involving a previously undiagnosed opportunistic infection after ART is started. Diseases associated with IRIS include cryptococcal meningitis, tuberculosis, visceral leishmaniasis, *Pneumocystis* pneumonia.

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2.15 Hepatitis

The following viruses are recognized as causing viral hepatitis:

Hepatitis A virus (HAV)
Hepatitis B virus (HBV)
Hepatitis C virus (HCV)
Hepatitis D virus (HDV)
Hepatitis E virus (HEV)

Note: Hepatitis can also occur with other viral and bacterial infections, including yellow fever, Ebola virus disease, Marburg disease, Rift Valley fever, leptospirosis, relapsing fever and infection with herpesviruses HHV-5 (cytomegalovirus) and HHV-4 (Epstein-Barr virus). It can also be caused by alcohol excess, certain hepatotoxic drugs, ingestion of herbal remedies and toxins including aflatoxin (carcinogenic mycotoxin contaminating crops).

Infection with hepatitis viruses can be asymptomatic, chronic or acute. Symptoms of acute infection include jaundice, dark urine, fever, headache, pain in the muscles and joints, nausea, vomiting, extreme fatigue and an enlarged painful liver. Recovery from viral hepatitis is usually slow. Chronic hepatitis and a carrier state may follow infection with HBV, HCV and HDV. Chronic hepatitis can lead to cirrhosis of the liver and liver cancer (see later text).

Infection with one type of hepatitis virus does not give protection against infection with other hepatitis viruses. Safe and effective vaccines are available to prevent HAV, HBV (also protects against HDV) and HEV.

WHO Health Sector Strategy on Viral Hepatitis¹

The 2022 WHO Strategy for viral hepatitis proposes the elimination of chronic HBV and HCV as public health threats by 2030 with the following targets:

- Reducing new HBV infections per year from 1.5 million in 2020 to 170 000 by 2030 and HCV infections from 1.57 million in 2020 to 350 000.
- Reducing HBV deaths per year from 820 000 in 2020 to 310 000 by 2030 and HCV deaths from 290 000 in 2020 to 140 000.
- Increasing the percentage of people being diagnosed with HBV and HCV from 30% in 2020 to 90% by 2030.
- Increasing the number of people being treated for HBV from 30% in 2020 to 80% by 2030 and the number of people with HCV being cured from 30% in 2020 to 80% by 2030.

To achieve these targets requires increased access to hepatitis testing and treatment. Decentralised testing and self-testing is becoming possible with the availability of rapid point-of-care HBV and HCV tests.

Features of hepatitis viruses

<i>Virus</i>	<i>Transmission</i>	<i>Carrier state</i>	<i>Vaccine</i>
HAV	Faecal-oral	No	Yes
HBV	Blood, body fluids close contact, MTC	Yes	Yes
HCV	As for HBV	Yes	No*
HDV	As for HBV	Yes	Yes
HEV	Faecal-oral	No	Yes/No**

MTC: Mother-to-child transmission

* Vaccines are under development.

** A vaccine has been licenced in China.

Notes: HBV is a DNA virus, HAV, HCV, HDV, HEV are RNA viruses.

HDV can only be found in those infected with HBV.

Differentiating hepatitis jaundice from prehepatic and posthepatic jaundice

Clinical chemistry investigations which are helpful in differentiating hepatocellular jaundice caused by viral hepatitis from prehepatic (haemolytic) jaundice and posthepatic (obstructive) jaundice are described in Section 3.4 (Liver function tests).

In hepatitis, serum aminotransferase enzyme activities (ALT and AST) are increased. In the pre-icteric stage of viral hepatitis, urobilinogen can be found in urine and when there is jaundice, serum bilirubin levels are increased and bilirubin is present in the urine. Many viral hepatitis infections however are anicteric (without jaundice) but aminotransferase activity is increased.

Measurement of serum albumin can provide information on the severity of hepatitis.

HEPATITIS A

HAV is a RNA single stranded, non-enveloped picornavirus.

Transmission and distribution

HAV has a worldwide distribution. The virus is excreted in faeces. Infections occur following the ingestion of HAV in contaminated food or water or from faecally contaminated hands in areas where sanitation is inadequate, food and water supplies are unsafe and personal hygiene poor. The virus can also be transmitted by close contact with an infectious person.

Clinical features

HAV does not cause chronic liver disease. In endemic areas most infections occur in childhood, are asymptomatic and confer life-long immunity. In low transmission areas, epidemics are more common and can be prolonged. The incubation period is about 2–4 weeks. Adults usually develop hepatitis with jaundice. The disease is usually self-limiting but relapses can occur. Occasionally it causes acute liver failure which can be life-threatening². There is no carrier state. A vaccine is available to protect against HAV (not licensed for children under 1 year of age). HAV can withstand inactivation by food production processes used to inactivate most bacterial pathogens.

LABORATORY DIAGNOSIS

Hepatitis A can be diagnosed serologically by detecting HAV-specific IgM antibody in serum at the onset of jaundice (not usually performed in district laboratories). It persists for about 10 weeks and may be detectable up to 6 months after the onset of symptoms.

HAV IgG antibody gradually replaces HAV IgM antibody and usually persists for several years. PCR tests have been developed for detecting HAV RNA in faecal samples at the early stage of infection (not performed routinely).

HEPATITIS B

HBV is a double stranded (with single stranded regions) enveloped hepadnavirus. It carries hepatitis B core antigen (HBcAg), surface antigen (HBsAg), secreted protein antigen (HBeAg) and viral DNA.

Transmission and distribution

HBV occurs globally, causing both chronic and acute liver disease. WHO reports that the highest number of people chronically infected with HBV are in the Western Pacific Region (116 million) and the Africa Region (81 million)³.

In endemic areas most hepatitis B is transmitted from mother-to-child during birth and delivery or from an infected infant to another child in the first few years of life following close contact. Other routes of transmission include:

- Sexual transmission particularly among unvaccinated sex workers and people with multiple sex partners.
- Transfusion of infected blood or blood products when donor blood is not screened for HBV.
- Transfer of HBV via wounds or cuts.
- Reuse of HBV contaminated needles, syringes, razors and instruments used in tattooing, acupuncture and tribal ceremonies.
- Needlestick and “sharps” injuries among healthcare workers.

HBV can survive up to 7 days in the environment.

Clinical features

Infection with HBV is usually asymptomatic and clears in about 95% of healthy immunocompetent people. Less commonly infection causes acute disease with jaundice, fatigue, nausea, vomiting and abdominal pain. Acute disease can progress to chronic liver disease with risk of developing cirrhosis, liver failure and hepatocellular cancer. Chronic HBV can be treated with antivirals to suppress HBV replication, reduce liver inflammation and slow progression to cirrhosis and hepatocellular cancer. Most people remain on treatment for life. Patients with chronic infection can become HBV carriers.

HBV can be prevented by hepatitis B vaccination which provides 98–100% protection against HBV infection. Protection lasts for at least 20 years and is probably life-long. WHO recommends that all infants be vaccinated as soon as possible after birth, preferably within 24 hours, followed by 2 or 3 further hepatitis B vaccine doses at least 4 weeks apart³. Antiviral prophylaxis is recommended for the prevention of hepatitis B transmission from mother to child.

HBV-HIV coinfection: About 1% of persons with HBV are also infected with HIV. Coinfection increases progression to cirrhosis and liver cancer particularly in those with a low CD4 cell count. First line

treatment for HIV includes tenofovir which is also active against HBV.

LABORATORY DIAGNOSIS

Serological response in HBV hepatitis

HBsAg: This antigen is present in serum in the incubation stage of infection and detectable at high levels during acute and chronic HBV infection. It falls to undetectable levels during the recovery stage. Persistence of HBsAg for longer than 6 months indicates chronic hepatitis with increased risk of cirrhosis and liver cancer in later life.

HBeAg: This antigen is present during acute and chronic hepatitis B infection when the virus is replicating. Its presence is associated with increased infectivity and greater risk of virus transmission. HBeAg is not produced by variants of HBV. Antibodies to HBeAg form early in infection or seroconversion may be delayed in chronic HBV infection.

Anti-HBc: IgM antibody to hepatitis B core antigen (IgM anti-HBc) is present in acute hepatitis B infection and is a useful marker of recent infection. It becomes negative about 6 months after infection. In most people with chronic infection, IgG anti-HBc replaces IgM anti-HBc. In the absence of IgM anti-HBc the presence of IgG anti-HBc indicates past infection.

Anti-HBs: Antibody to HBsAg is the last serological marker to form, appearing in the convalescence stage. It indicates recent infection or past immunization. Following immunization, only anti-HBs is present.

Acute and chronic hepatitis B can be diagnosed by detecting HBsAg qualitatively in serum, plasma or whole blood.

Testing for chronic HBV infection⁴

WHO recommends HBsAg testing (linked to prevention, care and treatment services):

- when chronic HBV infection is suspected clinically,
- for pregnant women,
- for adults and adolescents from high HBV seroprevalent areas or who have a history of exposure and/or high risk behaviours for HBV infection,
- for sexual partners, children and other family members and close contacts of those with HBV infection,
- for healthcare workers, with hepatitis B vaccination offered to those not previously vaccinated.

A rapid HBsAg test is most frequently used to detect HBsAg qualitatively in serum, plasma, or whole blood. In blood transfusion centres, donor blood is screened for HBsAg using an enzyme immunoassay (ELISA) or rapid test depending on test volume, available resources and whether donors are screened prior to donation. Donors testing positive

for HBsAg should have access to prevention, care and treatment services.

Most rapid HBsAg tests are IC lateral flow tests that do not require refrigerated transport. Recently developed rapid tests have high sensitivity and specificity. An example of an IC rapid test prequalified by WHO in 2018 for the detection of circulating HBsAg is *VIKIA HBsAg*.⁵

Note: Also prequalified by WHO in 2017 for the detection of HBsAg is the rapid test *SD BIOLINE HBsAg WB* manufactured by Standard Diagnostics Inc.

VIKIA HBsAg

VIKIA HBsAg is manufactured by bioMérieux SA. It is a cassette IC lateral flow test using monoclonal and polyclonal antibodies specific to HBsAg. It is validated for detecting the main subtypes ad and ay in serum, plasma, or whole blood using a fingerstick or EDTA sample. Test time is 30 minutes.

The shelf-life of *VIKIA HBsAg* is 25 months from date of manufacture. It requires storage at 4–30 °C. The test is available as a 25 test pack. Each kit contains individually sealed test cassettes with pipettes and dropper bottle containing phosphate buffer. The sensitivity of the test compared to ELISA using whole blood is reported as 98.92% and for serum or plasma as 99.05%.⁵ Specificity of the test has been reported for whole blood as 99.79% and for serum or plasma as 99.80%.⁵

Test procedure

1 Dispense 75 µL of fingerstick or EDTA whole blood or 3 drops of serum or plasma (using the pipette provided) into the sample well (S).

Serum and plasma samples can be kept at 15–37 °C for up to 4 hours or stored at 2–8 °C for up to 5 days.

Caution: Hepatitis viruses are *highly infectious*, particularly HBV which is 50–100 times more infectious than HIV. Handle samples with care and minimize procedures which create aerosols.

2 Add 1 drop of buffer.

3 Read test results after 30 minutes. Do not read the tests before 30 minutes or after 60 minutes.

Test results

A positive HBsAg test is shown by a pink band in the Test (T) area of the viewing window and a blue band in the Control (C) area.

A negative HBsAg test is shown by a blue band in Control (C) area only.

An invalid test is shown by no blue band in the Control area.



Plate 2.69 Positive and negative *VIKIA HBsAg* test results. Courtesy of bioMérieux SA www.biomerieux-diagnostics.com

Positive and negative *VIKIA HBsAg* test results are shown in Plate 2.69.

Limitations of VIKIA HBsAg

A false negative test (of particular concern when screening donor blood) can occur when:

- The concentration of HBsAg in the sample is below the detection limit of the test, e.g. in the early stage and recovery stage of acute HBV infection and during asymptomatic chronic infection.

In the window period, IgM antibody to core antigen (anti- HBC) can be detected.

- The sample contains an HBsAg variant (occult HBV infection) not detected by the antibodies used in the test.

Molecular tests to detect HBV DNA can be used to detect HBsAg variants (in laboratories with facilities for nucleic acid testing).

- The sample contains both antigen HBsAg and antibody anti-HBs, forming undetectable antigen-antibody complexes.

Note: Greater coverage of recently developed effective recombinant HBV vaccines has the potential to reduce the number of blood donors with transmissible HBV.

Quality control

Use only reliable tests, e.g. those that have been WHO prequalified. Ensure validated SOPs are available and adequate training is provided on how to perform tests and interpret test results.

New batches of test kits should be checked using known positive and negative control samples. Do not

open sealed tests before they are ready to be used. Follow exactly the manufacturer's test procedure particularly when to read test results. Do not use test kits beyond their expiry date. Store test kits correctly.

Whenever possible, participate in an external quality assessment scheme which includes hepatitis testing.

ELISAs to detect HBsAg

ELISAs that have been prequalified by WHO (up to 2019) to screen donor blood for HBsAg include:

- *bioelisa HBsAG 3.0* manufactured by Biokit SA, prequalified in 2016.
- *DS-EIA-HBsAg-0,01* manufactured by RPC Diagnostic Systems, prequalified in 2016.
- *Murex HBsAg Version 3*, with *Murex HBsAg Confirmatory Version 3*, manufactured by DiaSorin S.p.A., prequalified in 2014.

Non-invasive assessment of liver fibrosis

In chronic hepatitis, liver cell damage can occur with fibrosis which can lead to cirrhosis. In district laboratories liver fibrosis can be assessed by using the aminotransferase (AST)-to-platelet ratio index (APRI). AST levels rise and platelet numbers decrease with the progression of liver disease. The APRI is also helpful in assessing the choice and duration of therapy.

The APRI is calculated by dividing the AST in IU/L by the AST upper limit of normal (ULN) usually set at 40 or 42 IU/L, multiplying the result by 100 and dividing by the platelet count.

$$\text{APRI} = \frac{\text{AST (IU/L)}}{\text{Platelet count (10}^9\text{/L)}} \times 100$$

Interpretation of APRI

An APRI score of less than 0.5 indicates little or no fibrosis.

An APRI score greater than 1.5 is an indicator of cirrhosis.

Studies have shown that an APRI score greater than 0.7 has a sensitivity of 77% and specificity of 72% for predicting significant fibrosis.

Note: The measurement of AST is described in Section 3.4 and counting platelets in Section 3.1.

HEPATITIS C

HCV is a RNA single stranded enveloped flavivirus. It shows considerable genomic variation with at least six main genotypes and many subgenotypes which vary in their geographical distribution and influence treatment regimens. HCV genotypes 1–3 are distributed worldwide, genotype 4 is prevalent in central and west Africa, north Africa and the Middle East, genotype 5 is found in South Africa and genotype 6 in Hong Kong.

Transmission and distribution

HCV causes both chronic and acute liver disease. Globally 58 million people are estimated to be chronically infected with HCV with about 1.5 million new infections each year. WHO estimates that in the Eastern Mediterranean Region and European Region there are 12 million people in each Region chronically infected with HCV⁶. In South-East Asia and Western Pacific there are 10 million chronic infections in each region, 9 million in the Africa Region and 5 million in the Region of the Americas⁶.

Hepatitis C is a blood borne virus with most HCV infections occurring in tropical countries by using contaminated needles during injection procedures and by transfusing unscreened blood and blood products. Transmission can also occur among those who inject drugs and also during tribal ceremonies, tattooing, body-piecing, scarification and circumcision. Other less common routes of HCV transmission include mother-to-child transmission (HCV cannot be transmitted in breast milk), and transmission among men who have sex with men with increased risk among those coinfecting with HIV.

Clinical features

The incubation period for HCV is usually 2–8 weeks but can be up to 6 months. Infection is asymptomatic in about 80% of those infected and often only diagnosed at a late stage when there are symptoms of liver disease. Acute infection can cause jaundice, fever, fatigue, nausea, vomiting, abdominal pain and joint pain.

In 15–45% of infected persons, clearance of acute HCV infection usually occurs within 6 months. In 55–85% of persons, HCV persists after 6 months causing chronic hepatitis C. Left untreated chronic HCV infection can lead to liver cirrhosis, liver failure and liver cancer developing in later life, accelerated in those coinfecting with HIV. Antiviral drugs can cure more than 95% HCV infections. In the absence of an HCV vaccine, measures to prevent HCV infection are particularly important.

Note: Information on interventions to prevent HCV infection and the care and treatment of those with hepatitis C can be found in the 2022 WHO *Hepatitis C Fact sheet*.⁶

LABORATORY DIAGNOSIS

The diagnosis of acute and chronic HCV infection and screening of blood for transmissible infection is by:

- Detecting antibodies to HCV to identify people who have been infected with HCV.
- Confirming active chronic infection requiring treatment by testing for HCV RNA.

A positive HCV antibody test should be followed automatically by an HCV RNA test using the previously collected sample or new capillary blood sample.

WHO HCV testing recommendation⁷

To provide greater access to HCV testing and provide same day diagnosis and treatment, WHO recommends decentralising and integrating HCV testing in peripheral health or community-based facilities using rapid point-of-care tests and trained non-specialist staff. Ideally HCV antibody testing followed by an HCV RNA test should be performed at the same site.

HCV self-testing is recommended by WHO as an additional approach to HCV testing services, requiring linkage to facilities that can confirm active infection and provide treatment, care and support⁸.

As in chronic HBV infection, it can also be helpful in chronic HCV to assess liver cell damage by calculating the AST-to-platelet ratio index, i.e. APRI (see previous text).

HCV antibody testing

HCV antibody testing does not distinguish between IgM and IgG antibody and does not distinguish between acute, chronic or resolved past infection. WHO recommends that HIV antibody screening linked to care and treatment services be offered to individuals with suspected chronic HCV infection or who are part of a population with high HCV seroprevalence or have a history of exposure and/or high risk behaviour for HCV infection.

In acute hepatitis C, HCV antibody can usually be detected 6–24 weeks following exposure to infection. Antibodies persist throughout life and can be detected following recovery and in chronic HCV infection. They do not protect against reinfection. In HIV coinfection, antibody levels may be low or undetectable in those with immunosuppression (untreated HIV infection). HCV RNA can usually be detected.

In blood transfusion centres, donor blood is anti-HCV screened using a rapid test when donors are tested individually prior to donation or by ELISA when screening blood in batches after it has been donated.

An example of a rapid HCV antibody test prequalified by WHO in 2019 is *Rapid Anti-HCV Test*.⁹

Note: Other WHO prequalified HCV rapid tests that have been shown to perform well include *Bioline HCV* and *Ora-Quick HCV Rapid Antibody Test*.

Rapid Anti-HCV Test

Rapid Anti-HCV Test is manufactured by InTec Products. It is a lateral flow IC test that uses recombinant HCV antigen (containing core, NS2, NS3, NS4, NS5 segments) to detect qualitatively HCV antibody in fingerstick whole blood, serum or plasma (EDTA, heparin or sodium citrate whole blood). The test is validated to test genotypes 1–6. Test time is 15 minutes.

The shelf-life of *Rapid Anti-HCV Test* is 24 months from date of manufacture. It requires storage at 2–30 °C. The test is available as a 25 or 40 test kit. Test kit Code ITPWO1153-TC40 (for 40 tests) contains individually sealed test cassettes, sample diluent (2 bottles), sample dropper, sterile safety lancets and alcohol swabs. The final sensitivity of the test has been reported by WHO as 100% and specificity as 99.7%.⁹

Test procedure

- 1 Dispense 1 drop of free-flowing capillary blood into the Sample port (S) using the sample dropper provided or 10 µL of serum or plasma using a micropipette.

Note: When not tested immediately, serum or plasma can be stored at 2–8 °C for up to 7 days. Store at –18 °C for long term storage.

- 2 Holding the sample diluent bottle vertically, dispense 2 drops of diluent into the Diluent port (D).

Note: The sample diluent should be used within 8 weeks after opening.

- 3 Read the test results between 15–20 minutes (not after 20 minutes).

Positive Anti-HCV Tests

Negative Anti-HCV Test



Plate 2.70 Test results for InTec *Anti-HCV Test*.
Courtesy of InTec Products.

Test results

A positive *Rapid Anti-HCV Test* is shown by a purple-pink band in both the Test (T) area and Control (C) area of the viewing window. Even a faint coloured band in the Test area indicates a positive test. The intensity of colour of the Test band does not necessarily correlate with the antibody level.

A negative *Rapid Anti-HCV Test* is shown by a purple-pink band in the Control area only.

Positive and negative *Rapid Anti-HCV* test results are shown in Plate 2.70.

Quality control: As described previously for HBV testing.

Rapid Anti-HCV Test video

An excellent YouTube video is available showing how to perform *Rapid Anti-HCV Test*. Access by entering in Google, "InTec HCV Rapid test instruction YouTube".

Limitations of Anti-HCV Test

A negative *Anti-HCV Test* does not exclude the possibility of HCV infection. A negative test result can occur as a result of:

- Levels of HCV antibody below the detection limit of the test, e.g. seroconversion ("window" period) or patient with HIV immunosuppression.
- Sample contains antibodies that do not react with the specific antigens used in the test.
- Sample contains genotype(s) not validated for the test e.g. genotype 7.

Note: Incorrect test results may occur with samples that are haemolyzed, hyperlipaemic, highly icteric or contain rheumatoid factors.

ELISAs to detect HCV antibody

ELISAs that have been prequalified by WHO (up to 2019) to detect anti-HCV in donor blood include:

- *INNO-LIA HCV Score*, manufactured by Fujirebio Europe NV, prequalified in 2015.
- *Murex anti-HCV (version 4.0)*, manufactured by DiaSorin South Africa (pty), prequalified in 2015.
- *Bioelisa HCV 4.0*, manufactured by Biokit S.A, prequalified in 2015.

Identification of active HCV infection

Following detection of HCV antibody, further testing is required to differentiate between persons who are antibody positive but no longer infected with HCV and those with active infection requiring treatment. Active infection can be diagnosed by detecting HCV RNA or HCV core (p22) antigen in blood samples. Where available, detecting and quantifying HCV RNA using nucleic acid testing is the preferred test.

Dried blood spots (DBS) can be used when the equipment is available which has a validated method for processing DBS.

Point-of-care HCV RNA test

A point-of-care test has been developed by Cepheid and FIND to detect and quantify HCV RNA in finger-stick blood samples. The test is called *Xpert HCV VL Fingerstick*. It uses an *Xpert HCV VL* cartridge (see Plate 2.71) and a *GeneXpert* instrument the same as that used for diagnosing tuberculosis described in Section 2.11.

Xpert HCV VL automatically detects and quantifies HCV RNA by RT-PCR from 100 µL of capillary blood. The sample is dispensed directly into the cartridge chamber. No pretreatment of the sample is required. Test results are available within 1 hour. The cartridge does not require refrigerated transport or storage. The sensitivity and specificity of *Xpert HCV VL Fingerstick* have been reported as 100%.¹⁰ WHO prequalification is pending.



Plate 2.71 *Xpert HCV VL Fingerstick* cartridge. Courtesy of Cepheid www.cepheidinternational.com

HEPATITIS D

HDV is an incomplete RNA single stranded non-enveloped delta virus. HDV depends on the surface antigen of HBV (HBsAg) to replicate and is therefore only found in those infected with HBV.

Transmission and distribution

HDV is only found among HbsAg positive people with an estimated prevalence of 4.5%. In a 2020 study, 12 million people were estimated to have been infected with HDV¹¹. Prevalence among HbsAg positive people was found to be highest in Mongolia, the Republic of Moldova and countries in Western and Middle Africa. High infection rates were

found among those who inject drugs, haemodialysis recipients, men who have sex with men, commercial sex workers and those with HCV or HIV¹¹.

Clinical features

Chronic HBV carriers and people who are not immune to HBV are at risk of infection with HDV. HDV infection can occur as an acute coinfection when a person becomes infected with HBV and HDV at the same time. This can lead to mild to severe hepatitis and is usually self-limiting. HDV coinfection is prevented by HBV vaccination. As HBV immunization coverage increases, fewer HDV infections are being reported..

When HDV infects people with existing chronic HBV, this can cause a superinfection which can lead to severe disease. In 70–90% of people, superinfection causes rapid progression to cirrhosis and an increased risk of hepatocellular cancer. Among HbsAg positive people, HDV causes an estimated 18% of cirrhosis and 20% of hepatocellular cancer¹¹. HBV immunization does not protect against HDV in those already infected with HBV.

Note: Further information on the prevention and treatment of HDV infection can be found in the WHO 2022 hepatitis D Fact sheet¹².

LABORATORY DIAGNOSIS

HDV can be diagnosed by detecting IgG and IgM antibody in serum by immunoassay. A positive IgM test indicates ongoing viral replication. Active HDV infection is confirmed by detecting HDV RNA in serum by PCR. HDV antibody tests and HDV RNA tests are usually available only in Public Health Reference Laboratories.

HEPATITIS E

HEV is a RNA single stranded non-enveloped hepevirus. There are at least four genotypes: G1-G4. Genotypes 1 and 2 are human viruses and genotypes 3 and 4 are zoonotic with pigs being the main reservoirs.

Transmission and distribution

HEV is transmitted by the faecal-oral route with most infections (genotypes 1 and 2) occurring via faecally contaminated water supplies due to poor sanitation and lack of safe water supplies. Epidemics frequently occur following flooding during the rainy season. Epidemics are likely to increase due to climate change.

In recent years serious waterborne HEV outbreaks due to genotype 1 have occurred in refugee camps, emergency situations and in conflict areas due to lack of clean water and adequate sanitation. Less commonly, HEV is transmitted by transfusion of infected blood, by mother-to-child transmission, or by ingesting HEV in uncooked or undercooked meat or meat products, e.g. pork liver (genotypes 3 and 4).

HEV has a worldwide distribution with most infections occurring in east and south Asia with seroprevalence estimated at 17–42% in South-East Asia and above 30% among adults in India, Bangladesh, China and Malaysia¹³.

Clinical features

The incubation period for HEV is 2-10 weeks. In endemic areas, most infections occur in young people with symptoms of mild hepatitis and resolve in 1–6 weeks in persons with normal immune responses. It is estimated 20 million HEV infections occur annually with 3.4 million symptomatic infections¹⁰.

Occasionally HEV infection causes severe disease with liver failure. Chronic HEV infection is reported in people with immunosuppression, following organ transplantation, treatment with immunosuppressive drugs and infection with HEV genotypes 3 or 4¹⁴.

HEV infection in pregnant women, particularly in the second or third trimester, can be severe and life-threatening (20–25% mortality rate) with liver failure and excessive bleeding. Miscarriage, premature delivery and still birth can occur.

In 2022, the *Hecolin* (Chinese developed) HEV vaccine was used successfully to control an outbreak of hepatitis E in a displaced persons camp in South Sudan, vaccinating around 25 000 people, including pregnant women¹⁵.

Note: Further information on HEV can be found in the WHO 2022 Hepatitis E Fact Sheet¹⁴ and management of epidemics can be found in the WHO publication: *Waterborne outbreaks of hepatitis E: recognition, investigation and control*¹⁶.

LABORATORY DIAGNOSIS

HEV infection can be diagnosed by detecting specific IgM HEV antibodies in serum, plasma or whole blood about 2–6 weeks following infection using ELISA or a rapid HEV IgM rapid test. There are currently no WHO prequalified HEV IgM rapid antibody tests. In low prevalence areas, HEV RNA tests are used to diagnose chronic infections.

HEV IgM Rapid Test

An example of a rapid test to detect IgM antibodies in HEV infection is *HEV IgM Rapid Test* manufactured by Beijing Wantai Biological. It is an IC lateral flow cassette test that detects qualitatively anti-HEV in serum, plasma or whole blood. The test has an 18 month shelf-life from date of manufacture and requires storage at 2–8 °C. It is available as a 10 test kit. Each test kit contains individually sealed test cassettes, sample dilution tubes and sample dispensers. Test time is 5–10 minutes.

The sensitivity of *HEV IgM Rapid Test* in immunocompetent patients has been reported as 90.0% and in immunocompromised patients as 73.3%.¹⁷ Specificity has been reported as high.

Test procedure

- 1 Using the sample dispenser provided, add 1 drop (approximately 15 µL) of whole blood into the sample dilution tube provided and mix completely.
- 2 Dispense 80 µL of the diluted sample into the Sample well of the test cassette.
- 3 Read the test result after 5–10 minutes (do not read after 10 minutes).

Test results

A positive *HEV IgM Rapid Test* is shown by a mauve-pink band in the Test zone (T) of the viewing window and a mauve-pink band in the Control zone (C).

A negative *HEV IgM Rapid Test* is shown by a mauve-pink band in the Control zone (C) only.

An invalid test is indicated when there is no mauve-pink band in the Control zone.

Quality control: As described previously for HBV testing.

Limitations of test

- The intensity of colour of the Test band cannot be used to evaluate HEV IgM antibody levels.
- A false negative test result can occur when the antibody level is below the detection limit of the test, e.g. during the seroconversion period.

Note: When possible a negative *HEV IgM Rapid Test* should be further tested for HEV infection by using a molecular test to detect HEV RNA (requiring the facilities of a laboratory with nucleic acid testing facilities).

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2.16 Water and sanitation related infections

Safe water sanitation and hygiene (WASH) facilities are essential for good health, wellbeing, social and economic growth. Each year, WHO estimates that 829 000 people in low- and middle-income countries die as a result of inadequate water, sanitation and hygiene (WASH)¹.

Adequate safe water supplies are required in the home for drinking, cooking, hand washing, bathing, cleaning and laundering. Contaminated drinking water can cause water-borne infections (see later text). Not only must water be free from microbial contamination and chemicals that affect health, it should also be accessible at all times and meet local standards for taste, odour and appearance.

Water not being available at all times can lead to water being collected from polluted sources and sites where disease vectors breed. Hygienic toilets and safe management of waste disposal are required to prevent faecal contamination of water supplies. Young children lacking access to WASH facilities are frequently undernourished, have impaired cognitive function and poor school attendance records due to diarrhoeal disease. Lack of WASH services in



Plate 2.72 Cynthia, 6 years old with classmates washing hands at the handwashing station of their primary school in Mahavoky village, Belavabary commune, Moramanga district, Alaotra, Mangoro region, Madagascar, May 2017.
Photographer: Ernest Randriarimalala.
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healthcare facilities increase the risk of healthcare associated infections and disease to patients and staff.

Climate change causing unpredictable weather and extreme weather events is having an important effect on WASH. Access to water is becoming more difficult and costly, and more people are using water sources that are unsafe. Low rainfall and drought cause boreholes and springs to run dry while heavy rainfall can pollute wells and carry run-off and waste into community water supplies. Flooding increases the risk of water-borne diseases due to water supplies becoming contaminated, particularly when water points and latrines are not raised above flood levels. Salt water contamination from rising sea levels is increasing the salinity of ground water sources, making water undrinkable.

Water conservation, rain water harvesting, protecting water supplies, preventing sewage spills, filtering drinking water, replacing diesel powered water systems with solar-powered systems and the efficient management of WASH services can help to combat the effects of climate change.

United Nations Water and Sanitation Sustainable Development Goal 6

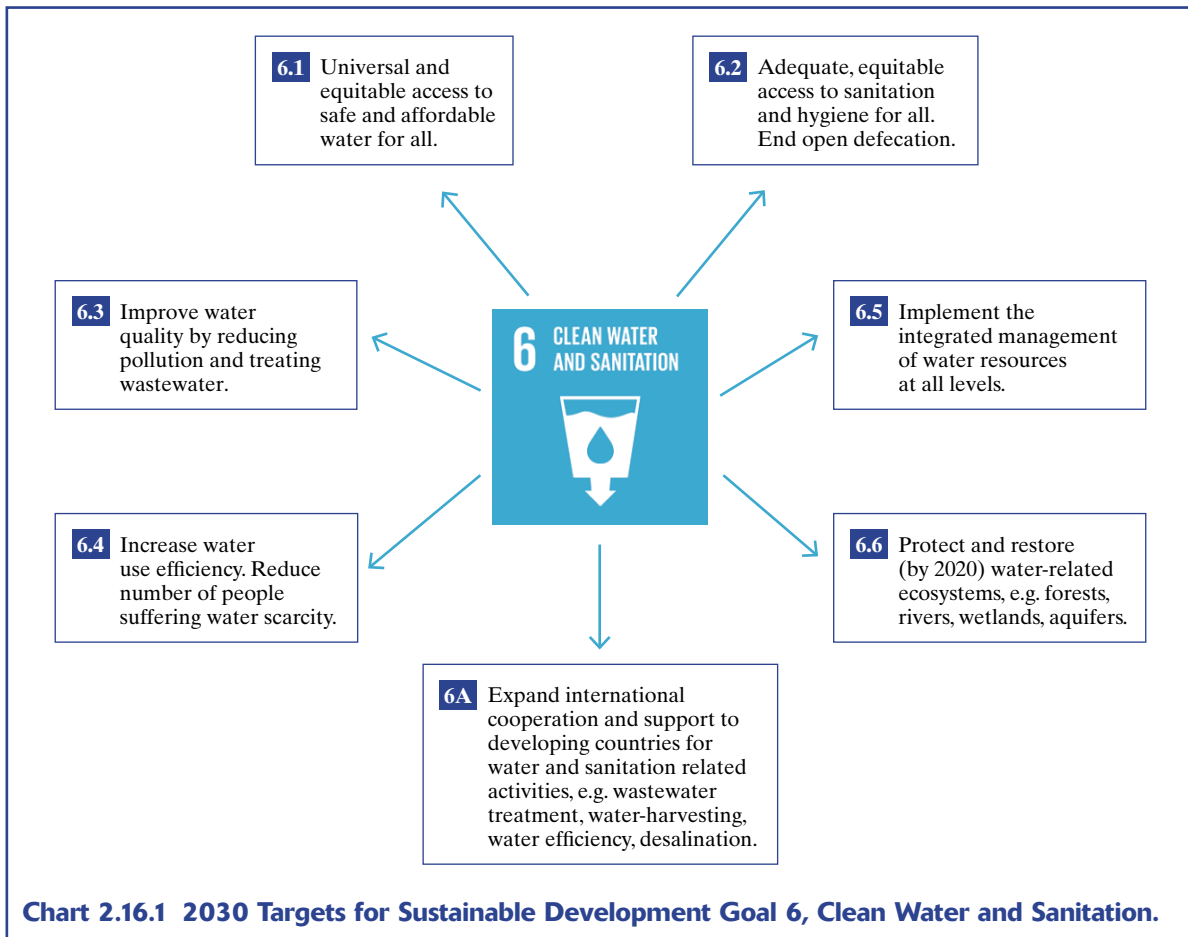
Ensuring availability and sustainable management of water and sanitation is one of the United Nations Sustainable Development Goals (SDG 6)² the targets of which include:

- By 2030, achieve universal and equitable access to safe and affordable drinking water for all.
- By 2030, achieve access to adequate and equitable sanitation and hygiene for all and end open defecation, paying special attention to the needs of women and girls and those in vulnerable situations including the elderly, refugees and those with disabilities.
- By 2030, improve water quality by reducing pollution, eliminating dumping and minimizing the release of hazardous chemicals and materials, halving the proportion of untreated wastewater and substantially increasing recycling and safe reuse globally.

Other targets addressed by SDG 6: Water scarcity, management of water resources, protection and restoration of water-related ecosystems, community participation in improving water harvesting and desalination. Further information can be found on website:

<https://sustainabledevelopment.un.org/sdg6>

SDG 6 is closely linked to other SDGs, particularly, SDG 3 to ensure healthy lives and promote well-being for all ages, with one of the targets being combating water-borne diseases.



Transmission of water and sanitation related infections

There are four main routes by which water and sanitation related infections are transmitted:

- **Water-borne route** in which humans become infected by ingesting pathogenic bacteria, viruses, or parasites in water polluted by human or animal faeces or urine.

Examples of water-borne diseases

<i>Bacterial</i>	<i>Parasitic</i>
Cholera	Amoebic dysentery
Bacillary dysentery (shigellosis)	Cryptosporidiosis
Typhoid	Giardiasis
Paratyphoid	Balantidiasis
Salmonellosis	<i>Viral</i>
<i>Campylobacter</i> enteritis	Rotavirus diarrhoea
<i>E. coli</i> diarrhoea	Hepatitis A and E
Leptospirosis	Poliomyelitis

All water-borne diseases can also be transmitted by other faecal-oral routes, e.g. ingestion of faecal contaminated food. Control is by preventing water supplies becoming contaminated by human or

animal faeces or raw sewage entering a water supply, e.g. during times of flooding.

- **Water-washed route** in which disease is associated with a scarcity of water for personal hygiene (bathing, handwashing), laundering clothes, cleaning of cooking utensils.

Examples of water-washed diseases

<i>Skin diseases</i>	<i>Louse-borne</i>
Scabies	Typhus
Yaws	Relapsing fever
Impetigo	<i>Faecal-oral diseases</i>
Skin ulcers	See list under Water-borne diseases
<i>Eye diseases</i>	
Trachoma	
Conjunctivitis	

- **Water-based route** which involves parasites that require a snail host, fish, or other aquatic animal in which to develop. Humans become infected by ingesting the infective forms or by the infective forms penetrating the skin.

Examples of water-based parasitic infections*Infective forms penetrate skin*

Schistosomiasis (cercariae released from snail, penetrate skin).

Infective forms ingested

Dracunculiasis (larvae ingested in crustacean).

Paragonimiasis (metacercariae ingested in crab or crayfish).

Clonorchiasis (metacercariae ingested in fish).

- *Insect vector route* in which humans become infected by being bitten by an insect vector which breeds in or around water.

Examples of water-related insect-transmitted infections*Mosquito-borne* *Other insects*

Malaria Trypanosomiasis (riverine tsetse flies)

Filariasis Onchocerciasis (blackflies)

Dengue

Yellow fever

Importance of WASH and neglected tropical diseases

Neglected tropical diseases (NTDs) are particularly prevalent when sanitation and hygiene are poor and water supplies are unsafe.² The importance of WASH in the prevention, control and care of NTDs is described in a 2019 WHO publication: *WASH and health working together. A how-to guide for Neglected Tropical Disease programmes* (pages 74–82 Tools and resources). Each of the 20 NTDs* are described in Chart form with the WASH aspects highlighted. The publication can be downloaded from:

<https://www.who.int/publications/i/item/9789241515009>

* **NTDs included in the above publication:** Buruli ulcer, Chagas disease, chromoblastomycosis, dengue, dracunculiasis, echinococcosis/hydatidosis, endemic treponematoses, food-borne trematode infections, human African trypanosomiasis, leishmaniasis (visceral/cutaneous), leprosy, lymphatic filariasis, onchocerciasis, rabies, scabies, schistosomiasis, snakebite envenoming, soil-transmitted helminthiasis, taeniasis/cysticercosis, trachoma.

LABORATORY INVESTIGATION OF WATER AND SANITATION RELATED INFECTIONS

The laboratory investigation of water and sanitation related infections includes:

- A sanitary inspection of an installation or water supply system.
- Water quality analysis.

SANITARY INSPECTION

A sanitary inspection is performed to:

- identify potential risks of contamination and any source of pollution,
- recommend appropriate remedial measures.

What is included in the sanitary inspection will depend on the type of system or installation, e.g. a well, borehole, spring or piped water supply. Appropriate, easy to understand forms should be used to help those carrying out the inspection and those responsible for deciding what action to take. The following is an example of a sanitary inspection form for a distribution network.

Example of a sanitary inspection form for a distribution network

Community _____ Department _____

Date ____ / ____ / ____ Water sample result _____

- | | |
|--|--------|
| 1 Is there any point of leakage between source and reservoir? | Yes/No |
| 2 If there are any, are their covers insanitary? | Yes/No |

If there is a reservoir:

- | | |
|--|--------|
| 3 Is the inspection cover or roof insanitary? | Yes/No |
| 4 Are any air vents insanitary? | Yes/No |
| 5 Is the reservoir cracked or leaking? | Yes/No |
| 6 Are there any leaks in the distribution network? | Yes/No |
| 7 Is there any sanitation within 10 metres of the pipeline (e.g. latrines, sewers, septic tanks or burial grounds?) | Yes/No |
| 8 Does water accumulate near tap-stands? | Yes/No |
| 9 Is the plinth cracked or eroded? | Yes/No |
| 10 Does the tap leak? | Yes/No |

Sanitary risk score _____/10

All the 'Yes' answers are added up to give a sanitary risk score out of 10

Recommendations: _____

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Note: A sanitary inspection cannot identify all sources of contamination (e.g. leaking pipes or well-linings), but it can help to identify the source(s) and point of entry of contamination which might be missed by water quality analysis alone. It can help

to identify potential problems before they cause contamination, often preventing the need for costly remedial work at a later stage. An inspection can check the proximity of a water supply to sources of pollution, e.g. latrines or refuse collection points. The importance of inspecting a water supply is illustrated in the following examples.

Examples

- Water from a well supplying a small community was shown to be heavily contaminated. When the site was inspected the sources of the contamination were obvious. The well was badly protected, with pigs, chickens and donkeys allowed around the well. There was no handpump. A bucket and rope used to collect the water was left on the muddy ground and spilt water seeped back into the well taking with it surface contamination. There was no concrete slab around the well (sloping away from it).
- A village is supplied with water from a large distribution network. The water coming from the outside taps of the houses was found to be of excellent quality, but many children had diarrhoeal disease. Investigation of the water supply found that each morning the water source was being diverted, cutting off the supply to villagers' homes. To ensure water was available, each evening the villagers collected the water in open containers. From these, animals were allowed to drink and scoops used to transfer the water were left on the ground. The water became contaminated and children and sometimes adults drinking from this stored contaminated water became sick.

WATER QUALITY ANALYSIS

Although water can contain unwanted chemicals (from natural sources, industrial and agricultural activities), the greatest risk to human health is from faecal contamination of water supplies. Serious ill-health can be caused by water becoming contaminated from faeces being passed or washed into rivers, streams or pools or being allowed to seep into wells or boreholes. The most important aspect of analysis is therefore to determine whether faecal contamination is present.

A bacteriological analysis of water can confirm whether a water supply has been faecally contaminated. In piped water distribution systems, a sanitary inspection will often not detect problems occurring during distribution, e.g. pipes buried underground might be damaged allowing in pollution. Analysis is also used to check the effectiveness of disinfection processes. It is a useful way of keeping communities interested in their water supplies and justifying requests to health authorities for improvements in water quality.

Collection of samples for water analysis

Samples of water for bacteriological testing must be collected in sterile bottles and care *must* be taken to prevent accidental contamination of the water during its collection.

Sampling bottles

Glass bottles used for water sampling should have a capacity of at least 200 mL. They should be fitted with ground glass stoppers or screw caps. The stopper or cap and neck of the bottle should be protected from contamination by a suitable cover either of paper or thin aluminium foil. Silicon rubber liners, that will withstand repeated sterilization at 160 °C, should be used inside screw caps. After being sterilized the bottle should not be opened before the sample is collected.

Neutralizing chlorine in water samples

When the water to be examined is likely to contain chlorine or chloramine, sufficient sodium thiosulphate ($\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$) to neutralize these substances must be added to each bottle as follows:

Add 100–200 μL (0.1–0.2 mL) sodium thiosulphate 30 g/L (3% w/v) to each bottle of 200 mL capacity before it is sterilized. This will give a concentration of approximately 18 mg/litre of water.

Information to be supplied with water samples

Each water sample should be given a code number and the following information should accompany the sample (preferably using a standardized form):

- Reasons for examination, e.g. whether a routine sample or otherwise.
- Source from where the water has been collected, e.g. whether from a well, spring, lake, reservoir, or piped supply. The exact place from where the water was taken should also be stated.
- State whether the water has been filtered, chlorinated, or treated in some other way.
- *Sample from a house tap:* Mention whether the water was drawn from a cistern or direct from the main.

Sample from a well: Give details of the well's depth, whether covered or uncovered, and whether recently constructed or altered.

Sample from a spring: Describe the stratum from which it issued and whether the sample was taken directly from spring or from a collecting chamber.

Sample from a river or stream: Mention the depth at which the sample was collected, whether from the side or the middle of the stream, whether the water level was above or below average,

and whether there had been heavy rainfall or flooding.

Sample from a lake or reservoir: Give the exact position and the depth at which it was collected.

- Temperature of the source of the sample.
- Mention any possible sources of pollution in the area and their approximate distance from the sampling point.
- Date and time when the sample was taken (and dispatched if sent to a Water Testing Laboratory).

Sampling

Hold the sterile bottle by the base in one hand. Use the other hand to remove the stopper and cover together. The stopper and cover should be retained in the hand while the bottle is filled and then they should be replaced together. To prevent contamination. The person collecting the water must not touch, or allow any surface to touch, the screw thread of the bottle neck or the inside of the cap. If the bottle becomes contaminated, it must not be used.

Sampling techniques: Details of sampling water from a tap, street hydrant, tubewell, open well, river, stream or other surface water can be found on pages 148–149 *Part 2 District Laboratory Practice in Tropical Countries*. Access by entering the title in Google and clicking on www.medbox.org

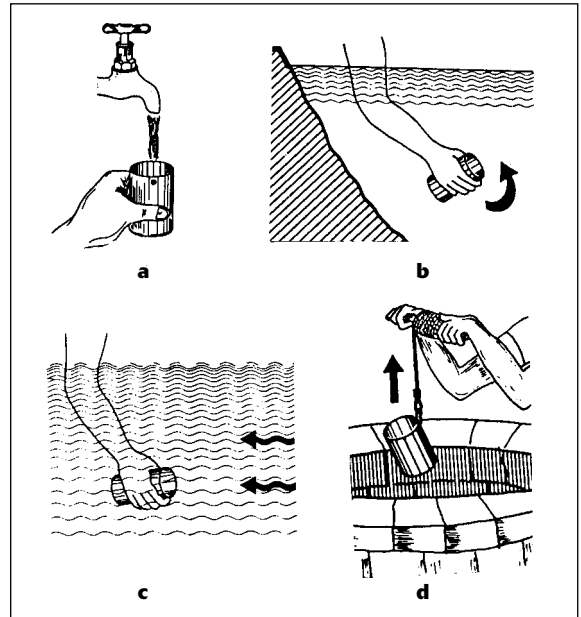
Transporting water samples to a Water Testing Laboratory

Immediately after collection, samples should be placed in an insulated cold box for transport to a water testing laboratory. Water samples should be examined as soon as possible on arrival and always within 6 hours of collection. Whenever possible, process water samples in the field.

Frequency of sampling

In large treatment plants, it should be a routine to sample water daily at each stage of treatment. In many tropical rural areas, however, untreated sources of water are used. In these situations, periodic sanitary surveys of the raw water should be carried out to establish the level of risk of epidemic waterborne disease to which the population is exposed. The survey should include an on-site inspection and evaluation of the water supply system, and a bacteriological analysis of the water.

The frequency of sampling water in distribution pipes, unchlorinated water supplies before distribution, and chlorinated water before distribution is as follows:



Water sampling techniques (a) Taking a sample from a tap. (b) Sampling from a lake, reservoir or other surface water source. (c) Taking a sample from a river or stream (against the current flow). (d) Sampling from a well or tank (not allowing the cup to touch the walls). *Courtesy of Robens Centre for Public and Environmental Health.*

Water in distribution pipes

It is inevitable that water quality deteriorates in distribution, mainly as a result of corrosion in pipes allowing leaks and infiltration. The larger the population served, the longer is the distribution system and therefore the greater the risk of contamination.

At least one sample per 5 000 population per month should be examined and every effort should be made to establish a random routine sampling procedure.

Unchlorinated water supplies before distribution

The World Health Organization has suggested that the maximum interval between successive samples for bacteriological analysis should be as follows:

Population served	Maximum interval
Less than 20 000 1 month
20 000–50 000 2 weeks
50 000–100 000 4 days

Chlorinated water supplies before distribution

Large supplies, serving populations of greater than 100 000, should be subjected to daily sampling for bacteriological analysis in conjunction with continuous chlorine residual recording. For rural supplies serving populations, often significantly less than 10 000, where even sampling for bacteriological

analysis at weekly intervals may be impractical, it is important that the chlorine in water leaving treatment plants should be checked at least once daily.

Bacteriological testing of water

Testing for the presence of normal faecal organisms in water is a way of determining whether a water supply is faecally polluted. To search directly for the presence of specific enteric pathogens in water samples is impractical for routine control purposes. When no normal faecal bacteria are detected in a water sample, it is probable that enteric pathogens (usually present in much smaller numbers) are also absent. It must be remembered, however, that some viruses and parasites survive longer and are more resistant to chlorination than some faecal bacteria used as indicators of pollution.

Faecal coliforms, referred as thermotolerant coliforms (incubated at 44 °C) are the most appropriate indicators of faecal pollution. More than 95% of thermotolerant coliforms isolated from water samples are *Escherichia coli*, the presence of which is proof of faecal contamination. It is less useful to test for total coliforms because they are not directly related to the presence of faecal contamination and so not to the risk of disease. The most valuable test for the routine quality control of water supplies is the thermotolerant coliform count.

Note: Examination of water for faecal streptococci and clostridia may sometimes be of value in confirming the faecal nature of pollution in doubtful cases.

Two principal techniques are used for counting faecal coliforms:

- Membrane filtration
- Multiple tube/most probable number (MPN)

Membrane filtration

In this technique, a 100 mL water sample or a diluted sample is filtered through a membrane filter. The membrane with the coliform organisms on it is then cultured at 44 °C in a Petri dish on a pad of membrane lauryl sulphate broth containing lactose and an indicator.

Following incubation for 18 hours, the membrane is inspected visually for lactose fermenting yellow colonies (see Plate 2.73). The number of yellow colonies is the number of thermotolerant coliforms/100 mL in the water sample. The count can also be expressed as the number of colony-forming units (CFU)/100 mL. When the number of

colonies are too numerous to count, the count is reported as “*Too numerous to count*” (indicative of gross contamination).

The membrane filtration technique where affordable is recommended for its accuracy, speed of results and because it can be performed in the field. It cannot however be used with turbid water samples due to membrane clogging.

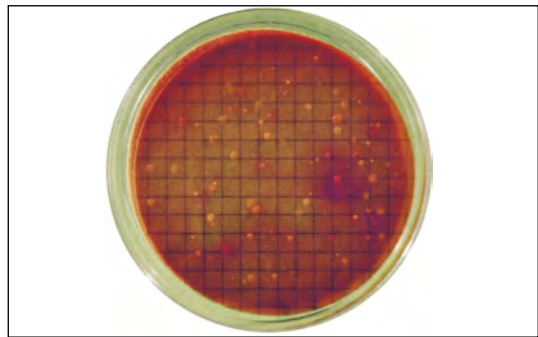


Plate 2.73 Membrane culture of a water sample after incubation at 44 °C for 24 h., showing yellow colonies of thermotolerant coliforms.

Multiple tube/most probable number (MPN)

In this technique a 100 mL water sample is distributed (five 10 mL amounts and one 50 mL amount) in bottles of sterile selective MacConkey broth (purple) containing lactose and an indicator. After incubation, the number of bottles in which lactose fermentation with acid and gas production has occurred are counted. Acid production is shown by a change of colour from purple to yellow and gas production by the collection of a bubble in the inverted Durham tube. The lactose is fermented by the coliforms in the water. By reference to probability tables the most probable number of coliforms in the 100 mL water sample can be estimated.

The membrane technique is more accurate than the multiple tube/MPN technique and this may be important when deciding whether a slightly contaminated unchlorinated water source is fit for consumption. The multiple tube/MPN technique suffers from a large sampling error. A further disadvantage is that it takes up to 48 hours to obtain a presumptive coliform count and confirmation may then be required.

Interpretation of faecal coliform count

A distinction needs to be drawn between samples from chlorinated and unchlorinated water supplies.

Chlorinated samples

At least 90% of all samples taken over a 12 month period should have a zero thermotolerant coliform count per 100 mL. The count should never exceed 5 per 100 mL.

When analyses reveal that these guidelines are not being met, the results should be reported immediately to the treatment plant superintendent who should investigate and rectify the cause of the treatment failure.

Unchlorinated samples

Most untreated water supplies contain faecal bacteria but in the case of protected ground water, e.g. springs, sealed wells, and tubewells, it should be possible to achieve very low levels of contamination. The following guidelines are therefore suggested:

Mean count* 44 °C, 100 mL	Category	Comments
0	A	Excellent
1–10	B	Acceptable: But make regular sanitary checks.
10–50	C	Unacceptable: Look for and correct structural faults and poor maintenance of pump and plinth. Then disinfect equipment and source.
More than 50	D	Grossly polluted: Look for alternative source, or carry out necessary repairs, and disinfect well.

*Guidelines can only be applied when *routine* survey data are available, e.g. 5–10 consecutive weekly samples.

Important: *A single laboratory examination of any water, whether raw or treated, and however favourable the result, does not justify the conclusion that all is well and that a water supply is suitable for drinking. Contamination is often intermittent and may not be revealed by the examination of a single sample or when samples are tested at lengthy intervals or not during both dry and wet seasons. The value of water analysis is dependent upon it being performed frequently at regular intervals, using tests which can be carried out reliably in the field.*

Note: To contribute to healthcare, water supply surveillance must be linked to improvement strategies.

WATER ANALYSIS IN THE FIELD

In remote settings in tropical countries it may not be possible to refer water samples for analysis to a

Water Testing Laboratory. In this situation a portable water testing kit for use by a community health worker or water surveillance officer can help to monitor the safety of community water supplies.

Portable water testing kit

An example of a portable water testing kit is the Wagtech *Potatest+* kit manufactured by Palintest. It weighs 13 kg and measures 57 × 43 × 24 cm.

Availability of Wagtech *Potatest+* testing kit

The kit is available from:

- Oxfam Supply Centre under the description *Water Testing kit, microbiological with battery*, Product Code FKB, website <https://supplycentre.oxfam.org.uk>
- Palintest under the description *Potatest+*, Product Code: PTW10005, Website: <https://www.palintest.com/en/products/wagtech-potatest>
The kit is also available from distributors in several tropical countries (contact Palintest for details).

The Wagtech *Potatest+* water testing kit contains all the supplies needed to perform 200 tests and equipment for testing water samples in the field including a battery-operated, digital, self-calibrating incubator, 20 reusable aluminium Petri dishes and a membrane filtration unit for filtering water samples (see Plate 2.74).

Also included in the kit is a *Safety Pack* for testing water supplies in the field which contains Jackson turbidity tubes, pH sensor, chlorine testing comparator and a deionizer pack to provide distilled water for making the membrane lauryl sulphate broth (MLSB).

A well written and illustrated User Manual, available in four languages, is provided together with step-by-step pictorial guides. A video showing the contents of the kit can be seen on the Oxfam Supply Centre website (see previous text).

The equipment needed to sterilize the aluminium Petri dishes and culture medium is not supplied with the kit (a portable steam sterilizer, i.e. pressure cooker, is available from Oxfam Supply Centre). Disposable sterile Petri dishes containing sterile culture medium (MLSB), called *NutriDiscs*, sealed in packs of 10, are available as an optional extra from Palintest, Code PTW 10064 (*NutriDiscs* for *E. coli* and faecal coliforms). Up to 7 *NutriDiscs* can fit inside the incubator.

Other tests used in water quality analysis

These include:

- Testing chlorine treated water supplies for residual chlorine (mg/L) to assess the effectiveness of chlorine disinfection. To ensure complete disinfection, chlorine needs to be added to the water



Plate 2.74 Palintest *Patatest+* water testing kit. Palintest Code PTW 10005 (Oxfam Code: FKB)
 Courtesy of Palintest. www.palintest.com/en/products/wagtech-potatest

to leave a slight excess. The amount of free residual chlorine in the water should normally be greater than 0.2 mg/L and less than 1 mg/L. The amount will depend on the distance from source to distribution and on the ambient temperature and pH of the water sample. The presence of free chlorine should be checked in all parts of the system.

The DPD (N,N-diethyl-1, 4-phenylenediamine) colorimetric technique is recommended for detecting residual chlorine in water samples. It can be simply performed in the field using a visual chlorine comparator or if available, a digital chlorine meter.

- Measuring the pH of the water. The pH can affect the efficiency of chlorine disinfection. Chlorination is more effective at below pH 7.0. The pH can be tested in the field using pH papers or a digital pH sensor.
- Testing the turbidity, or cloudiness, of water supplies. Turbidity can be caused by faecal pollution, chemicals, or particularly during the

rainy season, by mud, sand or silt sediments. It is not only an indication of possible microbial pollution but turbidity can also damage taps and valves and block filters.

Turbidity can be tested in the field using Jackson calibrated tubes (measuring in Turbidity Units) or if available, using a digital turbidity meter (measuring in Nephelometric Units). Groundwater should always be less than 5 Turbidity Units (TU). This is needed for effective disinfection. Ideally drinking water should have a turbidity value of less than 1 TU. Over 5 TU, turbidity can be seen by eye.

Note: When a chlorinated water supply contains more than 0.2 mg/L free chlorine and gives a turbidity reading of less than 5 TU, it is unlikely to contain faecal coliforms. There is no need to perform bacteriological analysis.

Chemical contamination of drinking water

Toxic chemicals can enter drinking water sources naturally or following agricultural, industrial, or

human settlement activities. Chemicals which can cause serious health problems include arsenic, fluoride and nitrate. Arsenic is naturally occurring in India, Bangladesh, Thailand, China, Tibet, Nepal, Vietnam, Cambodia, Myanmar and parts of South America. It occurs in deltaic sediments deposited from mountains, and in river, lake and coastal sandy deposits. Also occurs as a result of mining and manufacturing processes. Following long term exposure (5–15y), arsenic causes skin pigmentation changes, peripheral neuropathy, cancer of the skin, lungs, bladder, kidneys and peripheral vascular disorders.

Fluoride occurs naturally in groundwater in South East Asia and elsewhere, particularly in dry areas. It causes dental fluorosis, brittle bones and crippling painful deformities. Nitrate is usually present in drinking water due to the excessive use of fertilizers and agricultural practices which allow nitrate to enter surface and ground water. Unlike arsenic and fluoride, nitrate health problems occur after short term exposure, i.e. methaemoglobinaemia in bottle-fed infants.

Other water chemical tests will be required depending on the source and location of community water supplies, local activities affecting water quality and water treatment processes. Tests include water pH (see previous text), water hardness, conductivity, free and total chlorine (see previous text) and chemicals such as nitrite, nitrate, iron and aluminium. Chemical test strips, field testing comparators and battery operated meters are available from Lovibond Tintometer and other suppliers of water testing products.

When district laboratories are requested to assist in the chemical testing of water supplies, the local Water Testing Reference Laboratory should advise on test procedures and provide the necessary equipment and consumables.

Note: Further information on the chemical contamination of water supplies can be found in the WHO 2018 publication: *Developing drinking-water quality regulations and standards: general guidance with a special focus on countries with limited resources*.⁴

WHO evaluation of household water treatment technologies

A list of products evaluated by WHO in 2019 for the removal of microbial contaminants from drinking water (including chemical, solar, UV disinfection, ceramic and membrane filtration and flocculation-biofiltration, pages 48–49).

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2.17 Amoebic dysentery Giardiasis *Cryptosporidium*, *Cyclospora* *Cystoisospora* infections

Amoebic dysentery, giardiasis, *Cryptosporidium*, *Cyclospora* and *Cystoisospora* infections are caused by protozoan parasites.

AMOEBIC DYSENTERY

Amoebic dysentery is caused by *Entamoeba histolytica*, an invasive anaerobic parasite which reproduces asexually forming motile trophozoite forms (amoebae) and cyst forms by which the parasite is transmitted. The direct life cycle of *E. histolytica* is shown in Fig. 2.43. It is similar to the direct life cycle of *Giardia intestinalis*.

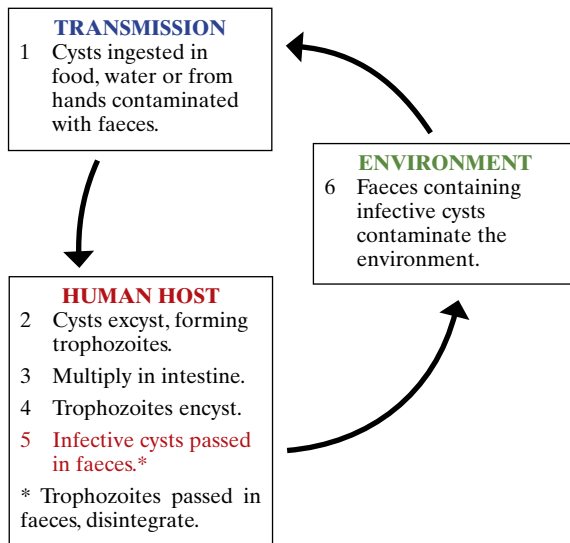


Fig. 2.43 Transmission and life cycle of *Entamoeba histolytica* and *Giardia intestinalis*.

Distribution and transmission

E. histolytica is endemic in many parts of tropical and subtropical Africa, Asia, Mexico, South Africa and China. Distribution is related to inadequate environmental sanitation and poor personal hygiene. *E. histolytica* is transmitted by the faecal-oral route with infective cysts being ingested in food, water or from hands contaminated with faeces.

Amoebic dysentery

Amoebic dysentery occurs when *E. histolytica* trophozoites invade the wall of the large intestine and multiply in the submucosa, forming large flask-shaped ulcers. The amoebae ingest red cells from damaged capillaries. Compared with bacillary dysentery, the onset of amoebic dysentery is less acute, lasts longer, there is usually no significant fever and fewer pus cells are found in samples. Without treatment dysenteric attacks may recur for several years.

Occasionally *E. histolytica* amoebae are carried to the liver in the portal circulation and form abscesses, usually in the right lobe. Amoebic liver abscesses are more common in adults than in children with a higher frequency in men (3 to 1 rate). There is pain and tenderness over the liver, wasting, and fever with chills and night sweats. Patients with large or multiple abscesses may become jaundiced and anaemic. There is usually a raised white cell count with neutrophilia.

Prevention and control of *E. histolytica* infection

- Preventing faecal contamination of the environment by using latrines and protecting water supplies from faecal contamination.
- Handwashing after defaecation and before eating.
- Covering food and water to prevent contamination from flies which can act as cyst carriers.
- Not eating green salads or other uncooked foods which may contain cysts, usually as a result of fertilization with untreated human faeces.
- Boiling drinking water (*E. histolytica* cysts are killed at 55 °C).
- Health education, particularly of food handlers, and also in schools and community health centres.

LABORATORY DIAGNOSIS

The laboratory diagnosis of amoebic dysentery is by:

- Finding motile *E. histolytica* trophozoites containing red cells in *fresh* (still warm) faecal samples or rectal scrape. Samples must be examined without delay or kept at body temperature otherwise the amoebae are difficult to identify because they lose their motility, extrude food vacuoles containing red cells and round up.

E. histolytica cysts in faeces

Infection with *E. histolytica* cannot be diagnosed by finding cysts in faeces. This is because the cysts of *E. histolytica* are morphologically identical to those of other *Entamoeba* species, including *E. dispar* a non-pathogenic species and *E. moshkovskii* of unknown pathogenicity.¹

- Detecting *E. histolytica* antigen in faecal samples.

Note: In Reference laboratories, *E. histolytica* infection can be diagnosed and differentiated from other *Entamoeba* species by polymerase chain reaction (PCR).

Examination of dysenteric samples for trophozoites of *E. histolytica*

- Using a wire loop or piece of stick, place a small amount of blood and mucus on one end of a slide. Without adding saline, cover with a cover glass and using a tissue, press gently on the cover glass to make a thin preparation.
- Place a drop of eosin reagent (Reagent No. 18) on the other end of the slide. Mix a small amount of the sample with the eosin and cover with a cover glass.

Value of eosin: Eosin does not stain living amoebae but provides a pink background which can make the motile amoebae easier to detect.

- Examine immediately the preparations microscopically, first using the 10× objective with the condenser iris closed sufficiently to give good contrast. Use the 40× objective to identify motile *E. histolytica* trophozoites.

Cysts of *E. histolytica*

As explained previously, the cysts of *E. histolytica* are morphologically identical to those of *E. dispar* and *E. moshkovskii*. When seen in faecal samples

Trophozoite of *E. histolytica*

- Average size is about 25 × 20 μm.
- Shows active amoeboid movement (directional) in fresh warm sample.
- In dysenteric samples, the amoebae contain ingested red cells. This is diagnostic of *E. histolytica*.
- Single nucleus is present which has a central karyosome (not always visible).

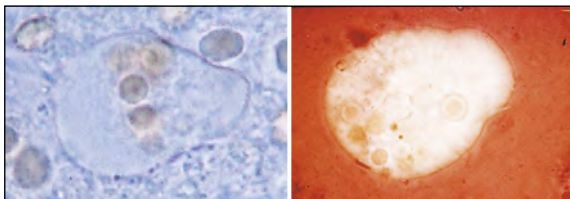


Plate 2.75 Left: *E. histolytica* trophozoite in dysenteric faecal sample. Note the ingested red cells (diagnostic feature). Right: *E. histolytica* trophozoite containing red cells as seen in an eosin preparation.

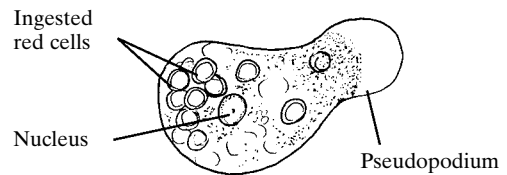


Fig. 2.44 Morphology of *E. histolytica* trophozoite.

they should be reported as: Cysts resembling *E. histolytica* detected. Further investigations are required to confirm *E. histolytica* infection such as the *E. histolytica* specific faecal antigen test (see page 212) or where available, molecular testing.

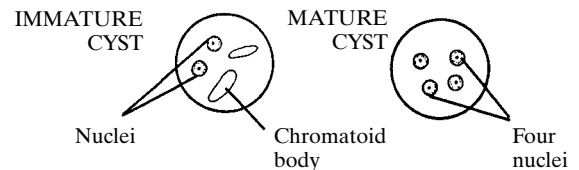


Fig. 2.45 Morphology of immature and mature *E. histolytica* /*E. dispar*/*E. moshkovskii* cysts.

Cysts of *E. histolytica*/*E. dispar*/*E. moshkovskii*

- Round, measuring 10–15 μm.
- Contain, 1, 2 or 4 nuclei with a central karyosome (special staining techniques are required to show nuclear structure).
- Chromatoid bodies (aggregations of ribosomes) can be seen particularly in immature cysts. They do not stain with iodine but can be stained with Burrow's stain, Sargeant's stain and acridine orange.

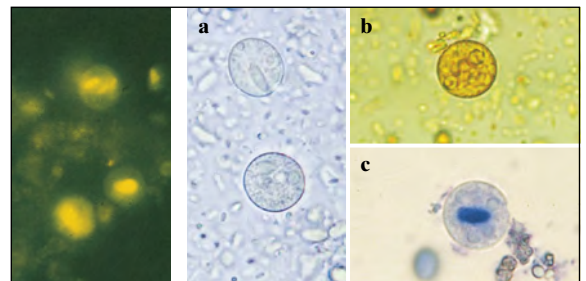


Plate 2.76 *E. histolytica*/*E. dispar*/*E. moshkovskii* cysts showing chromatoid bodies. **a** Immature single nucleus cyst in saline, **b** Iodine stained mature 4 nuclei cyst, **c** Burrow stained chromatoid body in cyst. Left Acridine orange stained chromatoid bars.

Staining of chromatoid bodies

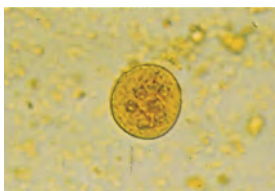
The simplest technique is to make a saline suspension of faeces in a small tube, add 2 or 3 drops of Burrow's stain (Reagent No 11) or Sargeant's stain (Reagent No 37) and mix. Leave the suspension overnight or at least 6 hours. Examine microscopically for stained chromatoid bars (blue if using Burrow's stain, green if using Sargeant's stain).

If facilities for fluorescence microscopy are available, acridine orange can be used to demonstrate chromatoid bars in *E. histolytica*/*E. dispar*/*E. moshkovskii*, see Plate 2.76.

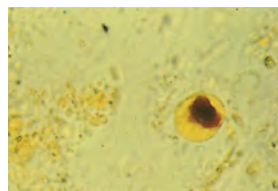
Differentiation of cysts that can be found in faeces

Species	Size	Nuclei	Chromatoid body	Glycogen inclusion
CYSTS				
<i>Entamoeba histolytica</i> *	10–15 µm	1–4	Present (immature cyst)	Diffuse
<i>Entamoeba hartmanni</i>	7–9 µm	1–4	Present (immature cyst)	Diffuse
<i>Entamoeba coli</i>	15–30 µm	1–8	Rare Needle-like	Diffuse
<i>Iodamoeba buetschlii</i>	9–15 µm	1	–	Compact mass
<i>Endolimax nana</i>	7–9 µm	4 Hole-like	–	–
<i>Chilomastix mesnili</i>	8–9 µm	1	–	–

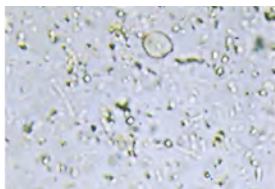
* Morphologically identical to *E. dispar* and *E. moshkovskii*



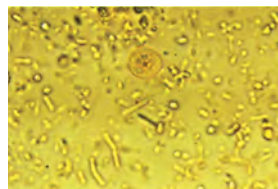
Entamoeba coli cyst, 15–30 µm. Iodine stained showing nuclei (up to 8 may be present).



Iodamoeba buetschlii cyst, 9–15 µm. Iodine stained showing brown glycogen vacuole.



Chilomastix mesnili cysts, 8–9 µm, lemon-shaped. Saline preparation showing single nucleus.



Endolimax nana cyst, 7–9 µm, round to oval. Iodine stained showing 4 hole-like nuclei.

Blastocystis hominis

This anaerobic intestinal protozoan can be found in faeces and is sometimes confused with cysts. It is usually non-pathogenic but may cause acute enteritis in persons with immunodeficiency (e.g. HIV infection).

Appearance: *B. hominis* is round and about the size of an *E. coli* cyst but shows great variation in size. It has peripheral cytoplasm, a central vacuole but no nucleus. It can be recognized from the granules which form a ring around the periphery. The granules stain with iodine.

Detection of *E. histolytica* antigen in faeces using *E. histolytica* Quik Chek

E. histolytica Quik Chek is a rapid enzyme immunoassay for the qualitative detection of *E. histolytica* adhesin (specific antigen secreted by *E. histolytica*) in unfixed faecal samples. The test detects only *E. histolytica* and therefore enables differentiation from other *Entamoeba* species. It is reported as having a sensitivity of 98% and specificity of 100% when compared against ELISA faecal antigen immunoassays.² The device contains two antibody strips, one with monoclonal antibody against *E. histolytica* adhesin which binds to antigen-conjugate complexes in the faecal sample and the other strip is a positive control line which binds conjugate.

Test procedure

Faecal sample is added to a tube containing diluent and conjugate. This is added to the sample well in the cassette. Following 15 minutes incubation at room temperature, the reaction window is washed with buffer and substrate is added. After a further 10 minutes incubation, the test result is read in the reaction window.



Positive Test

Blue line in both the Test (T) and Control (C) areas.

Negative Test

Blue line in the Control (C) area only.

The test procedure must be followed exactly and tests stored at 2–8 °C. The test takes 30 minutes to perform. A 25 test kit is available.

Note: Details of shelf-life, and cost can be obtained from the manufacturer Techlab Inc. A demonstration video is available from Techlab.

Website www.techlab.com

E-mail techlab@techlab.com

GIARDIASIS

Giardiasis is caused by *Giardia intestinalis* (previously known as *Giardia duodenalis* or *Giardia lamblia*). It reproduces asexually forming trophozoites which parasitize the small intestine and cyst forms by which the parasite is transmitted. The direct life cycle of *G. intestinalis* is similar to that of *E. histolytica* (see Fig 2.43).

Distribution and transmission

G. intestinalis has a worldwide distribution. It is particularly common in the tropics and subtropics in areas where water supplies and the environment become faecally contaminated. In endemic areas young children are frequently infected. *G. intestinalis* is transmitted by the faecal-oral route.

Giardiasis

Although many infections, particularly in adults are without symptoms, *G. intestinalis* can cause abdominal pain, severe diarrhoea, flatulence, vomiting, weight loss, malabsorption with lactose intolerance, and in children, impairment of growth. Symptoms can be severe particularly in children under 3 years of age, and in the undernourished. Those with reduced immune responses, gastrointestinal disorders or intestinal bacterial infections, tend to be more susceptible to *Giardia* infection.

Prevention and control of *G. intestinalis* infection

Giardiasis may be prevented by improving environmental sanitation and personal hygiene to prevent food, water, and hands becoming contaminated with faeces containing cysts.

The cysts are not killed in food or water stored at 4–6 °C. Like the cysts of *E. histolytica*, those of *Giardia* are resistant to the concentrations of chlorine normally used to treat domestic water supplies.

LABORATORY DIAGNOSIS

Faeces containing *G. intestinalis* have an offensive odour, are often pale coloured, fatty and float in water.

The laboratory diagnosis of giardiasis is by:

- Finding motile *G. intestinalis* trophozoites in fresh diarrhoeic samples particularly in mucus. They are often difficult to detect. Several samples collected at different times may need to be examined. A Giemsa or Field stained faecal smear can be used to confirm the identity of the flagellates.
- Finding *G. intestinalis* cysts in more formed faecal samples. The cysts are excreted irregularly. Often large numbers may be present for a few days

followed by fewer numbers for a week or more. Several samples may need to be examined and a concentration technique used.

Trophozoite of *G. intestinalis*

- Small pear-shaped flagellate with a rapid tumbling and spinning motility, often likened to a falling leaf.
- Measures 12–15 × 5–9 μm (see Appendix V to compare size of *G. intestinalis* with other intestinal protozoa).
- Has a large concave sucking disc on the ventral surface (attaches trophozoite to the intestinal mucosa).
- It has four pairs of flagella, two axonemes, and two nuclei which stain well (Giemsa or Field technique).
- A single or two curved median bodies are present.

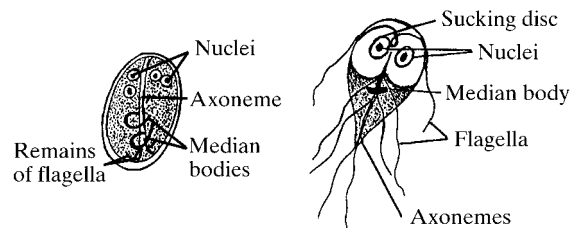


Fig. 2.46 Morphology of *G. intestinalis*.
Left: cyst. Right: trophozoite.

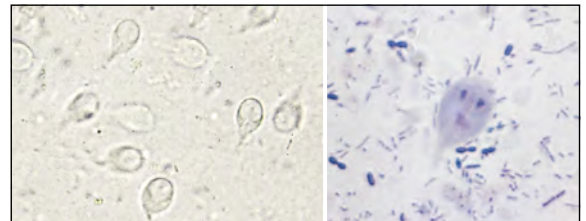


Plate 2.77 Left: *G. intestinalis* trophozoites in saline preparation, Right: Field stained trophozoite as seen with 100× objective.

Duodenal aspirates

Occasionally giardiasis can be diagnosed by detecting *G. intestinalis* trophozoites in duodenal contents. This should only be considered when giardiasis is suspected clinically and no parasites are detected after examining several faecal samples.

Note: The *Enterotest* (string test) is infrequently used because of its high cost.

Differentiation of *Giardia intestinalis* from non-pathogenic flagellates

Non-pathogenic flagellates that can be found in faeces that require differentiation from *G. intestinalis* include:

- *Chilomastix mesnili*
- *Pentatrichomonas hominis*
- *Enteromonas hominis*
- *Retortamonas intestinalis*

Trophozoites of the non – pathogenic flagellates can be easily differentiated from *G. intestinalis* by their shape and movement (in a fresh sample) and because they have only one nucleus and fewer flagella. The only other small trophozoite that has two nuclei is the flagellate *Dientamoeba fragilis* but this organism has no flagella or median bodies and looks like a small (9–15 µm) amoeba.

Cysts are produced by *C. mesnili*, *E. hominis* and *R. intestinalis*. They can be easily differentiated from those of *G. intestinalis* because they are smaller (under 8 µm) and do not contain the remains of flagella. The cysts of *E. hominis* are oval in shape and have four nuclei (but not grouped to one end). *C. mesnili* cysts are lemon-shaped. The cysts of *R. intestinalis* are pear-shaped.

Cyst of *G. intestinalis*

- Small and oval measuring 8–12 × about 6 µm.
- Internal structures include four nuclei grouped at one end (sometimes difficult to see), axonemes, median bodies, and remains of flagella. These structures can be seen in unstained preparations but are more clearly defined in an iodine preparation.

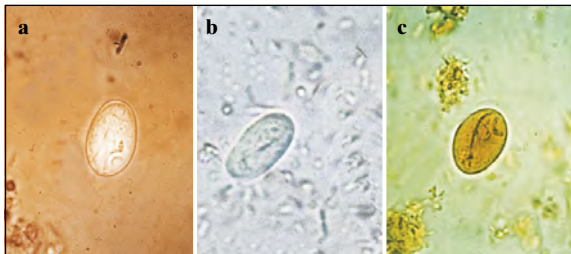


Plate 2.78 Cysts of *G. intestinalis* a Eosin preparation, b Saline preparation, c Iodine preparation.

Concentration of *Giardia* cysts

G. intestinalis cysts can be concentrated using the modified formol ether/ethyl acetate technique described in Appendix V.

Alternatively, the cysts can be concentrated using a zinc sulphate flotation technique as described in Appendix V. A drop of Sargeant's stain (Reagent No 37) can be added to the preparation to make detection of the cysts easier.

Rapid *Giardia* faecal antigen immunoassays

Rapid *Giardia* faecal antigen tests are of value when microscopy is not available or when after the examination of up to three faecal samples, microscopy fails to detect the parasites and clinical symptoms persist (the majority of *Giardia* infections can be diagnosed microscopically).

Giardia rapid antigen tests are usually available as tests that detect both *Giardia* and *Cryptosporidium*. Examples include:

- *ImmunoCard STAT Crypto/Giardia*, manufactured by Meridian Bioscience Inc. www.meridianbioscience.com
It is supplied as a 30 test kit, requiring 2–8 °C storage.
- *Xpect Giardia/Cryptosporidium*, manufactured by: Remel Inc. (part of Thermo Scientific) www.remel.com
It is supplied as a 20 test kit, requiring 2–8 °C storage.
- *Quik Chek Giardia/Cryptosporidium*, manufactured by Techlab Inc. www.techlab.com
It is supplied as a 25 test kit, requiring 2–8 °C storage.

The rapid tests have been shown to be both sensitive and accurate for the diagnosis of active giardiasis.³

CRYPTOSPORIDIUM, CYCLOSPORA CYSTOISOSPORA INFECTIONS

Cryptosporidium, *Cyclospora* and *Cystoisospora* infections are caused by intracellular oocyst-forming protozoan parasites belonging to the Apicomplexa subgroup. The parasites have a complex life cycle involving an asexual reproductive stage (schizogony) and a sexually development cycle (sporogony) as shown in Fig. 2.47. With *Cyclospora* and *Cystoisospora*, the oocyst contains sporozoites

enclosed in sporocysts. With *Cryptosporidium* the oocyst contains sporozoites not enclosed in sporocysts. *Cryptosporidium* has recently been reclassified from the Coccidia to a new subclass, Cryptogregarina.⁴

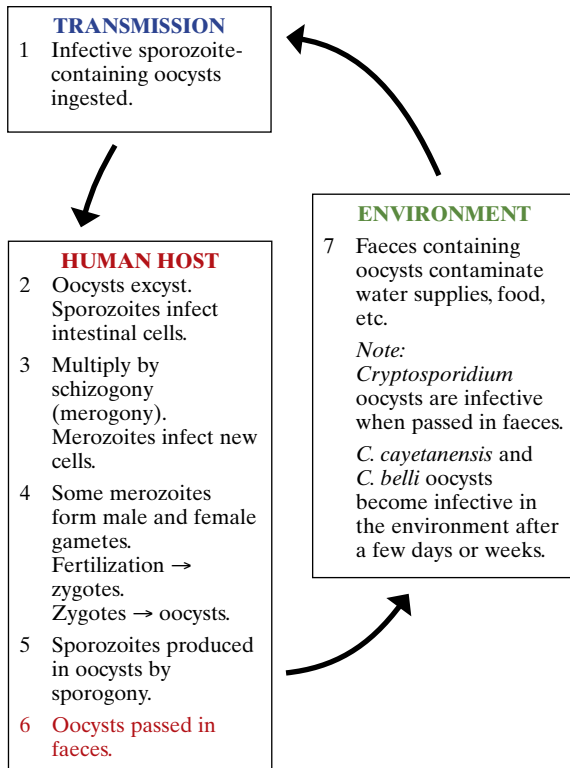


Fig 2.47 Transmission and life cycle of *Cryptosporidium*, *Cystoisospora belli*, and *Cyclospora cayetanensis*.

CRYPTOSPORIDIUM

Distribution and transmission

Cryptosporidium has a world-wide distribution with a high prevalence in tropical countries. Most infections are caused by *C. hominis* which predominately infects humans and *C. parvum* which is widely distributed in the environment infecting humans and a wide range of animals. Other species have been reported in immunosuppression. Animal hosts are important sources of *C. parvum* infections.

Cryptosporidium is transmitted through contaminated water or food or following person to person contact. When passed in faeces, oocysts are immediately infective. Auto-infection can occur when oocysts sporulate in the intestine. Those at greatest risk of infection are immunocompromised and malnourished persons and infants.

Cryptosporidiosis

Most *Cryptosporidium* infections cause self-limiting diarrhoeal illness. In persons with low CD4 T cell counts, *Cryptosporidium* can cause severe, prolonged, life-threatening diarrhoeal disease, respiratory and disseminated disease. It is an important pathogen in advanced HIV disease (see p. 190). In sub-Saharan countries and South Asia, *C. parvum* has been reported as causing severe diarrhoea in young children.

Prevention and control of *Cryptosporidium* infections

Cryptosporidium infection can be prevented by improving environmental sanitation and personal hygiene to prevent water, food and hands becoming contaminated with faeces containing oocysts. Cattle and other animals can also be sources of infection.

Cryptosporidium oocysts are particularly resistant to disinfectants, including chlorine at concentrations normally used to treat water supplies. They can survive in the environment for prolonged periods. In tropical countries, infection rates are highest in the warm wet season.

LABORATORY DIAGNOSIS

Laboratory diagnosis of *Cryptosporidium* infection is by:

- Examining faecal smears for acid fast oocysts following formol ether/ethyl acetate concentration and staining by the modified Ziehl-Neelsen technique.
- Demonstrating oocysts in faecal smears by fluorescence microscopy following auramine phenol staining.
- Detecting *Cryptosporidium* antigen in faecal samples. Antigen testing is significantly more sensitive than microscopy.

Modified Ziehl-Neelsen (ZN) technique

In unstained direct wet faecal preparations the small oocysts of *Cryptosporidium* are difficult to differentiate from yeasts and other small spherical structures found in faeces. The oocysts do not stain with iodine. Examination of a modified ZN stained smear can help to detect the oocysts.

Reagents

- Carbol fuchsin stain* No. 12
- Malachite green or methylene blue stain* No. 27
- Acid alcohol 1% v/v* No. 3

*Same as used in the ZN technique for staining AFB in sputum (see Section 2.11).

This can be made by diluting 3% acid alcohol (Reagent No. 3) as used in the ZN technique for staining AFB, i.e. add 30 mL of the 3% acid alcohol to 60 mL of distilled or deionized water and mix.

Procedure

- 1 Prepare a smear from the sediment obtained by the formol ether/ethyl acetate concentration technique, see Appendix V. Air-dry the smear. Fix the smear with methanol for 2–3 minutes.
- 2 Stain with *unheated* carbol fuchsin for 15 minutes. Wash off the stain with water.
- 3 Decolorize with 1% acid alcohol for 10–15 seconds. Wash off with water.
- 4 Counterstain with 0.5% malachite green (or methylene blue) for 30 seconds. Wash off with water and stand the slide in a draining rack for the smear to dry.
- 5 Examine the smear microscopically for oocysts, using a low power magnification to detect the oocysts and the oil immersion objective to identify them.

Oocysts of *Cryptosporidium*

In modified ZN stained smear:

- Small, round to oval, pink red stained bodies measuring 4–6 μm , as shown in Plate 2.79 *Right*.

Note: Older oocysts stain palely.

- Some oocysts may contain a single deeply stained red dot.

Note: Yeasts and faecal debris often stain pale red but these structures can usually be distinguished from cryptosporidia. Some bacterial spores are also acid fast but most of these are too small to cause confusion.

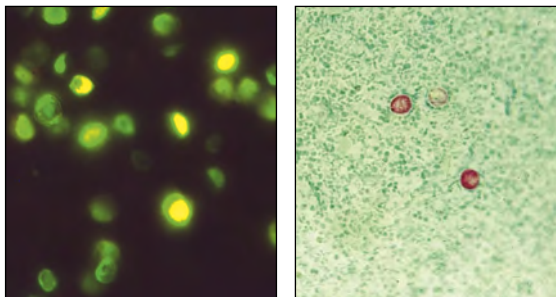


Plate 2.79 *Cryptosporidium* oocysts in faecal smear as seen with 40 \times objective.

Right: Modified Ziehl-Neelsen stained smear.

Left: Auramine 0 stained smear. Nucleic acid in oocysts fluoresces bright yellow.

Auramine phenol technique

The auramine phenol technique used to detect acid fast bacilli (AFB) in sputum can also be used to demonstrate *Cryptosporidium* oocysts in faeces.

Oocysts of *Cryptosporidium*

In auramine phenol stained sputum smear

- Small round to oval oocysts, 4–6 μm .
- Intracellular nucleic acid fluoresces yellow as shown in Plate 2.79 left.

Note: Yeast cells do not fluoresce, making it easier to detect the oocysts.

***Cryptosporidium* faecal antigen tests**

Commercially available *Cryptosporidium* faecal antigen tests include direct immunofluorescence assays, enzyme immunoassays, and rapid immunochromatographic (ICT) cartridge and strips tests. Sensitivity and specificity vary depending on the assay method and test kit used. Some tests are affected by binding substances and inactivating enzymes which may be present in faecal samples.

The rapid ICT antigen tests are simple to perform and all the items needed to perform the test are provided in the test kits. To avoid false test results, the manufacturer's instructions must be followed exactly, including the storage and expiry dates of test kits, preparation of the faecal sample and reading times of tests. Weak reactions can be difficult to interpret.

Examples of ICT tests for detecting *Cryptosporidium* antigen in faeces (as *Cryptosporidium*/*Giardia* combined tests) can be found on page 214.

CYCLOSPORA**Distribution and transmission**

Cyclospora infection is caused by *Cyclospora cayetanensis*. Humans are the only known hosts. *C. cayetanensis* has a world-wide distribution with high prevalences in tropical and subtropical countries. In several countries outbreaks of cyclosporiasis have been linked to the consumption of imported contaminated fruit, leafy greens and herbs. The life cycle of *C. cayetanensis* is similar to that of *Cryptosporidium* and *Cystoisospora* (see Fig. 2.47).

C. cayetanensis is transmitted by ingesting mature infective oocysts in faecally contaminated water or food. When excreted in faeces, the oocysts are immature. They become sporulated infective oocysts in the environment within 1–2 weeks.

Cyclosporiasis

Infection with *C. cayetanensis* causes watery diarrhoea, often

intermittent with abdominal cramps, flatulence, fatigue and low grade fever. In endemic areas young children are frequently infected and infections are often seasonal. In immunocompromised persons with low CD4 T cell counts diarrhoea can be persistent and symptoms more severe.

The prevention and control of *C. cayetanensis* infection is similar to that described for cryptosporidiosis. The oocysts of *C. cayetanensis* are resistant to pesticides, disinfectants and sanitisiers.

LABORATORY DIAGNOSIS

C. cayetanensis infection is diagnosed by:

- Identifying oocysts in fresh unstained wet faecal preparations.
Note: Staining faecal smears by the modified ZN technique is not recommended because the oocysts often remain unstained, or stain poorly and can appear distorted, making identification difficult.
- Detecting oocysts following concentration using the formol ether/ethyl acetate faecal concentration technique described in Appendix V.
- Using UV light at wavelength 340–360 nm to demonstrate the autofluorescence of oocyst walls when facilities for fluorescence microscopy are available.

Oocysts of *Cyclospora cayetanensis*

Saline preparation:

- Spherical, measuring 8–10 µm in diameter (larger than *Cryptosporidium*).
- Contain a central refractile morula-like structure as shown in Plate 2.80 *Right*.

Note: The oocysts do not stain with iodine.

Fluorescence preparation:

- Spherical oocysts, measuring 8–10 µm.
- Cyst wall autofluoresces as shown in Plate 2.80 *Left*.

Note: Autofluorescence is also seen with *C. belli* but not with *Cryptosporidium* species.

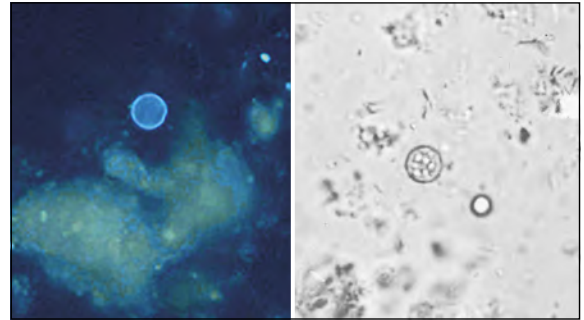


Plate 2.80 *Right*: Oocyst of *C. cayetanensis* in unstained faecal preparation.

Left: *C. cayetanensis* showing autofluorescence of oocyst wall.
Credit: CDC DPDx Image Library.

CYSTOISOSPORA

Distribution and transmission

Cystoisospora infection is caused by *Cystoisospora belli*. It is known to infect only humans. *C. belli* has a worldwide distribution with higher prevalences in tropical and subtropical regions. The life cycle of *C. belli* is similar to that of *Cryptosporidium* and *Cyclospora* (see Fig. 2.47).

C. belli is transmitted by the ingestion of mature oocysts in faecally contaminated water or food. When excreted in faeces the oocysts of *C. belli* are immature. They become mature sporulated infective oocysts in the environment within a few weeks. *C. belli* oocysts can survive in the environment for prolonged periods.

Cystoisosporiasis

Most infections with *C. belli* are rarely serious and are self-limiting. In untreated HIV infection, low CD4 T cell counts can cause chronic, profuse, watery diarrhoea with abdominal pain and malabsorption. Faecal samples may contain blood and mucus. Frequently there is eosinophilia.

The prevention and control of *C. belli* infection is similar to that of *Cryptosporidium* and *Cyclospora* infections.

LABORATORY DIAGNOSIS

C. belli infection is diagnosed by:

- Finding oocysts in a direct wet faecal preparation. Several faecal samples may need to be examined and a concentration technique used to detect the oocysts because they can be few in number and excreted intermittently.

Note: Some workers have shown that more *C. belli* oocysts can be recovered using a centrifugation sedimentation technique than by using the formal ether/ethyl acetate concentration technique.⁵

- Detecting acid fast oocysts in smears stained by the modified Ziehl Neelsen technique (see previous text). The granular centre of the oocysts stains red, often unevenly.
- When fluorescence microscopy facilities are available, using ultraviolet (UV) light at wavelength 340–360 nm to demonstrate the autofluorescence of oocysts in wet preparations. The oocyst and sporoblast/sporocyst walls, show autofluorescence.

Eosinophila

Eosinophila is a frequent finding in *C. belli* infections.

Oocysts of *Cystoisospora belli*

In unstained wet preparation:

- Oval, measuring 20–33 × 10–19 μm.
- Usually contains a granular zygote as shown in Plate 2.81.
- Occasionally more mature sporulated oocysts can be found (particularly if the sample is not fresh), containing two sporocysts each with four sporozoites as shown in Plate 2.81 (left).

Note: Iodine does not stain oocysts. In a modified ZN stained smear, there is a red-pink staining of the oocysts, often unevenly.

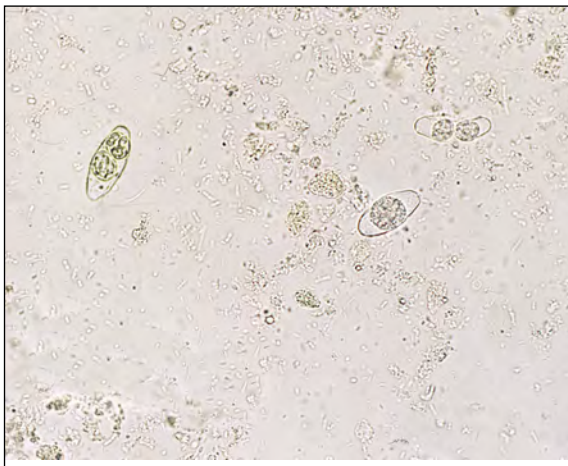


Plate 2.81 *C. belli* oocysts in a saline preparation. *Right:* Oocyst containing granular zygote. *Left:* Oocyst containing two sporocysts.

Important: *C. belli* oocysts are translucent. It is therefore important to ensure the microscope illumination is not too bright when examining wet preparations. Close the condenser iris sufficiently to give good contrast.

Centrifugation sedimentation technique⁵

- 1 Emulsify about 2 grams of faeces in 50 mL of water.
- 2 Filter the suspension through gauze.
- 3 Centrifuge the filtered suspension for 2 minutes at 400× g.
- 4 Discard the supernatant and resuspend the sediment in 10% v/v formalin.
- 5 Centrifuge for 2 minutes at 400× g. Discard the supernatant.
- 6 Mix the sediment and examine a wet preparation microscopically for oocysts, see Plate 2.81.
- 7 Make a faecal smear of the sediment and stain by the modified Ziehl-Neelsen technique described previously.

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2.18 Healthcare associated infections

A healthcare associated infection (HAI) refers to an infection acquired in a hospital or other healthcare facility that was not present or incubating at the time of a patient's admission, or an infection acquired in a hospital but appearing after a patient is discharged. HAIs also include occupationally acquired infections among healthcare and laboratory staff.

Note: HAIs are also referred to as nosocomial infections.

High risk patients

Patients at greatest risk of HAIs include:

- The very young and elderly with decreased resistance to infection.
- Those with reduced immune responses due to HIV infection, malignant disease, renal failure, immunosuppressive therapy, or diabetes.
- Patients undergoing invasive procedures, e.g. surgery, catheterization, intubation, ventilation, intravenous therapy, biopsies and endoscopy examinations.
- Those with open wounds, e.g. due to burns, conflict injuries, road traffic accidents or disaster injuries.
- Severely ill patients requiring prolonged hospital stay, particularly in crowded wards with poor infection control.

The most frequent HAIs are infections of surgical wounds, urinary tract infections and lower respiratory infections, with the highest prevalence of infections occurring in acute surgical and orthopaedic wards,

and intensive care units. HAIs increase significantly the cost of patient care with the need for extra drugs, further inpatient care, more investigations, and possible patient isolation.

INFECTION CONTROL TEAM

District hospitals should have an infection control team, comprising, as a minimum:

- a senior nurse,
- a senior clinician,
- a hospital administrator/manager,
- the senior laboratory technologist,
- the head of hospital cleaning,
- the hospital estates/building officer.

The control team should meet on a monthly basis as the infection control committee, and more frequently when there is a special problem such as a disease outbreak. In hospitals where no infection control team exists (though it should be set up as a priority), the laboratory should work closely with a specific nurse and doctor for infection issues.

It is the responsibility of the hospital infection control team to:

- Assess risks and priorities for infection control.
- Implement appropriate procedures for preventing and monitoring infections.
- Perform surveillance of HAIs.
- Compile and keep up-to-date an infection control manual for hospital personnel.
- Investigate and document outbreaks.

The infection control team should also provide guidance on antimicrobial prescribing, train staff in infection prevention and control procedures, provide continuing education of HAIs, and budget for infection control activities.

PRIORITIES FOR INFECTION CONTROL

These include:

- Hand-washing, other aseptic techniques and personal hygiene.
- Hospital cleaning, maintenance, and laundry services.
- Management and disposal of clinical and hazardous waste.

- Procedures for disinfection, sterilization and decontamination.
- Sources of water and its quality.
- Adequacy and safety of patient washing and sanitary facilities.
- Food preparation and kitchen hygiene.
- Patient and visitor awareness of infection control.
- Isolation facilities for highly infectious patients.
- Control of mosquitoes and other insects, rodents and other pests.
- Procedures for identifying and managing staff infections.

Note: Further information on infection control programmes can be obtained from WHO publications: *Prevention of hospital-acquired infections. A practical guide*¹ and *Improving infection prevention and control at the health facility*².

PATHOGENS CAUSING HAIs

Only limited data are available on the pathogens causing HAIs in tropical areas and there is a great need for surveillance and information. Although methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant enterococci (VRE) and *Clostridium difficile* do occur, these may not be the principal pathogens as they are in industrialized area hospitals.

Increasingly multi-resistant Gram-negative bacteria, particularly extended spectrum beta-lactamase and carbapenemase-producing *E. coli*, *Klebsiella* species, *Enterobacter* species and *Pseudomonas* species, are responsible for healthcare infections in tropical district hospitals. They may occur as post operative wound, respiratory, urinary tract (particularly catheter-related), and blood stream infections. They may be a particular problem in neonatal infections, particularly if the hospital has a special care baby unit, where the risk of transmission is high.

Among Gram-positive bacteria, Group A streptococci and *S. aureus* are important causes of surgical wound infections and rarely, but with severe results, *Clostridium perfringens*.

Multi-resistant Gram-negative bacteria may be resistant to all available antimicrobials, including carbapenems such as imipenem, leaving few options for treatment. Laboratory detection and monitoring of these pathogens are essential.³

In areas where tuberculosis is prevalent, hospital transmission of multi-drug resistant tuberculosis is a risk. Transmission may occur from undiagnosed patients, from an infected staff member, or from visitors.

Transmission of blood-borne viruses hepatitis B, hepatitis C and HIV may occur with unscreened blood transfusions or when using contaminated syringes and needles. Highly infectious blood-borne viruses such as Ebola and Marburg may be transmitted in healthcare settings where strict patient isolation is not possible.

Occasionally, in crowded hospitals with poor hygiene or inadequate water/hygiene facilities, enteric pathogens including *Salmonella* and *Shigella* species, *V. cholerae* and hepatitis A and E viruses may be transmitted.

Unusual environmental Gram-negative bacteria including *Vibrio vulnificus*, *Aeromonas* species, *Burkholderia pseudomallei* and environmental fungi may be transmitted by patients affected by flood or earthquake disasters, or with untreated wounds in conflict areas. Regional Public Health laboratory advice should be sought if this situation occurs.

COVID-19: Guidance on how to prevent and control SARS-CoV-2 HAIs can be found in the WHO publication *Infection prevention and control during health care when coronavirus disease (COVID-19) is suspected or confirmed*⁴.

TRANSMISSION OF HAIs

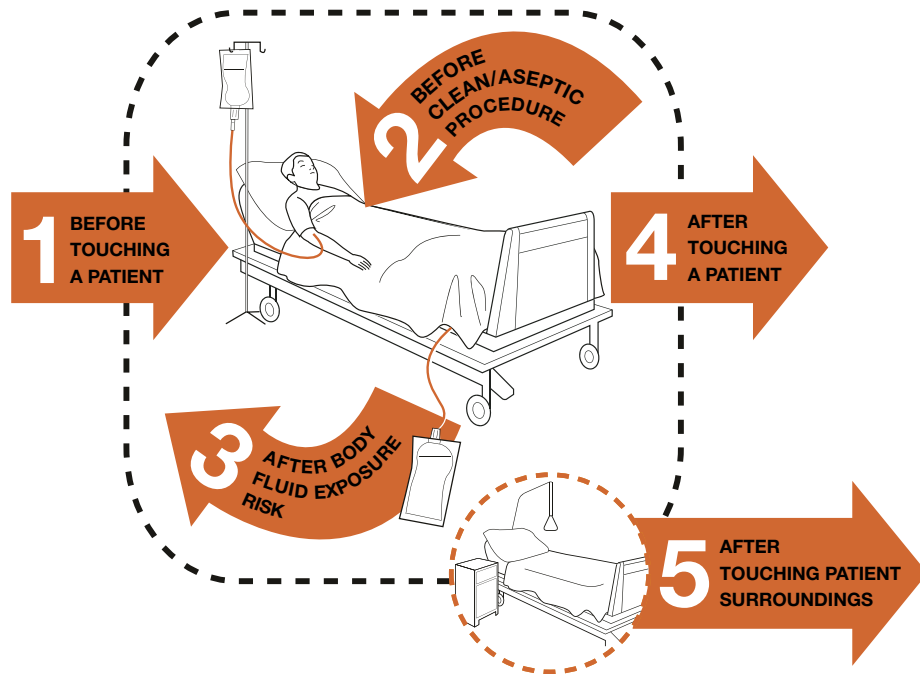
The following are the main routes by which HAIs are transmitted:

- From another patient or member of staff (exogenous cross-infection), through direct contact between patients, or from staff during patient care, e.g. from hands, clothes, nose and throat.

Person to person transmission can be minimized by effective hand decontamination and other aseptic techniques, good personal hygiene, appropriate use of gloves, masks, gowns and ensuring injection practices are safe.

- From the normal microbial flora of a patient (endogenous infection). For example, Gram-negative bacteria in the digestive tract frequently cause surgical site infections following abdominal surgery.

Your 5 Moments for Hand Hygiene



1	WHEN?	Clean your hands before touching a patient when approaching him/her.
	WHY?	To protect the patient against harmful germs carried on your hands.
2	WHEN?	Clean your hands immediately before performing a clean/aseptic procedure.
	WHY?	To protect the patient against harmful germs, including the patient's own, from entering his/her body.
3	WHEN?	Clean your hands immediately after an exposure risk to body fluids (and after glove removal).
	WHY?	To protect yourself and the health-care environment from harmful patient germs.
4	WHEN?	Clean your hands after touching a patient and her/his immediate surroundings, when leaving the patient's side.
	WHY?	To protect yourself and the health-care environment from harmful patient germs.
5	WHEN?	Clean your hands after touching any object or furniture in the patient's immediate surroundings, when leaving – even if the patient has not been touched.
	WHY?	To protect yourself and the health-care environment from harmful patient germs.

Fig 2.48 Hand Hygiene in health care.

Reproduced with permission of the World Health Organization from *WHO guidelines on hand hygiene in health care* (see Further Reading).

Minimizing invasive procedures can help to reduce endogenous infections.

- From the healthcare environment. For example, HAIs can be transmitted by contaminated water, food, disinfectant solutions, and infusions, on contaminated instruments, equipment, waste containers, dressings, bed linen and other supplies used in patient care, in dust particles and aerosols.

Environmental infections can be minimized by ensuring effective cleaning, correct sterilization and disinfection, safe handling, transport and disposal of hazardous waste, and monitoring the risk of infection from visitors to the healthcare facility.

Important: Hand decontamination has been proven to be the most effective measure in reducing the burden of HAIs.

Fig. 2.48 demonstrates the *WHO 5 moments of Hand Hygiene* that should be followed by all staff.

ROLE OF THE DISTRICT MICROBIOLOGY LABORATORY IN INVESTIGATING HAIs

The main functions of the district microbiology laboratory in the investigation and control of HAIs include:

- Providing written instructions on the collection, handling and transport of patients' samples and environmental samples.
- Identifying on site or by referral, pathogens causing HAIs and if possible, arranging for epidemiological typing of isolates in the Public Health Referral Laboratory when clusters of infection or outbreaks occur.
- Performing on site or by referral, antimicrobial susceptibility testing, reporting on the prevalence of drug resistance and providing advice on antimicrobial susceptibility of pathogens.
- Investigating outbreaks and identifying sources and routes of transmission in collaboration with the clinical staff/infection control team.
- Testing potential carriers and possible environmental sources.

- Notifying the clinical staff/infection control team of any unusual antimicrobial resistance patterns in organisms isolated from clinical samples.
- Monitoring the effectiveness of hospital sterilization and disinfection procedures using TST (Time, Steam, Temperature) control strips.
- Assisting medical and nursing staff to ensure safe working practices in clinical areas to prevent staff HAIs. Also, assisting the clinical staff/infection control team in training hospital staff in the awareness of HAIs and antimicrobial resistance.

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2.19 Acute febrile illness Measuring CRP and PCT

In district laboratories with limited facilities for bacterial culture, particularly blood culture, testing for biomarkers of infection combined with clinical findings may help to:

- differentiate bacterial from viral and other non-bacterial causes of acute febrile illness,
- diagnose sepsis at an early stage,
- monitor response to treatment,
- reduce the inappropriate use of antibiotics,
- decide which patients require referral for specialist care.

Host biomarkers

Biomarkers of systemic inflammation for which rapid point-of-care tests are available include:

- C-reactive protein (CRP)
- Procalcitonin (PCT)

Point-of-care tests being developed for other biomarkers

Information on other biomarker tests being developed and evaluated for differentiating the causes of acute febrile conditions can be found in the Unitaid publication, *Fever diagnostic technology landscape, 1st Edition, 2018*¹

TESTING FOR CRP

C-reactive protein (CRP) is an acute phase protein produced in the liver in response to inflammation and infection. It is a non-specific marker. Levels rise significantly and rapidly during bacterial disease and acute inflammation particularly in children. Also in tissue injury, e.g. following burns, necrosis, myocardial infarction. In bacterial infections, CRP levels return to normal with effective treatment. With most viral infections CRP levels are not raised or only slightly elevated.

Measurement of CRP has been found to be helpful in differentiating and managing non-malaria fevers and respiratory infections enabling antibiotics to be used more selectively.^{2,3} In studies carried out in South east Asia, measuring CRP was found to be a more useful biomarker than PCT in differentiating

viral and bacterial infections.^{4,5} Low CRP and WBC count were significant predictors of a viral infection (mainly dengue).⁵ Establishing locally CRP cut-off values increased specificity.

Measurement of CRP is helpful in differentiating viral meningitis from pyogenic meningitis. With clinical assessment it has also been found to be helpful in excluding pulmonary TB in HIV infected persons in TB-endemic areas.⁶ In HIV infection, CRP levels are not specifically associated with immune deficiency, the effects of antiretroviral therapy or disease progression.⁷

Measurement of CRP using *Abbott NycoCard Reader 11*

Abbott NycoCard reader CRP is an example of a point-of-care immunochemical test that measures CRP quantitatively. It has a measuring range of 8–200 mg/L for whole blood and a 5–120 mg/L for serum and plasma. It requires 5 µL of sample.

Test results are available within 3 minutes. They are read using the battery operated *Abbott NycoCard Reader 11* colour densitometer. The test procedure is shown in Plate 2.82.

NycoCard Reader CRP reagents (sample diluting fluid, conjugate, washing solution) have a shelf-life of 18 months from date of manufacture. The reagents require storage at 2–8 °C. The test is available as a 24 and 48 test kit.

The *NycoCard Reader 11* densitometer requires ordering separately. It is supplied with a Reader Pen, re-chargeable NiMH batteries (size AA, 1.2v) and battery charger. The *Reader* is equipped with a RS232 port for PC connection.

Note: The *NycoCard Reader 11* can also be used to measure HbA1c, urine microalbumin and D-Dimer (fibrin degradation products) using separate test kits.

*Interpreting CRP test results **

0–10 mg/L	Normal concentration in healthy adults.
10–40 mg/L	Possible mild inflammation, e.g. local bacterial infection, mild trauma.
40–80 mg/L	Increase usually indicating bacterial infection with inflammation. Occurs infrequently with viral infection.
> 100 mg/L	Significant bacterial infection with inflammation.

* Cut off values to assist in differentiating viral and bacterial infections should be decided locally.

- 1 Dilute sample using the Dilution Liquid Tube. Mix for 10 seconds.



- 2 Apply lyzed sample to test device. Allow to soak in.



- 3 Add 1 drop conjugate R2. Allow to soak in.



- 4 Add 1 drop Washing Solution R3. Allow to soak in.



- 5 Read test result from screen using whole blood or serum/plasma menu.



Plate 2.82 Quantitative measurement of CRP using Nycocard Reader 11. Courtesy of Axis-Shield. Available from www.alere.com

Standard Q Malaria/CRP Duo

Malaria/CRP Duo is a two cassette IC test developed by FIND and SD Biosensor Inc to help differentiate viral and bacterial infections in patients with fever who test negative for malaria in low malaria transmission areas.⁸ The malaria cassette detects *P. falciparum* specific HRP2 (separate P.f band) and *Plasmodium* pLDH (separate Pan band). The CRP cassette detects CRP levels > 20 mg/L. The test has a shelf-life of 24 months from date of manufacture and can be stored at 2–40 °C. It is available as a 25 test kit.

Test procedure

In the malaria test, 5 µL of whole blood is dispensed into the circular well. 3 drops of Malaria Assay diluent are added to the square well and the test result is read after 15 minutes.

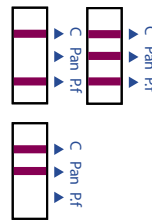
In the CRP test, 10 µL of whole blood is added to the Assay diluent tube, mixed using the dropper

provided and transferred to the square well of the cassette. The test result is read after 20 minutes.

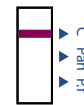
Field evaluations of the test in tropical countries are awaited.

MALARIA CASSETTE

Positive malaria test



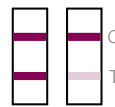
Negative malaria test



No pink-red band indicates an invalid test

CRP CASSETTE

CRP ≥ 20 mg/L



CRP not raised



No pink-red control band indicates an invalid test

Plate 2.83 *Standard Q Malaria/CRP Duo* test results. Courtesy of SD Biosensor

FebriDx CRP/MxA test

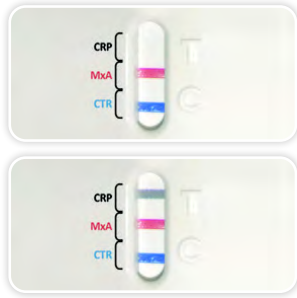
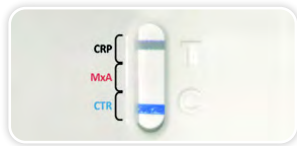
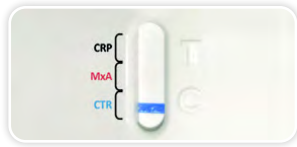
FebriDx is a two biomarker lateral flow rapid IC test manufactured by Lumos Diagnostics. It detects raised levels of CRP > 20 mg/L and MxA > 40 ng/mL. MxA (Myxovirus resistance A) is a non-specific protein marker raised in the blood in acute viral infection.

FebriDx has a shelf-life of 24 months from date of manufacture and can be stored at 4–25 °C. The test is available as a 20 test kit.

Test procedure

The test requires 5 µL of whole blood collected using the integrated lancet and blood collection tube. The blood is delivered to the test strip and the test activated by pressing the Buffer Release Button which adds buffer to the test.

The test result appears in the Test window after 10 minutes. Test results are shown in Plate 2.84.

Viral infection**Bacterial infection
or other cause of > CRP****CRP and MxA levels
not raised**

No blue control band indicates an invalid test

Plate 2.84 *Febridix* test results.
Courtesy of Lumos Diagnostics. www.febridix.com

Note: Evaluations of the performance of *Febridix* in tropical countries are awaited.

TESTING FOR PCT

Procalcitonin (PCT) is the glycoprotein precursor of the hormone calcitonin which regulates blood calcium and phosphate levels. In healthy adults, plasma PCT concentrations are below 0.1 µg/L. PCT levels rise when there is inflammation, produced in response to bacterial endotoxins and inflammatory cytokines.

PCT concentrations also increase following serious trauma and major surgery. PCT levels are usually low in viral infections, chronic non-bacterial causes of inflammation and autoimmune disorders.

Measuring PCT has been shown to be useful with clinical findings in diagnosing bacterial infected sepsis and assessing its severity. At a PCT concentration of > 0.5 µg/L, a patient is considered at risk of developing severe sepsis or septic shock.⁹ PCT is also a sensitive and specific biomarker for differentiating patients with systemic inflammatory response syndrome of non-bacterial origin from those with septic shock.

In a study carried out in India, patients with high PCT levels in severe trauma had poor outcomes and were at risk of developing complications.¹⁰

Compared to CRP, PCT is a more useful and accurate biomarker for diagnosing sepsis at an early stage, monitoring disease progression and assessing prognosis. PCT levels increase earlier than CRP levels (within 2–4 hours), reach a peak within 24–48 hours and following treatment return more quickly to normal.⁹

BRAHMS PCT direct test

Recently developed by BRAHMS GmbH and Thermo Fisher Scientific, *BRAHMS PCT direct* is a rapid point-of-care IC test that measures quantitatively PCT in 20 µL of capillary blood or venous EDTA whole blood over the range 0.22–10 µg/L.

Test results are read on a small portable *BRAHMS PCT direct* Reader and printed using the Reader's integral printer. Test time is 20 minutes.

BRAHMS PCT direct cassettes require storage at 2–8 °C and should be used with a humidity of 15–60%. Storage at room temperature should not exceed 24 hours. Test kits must not be used beyond their expiry date.

Test procedure using capillary blood

- 1 Fill the cassette completely with blood until the sample makes contact with the test membrane. Close the cassette.
- 2 Within 10 minutes of applying the sample, load the cassette into the drawer of the *BRAHMS direct* Reader and close the door.
- 3 After 20 minutes the PCT test result in µg/L will appear on the Reader display.
- 4 Print out the test result using the Reader printer. The test result can be transferred to a *Laboratory Information System (LIS)*.

Note: The test procedure is summarized in Plate 2.85 on page 226.



Plate 2.85 Upper: Summary of BRAHMS PCT direct test procedure. Courtesy of BRAHMS GmbH Thermo Fisher Scientific Inc. www.thermoscientific.com. procalcitonin Lower: B.R.A.H.M.S direct Reader' Copyright owner: B.R.A.H.M.S GmbH

Interpretation of BRAHMS PCT direct test results

Diagnosis of systemic bacterial infection (sepsis)¹¹

PCT µg/L

< 0.5 Local bacterial infection is possible. Systemic infection (sepsis) is unlikely.

Low risk for progression to severe sepsis.

Note: PCT levels below 0.5 µg/L do not exclude an infection. Localized infections (without systemic signs may be associated with such low levels). When PCT is measured early, values may still be low. PCT should be re-assessed 6–24 hours later.

≥ 0.5 Sepsis is possible but various non-infectious and < 2 causes are known to induce PCT.*

* Non-infectious causes of raised PCT are listed in the manufacturer's *Instructions for Use* which accompany the test¹¹.

Moderate risk for progression to severe sepsis. Patient should be closely monitored both clinically and by re-assessing PCT within 6–24 hours.

≥ 2 Sepsis is likely unless other causes are known. and < 10

High risk for progression to severe sepsis.

≥ 10 Important systemic inflammatory response almost exclusively due to severe bacterial sepsis or septic shock.

High likelihood of severe sepsis or septic shock.

Note: The maximum measuring range of BRAHMS PCT direct is 10 µg/L. Test results around 10 µg/L should be measured using a PCT test assay with a wider measuring range.

Diagnosis of lower respiratory tract infections¹¹

PCT µg/L

< 0.25 Bacterial infection unlikely. The use of antibiotics is discouraged.

0.25 to Bacterial infection is possible.

< 0.5 Possibly start antimicrobial therapy.

≥ 0.5 Suggestive of the presence of bacterial infection. Antibiotic treatment strongly recommended.

Quality control

The validity of the measured test is automatically checked by the BRAHMS direct Reader. A QC sample kit with two concentration levels of PCT is available from BRAHMS GmbH to check instrument performance. The controls should be run when a new batch of test cassettes is started.

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2.20 Measuring whole blood lactate

Lactate is formed from pyruvate as part of anaerobic glycolysis in response to the body's requirement for energy. Most of the lactate is cleared from the blood by the liver and kidneys. Raised levels of blood lactate (hyperlactaemia) can be found when oxygen is depleted or when the amount of lactate produced is more than can be metabolized by the liver.

Raised lactate levels in sepsis

Hyperlactaemia is associated with sepsis and septic shock with lactate levels being raised from both aerobic and anaerobic sources and decreased rate of lactate clearance. The condition has a poor prognosis.

Sepsis¹

Sepsis is defined as a life-threatening organ dysfunction caused by dysregulated host response to infection. It arises when the body's response to infection injures its own tissues and organs. If not diagnosed and managed promptly it can lead to septic shock, multiple organ failure and death. Diagnosing sepsis at an early stage and treating patients promptly are essential in reducing mortality.

A serum lactate concentration of > 2 mmol/L with persistent hypotension even after adequate fluid replacement is diagnostic of septic shock.

In maternity units, lactate testing can help to detect sepsis in pregnant women and assist in identifying patients requiring blood transfusion following postpartum haemorrhage. Maternal sepsis is a life-threatening condition defined as organ dysfunction resulting from infection during pregnancy, childbirth, post-abortion or postpartum period.² WHO estimates that 1 in 10 deaths associated with pregnancy and childbirth is due to maternal sepsis with over 95% occurring in low and middle income countries.³

High risk patients

Patients at increased risk of sepsis include:

- Hospitalized patients undergoing invasive procedures particularly when handwashing and other hygiene practices are not being followed.
- Newborn infants and young children.
- Patients infected with antimicrobial resistant pathogens.
- Persons with immunodeficiency e.g. with untreated HIV infection.

- Persons with liver cirrhosis, kidney disease, cancer or an autoimmune disease.
- Elderly people.

MEASUREMENT OF WHOLE BLOOD LACTATE

Whole blood lactate is measured to assist in the diagnosis of sepsis and septic shock (with clinical findings) and to assess and monitor severity of illness and response to treatment.

Lactate testing is also used to detect lactic acidosis in patients with HIV infection being treated with nucleoside reverse transcriptase inhibitors (NRTIs).

Note: Increasingly, measuring the bacterial infection and systemic inflammation biomarker procalcitonin (PCT) is being used to detect sepsis at an early stage and monitor disease progression (see Section 2.19).

Measuring blood lactate using *StatStrip Lactate Express* meter

StatStrip Lactate Express meter, manufactured by Nova Biomedical, is an example of a point-of-care battery operated meter that measures whole blood lactate rapidly (13 second test time) in a small volume of whole blood (0.06 μ L). The meter measures lactate electrochemically using an enzyme coated biosensor test strip. All the information needed to perform the test is contained in the pre-calibrated biosensor strip.

StatStrip Lactate Express measures and corrects for substances that can interfere with test results such as haematocrit, ascorbic acid, uric acid and acetaminophen (Paracetamol).

The lactate measuring range of the meter is 0.3–20.0 mmol/L. Test results are provided as plasma equivalent values in mmol/L. Up to 400 tests can be stored. The meter is equipped with a USB port for connection to a computer. It operates from a 3V Li replaceable battery, providing power for about 600 tests. It has automatic shut-off when not in use. The display incorporates a low battery indicator.

Procedure

- 1 Immediately before use, insert a lactate biosensor strip in the test strip port (see Plate 2.86).
- 2 Touch the end of the strip with a drop of free-flowing capillary blood until the test strip fills. A quick beep sounds when sufficient blood has been added to the strip.

- 3 Test results are displayed after 13 seconds. A long beep sounds when the test result is ready. The test result is automatically saved.
- 4 Remove the strip by using the test strip ejector (see Plate 2.86) and discard it into a biohazard waste container.



Plate 2.86 *StatStrip Lactate Express* meter, manufactured by Nova Biomedical
Courtesy of Nova Biomedical.

Quality control

High, medium and low lactate control solutions are available from the manufacturer. Automatic electronic function checks are used to verify correct meter operation. There are also built-in error codes covering meter performance, strip and sampling errors.

StatStrip Lactate Express can be operated at 15–40 °C, 10–90 % relative humidity and at altitudes up to 4 500 m (15 000 feet). The test strips must be kept in tightly closed containers, protected from direct sunlight and stored at 15–30 °C (not refrigerated). The strips have a 24 month shelf-life from manufacture and 3 months after opening. They are supplied 25 strips/vial.

StatStrip Lactate Express has been shown to perform reliably when tested against laboratory analyzers.⁴

Reference interval for whole blood lactate

The following is a reference interval for whole blood lactate using *StatStrip Lactate Express*:

Lactate 0.7–2.5 mmol/L

Increased lactate levels

Raised lactate levels (hyperlactaemia) can be found

in critically ill patients, particularly those with sepsis, septic shock, other forms of shock, trauma, severe heart failure and hypoxia (insufficient oxygen reaching body tissues).

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Section 3 Laboratory investigation of non-communicable diseases

3.1 Investigation of anaemia

3.2 Diagnosis and monitoring of diabetes mellitus

Screening for raised lipids

3.3 Investigation of kidney disease

3.4 Investigation of liver disease

3.1 Investigation of anaemia

The World Health Organization defines anaemia as a condition in which the number of red blood cells or their oxygen-carrying capacity is insufficient to meet physiologic needs which vary by age, gender, altitude, smoking and pregnancy status.*

* WHO Technical meeting. *Use and interpretation of haemoglobin concentration for assessing anaemia status in individuals and populations*. 2017, WHO.

Anaemia is an indicator of poor health and poor nutrition and is particularly prevalent in tropical countries with pregnant and lactating women, infants and young children being at greatest risk. It is diagnosed by measuring haemoglobin (iron-containing protein in red cells that transports oxygen in the blood from the lungs to body tissues).

Anaemia is likely to be present when the haemoglobin concentration falls below:

Newborn infants	140 g/L (14.0 g/dL)
Child 6 months – 6 years	110 g/L (11.0 g/dL)
Child 6 – 14 years	120 g/L (12.0 g/dL)
Non-pregnant women	120 g/L (12.0 g/dL)
Pregnant women	110 g/L (11.0 g/dL)
Men and adolescent boys	130 g/L (13.0 g/dL)

Note: Figures are taken from *Manson's Tropical Diseases*, 22nd ed, 2008, Saunders Elsevier.

Anaemia can develop:

- Following blood loss.
- When the normal production of red cells is reduced.
- When red cells are destroyed (haemolyzed) prematurely.

Often these mechanisms overlap with several factors contributing to a person becoming anaemic. Anaemia due to acute blood loss causes more

serious symptoms than anaemia of slow onset (compensated anaemia). Low haemoglobin levels may cause fewer symptoms in anaemia associated with increased 2,3-DPG in red cells, e.g. sickle cell anaemia. Haemoglobin levels may be high in patients with burns and severe dehydration due to changes in plasma volume.

Causes of anaemia in tropical countries

The following are the commonest causes of anaemia in tropical countries:

- Lack of essential nutrients and increased requirement for nutrients during pregnancy and by infants and young children:
 - iron deficiency (commonest cause of anaemia),
 - folate, vitamins A, C, B12 deficiencies,
 - protein deficiency.
- Parasitic infections, particularly:
 - malaria,
 - hookworm disease,
 - visceral leishmaniasis,
 - schistosomiasis.
- Inherited haemolytic anaemias:
 - sickle cell disease,
 - thalassaemia syndromes,
 - G6PD deficiency (haemolytic crisis).
- Bacterial and viral infections:
 - tuberculosis,
 - pneumonia,
 - bartonellosis,
 - bacteria causing sepsis,
 - HIV disease and associated opportunistic infections,
 - Viral haemorrhagic fevers, particularly outbreaks of Ebola virus disease and Marburg virus disease.
- Abnormal bleeding associated with:
 - obstetric complications such as ectopic pregnancy, placenta praevia, post-partum haemorrhage,

- injuries, traffic accidents, assault,
 - menorrhagia,
 - burns,
 - venomous snake bite,
 - peptic ulcer and gastrointestinal malignancies.
- Chronic kidney disease.
 - Lymphoma and leukaemia.

The mechanisms of anaemia can be summarized as follows:

MECHANISMS of ANAEMIA

Blood loss

- Acute bleeding, e.g. from wounds, surgical, ectopic pregnancy, placenta praevia.
- Chronic blood loss, e.g. hookworm infection, schistosomiasis, gastrointestinal bleeding, menorrhagia, PPH.

Decreased red cell production

- Lack of essential nutrients, e.g. deficiencies of iron, folate, vitamin B₁₂, protein.
- Depressed bone marrow activity, e.g. anaemias associated with chronic disease such as tuberculosis, HIV disease, chronic nephritis, chronic hepatitis, connective tissue disorders, malignant disease, leukaemias.
- Due to drugs, chemicals, ionizing radiation, some viruses.
- Thalassaemia syndromes.

Increased red cell destruction (haemolysis)

- Inherited haemolytic anaemias:
 - Haemoglobinopathies, e.g. sickle cell diseases, thalassaemia syndromes.
 - Red cell enzyme deficiencies, e.g. G6PD deficiency, pyruvate kinase deficiency.
 - Red cell membrane defects e.g. hereditary spherocytosis.
- Non-immune acquired haemolytic anaemias:
 - Infections, e.g. malaria, African trypanosomiasis, meningococcal septicaemia, *C. perfringens* infection, bartonellosis.
 - Pre-eclampsia and HELLP syndrome (haemolysis, elevated liver enzymes, low platelet count).
 - Conditions which cause disseminated intravascular coagulation (DIC).
 - Haemolytic uraemic syndrome.
 - Hypersplenism and splenomegaly, e.g. visceral leishmaniasis, hyper-reactive malaria, splenomegaly, myelofibrosis.
 - Burns
 - Venomous snake and spider bites.
 - Chemicals, oxidant drugs, local herbal remedies.
 - Paroxysmal nocturnal haemoglobinuria.

- Immune acquired haemolytic anaemias (DAT positive):
 - Haemolytic disease of the newborn.
 - Haemolytic blood transfusion reaction.
 - Warm reactive autoantibody, e.g. drug-induced chronic lymphatic leukaemia, lymphoma, systemic lupus erythematosus.
 - Cold reactive autoantibody, e.g. *M. pneumonia* infection, lymphoma.
 - Paroxysmal cold haemoglobinuria.

Morphological classification of anaemia

Based on red cell size and haemoglobin content, anaemia can be classified morphologically as:

- Normocytic normochromic anaemia
- Microcytic hypochromic anaemia
- Macrocytic anaemia
- Dimorphic anaemia

Normocytic normochromic anaemia: The red cells are of normal size, about 8 µm in diameter and adequately haemoglobinized, as shown in Plate 3.14 (see later text). The cells contain a small area of central pallor, no more than one third of the cell's diameter, due to the biconcavity of red cells.

Conditions associated with normocytic normochromic anaemia include:

- acute blood loss,
- haemolytic anaemias,
- inflammation,
- kidney disease,
- autoimmune diseases,
- aplastic anaemia,
- leukaemia, myelomatosis, myelofibrosis.

Note: In normocytic normochromic anaemia the MCV and MCH are normal (see later text).

Microcytic hypochromic anaemia: The red cells are smaller than normal having a diameter of usually less than 6.5 µm. They stain palely due to reduced haemoglobin. The cells show an increased area of central pallor as shown in Plate 3.15 (see later text).

Conditions associated with microcytic hypochromic anaemia include:

- iron deficiency anaemia,
- thalassaemia syndromes,
- chronic disease, e.g. tuberculosis, HIV disease,
- rheumatoid arthritis, malignant conditions,
- lead toxicity,
- sideroblastic anaemia (rare hereditary or acquired condition in which haemoglobin is deficient due to large amounts of iron in the red cells).

Note: In microcytic hypochromic anaemia, the MCV and MCH are low (see page 234).

Macrocytic anaemia: The red cells are larger than normal, having a diameter greater than 8 µm as shown in Plate 3.16 (see later text).

Conditions associated with macrocytic anaemia include:

- megaloblastic anaemia caused by vitamin B12 deficiency or folic acid deficiency, (macrocytes are typically oval),
- chronic liver disease (macrocytes are typically round),
- haemolytic anaemia or acute blood loss with reticulocytosis.

Dimorphic anaemia: Blood films show two different populations of red cells, for example:

- microcytic hypochromic cells with normocytic normochromic cells as seen in iron deficiency anaemia following blood transfusion,
- microcytic hypochromic cells with macrocytic cells which can be seen when the patient has both iron and folic acid deficiencies.

Note: In dimorphic anaemia the RBC histogram shows a bimodal peak.

Tests to investigate anaemia in district laboratories

- Measurement of haemoglobin.
- Reporting red cell parameters when a haematology autoanalyzer is available.
- Blood film examination.
- Reticulocyte count.
- Sickle cell test.
- Screening for G6PD deficiency.
- Detection of malaria parasites (see Section 2.1).
- Investigation of visceral leishmaniasis (see Section 2.3).
- Microscopy of faecal sample to detect hookworm eggs and schistosome eggs (see Sections 2.5, 2.6).
- Testing faeces for occult blood (see end of this Section).

- HIV testing (see Section 2.14).
- Measurement of serum/plasma creatinine and calculating albumin creatinine ratio to investigate chronic kidney disease (see Section 3.3).
- Examination of urine microscopically for RBCs and casts and chemically to investigate jaundice, kidney disease and haemoglobinuria (see Section 3.3).

Referral of tests

Depending on patient clinical history, suspected diagnosis and treatment, and the facilities of the haematology referral laboratory, the following tests may be requested by clinicians:

- Haemoglobin electrophoresis to diagnose haemoglobinopathies and thalassaemia.
- Examination of bone marrow films to diagnose megaloblastic anaemia and investigate leukaemias.
- Tests to assist in the treatment of iron deficiency.
- Tests to investigate haemolytic anaemias.

MEASURING HAEMOGLOBIN

Measurement of haemoglobin is used to diagnose anaemia and its severity and to monitor an anaemic person's response to treatment. The test is also performed to check the haemoglobin level of a blood donor prior to donating blood.

Haemoglobin values are lower in women than men due to menstrual loss and the influence of hormones on erythropoiesis. Values fall during pregnancy (particularly in the 2nd trimester) due to an increase in plasma volume and iron requirement of the foetus.

In district hospital laboratories haemoglobin is measured using:

- A haematology autoanalyzer where the work load is sufficiently high, service engineers and consumables are available, electricity supplies are sufficiently stable, and staff training can be provided.
- A direct reading calibrated haemoglobin meter, e.g. *HemoCue 201+*.
- A filter colorimeter calibrated by the user to measure haemoglobin.

Note: A visual reading haemoglobin method such as the *Haemoglobin Colour Scale* is frequently used to check the haemoglobin of blood donors where photometric techniques are not available.

Assessing anaemia using the Packed Cell Volume (PCV)

Compared with measuring haemoglobin, the PCV is a less reliable and controllable method of assessing anaemia. When used, it is important to ensure capillary tubes are completely sealed and the microhaematocrit centrifuge has sufficient electrical power and is designed to pack red cells completely. A reliable method of identifying samples must be used and care taken to dispose of glass capillaries safely.

Blood sample

Capillary blood or ethylene diamine tetra-acetic acid (EDTA) anticoagulated venous blood can be used.

Capillary blood: Ensure the skin is dry and the blood is free-flowing. Avoid excessive squeezing of the area which can dilute the sample with interstitial fluid.

Venous blood: Do not apply the tourniquet too tightly or for too long a period as this will cause venous stasis leading to a false increase in haemoglobin concentration. When available, use an EDTA (purple-top) collecting vacuum tube.

Vacuum-tube

This is a single use colour-coded blood collection tube system. When using a *Vacutainer*, one end of a double-ended needle is inserted in the vein. The other sheathed end pierces the cap of the *Vacutainer*. The vacuum in the tube draws the correct volume of blood into the tube. When collecting samples for several different tests it is important to collect samples in the correct order to minimize contamination of sterile samples and anticoagulant carry-over between tubes.

Order of collection: 1. Sample for blood culture, 2. Blue-top, sodium citrate 3.2%, 3. Red-top, serum tube, 4. Yellow-top, gel separator/clot inactivator tube, 5. Green-top, heparin, 6. Purple (lavender)-top, EDTA, 7. Gray-top, fluoride containing tube. *NEVER* pour blood from one vacuum tube to another.

When using a needle and syringe, avoid haemolyzing the sample by not withdrawing the blood too rapidly or moving the needle once it is in the vein. Remove the needle from the syringe before dispensing the blood into the EDTA container. Add the correct amount of blood to anticoagulant. Do not shake the blood but gently invert the container about six times to prevent the blood from clotting.

Stability of blood sample: Haemoglobin concentration is stable for 2–3 days when the EDTA sample is stored at 4–8 °C within 6 hours of being collected. Blood that has been refrigerated must be allowed to warm to room temperature (30 minutes) and be well mixed before being tested. Before measuring the haemoglobin, check the sample for clots. If clots are found the sample must not be used.

USING A HAEMATOLOGY AUTOANALYZER

Using an autoanalyzer, anaemia is diagnosed from measuring haemoglobin, calculating the haematocrit, counting and sizing red blood cells (RBCs) and using RBC parameters to obtain information on the size and haemoglobin content of red cells.



Using a haematology autoanalyzer.
Courtesy of Amref International University, laboratory unit.

Measuring haemoglobin

When an autoanalyzer is used, haemoglobin can be measured accurately, precisely and rapidly. A methaemoglobin photometric method is used. Most manufacturers use a non-hazardous reagent such as sodium lauryl sulphate. A non-ionic detergent is added for rapid cell lysis and to minimize turbidity.

Lipaemia, hyperproteinaemia, hyperbilirubinaemia, a high white blood cell count (i.e. > 100 00/μL) and red cells that resist lysis can interfere with haemoglobin readings, causing falsely raised values. Most analyzers compensate to some degree for turbidity. It is important to consult the manufacturer's *User Manual* regarding substances that may interfere with the accuracy of haemoglobin test results.

RBC count

Red cells are counted by impedance or light scatter analysis. RBC numbers are plotted on the Y-axis and RBC volume on the X-axis. The analyzer counts as

red cells those cells with cell volumes of 36–360 fL. Between 60–125 fL is used to calculate the mean cell volume and red cell distribution width. A left shift of the peak indicates microcytosis and a right shift indicates macrocytosis. A double population of red cells is shown by a bimodal peak of the curve.

The RBC count is affected by red cell agglutination and cold agglutinins which may cause a false decrease in the count. Fragile RBCs may lyse and cause incorrect RBC counts. The *User Manual* should be consulted regarding those factors that can affect the accuracy of RBC counts.

Red blood cell parameters

These include mean cell volume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and red cell distribution width (RDW).

MCV: This is the volume of the average red cell, usually measured directly. A guideline reference interval is 83–101 femtolitres (fL). The MCV is increased in macrocytic anaemias, chronic alcoholism, acquired aplastic anaemia and when there is reticulocytosis. A false MCV increase can occur with hyperlipidaemia, hyperglycemia, and red cell auto-agglutination. The MCV is reduced in microcytic anaemias, particularly iron deficiency anaemia, thalassaemia and anaemias of chronic diseases.

MCH: This gives the average amount of haemoglobin in an average red cell. It is calculated by dividing the haemoglobin concentration by the RBC count. A guideline reference interval is 27–32 picograms (pg). A reduced MCH is found in microcytic hypochromic anaemia and when the red cells are microcytic and normocytic. It is reduced in thalassaemia minor even when anaemia is mild. A raised MCH is found in macrocytic normochromic anaemia.

MCHC: This gives the average concentration of haemoglobin in an average red cell. It is calculated from the haemoglobin, MCV and RBC count ($MCHC = Hb/MCV \times RBC \text{ count} \times 100$). It can also be calculated from the haemoglobin and haematocrit ($MCHC = Hb / HCT$). A guideline reference interval is 315–345 g/L (adults). The MCHC is low in established iron deficiency anaemia and other conditions in which red cells are microcytic and hypochromic including anaemia of chronic disorders, lead toxicity and thalassaemias.

Unlike the manually calculated MCHC, the autoanalyzer computed MCHC is not a sensitive indicator of early iron deficiency. The MCHC is however a valuable indicator of the validity of a FBC measurement and should be carefully checked on each sample tested. In thalassaemia minor the MCHC is often normal. Red cell autoagglutination can cause a false increase in MCHC. A very high white cell count can cause a false decrease in MCHC.

RDW: This gives the quantitative indication of variation in the sizes of red cells, usually expressed as coefficient of variation (CV) of red cell distribution (seen as anisocytosis in blood films). The greater the RDW, the greater the variation in sizes of the red cells. A guideline reference interval is 11.6–14% CV. The RDW is increased in iron deficiency anaemia but not in beta-thalassaemia minor and anaemia

of chronic disease. A common cause of increased RDW and MCV is reticulocytosis.

Important: A stained blood film should always be examined when the RDW is raised. A normocytic normochromic anaemia with raised RDW is frequently seen as dimorphic anaemia in blood films, e.g. following blood transfusion.

Based on the RBC histogram and red cell parameter values, anaemia can be classified as:

- Normocytic normochromic anaemia with normal RDW
- Normocytic normochromic anaemia with raised RDW
- Microcytic hypochromic anaemia with normal RDW
- Microcytic anaemia with raised RDW
- Macrocytic anaemia

White blood cell information

Autoanalyzers provide a total white blood cell count (WBC) and either a 3-part or 5-part WBC differential count (3-part differential analyzers are commonly used in district laboratories).

The 3-part differential divides WBCs into lymphocytes having a cell volume of 35–100 fL, monocytes, eosinophils and basophils (mid-size cells) having cell volumes of 100–150 fL and neutrophils having a cell volume of 150–300 fL. The cell volumes correspond to the sizes of cell nuclei.

WBC total count reference interval

The following is a guideline reference interval for a WBC total count:

Children at 1 year	6.0–18.0 × 10 ⁹ /L
Children 4–7 year	5.0–15.0 × 10 ⁹ /L
Adults of non-African origin	4.0–10.0 × 10 ⁹ /L
Adults of African origin	2.6–8.3 × 10 ⁹ /L
Pregnant women	Up to 15 × 10 ⁹ /L

Leucocytosis: The main causes of a raised WBC count are:

- Acute bacterial infections.
- *Note:* Acute infections in children can cause a sharp rise in a WBC count.
- Inflammation and tissue necrosis, e.g. burns, fractures and trauma, arthritis, tumours.
- Metabolic disorders, e.g. uraemia, diabetic coma, eclampsia.
- Poisoning, e.g. chemical, some drugs, snake venoms.
- Acute haemorrhage.
- Leukaemia.

Leucopenia: The main cause of a low WBC count are:

- Bacterial infections such as brucellosis, typhoid, relapsing fever, miliary tuberculosis, rickettsial infections, septicæmia.
- Viral infections including HIV, measles, rubella, chicken pox, hepatitis.
- Parasitic infections, e.g. leishmaniasis.
- Some drugs and chemicals.
- Aplastic anaemia, megaloblastic anaemia, acute leukaemia.
- Splenomegaly.
- Anaphylactic shock.

Most autoanalyzers flag the presence of nucleated red cells, reactive lymphocytes, myelocytes, promyelocytes and significantly increased numbers of eosinophils.

The WBC count may give incorrect test results when abnormal unlyzed red cells greater than 35 fL are present, when the sample is haemolyzed, contains nucleated red cells, cryoglobulins or small clots and fibrin strands. The *User Manual* should be consulted regarding other factors that may affect the reliability of the total and differential WBC count.

Platelet information

Autoanalyzers provide a platelet count measured by electrical impedance. A guideline reference interval is $150\text{--}410 \times 10^9/\text{L}$. The platelet histogram shows platelet cell volume in fL on the X-axis and relative platelet frequency on the Y-axis. The analyzer counts as platelets particles that are 2–25 fL.

Interpretation of platelet counts

In health there are about $150\text{--}410 \times 10^9/\text{L}$ platelets. A lower platelet count can occur in pregnancy.

Thrombocytosis: The main causes of a raised platelet count are:

- Iron deficiency anaemia, associated with active bleeding.
- Chronic myeloid leukaemia, myelofibrosis.
- Tumours.
- Acute inflammatory disease, burns, trauma.
- Haemorrhage.
- Sickle cell disease associated with hyposplenism.
- Following splenectomy.

Thrombocytopenia: A low platelet count can lead to bruising and bleeding. The main causes are:

Reduced platelet production

- Infections, e.g. brucellosis, typhoid.
- Megaloblastic anaemia.
- Aplastic anaemia.
- Drugs, e.g. aspirin, cytotoxic drugs, quinine.
- Chemicals, some herbal remedies, alcoholism.
- Leukaemia, lymphoma, myelomatosis.

Increased destruction or consumption of platelets

- Infections, e.g. acute falciparum malaria, dengue haemorrhagic fever, visceral leishmaniasis.
- DIC (disseminated intravascular coagulation).
- Hypersplenism.
- Immune destruction of platelets e.g. ITP, (idiopathic thrombocytopenic purpura), onyala, SLE (systemic lupus erythematosus), other connective tissue disorders, chronic lymphatic leukaemia, lymphoma and HIV disease. Also exposure to some drugs and herbal remedies.

Platelet parameters calculated by most analyzers include mean platelet volume (MPV) and platelet distribution width (PDW).

MPV: This gives the average size of platelets with a guideline value of 7–10 fL. An MPV greater than 10 fL indicates the presence of immature platelets. An increased MPV is associated with myocardial infarction. An MPV is raised in sickle cell disease, ITP and following splenectomy.

An MPV less than 7 fL indicates the presence of small platelets as occurs when platelet production in the bone marrow is reduced or there is immune destruction of platelets. It occurs with aplastic anaemia, megaloblastic anaemia, chemotherapy and thalassaemia trait.

PDW: This measures variation in the size of platelets. An increased PDW occurs in megaloblastic anaemia, chronic myeloid leukaemia and following chemotherapy.

Blood film requirement

Abnormal autoanalyzer test results and/or flagging, indicate the need to examine a stained blood film. Examination of a blood film is also required when clinical findings suggest:

- malaria,
- sickle cell disease or other haemoglobinopathy,
- G6PD deficiency,
- infectious mononucleosis,
- leukaemia or lymphoma,
- splenomegaly or lymphadenopathy,
- kidney disease.

Also, when a patient is jaundiced or receiving treatment for a blood disorder.

District laboratory staff require the support of a specialist haematology laboratory to investigate and report serious blood disorders.

USING A HAEMOGLOBIN METER

An example of a direct read-out haemoglobin meter is the *HemoCue 201+*. It measures haemoglobin in undiluted blood using dry reagents in single use microcuvettes. It is factory calibrated against the haemoglobin cyanide (HiCN) international reference method. No user calibration is required.

Operating principle of *HemoCue 201+*

A modified azidemethaemoglobin reaction is used. Sodium deoxycholate haemolyzes the red cells, releasing haemoglobin. Sodium nitrite converts the haemoglobin iron from the ferrous to the ferric state, forming methaemoglobin. This combines with sodium azide to form azidemethaemoglobin which is measured at two wavelengths, 570 nm and 880 nm. The second wavelength compensates for turbidity in the sample.

HemoCue 201+ can be operated from mains electricity using the AC adapter provided or from four AA batteries (not provided). When using batteries, the analyzer turns off automatically after

5 minutes when not in use. When battery power is running low, the display shows a low battery symbol.

Note: Test results can be printed using the HemoCue printer (optional accessory). The analyzer can store and recall up to 600 test results.

Quality control of HemoCue 201+

Every time the analyzer is turned on, it performs automatically a selftest to verify the performance of the optronic unit. The selftest is repeated every second hour as long as the analyzer remains switched on. Inbuilt error codes alert the user of any electronic or optronic fault, when to replace batteries or when a test result is outside the measuring range of the analyzer (0–25.6 g/dL). Regular cleaning and maintenance of the analyzer, as instructed by the manufacturer, are essential.

Liquid quality controls (2x1mL pack size) are available from HemoCue covering high values (*HemoTrol High*), normal values (*HemoTrol Normal*) and low values (*HemoTrol Low*). A quality control check should be performed daily, every time a new batch of microcuvettes is opened or when a test result does not match clinical findings.

Precision and accuracy of HemoCue 201+ analyzer

Performance evaluations are variable with some studies showing optimal performance and others indicating test results are more accurate when using venous blood. Adequate training in the use of the analyzer has been shown to be important. Inaccurate capillary test results may occur due to poor blood collection technique or less commonly, due to a patient's circulatory disorder.

Use and storage of analyzer and microcuvettes

The analyzer can be used between 15–30 °C and relative humidity < 90 %. Microcuvettes require storage at 15–30 °C (do not refrigerate). To prevent deterioration in hot humid climates, ensure a microcuvette container is tightly closed immediately after use. Microcuvettes are available, in 4 tube × 50 pack or individually wrapped in boxes of 25. They are stable for 2 years from date of manufacture. Once a container is opened the microcuvettes are stable for 3 months.

Other HemoCue haemoglobin analyzers

HemoCue 201+ requires differentiating from the *HemoCue 201 DM* analyzer which operates from mains electricity and a built-in rechargeable lithium ion battery, recharged from AC mains electricity using a docking station. It cannot be used with AA batteries like the *HemoCue 201+*. The *HemoCue 301* analyzer uses microcuvettes that do not

contain reagents to convert haemoglobin to azidemet haemoglobin. It is unsuitable for testing haemoglobin lysates in EQA programmes.

External Quality assessment (EQA)

Participation in a proficiency testing programme is required if a laboratory is to be sure a *HemoCue 201+* meter is providing accurate test results for the measurement of haemoglobin.



HemoCue 201+ haemoglobin meter.
Courtesy of HemoCue AB www.hemocue.com

USING A FILTER COLORIMETER

In peripheral laboratories, haemoglobin may be measured colorimetrically using the following manual techniques:

- Haemiglobincyanide (HiCN) technique,
- Alkaline haematin D (AHD) technique.

Haemiglobincyanide technique

This is recommended by the International Committee of Standardization in Haematology (ICSH) because it measures all forms of haemoglobin except sulfhaemoglobin and stable HiCN standards are available for instrument calibration. It does, however have disadvantages when used in tropical countries (see following text).

Principle of the HiCN method

Whole blood is diluted 1 in 200 in a modified Drabkin's solution which contains potassium ferricyanide and potassium cyanide. The red cells are haemolyzed and the haemoglobin is oxidized by ferricyanide to methaemoglobin. This is converted by the cyanide to stable haemiglobincyanide (HiCN). Absorbance of the HiCN solution is read

at wavelength 550 nm in a filter colorimeter. The absorbance obtained is compared with that of a reference HiCN standard solution. Haemoglobin values are obtained from tables prepared from a calibration graph.

Quality control of HiCN technique: A control haemolysate should be used daily to check the performance of the colorimeter and quality of the Drabkin's reagent. When a control haemolysate or preserved whole blood control is not available, the minimum control of haemoglobin tests using the HiCN technique must include:

- Daily use of a HiCN reference standard (as used to calibrate the colorimeter) to check the colorimeter is functioning correctly.
- Visible and photometric check of Drabkin's diluting fluid for signs of deterioration, particularly turbidity which is a common problem in tropical countries. When measured against a water blank with a yellow-green filter in place (wavelength 550 nm), Drabkin's fluid should give a zero reading. The pH of the fluid should be pH 7.0–7.4. If deterioration is indicated, the fluid should not be used. Fresh Drabkin's reagent must be prepared. During the 'hot' season, Drabkin's fluid is best stored refrigerated. It must be allowed to warm to room temperature before being used.

Alkaline haematin D technique

The alkaline haematin D-575 technique using a filter colorimeter is as accurate as the HiCN technique but less expensive and uses a stable diluting reagent that does not contain toxic potassium cyanide. It is recommended for use in tropical countries. A commercially available AHD standard solution is used to calibrate the method. All forms of haemoglobin are converted into stable alkaline haematin D-575.

Principle of the alkaline haematin D method

Whole blood is diluted in a non-ionic, alkaline detergent diluting fluid (AHD reagent) which contains sodium hydroxide and Triton X-100 (or equivalent). The red cells are haemolyzed and the haemoglobin is converted to alkaline haematin D-575 which is a stable coloured compound. Absorbance of the alkaline haematin D-575 solution is measured at wavelength 580 nm in a filter colorimeter. The absorbance obtained is compared with that of a stable AHD chlorohaemin standard. Haemoglobin values are obtained from tables prepared from a calibration graph.

Further information on the AHD technique

Details of measuring haemoglobin by the AHD technique can be found in the new edition of *Practical Laboratory Manual for Health Centres in Eastern Africa*, Carter J Y, Orgenes L, 2nd ed, 2020. Amref International University.
E-mail: enquiry@amref.ac.ke

NON-INVASIVE TECHNOLOGIES TO MEASURE HAEMOGLOBIN

Non-invasive technologies to measure haemoglobin include:

- *Pronto – 7* (Masimo Corporation, Irvine, USA)
- *NBM 200* (OrSense Co, Israel)
- *Haemospect* (MBR Optical Systems GmbH, Germany)
- *TouchHb* (Biosense Technologies, India)

Pronto-7

This technology uses co-oximetry to calculate haemoglobin transcutaneously. A multi-wavelength sensor using near-infrared spectroscopy is placed over the finger tip. Three measurements are displayed within 40 seconds: total haemoglobin, oxygen saturation rate, pulse rate. The rechargeable battery lasts for up to 2 hours. A perfusion index is also displayed (adequate perfusion rate is required for assessing haemoglobin). Using *Pronto-7*, haemoglobin referred to as SpHb can be monitored continuously, e.g. in surgical and trauma units to detect blood loss and identify the need for blood transfusion.

NBM 200

This technology uses occlusion spectroscopy to determine haemoglobin in an underlying blood vessel. A ring-shaped multi-wavelength sensor is fitted to the finger to temporarily stop blood flow. The sensor measures light transmitted through the finger between wavelengths 600–1500 nm. The differential light absorption before and after blood flow obstruction is used to determine haemoglobin. The device is battery operated and displays total haemoglobin, oxygen saturation and pulse rate.

Haemospect

This is a handheld device in which a sensor placed on the fingertip of the middle finger projects a white light into the underlying tissue. The light reflected back is analyzed and processed electronically to measure haemoglobin in tissue capillaries. Readings include total, oxygenated and deoxygenated haemoglobin.

TouchHb

This battery operated handheld device uses light to measure haemoglobin from an eyelid scan. *TouchHb* quantifies conjunctival pallor caused by the reduced amount of oxyhaemoglobin in the blood. The test result is displayed in about 60 seconds. The device can be linked to a smartphone via Bluetooth.

Haemoglobin values from non-invasive techniques can be affected by skin tones, high levels of bilirubin, haemoglobinopathies, ambient light, patient movement and metallic nail polish. Non-invasive technologies however are ongoing with more reliable, verifiable and less costly sensors being developed and adapted for use in resource limited countries, e.g. to monitor haemoglobin levels in pregnant women and children and to screen blood donors. Field studies in low resource countries are awaited.

Applications using smartphone cameras with light source are also being researched to analyze haemoglobin by measuring light absorption at different wavelengths.

HAEMOGLOBIN REFERENCE INTERVALS FOR SOME TROPICAL COUNTRIES

Adults

COUNTRY	MEN g/dL	WOMEN g/dL
Togo, 15–58 y ^a	10.0–18.4	10.3–17.1
Tanzania, 19–48 y ^a	13.7–17.7	11.1–15.7
Ghana, 18–59 y ^b	11.7–16.5	9.1–14.0
Uganda, > 24 y ^a	11.1–16.8	10.1–14.3
Botswana, 18–66 y ^c	11.9–17.1	9.3–16.0
Mali, 18–59 y ^a	12.4–17.6	12.0–14.9
Ethiopia, 18–65 y ^d	13.6–19.8	12.0–18.0
Burkina, 18–78 y ^a	11.3–15.6	9.8–13.5
South Africa, av 41 y ^e	13.4–17.5	11.6–16.4
India, < 40 y ^f	12.6–17.1	9.6–14.3

References: ^a Kone B *et al.* Establishing reference ranges of haematological parameters from Malian healthy adults. *J Blood Lymph*, 2017;7:1. The Paper also includes reference intervals for other African countries. ^b Dosoo D K *et al.* Haematological & biochemical reference values for healthy adults in the middle belt of Ghana. *Plos One*, 2012, 7 (4): e36308 doi 10.1371. ^c Madisa M *et al.* Immunohaematological reference values for HIV-negative healthy adults in Botswana. *PubMed*, 2012 Jan, doi10.4102/ajilm.v.1i1.5. ^d Wondemagegn M *et al.* Haematological & CD4 + T cells reference ranges in healthy adult populations in Gojjam zones in Amhara region, Ethiopia. *PLos One* 2017;12(7):e0181268. ^e Lawrie D *et al.* Local reference ranges for full blood count and CD4 lymphocyte count testing. *S Afr Med J*, 2009, 99:243–248. ^f Shrivlekha S *et al.* Haematological & biochemical parameters in apparently healthy Indian population: defining reference intervals. *Indian J Clin Biochem*. 2014, July.

Healthy newborns and young children

COUNTRY		g/dL
Pakistan	Newborns,	12.0–17.9 ^g
Thailand	Infants, 1 y	10.3–15.0 ^h
	6–8 y	10.2–16.4
	10–12 y	11.0–16.0
Nigeria	Newborns,	13.5–18.0 ⁱ
	1–5 y	10.5–14.0
	5–9 y	11.5–13.5
	9–14 y	12.5–14.5

References: ^g Qaiser D H *et al.* Haematological reference values for full term healthy newborns of Karachi, Pakistan. *J Pakistan Med Assoc.* 2009, September. ^h Viprasit V *et al.* Haematological parameters and red blood cell indices in healthy Thai children: A Revision for 2005. *J Med Assoc Thai*, 2005 Vol. 88 Suppl. 8. ⁱ Buseri FI *et al.* Reference values haematological indices of infants, children, and adolescents in Port Harcourt, Nigeria. *Pathology and Laboratory Medicine International*, downloaded from <https://www.dovepress.com> by 90.213.43.199 on 23 May 2018.

Low haemoglobin levels

The common causes of anaemia in tropical countries are listed at the beginning of this Section.

Raised haemoglobin concentration

Causes include polycythaemia in which red blood cell production is increased in response to low blood oxygen levels as can occur in chronic lung disease, heart failure, excessive smoking (carbon monoxide exposure) and at high altitude. Less common causes are bone marrow disease (primary polycythaemia), kidney and liver cancer. Haemoglobin levels may be high in patients with burns or severe dehydration due to changes in plasma volume.

REPORTING BLOOD FILMS

The reliable reporting of blood films requires films to be made, fixed and stained correctly using standardized procedures. District laboratory staff require training in the recognition and reporting of normal blood cell morphology and changes which may occur in the appearances of WBCs, RBCs and platelets, particularly in anaemias, infections and blood disorders. A well interpreted blood film can provide, at low cost, considerable information about a patient's condition.

Note: Malaria parasites, trypanosomes, microfilariae, borreliae, *Babesia* and *Bartonella* can also be seen in thin blood films. They are however usually best detected in thick blood films.

External quality assessment (EQA)

Whenever possible, district laboratories should participate in an EQA programme which includes blood film reporting, and haemoglobin measurement. EQA can help laboratories identify and correct errors and interpret test results.*

* The East African Regional External Assessment Scheme (www.eareqas.org) focuses on strengthening clinical and laboratory diagnostic services at primary healthcare level and educating laboratory staff. It includes blood film reporting and measuring haemoglobin.

Blood sample

A thin blood film can be made from free-flowing capillary blood or EDTA anticoagulated venous blood. Estimating platelet numbers and interpreting platelet morphology is less reliable when using capillary blood because platelets tend to clump.

Important: Blood films from EDTA anticoagulated venous blood must be made with as little delay as possible (within 1 hour of collection) to prevent morphological changes making it difficult to identify blood cells correctly. Changes occur soon after the blood is collected when it is stored at room temperature (18–25 °C), more marked at higher

temperatures, and within 3 hours when the blood is stored at 4–8 °C. Changes include:

- Neutrophil degeneration with neutrophils becoming more irregular in shape, nuclear lobes separating, vacuoles appearing in the cytoplasm. Granules may also be lost.
- Segmentation (budding) of the nucleus of monocytes and lymphocytes and vacuoles appearing in the cytoplasm.
- RBCs becoming crenated and spherocytic and platelets disintegrating.

Note: Manual or automated cell counts, reticulocyte counts and haematocrit change little in EDTA blood at 4–8 °C when stored for up to 24 hours. At room temperature changes start to occur after about 2 hours. Haemoglobin concentration is stable for 2–3 days when EDTA blood is stored at 4–8 °C provided there is no haemolysis.

Preparation, fixing and staining of thin blood films

Monitoring the quality of blood films forms part of quality control.

Features of a well-made film

- Not too thick, too thin or too long, avoided by not using too large a drop of blood and when the patient is anaemic, spreading the blood at a steeper angle and more quickly.
- Free from lines and holes, avoided by using a clean grease-free slide and spreading the blood smoothly.
- Has a smooth tail, achieved by using a clean spreader which does not have a chipped end.

Methanol (methyl alcohol) fixing of thin blood films

When *completely dry*, fix the blood film by adding 1–2 drops of water-free absolute methanol * and allowing it to dry on the film.

Water-free methanol: Water-free methanol is essential for the correct fixing of thin blood films. In humid climates it is particularly important to ensure moisture is prevented from entering the methanol by using a dispensing container that can be closed completely when not in use e.g. TK bottle. Also, ensuring the cap of stock methanol bottles is tightly closed after use.

When absolute methanol is not available, absolute ethanol (ethyl alcohol) can be used but this is more expensive and usually less available than methanol. Methanol described as “*Technical*” grade is not suitable for fixing thin blood films (nor for making Romanowsky stains).

Note: Plates showing a well-made blood film and the damage to blood cells which occurs when moisture containing methanol is used to fix blood films can be found in subunit 8.7 Part 2 *District Laboratory Practice in Tropical Countries*. Access on-line by entering the title in google and clicking on the PDF entry fac.ksu.edu.sa. Use the left hand column to access subunit 8.7, Blood films.

Staining thin blood films

In most district laboratories thin blood films are stained manually using Leishman or Wright’s Romanowsky stain which stains blood cells differentially.

Poorly inconsistently stained blood films are mainly due to:

- Using poor quality stains or allowing moisture to enter stock stains.
- Using buffered water of incorrect pH, i.e. not at pH 6.8.
- Allowing stain deposit to form on blood films by tipping off the stain instead of washing it off.
- Not using quality control blood films to check the staining reactions of newly made batches of stain, and to decide the optimum staining time.
- Not using a standardized staining procedure.

Note: Suggested procedures for Leishman and Wright’s staining of thin blood films can be found in Appendix VI.

MANUAL WBC DIFFERENTIAL COUNT

A differential WBC count provides information on the different white cells present in the circulating blood, i.e. neutrophils, lymphocytes, monocytes, eosinophils, basophils. The absolute number of each type of WBC is calculated from the total WBC count and expressed as cells per litre of blood. A WBC differential also provides information on the morphology of red cells and platelets.

When the blood film is prepared from EDTA anticoagulated venous blood, it is usually possible to estimate whether platelets are normal, increased or decreased in number. The red cell to platelet ratio is normally 10–15:1. Large platelets can be seen in bone marrow disorders and hyposplenism. See previous text for the causes of increased and decreased platelet numbers.

Important: First use the 10× objective to select a part of the blood film that is well stained, the WBCs are

well distributed and the red cells are just overlapping. Using the 40× objective, systematically examine the film, counting up to a total of 100 WBCs, preferably using a differential WBC counter. Calculate the absolute number of each WBC by multiplying the number of each cell counted (expressed as a decimal fraction) by the total WBC count.

Example: Percentage of neutrophils counted = 80%
Total WBC count = $8.6 \times 10^9/L$
Absolute neutrophil count: $0.80 \times 8.6 = 6.9 \times 10^9/L$

Use the 100× objective to identify malaria parasites (see Section 2.1) and other RBC inclusions.

Differential WBC reference interval (guideline values, to be checked locally)

	Absolute number	Percentage
ADULTS		
Neutrophils	$1.5-7.5 \times 10^9/L$	(40–75%)
Lymphocytes	$2-4.0 \times 10^9/L$	(21–40%)
Monocytes	$0.2-1.0 \times 10^9/L$	(2–10%)
Eosinophils	$0.02-0.6 \times 10^9/L$	(1–6%)
Basophils	$0.01-0.1 \times 10^9/L$	(0–1%)
CHILDREN (2–6 y)		
Neutrophils	$1.5-6.5 \times 10^9/L$	(20–45%)
Lymphocytes	$0-8.5 \times 10^9/L$	(45–70%)
Monocytes	$0.1-1.0 \times 10^9/L$	(2–10%)
Eosinophils	$0.3-1.0 \times 10^9/L$	(1–6%)
Basophils	$0.01-0.1 \times 10^9/L$	(0.1–1%)

Note: In an adult, lymphocytes are mainly of the small type, whereas in a child large lymphocytes predominate. Neutrophil counts are lower in Africans and Afro-Caribbean people.

Microscopical appearance of WBCs

CELL	MORPHOLOGY
Neutrophil 12–15 μm	3–5 lobed nucleus, dark mauve staining. Pale staining cytoplasm with small granules.
Small lymphocyte 10–12 μm	Compact, dark mauve staining nucleus with clumped chromatin. Thin rim of blue cytoplasm surrounds the nucleus.
Large lymphocyte 12–16 μm	Round or oval, sometimes indented nucleus. Pale blue cytoplasm which may contain small granules.
Monocyte 15–20 μm (largest cell)	Round, indented or folded nucleus which stains mauve and has a delicate chromatin pattern. Cytoplasm stains grey-blue, contains <i>fine</i> granules and may contain vacuoles.
Eosinophil 12–17 μm	Bi-lobed nucleus which stains dark mauve. Cytoplasm contains orange-red granules and occasionally vacuoles.
Basophil 10–12 μm	Bi-lobed nucleus, usually obscured by granules. Cytoplasm contains large dark blue-violet granules. Basophils are not often seen.

NORMAL WBC MORPHOLOGY

40× Leishman stained

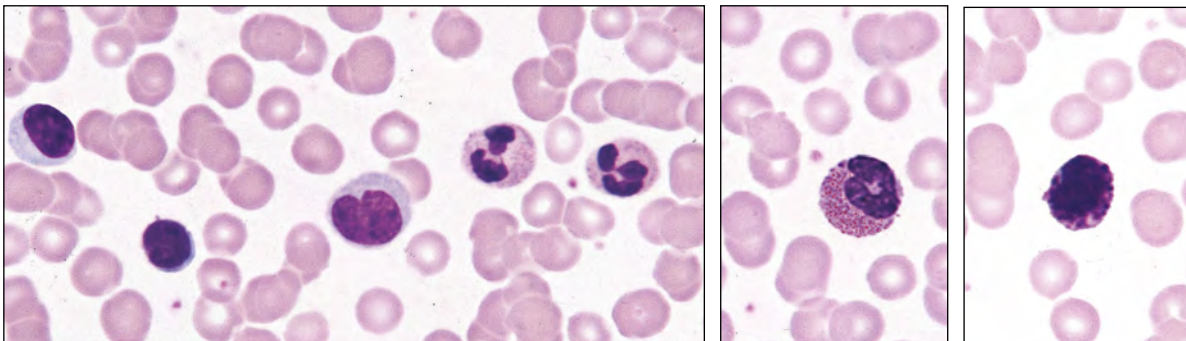


Plate 3.1 Normal blood film showing from left to right: a large lymphocyte, small lymphocyte, monocyte and two neutrophils. The small mauve-staining particles between cells are platelets. Plate 3.2 An eosinophil. Plate 3.3 A basophil.

INCREASED AND DECREASED WHITE CELLS

Neutrophilia: Causes of increased neutrophils include:

- Severe bacterial infections with cytoplasmic toxic granulation, e.g. abscesses, wound infections, meningitis, pneumonia.
- Sepsis (with low platelet count).
- Metabolic disorders and malignant diseases.
- Inflammatory conditions.
- Myeloid leukaemia.
- Snake envenomation.
- Polycythaemia (with raised haematocrit).
- During pregnancy and delivery.
- Reactions to some drugs, e.g. corticosteroid therapy.

Neutropenia: Causes of decreased neutrophils include:

- Bone marrow failure.
- Bacterial infections, e.g. brucellosis, typhoid, millitary tuberculosis.
- Viral infections.
- Splenomegaly.
- Cancer chemotherapy and some drugs.
- Vitamin deficiencies.

Lymphocytosis: Causes of increased lymphocytes include:

- Infections in children, e.g. whooping cough, rubella, mumps, measles, chicken pox.
- Bacterial infections, e.g. brucellosis, typhoid fever, chronic tuberculosis, secondary syphilis.
- Infections mononucleosis.
- Cytomegalovirus infection.
- Toxoplasmosis.
- Lymphocytic leukaemia and lymphoma.

Note: Atypical (reactive) lymphocytes may be seen in blood films in malaria and viral infections, e.g. infectious mononucleosis and whooping cough (see Plate 3.6).

Lymphopenia: Causes of reduced lymphocytes include:

- HIV disease.
- Some acute viral infection.
- Hodgkins disease.
- Autoimmune disorders.

Monocytosis: Causes of increased monocytes include:

- Chronic bacterial infections, e.g. tuberculosis, subacute bacterial endocarditis, brucellosis.
- Parasitic infections, e.g. visceral leishmaniasis, African trpanosomiasis.
- Chronic myelomonocytic leukaemia and lymphomas.

Eosinophilia: Causes of increased eosinophils include:

- Helminth infections, e.g. hookworm infection, filariasis, trichinellosis, schistosomiasis, strongyloidiasis.
- Allergic conditions, e.g. asthma, hay fever, urticaria, food allergies, drug allergies.
- Skin diseases including psoriasis, dermatitis.
- Pulmonary eosinophilia.
- Lymphoma and leukaemia.
- Connective tissue disorders.

Basophilia: Causes of increased basophils (rare) include:

- Leukaemia.
- Some allergic disorders.
- Some endocrine diseases.

ABNORMAL WBC MORPHOLOGY

Note: Further information on blood disorders can be found in Part 2 *District Laboratory Practice in Tropical Countries*, Subunit 8.2, pages 272–294. The publication can be accessed by entering the title in Google and clicking on the entry Medbox.

40× Leishman stained

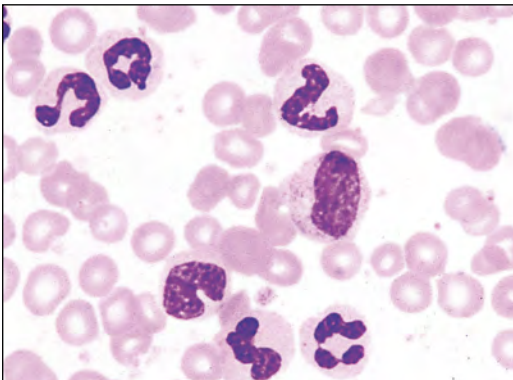
Infection blood films

Plate 3.4 Acute bacterial infection with increased neutrophils and immature cells: band cells and metamyelocytes (left-shift).

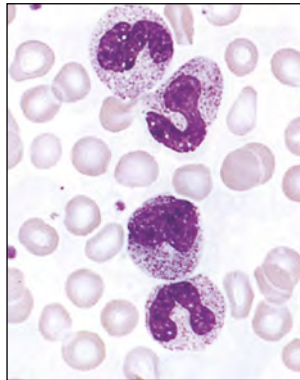


Plate 3.5 Blood film showing left-shift of neutrophils with cytoplasmic toxic granulation.

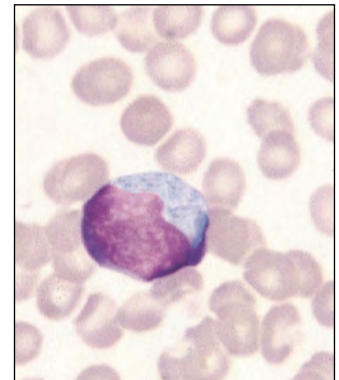


Plate 3.6 Atypical lymphocyte (reactive lymphocyte) seen in viral infections, malaria, whooping cough.

Leukaemia blood films

Leukaemia is a malignant disorder of haematopoietic tissues. Increased numbers of abnormal white cells are produced and present in the bone marrow and peripheral blood.

Clonal proliferation of leukaemic cells and tissue infiltration impair normal blood cell production. Based on whether the disease is acute (clinically aggressive) or chronic (slowly progressive) and whether the leukaemic cells are of myeloid or lymphoid origin, leukaemia can be broadly classified into:

- Acute myeloid leukaemia (AML)
- Acute lymphoblastic leukaemia (ALL)
- Chronic myeloid leukaemia (CML)
- Chronic lymphocytic leukaemia (CLL)

In most but not all forms of leukaemia, the total WBC count is raised with very high counts in CML and CLL.

Differentiation of CML and leukaemoid reactions

In leukaemoid reactions the WBC count is rarely over $60 \times 10^9/L$, and there is a left-shift of the neutrophils with toxic granulation.

Important: When a serious blood disorder such as leukaemia is suspected, an experienced haematologist should be consulted. The facilities of a specialist haematology referral laboratory are required to differentiate leukaemias.

40× Leishman stained

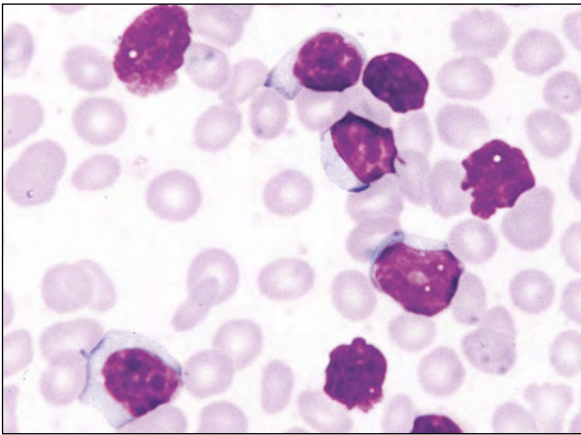


Plate 3.7 Film from a patient with chronic lymphocytic leukaemia. Note the smear cells (damaged lymphocytes).

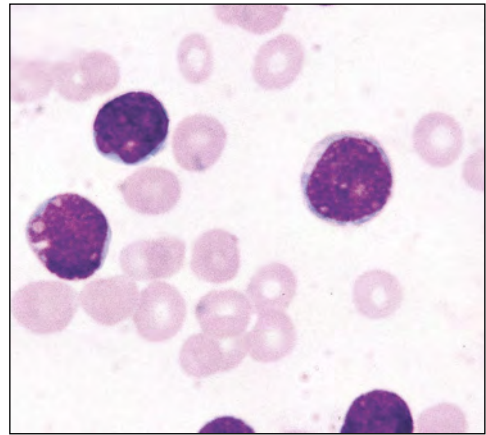


Plate 3.8 Film from a patient with acute lymphoblastic leukaemia showing blast cells.

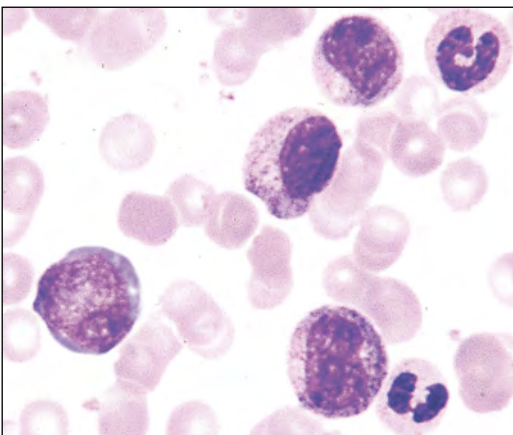


Plate 3.9 Film from a patient with chronic myeloid leukaemia showing early myeloid cells.



Plate 3.10 Myeloblasts showing Auer rods in cytoplasm and in the nucleus (diagnostic of AML).

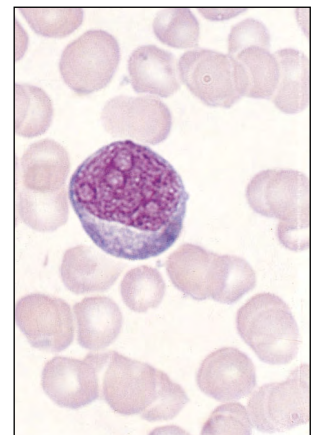


Plate 3.11 Blast cell showing clear nucleoli as seen in a patient with acute leukaemia.

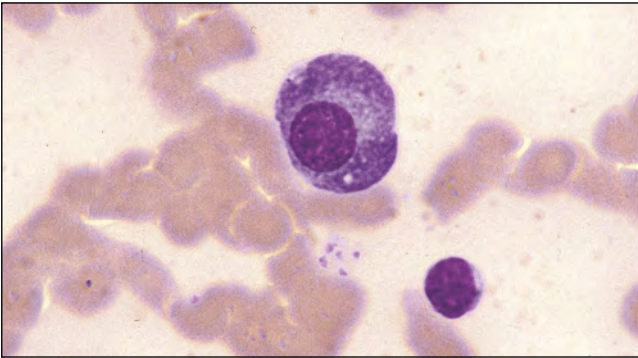
Myelomatosis blood film

Plate 3.12 Film from a patient with myelomatosis, showing a malignant plasma cell, marked rouleaux of red cells, background paraprotein staining.

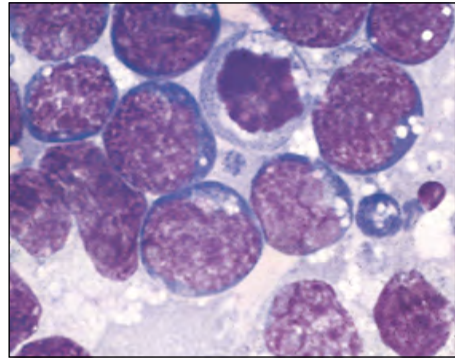
Burkitt's lymphoma film

Plate 3.13 Imprint (touch) smear showing Burkitt's lymphoma cells with nucleoli and vacuoles. (Giemsa stained)

RBC MORPHOLOGY

40× Leishman stained

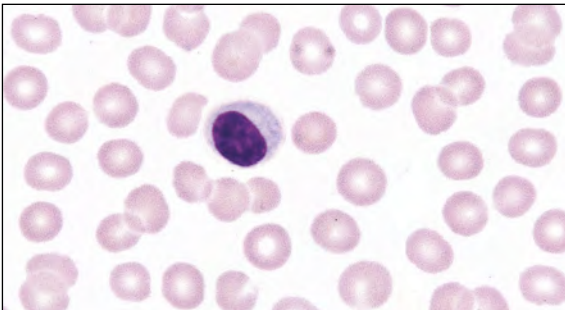
Anaemia blood films

Plate 3.14 Normocytic (about 8 μm in diameter) normochromic red red cells with an area of central pallor, no more than a third of the cell's diameter.

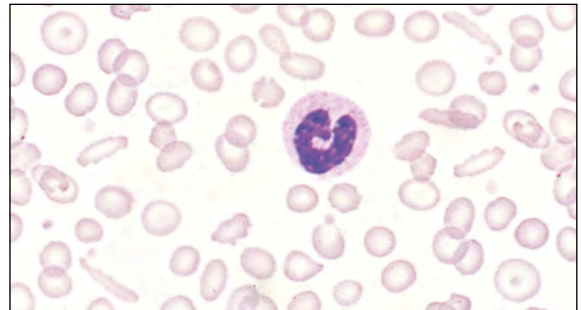


Plate 3.15 Film from a patient with iron deficiency anaemia showing microcytic hypochromic red cells. Note the pencil-shaped cells and occasional target cell.

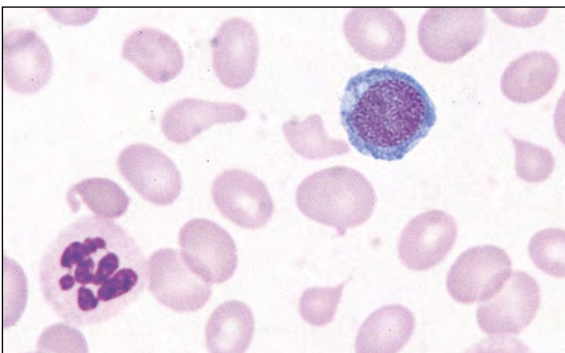


Plate 3.16 Film from a patient with macrocytic anaemia due to folic acid deficiency, showing oval macrocytes, a megaloblast (right) and a hypersegmented neutrophil.

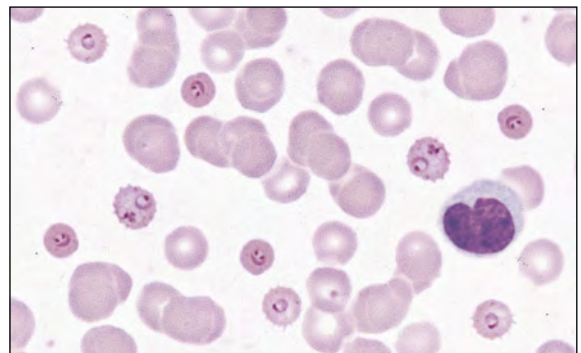


Plate 3.17 Blood film from a patient with *P. falciparum* infection, one of the commonest causes of anaemia in tropical countries, particularly in young children.

Sickle cell anaemia

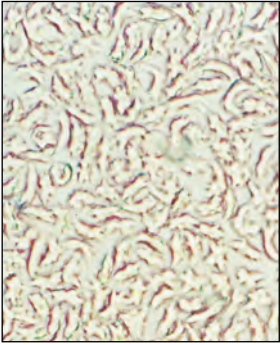


Plate 3.18 Positive sickle cell slide test, indicating the presence of Hb S in the red cells. The test does not distinguish between sickle cell trait and sickle cell anaemia. Examine a stained blood film.

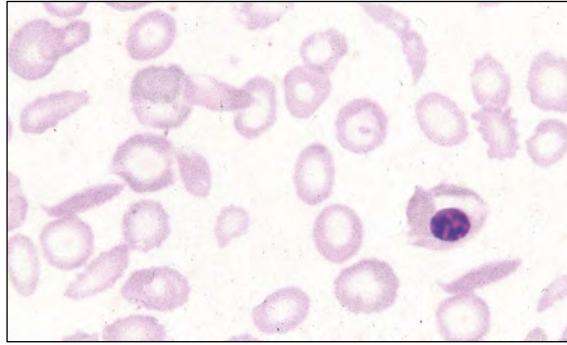


Plate 3.19 Film from a child with sickle cell disease (β^S/β^S) showing sickle cells (elongated and crescent-shaped) a nucleated red cell, target cells and polychromasia. Sickle cells can also be seen in blood films from patients with sickle cell/ β thalassaemia and HbSC disease. Refer a blood sample for haemoglobin electrophoresis.

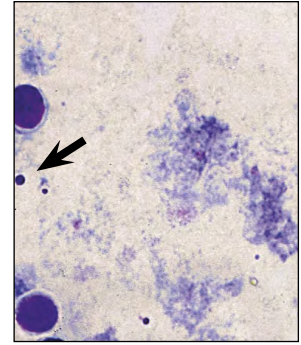


Plate 3.20 Thick film from a patient with sickle cell disease and malaria. Note the blue stippling (reticulocytosis) and nucleus of a nucleated RBC to left of *P. falciparum* trophozoite.

Haemoglobin C disease

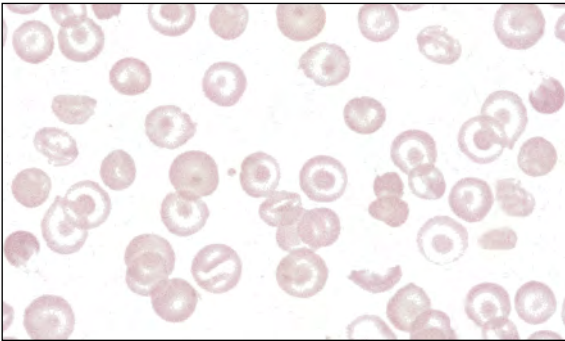


Plate 3.21 Film from a West African patient with haemoglobin C disease (β^C/β^C) showing many target cells and occasional cells containing crystals of precipitated HbC.

Thalassaemia major

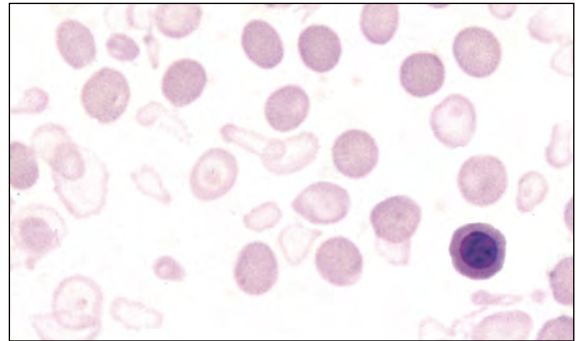


Plate 3.22 Film from a patient with *beta* thalassaemia major showing marked poikilocytosis with red cell fragments, hypochromic cells, many target cells and nucleated red cells. The well haemoglobinized cells are transfused red cells.

G6PD deficiency haemolysis

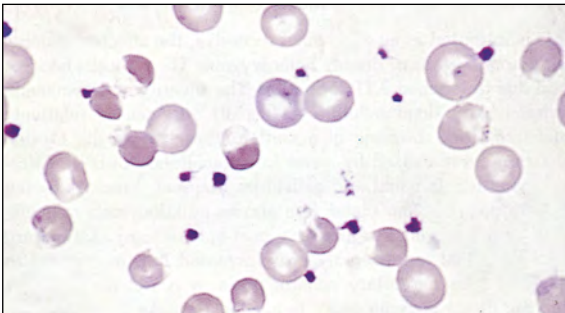


Plate 3.23 Film from a G6PD deficient patient with haemolytic anaemia due to drug-induced oxidant stress. Marked polychromasia, half-ghost cells (irregular distribution of haemoglobin) and red cells with bitten out margins can be seen.

Schistocytes

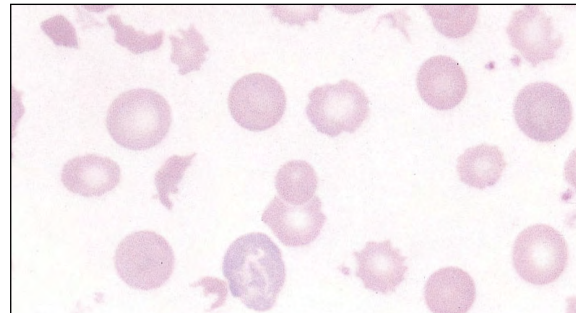


Plate 3.24 Film showing schistocytes (red cell fragments), some with projections (spicules) and polychromasia. They may be seen in uraemia, severe burns, DIC and in response to toxins and some drugs.

Spherocytes

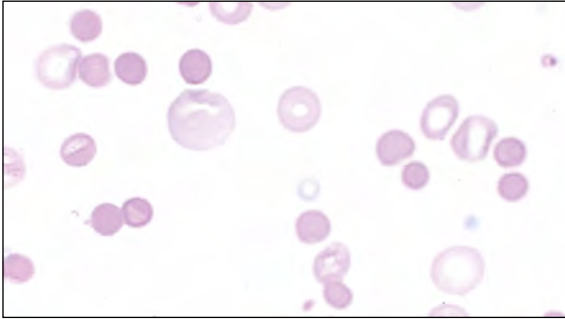


Plate 3.25 Film showing spherocytes (small densely stained microcytes). They may be seen in severe burns, haemolytic anaemia (warm-autoantibody) snake envenomation, haemolytic disease of the newborn, *C. perfringens* infection, bartonellosis, and hereditary spherocytosis.

Reticulocytes

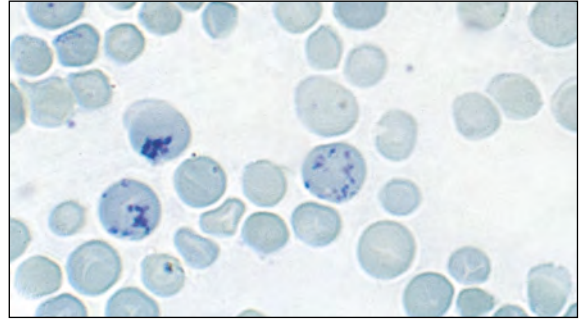
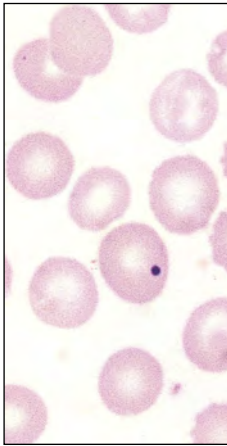


Plate 3.26 Reticulocytes containing dark blue-purple reticulin granules in a New methylene blue-stained preparation (similar when stained with cresyl blue). Reticulocytosis may be seen in haemolytic anaemias, following blood loss, megaloblastic anaemia and following therapy for iron deficiency anaemia.

Howell-Jolly body



Heinz body

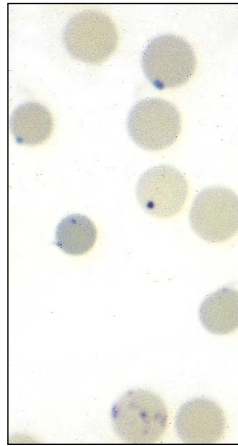


Plate 3.27 *Left* Howell-Jolly body (nuclear remnant) in red cell. May be seen in hyposplenism, post-splenectomy, megaloblastic anaemia, thassaemias, haemolytic anaemias. *Right* Heinz bodies in red cells (crystal violet stained). May be seen in G6PD deficiency (haemolysis), some thassaemias, and post-splenectomy.

Haemolytic disease of the newborn

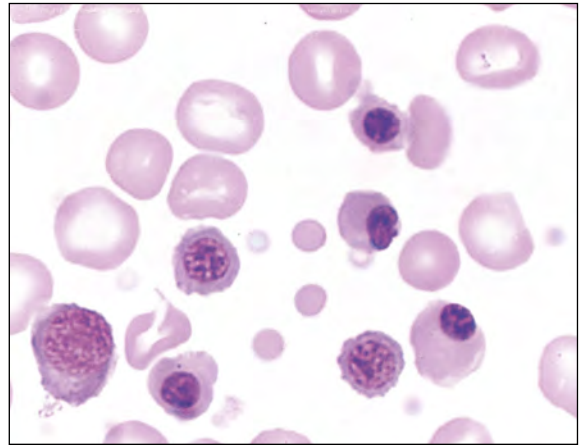


Plate 3.28 Film from an infant with haemolytic disease of the newborn (HDN) showing many nucleated red cells, polychromasia and few spherocytes. In ABO HDN, spherocytosis may be marked whereas in Rhesus HDN spherocytosis is usually less prominent.

Basophilic stippling

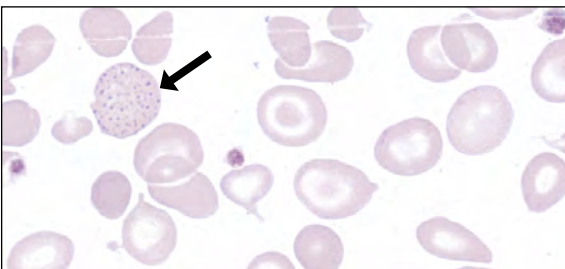


Plate 3.29 Basophilic stippling in a red cell which can be seen in thalassaemia, haemolytic anaemias, liver disease and lead toxicity. Film from patient with HbAE Bart's disease.

Haemoglobin H inclusions

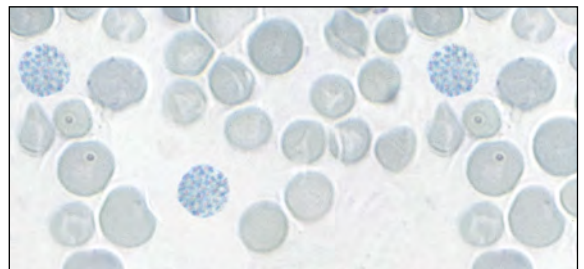


Plate 3.30 Cresyl blue stained preparation showing pale stained Hb H red cell inclusions as seen in Hb H (β_4) disease (form of alpha thalassaemia).

TESTING FOR OCCULT BLOOD

Requests for occult blood testing are usually made to investigate the cause of iron deficiency anaemia or to assist in the diagnosis of bleeding lesions of the gastrointestinal tract, e.g. peptic ulcer, carcinoma, or diverticulosis.

The following tests are used to detect occult blood in faeces:

- Guaiac chemical tests that detect the heme component of haemoglobin based on a non-specific peroxidase reaction. Other substances with peroxidase activity that may be present in the diet and some medications may also react in the test, causing false positive reactions. Specificity can be improved by dietary restrictions and testing several faecal samples.
- Immunochemical occult blood tests that are highly specific for human haemoglobin using monoclonal and polyclonal antibodies to identify selectively the globin component of haemoglobin with high sensitivity. These tests are recommended in preference to guaiac tests. No dietary or medication restrictions are required to perform immunochemical occult blood tests.

Hemosure faecal immunochemical occult blood test

Hemosure is an example of a qualitative one step immunochemical faecal cassette test with a lower limit sensitivity of 50 µg human haemoglobin per gram of faeces. The test takes 5–10 minutes to perform.

Principle of faecal immunochemical test

Labelled antibody-dye conjugate binds to haemoglobin forming an antibody-antigen complex. This complex binds to anti-haemoglobin antibody in the test (T) reaction zone, forming a pink-rose coloured band. A pink-rose band forms in the control (C) zone demonstrating the test is functioning correctly.

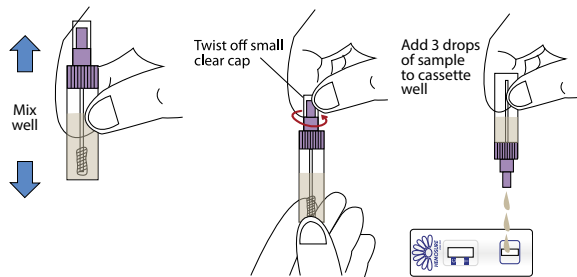
Sample

No dietary or medication restrictions are required before collecting a faecal sample. A sample should not be tested when a patient has menstrual bleeding, urinary bleeding, constipation bleeding or bleeding haemorrhoids.

After adding sample to the collecting tube, immediate testing is recommended or the tube can be kept at 2–8 °C for up to 30 days.

Procedure

- 1 Remove the applicator stick from the collecting tube supplied and insert it six times in different areas of the faecal sample.
- 2 Return the applicator stick to the collecting tube and shake the tube to ensure the sample is well mixed with the extraction buffer in the tube.
- 3 Add 3 drops of well-mixed sample to the sample well of the test cassette. Start timing the reaction.
- 4 Read the test within 5–10 minutes. *Do not read it after 10 minutes.*



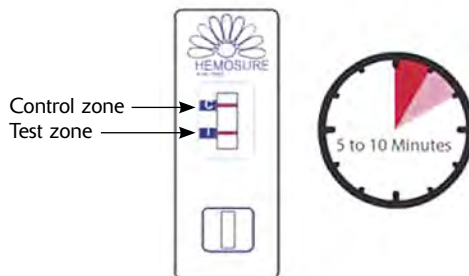
Procedure for performing *Hemosure* occult blood test.

Test results

Positive test: Rose-pink band in the test (T) zone.
Rose-pink band in the control (C) zone.

Negative test: Rose-pink band in the control (C) zone only.

Invalid test: No pink-rose band in the T or C zone.



Positive *Hemosure* immunochemical occult blood test.
Courtesy of Hemosure Inc. www.hemosure.com

Quality control

Hemosure test includes a positive procedural control indicating reagents and test have performed correctly.

Proficiency test evaluation has shown *Hemosure* to have a sensitivity of 94.4% and specificity of 91.2%.

Hemosure can be stored at 2–30 °C and is stable until the expiry date shown on the label. The test has a shelf-life of 24 months from date of manufacture. Each cassette is individually sealed in a foil pouch.

An occult blood test is not intended as a replacement for other diagnostic procedures when screening for colorectal cancer. A negative test can occur when there is intermittent bleeding. When clinically indicated, further faecal samples should be tested.

Interpreting occult blood test results

The commonest causes of positive occult blood tests in tropical and other developing countries are hookworm infection, peptic ulcer, colitis and bleeding from oesophageal varices due to cirrhosis of the liver.

Other causes include carcinoma in the gastrointestinal tract, erosive gastritis due to alcohol or drugs, or swallowed blood from recurrent nosebleeds.

Parker M et al. An evaluation of haemoglobin measurement tools and their accuracy and reliability when screening for child anaemia in Rwanda: A randomized study. *Plos ONE*, 2018.

doi.org/10.1371/journal.pone.0187663

Bain B J, Bates I, Lathan M A, Dacie and Lewis Practical Haematology. 2017, 12th ed. *Elsevier Health*. ISBN 9 780702 066962

Sandis-Gomar F et al. Haemoglobin Point-of-care testing – The HemoCue system. *Sage Journals*, Sept 2012. Access electronically by entering the title in Google.

M'baya B et al. Validation of the haemoglobin colour scale for screening blood donors in Malawi. *Malawi Medical Journal*, Vol 26, issue 2, pp 30–33, 2014. Access on-line by entering the title in Google.

FURTHER READING

Cheesbrough M Part 2 *District Laboratory Practice in Tropical Countries* 2010 2nd edition update. co-published Cambridge University Press, Tropical Health Technology.*

*Access on-line by entering the title in Google and clicking on the entry MEDBOX.

Further information on blood disorders

- Laboratory diagnosis of anaemia in tropical countries, described in subunit 8.2, pages 277 – 280.
- Thalassaemias, described in subunit 8.2, pages 280 – 283.
- Diseases caused by Haemoglobin S, Haemoglobin C, and Haemoglobin D^{PUNJAB}, described in subunit 8.2, pages 283 – 285.
- G6PD deficiency, hereditary red cell membrane disorders and polycythaemia, described in subunit 8.2, pages 285 – 286. Screening for G6PD deficiency, described in subunit 8.9, pages 333 – 334.
- Leukaemias, leukaemoid reactions, lymphomas, myelomatosis, and Waldenstroms macroglobulinaemia, described in subunit 8.2, pages 290 – 294.
- Haemolytic disease of the newborn, described in subunit 9.4, pages 376 – 378.
- The sickle cell slide test and Hb S solubility filtration test, described in subunit 8.10, pages 335 – 337.
- A reticulocyte count, described in subunit 8.10, pages 331 – 332.
- Using the *Haemoglobin Colour Scale*, described in subunit 8.4, page 306.

3.2 Diagnosis and monitoring diabetes mellitus

Screening for raised lipids

Diabetes mellitus is a chronic condition that occurs when there are raised levels of glucose in the blood because the body cannot produce any or enough of the hormone insulin or use insulin effectively.¹

Insulin regulates the amount of glucose in the blood, the rate at which glucose is taken up by the tissues and enters body cells, and the conversion of glucose to glycogen. When insulin is absent or ineffective, blood glucose levels rise.

High levels of glucose in the blood is called hyperglycaemia. Symptoms include excessive thirst and dry mouth, polyuria (frequent urination), fatigue, constant hunger, blurred vision and often unexplained loss of weight. Uncontrolled hyperglycaemia can damage nerves and blood vessels and lead to life-threatening conditions including diabetic ketoacidosis, cardiovascular disease with stroke and heart attacks, renal damage resulting in kidney failure, eye damage with loss of vision, foot ulcers, limb amputation and increased risk of infections which are often slow to heal.

In 2021 WHO launched the *Global Diabetes Compact* with the goals of reducing the risk of diabetes and ensuring that all people with diabetes have access to equitable, comprehensive, affordable and quality treatment and care (see Further Reading).

Types of diabetes

Type 1 diabetes

In this form of diabetes there is autoimmune destruction of the insulin-producing beta cells in the pancreas, necessitating insulin treatment to sustain life. The cause is not known. Viral infection, diet or other environmental factors in genetically susceptible persons are thought to be involved. The onset of symptoms may be rapid.

Globally 5–10% of diabetes is type 1 and is the commonest form in children and young adults in European countries. It is less common in tropical countries and often occurs at a later age. A rare form of hereditary type 1 diabetes has been reported in African and Asian people in which there is insulin deficiency but no evidence of autoimmunity.

Type 2 diabetes

This is the commonest form of diabetes. It is the result of reduced insulin production and the body's ineffective use of insulin referred to as insulin resistance. Immune processes may also be involved. Type 2 diabetes is often detected only when complications have developed. Commonly affecting adults, type 2 diabetes is being seen increasingly in children.

Type 2 diabetes is common in some populations in tropical countries with individual and ethnic genetic factors contributing to susceptibility. Overweight and obesity, unhealthy diet, physical inactivity and smoking are among the main risk factors for type 2 diabetes. In India, type 2 diabetes is frequently seen in young adults with low levels of obesity.

In tropical countries, urbanization, more sedentary lifestyles and dietary changes are contributing to the increasing prevalence of type 2 diabetes.

Gestational diabetes

In this form of diabetes which occurs in pregnancy, blood glucose values are above normal but below those diagnostic for diabetes. It increases the risks to mother and child of complications during pregnancy and at delivery, including high blood pressure, congenital malformations, stillbirth, pre-eclampsia, difficult birth, low birth weight and lower blood glucose levels in the newborn. Gestational diabetes usually resolves after pregnancy but can recur in subsequent pregnancies. There is a risk of mother and child developing type 2 diabetes later in life. Occasionally type 1 diabetes can develop following autoimmune gestational diabetes.

Other forms of diabetes

These include neonatal diabetes and maturity-onset diabetes of the young (monogenic disorders), and secondary diabetes, caused by pancreatic disease, some hormonal disorders, e.g. Cushing's disease, abnormalities of insulin or its receptors, and drug treatments, e.g. corticosteroids.

Conditions associated with diabetes

Diabetes and COVID-19

People with diabetes have an increased risk of infection with SARS-CoV-2 and the symptoms and complications of COVID-19 are often more serious. COVID-19 may also make diabetes control more difficult. In the early stages of the COVID-19 pandemic, diabetic services were disrupted, causing insulin shortages in some countries.

Diabetes and tuberculosis

Both type 1 and type 2 diabetes increase significantly the risk of developing active TB. There is also

evidence that in those with TB and diabetes, TB may progress more rapidly with more frequent chest and systemic symptoms particularly in those with uncontrolled hyperglycaemia.² Poor glycaemic control may also prolong smear and culture positivity and increase the risk of TB treatment failure.²

Type 2 diabetes and HIV

People living with HIV have been shown to be at increased risk of developing type 2 diabetes associated with the long-term effects of HIV infection and antiretroviral therapy combined with dietary and lifestyle factors.

Diabetes and melioidosis

Diabetes increases significantly susceptibility to melioidosis caused by *Burkholderia pseudomallei*. Sepsis and chronic infections are common in South East Asia, particularly in the rainy season in places of stagnant water, e.g. rice paddy fields.

Note: Other microbial pathogens for which diabetes mellitus is being reported increasingly as a risk factor in tropical countries can be found in the paper of Dunachie and Chamnan (see Further Reading).

Tropical chronic pancreatitis and fibrocalculous pancreatic diabetes³

Tropical chronic pancreatitis (TCP) has been reported from Africa, South America, and South East Asia with the highest prevalence in southern India. It affects adults (usually before 40 y), causing pain due to pancreatic calculi and progressive pancreatic damage resulting in malabsorption and fibrocalculous pancreatic diabetes (FCPD) developing later in life. Men are more commonly affected than women.

FCPD shows features of both type 1 and type 2 diabetes. There is usually marked hyperglycaemia with insulin resistance but without ketoacidosis. Most patients require insulin. There is an increased risk of pancreatic cancer. The causes of TCP are not fully understood. Earlier reports of TCP linked the condition to malnutrition as most patients were undernourished. Possible causes of TCP include genetic factors, micronutrient deficiencies, environmental toxins and increases in alcoholic intake.

Tropical diabetic hand syndrome (TDHS)

This condition associated with poorly controlled diabetes has been reported from India and several African countries. The condition, often originating from an insect bite, causes cellulitis, swelling and ulceration of the hand, often progressing to severe hand sepsis and gangrene.

Distribution and prevalence of diabetes

Globally the number of people with diabetes is increasing with three quarters of people living in low and middle income countries as shown in the world map reproduced from the International Diabetes Federation 2017, 8th edition of the *IDF Diabetes Atlas* shown on the following page.

Tests used to diagnose and monitor diabetes

In district laboratories, the following tests are used to diagnose and monitor diabetes:

- Measurement of blood glucose.
- Measurement of glycated haemoglobin (HbA1c).
- Urine biochemical strip tests to detect, glycosuria, ketonuria, and protein, described in Section 3.3.
- Urine albumin creatinine ratio (ACR) to detect early chronic kidney disease, described in Section 3.3.

MEASURING BLOOD GLUCOSE

Blood glucose measurement is used to detect patients with impaired glucose tolerance and to diagnose and monitor patients with diabetes.

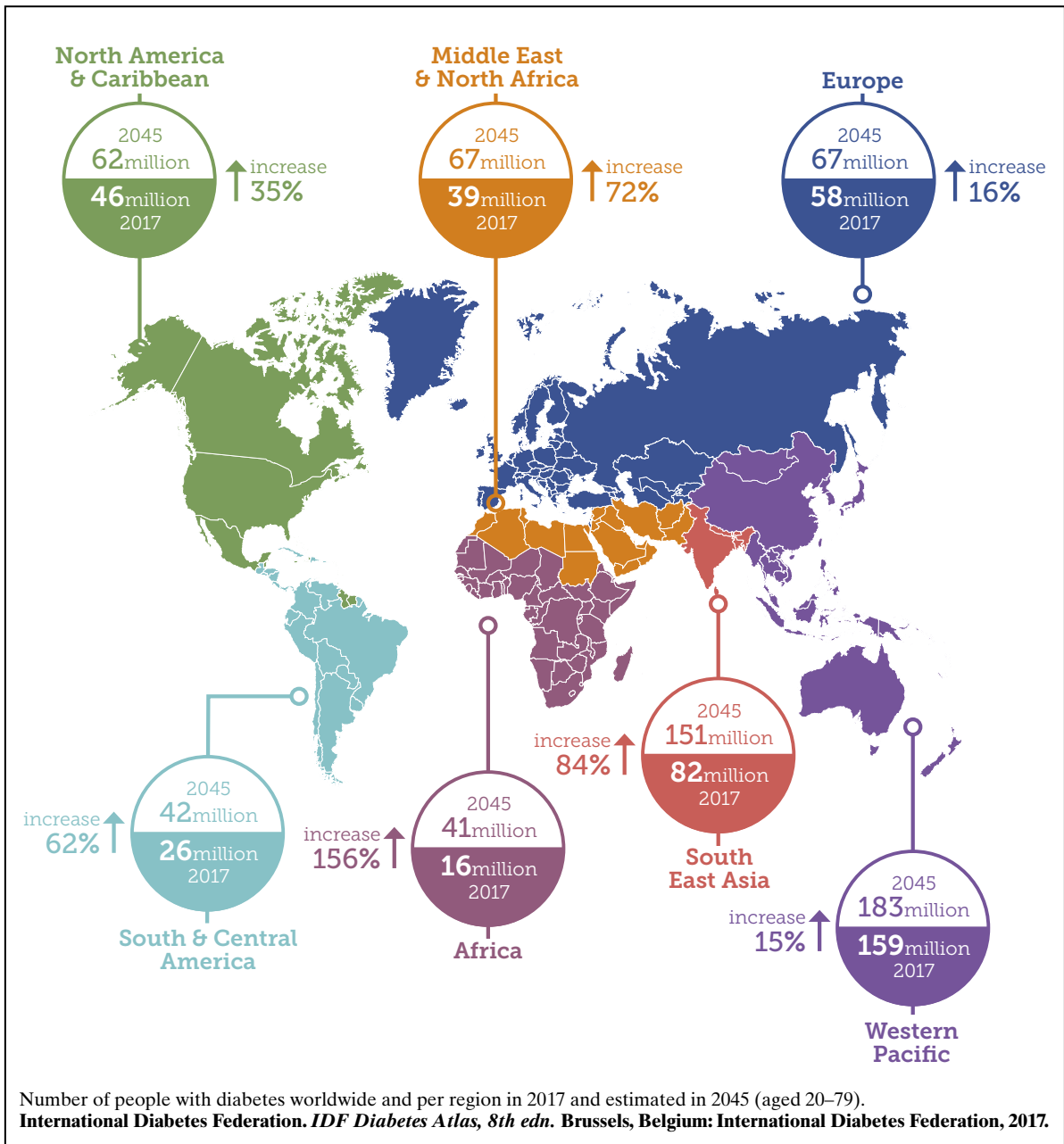
Analyte: Glucose is the main end product of carbohydrate digestion. Oxidation of glucose by the glycolytic and tricarboxylic acid pathways provides the chemical energy needed for cellular activity. When not required for the body's immediate energy needs, glucose is converted to glycogen and stored in the liver and muscles (glycogenesis). Excess glucose is oxidized to fatty acids and stored as fat in the tissues. If needed, glucose can also be formed from fats and protein (gluconeogenesis). An increase in the breakdown of fats to provide energy, results in an increase in the production of ketones.

Sample: Fasting plasma from anticoagulated (lithium heparin) venous blood, with plasma separated from cells within 30 minutes of blood collection, is the preferred sample. Most glucose meters measure glucose in capillary whole blood.

Fluoride oxalate by itself has been shown to be a poor inhibitor of glycolysis, failing to prevent glycolysis for the first 3–4 hours after blood is collected. Citrated sodium fluoride is an effective inhibitor of glycolysis, e.g. *Terumo Venosafe Glycemia* tubes, *Sarstedt S-Monovette* and *GlucoExact* tubes.

Important: Measurement of glucose must be carried out on the day and time requested. Collection times are often related to the administration of oral glucose, insulin treatment, or both. Provision must be made for urgent glucose tests which may be necessary outside normal laboratory working hours.

Terms used to describe the collection of blood glucose samples
Fasting plasma glucose: Refers to venous blood collected after a period of no food intake. For adults the fasting time should



not be less than 8 hours and for children not less than 6 hours. The drinking of plain water is permitted.

2 hour plasma glucose: Refers to anticoagulated venous blood collected 2 hours after the ingestion of 75 g oral glucose load.

Random plasma glucose: Refers to anticoagulated venous blood collected at any time, regardless of food intake.

Measuring plasma glucose using reagent test kits

Most semi-automatic chemistry analyzers use reagent test kits that measure plasma glucose

enzymatically using glucose oxidase or hexokinase. Both methods have been shown to provide reliable test results. The oxidase method is more frequently used because glucose oxidase reagent has excellent stability whereas hexokinase reagent is not as stable particularly in warm surroundings. Test results using the glucose oxidase method are read at 505 nm whereas hexokinase tests require reading in the UV range at 340 nm. Hexokinase reagent test kits are considerably more expensive.

Glucose oxidase enzymatic method

Glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid. The formed hydrogen peroxide is detected by a chromogenic oxygen acceptor, 4-aminophenazone in the presence of peroxidase. The intensity of colour formed is proportional to the glucose concentration in the sample.

The reagent and glucose standard provided in glucose oxidase test kits are ready to use. They require storage, in tightly capped containers at 2–8 °C, protected from light and contamination. In the assay procedure, reagent is added to the sample and standard. Following incubation for 10 minutes at 37 °C or 20 minutes at room temperature (15–25 °C), test results are read at 505 nm. The colour is stable for 30 minutes.

Quality control: A quality control glucose sample must be run with each batch of tests. High levels of ascorbic acid, lipaemia and some drugs may interfere with test results (refer to manufacturer's literature).

Oral glucose tolerance test

Occasionally a glucose tolerance test (GTT) is required to diagnose impaired glucose tolerance. The test should not be necessary in children. Ideally a GTT should be performed in the morning. The patient should be instructed not to eat, drink (except plain water) or smoke for 10–16 hours before the test. Ideally the patient should have no other disease at the time the GTT is performed. A note should be made of any drug treatments.

Procedure

- 1 Prepare a GTT chart for the patient on which to record collection times and test results.
- 2 Collect a fasting venous blood sample into a bottle or tube containing citrated sodium fluoride. Label the container 'fasting blood' and time collected.
- 3 Give the patient 75 g of glucose (D-glucose monohydrate) in 250–300 mL water, to be drunk in 5 to 15 minutes. To reduce nausea a few drops of lemon juice may be added to the water.
- 4 Make a note of the time and enter on the GTT chart the time at which the next blood sample is to be collected, i.e. 2 hours after the glucose water has been drunk.
- 5 Instruct the patient to rest quietly and not to eat, drink, exercise, smoke, or leave the hospital during the test. Inform the patient when the test will be completed.

Important: If the patient should feel faint, very nauseated or begin to perspire excessively, call a medical officer.

- 6 Collect the second blood sample at the correct time, labelling the container with the collection time. Measure the plasma glucose concentration in each of the blood samples.

- 7 Enter the patient's results on the GTT chart if the value of the control serum is acceptable.

Collection times	Plasma glucose results
Fasting mmol/L
2 hours mmol/L

REFERENCE INTERVALS FOR GLUCOSE

The reference intervals for blood glucose are:

- fasting blood glucose: 3.5–5.5 mmol/L
- 2 hours after eating: up to 7.8 mmol/L

The following are the current WHO recommendations for the diagnostic criteria for diabetes mellitus and intermediate hyperglycaemia⁴:

Diabetes

A diagnosis of diabetes is made when ONE of the following criteria are met:

- Fasting plasma glucose: ≥ 7.0 mmol/L (126 mg/dL), or
- 2-hour plasma glucose: ≥ 11.1 mmol/L (200 mg/dL) 2 hours after ingestion of 75 g oral glucose load, or
- HbA1c (type 2 diabetes): ≥ 48 mmol/mol ($\geq 6.5\%$)

Impaired glucose tolerance (IGT)

A diagnosis of IGT is made when BOTH of the following criteria are met:

- Fasting plasma glucose: < 7.0 mmol/L (126 mg/dL) and
- 2-hour plasma glucose: ≥ 7.8 mmol/L (140 mg/dL) and < 11.1 mmol/L (200 mg/dL) 2 hours after ingestion of 75 g oral glucose load.

Gestational diabetes (GDM)

A diagnosis of GDM is made when ONE or MORE of the following criteria are met:

- Fasting plasma glucose: 5.1–6.9 mmol/L (92–125 mg/dL).
- 1-hour plasma glucose: ≥ 10.0 mmol/L (180 mg/dL) 1 hour after ingestion of 75 g oral glucose load.
- 2-hour plasma glucose: 8.5–11.0 mmol/L (153–199 mg/dL).

Other causes of raised glucose levels

Hyperglycaemia may accompany pancreatic disease and some endocrine disorders such as thyrotoxicosis and Cushing's syndrome. Steroid therapy may also cause hyperglycaemia.

Transient hyperglycaemia often occurs following severe stress, e.g. after surgery, injury, shock, infections, or severe burns. It is important to check that hyperglycaemia does not persist when the intercurrent condition resolves or steroid treatment is withdrawn.

Decreases in glucose levels

A low blood glucose level is called hypoglycaemia. Persistent occurrences of hypoglycaemia with glucose levels less than 2.2 mmol/L accompanied by symptoms such as fainting, fits, sweating, hunger, pallor, confusion, or violence, should be investigated.

Causes of hypoglycaemia include severe malnutrition, severe liver disease, alcoholic excess, insulin secreting tumours, Addison's disease, and treatment with certain drugs. Commonly, however, markedly reduced blood glucose levels occur following the overtreatment of diabetes.

Neonatal hypoglycaemia

Newborn infants may suffer hypoglycaemia when blood glucose levels fall below 1.1 mmol/L. Infants particularly at risk are underweight poorly nourished babies, twins, premature infants, and babies born of diabetic mothers. It is important to detect hypoglycaemia of the newborn because without treatment brain damage may occur.

Malaria associated hypoglycaemia

In severe malaria, hypoglycaemia is a common finding and can increase mortality particularly in young children. Hypoglycaemia can also occur in those being treated with quinine and quinidine.

False glucose values

A falsely high glucose level will result if a blood sample is collected from an arm receiving a glucose (dextrose) intravenous (i.v.) infusion. A falsely low value may be obtained if the plasma is markedly icteric or the sample is an unspun serum sample.

BLOOD GLUCOSE METERS

Blood glucose meters are widely used by diabetic patients and in health units to monitor diabetic control. They are not suitable for diagnosing diabetes. Unlike laboratory analyzers that measure glucose in

plasma (requirement of diabetes diagnosis), blood glucose meters measure glucose in whole blood using enzyme reagent strips.

Glucose values in whole blood samples are 10–15 % lower than in plasma and are affected by haematocrit (packed cell volume). Recently developed glucose meters record plasma equivalent test results using a conversion factor. When a glucose meter does not report plasma equivalent test results, glucose meter readings can be multiplied by a factor of 1.11 to convert concentration in whole blood to the equivalent concentration in plasma (IFCC recommendation⁵). Patients with blood glucose test results suggestive of diabetes require referral for accurate venous blood (plasma) glucose testing and measurement of HbA1c (see following text).

Accuracy of glucose meters and technological advances

The accuracy of blood glucose meters depends on the meter and strip technology used, how well the test is performed and how correctly test strips are stored. All meters manufactured after 2016 should comply with ISO:15197:2013 accuracy standards for blood glucose meters*.

* The standards require 95% of blood glucose meter results to be within + 0.83 mmol/L of laboratory test results at glucose concentrations of under 5.6 mmol/L and within 20% of laboratory results at concentrations of 5.6 mmol/L or more.

The increased accuracy required by the new ISO standards reflect technological advances which have enabled manufacturers to eliminate the effects of many of the substances and drugs that can interfere with test results. Most meters measure glucose electrochemically. Glucose reacts with enzymes (glucose oxidase or glucose dehydrogenase) contained in the reagents strips, producing an electric current. The current generated at the electrode is proportional to the glucose concentration in the sample. Most reactions take a few seconds and require a small volume of blood (1–2 µL).

While most meters are easy to use with on-screen instructions, inaccurate test results can be caused by users, e.g. not washing and drying hands before collecting a capillary sample, not following exactly the testing procedure, using strips that have expired, or by not storing strips in tightly closed containers at the correct temperature and out of direct sunlight.

The measuring range of most meters has also increased and also the number of tests that can be stored and reviewed. Various options and software are available for transmitting data to a computer,

smart phone or LIS. Not all meters can be used for testing several patients, some meters are for single patient use only (designated as personal meters by manufacturers).

The cost of meters and particularly strips and the reliability of supply are additional important factors when considering purchasing a glucose meter. Most glucose meters have a lifespan of up to 2 years providing they are correctly used and cleaned. Meter deterioration can be detected using glucose controls or when provided, a check strip.

When not to use a blood glucose meter

A glucose meter should not be used for measuring glucose in capillary blood in patients with circulatory failure or severe dehydration, are hypotensive, severely anaemic, or when receiving oxygen therapy. Glucose should be measured using a venous blood sample.

Neonatal cord blood samples are unsuitable for glucose meter testing. Other neonatal samples can be tested using recently manufactured meters that correct for haematocrit and cover the reference interval for newborn infants.

Quality control of blood glucose meters

Glucose meter manufacturers provide control glucose solutions for controlling meter and strip performance, covering high, medium, and low glucose ranges. Most meters also incorporate error codes, e.g. to alert the user of any meter malfunction, sampling error, defective strip, or low battery.

StatStrip Express 2 Glucose Meter

An example of the new generation of glucose meters that comply with ISO:15197:2013 accuracy standards is the *StatStrip Express 2 Meter* manufactured by Nova Biomedical and shown in Plate 3.31. The meter eliminates haematocrit error. Also eliminated are errors from samples that contain ascorbic acid, uric acid, bilirubin, maltose, galactose, xylose, paracetamol and a wide range of other drugs. It measures glucose accurately over the range 0.6–33.3 mmol/L (10–600 mg/dL) and provides plasma equivalent test results.

The meter requires 1.2 μ L of fresh capillary or venous blood and testing time is 6 seconds. It is operated from a 3V Li button battery (minimum 600 tests), and has automatic shut-off when not in use. Up to 400 tests can be stored. Data transfer software is available and connection to USB for transferring results to a computer. *StatStrip Express* has been shown to measure accurately whole blood glucose.⁶



Plate 3.31 *StatStrip Express 2 Glucose Meter.*
Courtesy Nova Biomedical www.novabiomedical.com

Note: Some manufacturers also make available glucose meters that test one or more other analytes with glucose (often called *Glucose Plus* meters), e.g. glucose with ketones, cholesterol, triglycerides, lactate, uric acid. Separate strips and controls are required for each analyte. Glucose is measured amperometrically and other analytes are usually measured by reflectance. Most *Glucose Plus* meters are expensive.

Continuous glucose monitoring

Continuous glucose monitoring (CGM) refers to day and night monitoring of glucose levels in interstitial fluid using a sensor positioned subcutaneously in the skin with attached transmitter that sends information to a receiver with screen display. The data is stored and can be viewed and analyzed for high and low glucose trends, e.g. following eating or exercising, enabling better management of diabetes and insulin treatment, particularly for those with type 1 diabetes and mothers with gestational diabetes.

The concentration of glucose in interstitial fluid is close to that in plasma but levels lag behind by 5–10 minutes. Blood glucose testing is therefore required during times of rapidly changing glucose levels and when considering changing insulin treatment. CGM systems are currently expensive. What is referred to as flash glucose monitoring is less costly. In this form of monitoring, data is obtained only when the sensor is scanned every 8 hours. The technology however is developing with increasingly less costly, longer life and more accurate sensors becoming available.

MEASURING GLYCATED HAEMOGLOBIN

Analyte: Glycated haemoglobin (bonding of glucose to haemoglobin) is formed by non-enzymatic glycation of the N-terminal valine of the beta chain of haemoglobin. It is referred to as HbA1c of which the main glycated fraction is HbA1c which forms about 5% of circulating haemoglobin. HbA1c is specifically glycated by glucose.

The glucose remains complexed to the haemoglobin molecule for the lifespan of the red blood cell and therefore the concentration of circulating glycated haemoglobin is a guide to the average blood glucose level over a period of the previous 2 months (average lifespan of red cells). The more glucose present in the blood, the higher the concentration of HbA1c.

Purpose of test: WHO recommends diagnosing diabetes by measuring HbA1c providing stringent quality assurance tests are in place, assays are standardized to criteria aligned to the IFCC reference value and there are no conditions present which preclude its accurate measurement.⁷ The test is also used to monitor blood glucose levels particularly in patients with type 2 diabetes whose glucose levels do not change markedly and in prediabetic patients to reduce the risk of diabetes-associated complications.

Analyte: Capillary blood or EDTA anticoagulated venous blood can be used. HbA1c is stable for up to 7 days when the sample is stored at 2–8 °C.

Limitations of HbA1c test

HbA1c should not be performed in the following situations:

- When a patient's haemoglobin is < 7.0 g/L or > 24 g/L.
- Person has recently donated blood or has a condition causing rapid red blood cell turnover, e.g. malaria or other condition causing haemolysis.
- During pregnancy.
- When type 1 diabetes is suspected, e.g. in young children.
- Sample is highly lipaemic.

Measuring HbA1c using DCA Vantage Analyzer

The *DCA Vantage analyzer* is an example of an analyzer that measures HbA1c in capillary or venous

whole blood, requiring only 1 µL of sample and a test time of 6 minutes. It enables testing to be carried out at point-of-care, providing rapid test results. Diabetes can be diagnosed at an early stage and patients advised on how to control their glucose levels without having to wait for the results of plasma glucose tests. HbA1c testing does not require venous blood collection and can be performed at any time.

Principle of method: The *DCA Vantage analyzer* measures HbA1c immunologically. Both total haemoglobin and HbA1c are measured, the ratio is calculated and the test reported as percent HbA1c. Total haemoglobin is measured using a potassium ferricyanide, thiocyanmethaemoglobin method and HbA1c measured by an inhibition latex agglutination assay.

The *DCA Vantage analyzer* assay method is traceable to the IFCC International reference method, is FDA approved and NGSP (National Glycohaemoglobin Standardization Programme) certified.



Plate 3.32 *DCA Vantage analyzer*.
Courtesy Siemens Healthcare www.healthcare.siemens.com

Note: The *DCA Vantage analyzer* can also be used to determine the urine albumin creatinine ratio (ACR), described in Section 3.3.

Procedure

The test uses an HbA1c reagent cartridge containing antibody latex, agglutinator, buffer solution and oxidant. The procedure is summarized as follows:

- 1 Using the glass capillary and capillary holder provided, fill the capillary with capillary or venous whole blood (1 µL). Wipe the outside of the capillary.

Once the capillary is filled, analysis must begin within 5 minutes.

- 2 Insert the capillary holder into the reagent cartridge and insert the cartridge into the cartridge compartment as shown in Plate 3.33.
- 3 Pull the pull-tab completely out of the reagent cartridge and close the compartment door. This starts the reaction which takes 6 minutes to complete.
- 4 Enter the patient's data and sample ID in the analyzer.
- 5 Record and print out the patient's test result using the built-in printer.
- 6 Remove and discard the reagent cartridge into a biohazard container.



Plate 3.33 Inserting HbA1c reagent cartridge with sample into cartridge compartment.
Courtesy Siemens Healthcare GmbH.

Analyzer HbA1c reporting range: 2.5–14.0 %

Results lower than 2.5% are displayed as < 2.5 % and results higher than 14.0 % as > 14.0 %.

Data transfer: DCA Vantage test results can be transferred to a computer, LIS or other data recording system. Up to 4000 test results can be stored.

Falsely decreased HbA1c test results can occur from patients with:

- More than 10% Hb F
- Hb S and Hb C
- Thalassemia
- Polycythaemia

Note: Bilirubin up to 340 $\mu\text{mol/L}$ does not interfere with the assay. Rheumatoid factor up to 1:5120 titre does not interfere with the assay. Most drugs prescribed to persons with diabetes do not interfere with the assay (see manufacturer's literature).

Interpreting HbA1c test results

Non-diabetics: 15.0–37.7 mmol/mol (3.0–5.6 %)

Increased risk of developing diabetes:
38.8–46.4 mmol/mol (5.7–6.4 %)

Diagnosis of diabetes mellitus:
> 47.5 mmol/mol (6.5%)

Note: mmol/mol is the IFCC recommended reporting unit.

Quality control

HbA1c reagent cartridges are best stored at 2–8 °C and used until the expiry date. Once opened, a reagent cartridge requires using within 1 hour. A calibrator card is provided to calibrate each lot of reagent cartridges. The analyzer is calibrated by the manufacturer. Two liquid HbA1c controls are available from the manufacturer, one normal, the other abnormal. Imprecision of the assay is reported as less than 3 %.

DCA Vantage performs electronic, mechanical and reagent system checks during each assay, including calibration verification. If an error occurs during an assay, an error message is displayed preventing the reporting of incorrect test results. Other built in error codes include sampling technique and cartridge handling errors.

SCREENING FOR RAISED LIPIDS

In low and middle income countries, the number of people with hyperlipidaemia (raised lipids levels) and at risk of cardiovascular disease is increasing, mainly due to urbanization, dietary changes and rising national incomes.

Risk of cardiovascular disease can be assessed by measuring the levels of total cholesterol, HDL cholesterol and triglycerides and calculating LDL cholesterol and non-HDL cholesterol.

- Raised HDL cholesterol is protective against heart disease and coronary artery disease. Low HDL cholesterol increases the risk of cardiovascular disease.
- Raised LDL cholesterol often with raised triglycerides increase the risk of stroke and coronary heart disease. Lower levels reduce the risk.
- Raised non-HDL increases the risk of cardiovascular disease. Lower levels reduce the risk.
- Raised triglyceride levels are associated with atherosclerosis.

Cholesterol: Made in the liver, cholesterol is transported as lipoprotein from the liver to body tissues. It is essential for cell membrane structure and is used to manufacture steroid hormones, vitamin D and bile acids. It is present in dairy products, eggs and meat. Cholesterol is only excreted by way of the liver.

HDL cholesterol: HDL cholesterol transports excess cholesterol from the tissues back to the liver for excretion in bile. It is often referred to as "good cholesterol" because it removes cholesterol from blood vessels, reducing the risk of heart and circulatory diseases.

Non-HDL cholesterol: This is calculated from subtracting HDL cholesterol from total cholesterol. It is considered more useful for assessing the risk of developing cardiovascular disease than calculating the ratio of total cholesterol to HDL cholesterol.

LDL cholesterol: LDL cholesterol delivers cholesterol from the liver to body cells. About 70% of plasma cholesterol occurs in this form. It is often referred to as "bad cholesterol" because in excess it can stick to the walls of blood vessels causing hardening and narrowing of vessels, increasing the risk of a stroke and heart attack.

Triglycerides: Stored in the body's fat cells, triglycerides are a source of cellular energy. In excess, triglycerides can be deposited in artery walls. After a high fat meal, blood plasma can appear milky white.

Measuring total cholesterol, HDL cholesterol and triglycerides using reagent test kits

Test kits are available for the measurement of cholesterol, HDL cholesterol and triglycerides. Most semi-automatic chemistry analyzers use enzymatic methods.

Sample: Serum is commonly used for lipid tests and for some analyzers, whole blood is used. A fasting sample is no longer considered a requirement for routine lipid investigations.

Serum should be separated from cells as soon as possible. Lipids are stable in samples for 1 week at 2–8 °C or for up to 1 month when stored frozen at –20 °C. The volume of sample required will depend on the analyzer being used.

Principle of cholesterol method: Cholesterol esters are hydrolyzed by cholesterol esterase, producing free cholesterol and fatty acids. Free cholesterol is oxidized by cholesterol oxidase, liberating hydrogen peroxide which is converted to water and oxygen by peroxidase. Oxygen is taken up by 4-aminophenazone

and joined to phenol, producing a red coloured dye which is measured at 505 nm wavelength. The intensity of colour formed is proportional to the cholesterol concentration in the sample. The colour is stable for at least 60 minutes. Measuring range and linearity depend on the analyzer used.

Principle of HDL cholesterol method: A two stage direct method is used. In the first stage a detergent reagent is used to block non-HDL lipoproteins from the enzymatic reactions of cholesterol esterase and cholesterol oxidase, leaving HDL cholesterol to react with the enzymes in the second stage of the assay.

Principle of triglyceride method: Following incubation with lipoprotein lipase, triglycerides are hydrolyzed to produce glycerol and free fatty acids. Glycerol is oxidized by glycerol oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In the presence of peroxidase, the hydrogen peroxide reacts with 4-aminophenazone and 4-chlorophenol to form a red coloured dye which is measured at 505 nm wavelength. The intensity of the colour formed is proportional to the triglyceride in the sample. The colour is stable for at least 30 minutes. Measuring range and linearity depend on the analyzer used.

Quality control

Reagents for all three tests require storage at 2–8 °C in tightly stoppered containers, protected from direct sunlight and contamination. For the cholesterol test, the working reagent is stable for 4 months at 2–8 °C. For the HDL cholesterol test, the reagents, once opened are stable for 4 weeks. Once reconstituted, cholesterol serum calibrators are stable for 2 weeks at 2–8 °C or for 3 months at –20 °C. For the triglyceride test, the working reagent is stable for 6 weeks at 2–8 °C.

Control sera should be run with each batch of tests. Control sera covering normal and pathological levels are available from manufacturers.

Interpreting cholesterol test results

Desirable total cholesterol levels

Healthy adults: < 5 mmol/L
Children: < 4.4 mmol/L
High risk people*: < 4.0 mmol/L

* Includes those with diabetes mellitus, high blood pressure, chronic heart disease, previous transient ischaemic attack, or with a family history of heart disease.

Note: Familial hypercholesterolaemia should be considered when the total cholesterol is > 9.0 mmol/L, and triglycerides are > 20 mmol/L.

Raised total cholesterol

Cholesterol levels are affected by stress, diet, age,

gender, hormonal balance and pregnancy. Higher levels are found in post-menopausal women after 50 years.

Causes of raised total cholesterol levels include:

- Various hyperlipidaemias
- Uncontrolled type 2 diabetes
- Obstructive liver disease
- Chronic kidney disease
- Hypothyroidism
- Drugs, including beta-blockers, corticosteroids, oestrogens and protease inhibitor antiretroviral drugs.

Desirable HDL cholesterol levels

Men: > 1 mmol/L

Women > 1.2 mmol/L

Desirable total cholesterol/HDL ratio (calculated)

Total cholesterol / HDL ratio = $\frac{\text{Total cholesterol}}{\text{HDL cholesterol}}$

Desirable ratio : Below 5.0

Desirable non-HDL cholesterol levels

Non-HDL cholesterol = Total cholesterol – HDL cholesterol.

This gives a measurement of lipoproteins that are atherogenic (involved in atherosclerosis).

Low risk: 4 mmol/L or less.

Desirable LDL cholesterol levels

LDL cholesterol = Total cholesterol – HDL cholesterol – triglycerides*

* Equation is applicable providing triglyceride levels are below 4.4 mmol/L.

Healthy adults: < 3 mmol/L

Children: < 2.8 mmol/L

High risk people*: < 2.0 mmol/L

* See above Total cholesterol text.

Interpreting serum triglycerides test results

Desirable triglyceride levels

Non-fasting sample: < 2.3 mmol/L

Fasting sample: < 1.2 mmol/L*

* A fasting sample is no longer considered a requirement for routine lipid investigations.

Note: Values are slightly lower in women compared to men.

Raised triglyceride levels

As for total cholesterol. High triglyceride levels can occur with diabetic ketoacidosis and liver disease and very high levels in pancreatitis. Antiretroviral

drugs that may increase triglyceride levels include Efavirenz and Stribid.

Cholesterol meters

Most cholesterol meters are intended for home use, usually estimating only total cholesterol and varying in cost, accuracy, stability of reagent strips, volume of blood sample required, complexity of use and quality control. Only limited information is provided on the meaning of test results and there is no opportunity for health assessment and support.

A point-of-care cholesterol meter for use in healthcare and hyperlipidaemia screening programmes needs to be affordable and verified in the clinical setting in which it will be used. The meter needs to measure accurately and precisely total cholesterol, HDL cholesterol, triglycerides and calculate LDL cholesterol using a small volume of whole blood. It needs to have test storage, printout and connectivity facilities. Test strips or reagent cartridges need to be easily available, have appropriate shelf-life and be sufficiently stable without the need for refrigeration. The meter must be simple to operate and maintain with adequate quality control.

Few healthcare cholesterol meters are available with the above specifications. Point-of-care meters currently in use include *CardioChek PA* and similar design meter, *Mission Cholesterol*, and *Cholestech LDX*, a small bench top analyzer.

CardioChek PA has the advantage that it is less expensive than *Cholestech LDX* and does not require refrigeration of reagent cartridges, however inconsistencies and inaccuracies in the measuring of specific lipid parameters and predicting overall cardiovascular risk have been reported in some studies.⁸ *CardioChek PA* measures total cholesterol, HDL cholesterol, triglycerides, LDL cholesterol (calc.), TC/HDL (calc.) and glucose. A sample volume of 40 µL is required for a complete lipid panel of tests (15 µL for individual lipid parameters).

Cholestech LDX has been mostly reported as providing accurate test results when compared with standard laboratory tests, however reagent cartridges require refrigeration at 2–8 °C including shipping in dry ice. They can be stored at room temperature for up to only 30 days. A sample volume of 40 µL is required. *Cholestech LDX* meter and its reagent cartridges are expensive for routine use.

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3.3 Investigating kidney disease

Urinalysis

Measurement of serum/plasma creatinine, estimated glomerular filtration rate (eGFR) and urine albumin creatinine ratio (ACR) with urine microscopy and biochemical urine tests, are used to investigate chronic kidney disease and acute kidney injury, and monitor disease progression and treatment.

MEASURING SERUM/PLASMA CREATININE

Analyte: Creatinine is a nitrogenous waste product formed from the metabolism of creatine in skeletal muscle. Up to 10% is derived from dietary sources, e.g. cooked meat. Creatinine is filtered from the extracellular fluid by the kidneys and excreted in the urine. Excretion of creatinine is mainly renal and in the absence of disease, relatively constant.

Purpose of test: Measurement of serum/plasma creatinine with eGFR is used to assess kidney function and monitor patients being treated with nephrotoxic drugs e.g. antiretroviral drug, Tenofovir. Creatinine is used in preference to measuring blood urea because it is less affected by age, dehydration and catabolic states e.g. fever, sepsis and internal bleeding. Creatinine levels are also less influenced by changes in diet such as low intake of protein (providing this is not prolonged).

Sample: Haemolysis-free serum or plasma from lithium heparin anticoagulated blood can be used (method dependent). Creatinine is stable for up to 48 hours when stored at 2–8 °C. The volume of sample required depends on the test method.

Measuring serum/plasma creatinine using reagent test kits

Methods to measure serum/plasma creatinine using chemistry analyzers and ready to use reagents are either kinetic Jaffé-based or enzymatic.

Kinetic Jaffé-based method

Creatinine reacts with picric acid in an alkaline medium, producing a yellow-red complex which is measured at 490–500nm. To minimize interference from non-creatinine chromogens associated with the

Jaffé-reaction, a kinetic technique is used in which measurements are taken at specific times, e.g. after 30 and 90 seconds. Spectral interference can also occur from bilirubin, haemoglobin and lipaemia. For most reagent test kits, the working reagent is stable for 15 days at 2–8 °C.

Enzymatic methods

Enzymatic methods are more specific and accurate with less interference from biological solutes, (particularly glucose). Assays, however can be affected by spectral interference and some drugs. The enzyme method used depends on the manufacturer of the reagent test kit and chemistry analyzer. The creatinine amidohydrolase, sarcosine oxidase, peroxidase method is commonly used. Creatinine amidohydrolase catalyzes the conversion of creatinine to creatine. The enzyme creatine aminohydrolase catalyzes the reaction to produce urea and sarcosine. The sarcosine is measured using the enzyme sarcosine oxidase to generate hydrogen peroxide which is used to oxidize an indicator. The reagents are stable until their expiry dates when stored at 2–8 °C Enzyme reagent test kits are considerably more expensive than kinetic Jaffé-based kits.

Note: A standardized reference material NIST SRM 967 is available for the calibration of serum/plasma creatinine assays.

Measuring serum/plasma creatinine using a POCT analyzer

An example of a POCT analyzer that measures creatinine in capillary blood using an enzymatic amperometric method and provides calculated eGFR, is the *Nova StatSensor* creatinine analyzer shown in Plate 3.34.

The biosensor test strips used in the analyzer are pre-calibrated and have a shelf-life of 12 months when stored at 2–8 °C and 3 months at ambient room temperature (packed 25 strips/vial). Only 1.2 µL of blood is required and test results are displayed after 30 seconds. The creatinine measuring range is 27–1056 µmol/L. The analyzer is operated from a 3.7V Li rechargeable battery, providing sufficient power for 40 tests. Spare batteries are available. The analyzer has automatic shut-off when not in use. *Nova StatSensor* can be interfaced with hospital and laboratory information systems. It has a built-in barcode scanner.

The analyzer is easy to operate (see Plate 3.34).

Performance of Nova StatSensor analyzer

StatSensor measures and corrects for varying



Plate 3.34 Nova StatSensor creatinine analyzer.
Courtesy Nova Biomedical. www.novabiomedical.com



1 Place a biosensor test strip in the meter.



2 Apply the strip to a drop of capillary blood.



3 Read test results after 30 secs:
Creatinine, $\mu\text{mol/L}$
eGFR $\text{mL/min}/1.73 \text{ m}^2$.

Photos: Courtesy Nova Biomedical

haematocrit levels. Accurate results are obtained throughout a broad range of haematocrit. The eGFR is calculated using MDRD and Cockcroft-Gault equations (see later text). Quality control strips are available, covering low, medium and high creatinine levels.

The *StatSensor* analyzer has been shown to provide reliable creatinine measurement across a clinically relevant range. Unreliable readings were observed in patients with high glucose levels. Use of the analyzer is not therefore recommended for diabetic patients with glucose levels $>11.0 \text{ mmol/L}$ (200 mg/dL).¹

Evaluations of the *Nova StatSensor Xpress* model have shown underestimation of high creatinine values (i.e. $>600 \mu\text{mol/L}$). Repeatability, inter-device reproducibility and between-run reproducibility using quality control reagents were found to be acceptable. The analyzer was found sufficiently accurate for detecting pathological values in patients (age >10 year) with moderate risk of misclassification.²

INTERPRETING SERUM/PLASMA CREATININE TEST RESULTS

Serum/plasma creatinine concentration is affected by age, gender, muscle mass, diet, exercise and some drugs. It is an insensitive marker of renal function and unsuitable for detecting early kidney disease. Levels may be within the reference limit even when there is significant kidney damage. GFR has to fall to almost half before a significant rise in serum/plasma creatinine occurs. Results need to be interpreted with other tests results, including estimated (e) GFR, urine microscopy for red cells and casts, urine tests for proteinuria, glycosuria, haematuria and albumin creatinine ratio (see later text).

eGFR

eGFR is calculated from the serum/plasma creatinine and corrects for age, gender, body mass and ethnic origin using the Cockcroft-Gault and Modification of Diet in Renal Disease (MDRD) Study equations.*

* The Cockcroft-Gault equation uses age, gender and body weight whereas the MDRD Study equation incorporates age, gender and ethnicity.

A simplified MDRD Study equation to obtain eGFR is programmed into most chemistry analyzers that measure serum/plasma creatinine. eGFR is expressed in $\text{mL/min}/1.73 \text{ m}^2$ with 1.73 m^2 referring to the mean body surface area for young adults. The modified MDRD equation is not suitable for children ($<18 \text{ y}$), pregnant women, amputees and

patients with unstable creatinine levels, extreme body size, muscle-wasting disease, malnutrition, and severe liver disease.

The MDRD equation is less accurate at eGFR >60 mL/min/1.73 m². In most healthy adults the eGFR is about 90 mL/min/1.73 m² and declines with age. eGFR forms the basis for staging chronic kidney disease with levels below 60 for 3 months or more being indicative of chronic kidney disease and levels below 15 mL/min/1.73 m² being found in life-threatening acute kidney injury. The modified MDRD equation has not been validated for the adjustment of drug doses. The Cockcroft-Gault equation is used.

Reference intervals for serum/plasma creatinine

The following is an approximate creatinine reference interval when using a kinetic Jaffé method. Lower values are found when using enzymatic methods. Reference intervals should be established locally.

Men: 60 – 130 µmol/L (0.7 – 1.4 mg/dL)

Women: 40 – 110 µmol/L (0.4 – 1.2 mg/dL)

To convert µmol/L to mg/dL, multiply by 0.011.

To convert mg/dL to µmol/L, divide by 0.011.

Note: Reference intervals are lower for children. Values depend on muscle mass.

Creatinine reference intervals for some tropical countries

COUNTRY	MEN µmol/L	WOMEN µmol/L
Nigeria	76.3 – 111.1	63.0 – 117.8 ^a
Kenya	62.0 – 106.0	51.0 – 91.0 ^a
Ghana	81.0 – 141.0	70.0 – 121.0 ^b
India <40 y	63.6 – 109.1	45.4 – 90.9 ^c

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Increases in serum/plasma creatinine

Serum/plasma creatinine levels rise when there is a fall in glomerular filtration rate as can occur in conditions causing:

- glomerulonephritis (inflammation of kidney glomeruli),

- pyelonephritis (bacterial infection of the kidney pelvis),
- acute tubular necrosis (damage to tubular epithelial cells),
- sepsis,
- urinary obstruction.

Uraemia: Describes the presence of excessive amounts of creatinine, urea and other nitrogenous waste products in the blood as can occur in chronic kidney disease. If untreated it can progress to acute kidney injury with electrolyte and metabolic abnormalities, coma and death. Burr cells can be found in the peripheral blood film.

In tropical countries kidney disease frequently occurs in young people due to parasitic, bacterial and viral infections³, including:

- *Parasitic:* Particularly severe falciparum, knowlesi and increasingly vivax malaria causing acute kidney injury, and *P. malariae* causing malaria nephropathy in children (4–8 y). Also, lymphatic filariasis and schistosomiasis with renal involvement.
- *Bacterial:* particularly leptospirosis, scrub typhus, renal tuberculosis, acute bacterial diarrhoeal disease, cholera, leprosy, post-streptococcal glomerulonephritis.
- *Viral:* including HIV disease, dengue haemorrhagic fever and dengue shock syndrome, hantavirus fever with renal syndrome, haemorrhagic fever viral infections, hepatitis B and C, acute viral diarrhoeal disease.

Other important causes of renal disease in tropical countries are diabetes, hypertension, dehydration, obstetric complications, treatment with nephrotoxic antiretroviral drugs, envenoming from snake and arthropod bites, genetic disorders (sickle cell disease, thalassaemia, G6PD enzyme deficiency), exposure to nephrotoxic chemicals, and toxic herbal remedies. Progression to acute kidney injury is often rapid due to unavailability of diagnostic and treatment monitoring facilities, lack of trained personnel, unavailability of safe essential drugs and lack of community awareness of the causes of kidney disease.

Chronic kidney disease of undetermined origin (CKDu)

This serious condition which rapidly progresses to loss of kidney function has been reported recently from India, Central America, Sri Lanka, South East Asia, Middle East and South America where it affects middle age men employed in agricultural work.⁴ Possible exposure to nephrotoxic chemicals in fertilisers or pesticides may be involved. Laboratory findings include a urine albumin creatinine ratio > 30 mg/g.

Note: Chronic kidney disease and acute kidney injury can cause hyperkalaemia (raised potassium level).

Decreases in serum/plasma creatinine levels

Diseases associated with muscle wasting and over-hydration can cause reduced creatinine levels.

ALBUMIN CREATININE RATIO

The albumin creatinine ratio (ACR) in mg/g is calculated by dividing the urine albumin concentration in milligrams by the creatinine concentration in grams.

Purpose of test: The ACR is used to detect glomerular kidney diseases at an early stage. It is used to screen patients at high risk of developing chronic kidney disease, e.g. patients with diabetes and hypertension. It can also help to diagnose early pre-eclamptic toxæmia during pregnancy.

Sample: Freshly collected morning urine is recommended. If unable to test within 2 hours, refrigerate the sample at 2–8 °C. Refrigerated samples can be stored for up to 1 week prior to testing. Allow the sample to return to room temperature and mix well before testing. Boric acid preservative does not interfere with the test. Samples are unsuitable that have tested positive for blood, protein or bacterial infection.

Determining ACR using urine albumin creatinine strip tests

Urine strip tests with testing areas for both albumin and creatinine can be used to obtain the ACR by measuring semi-quantitatively albumin and creatinine.*

* **Chemical reactions:** Albumin measurement is based on dye binding using a high affinity sulfonephthalein dye. At a constant pH the development of any blue colour is due to the presence of albumin. Creatinine measurement is based on the peroxidase-like activity of a copper creatinine complex that catalyzes the reaction of di-isopropylbenzene dihydroperoxide and 3',3',5',5' tetramethylbenzidine.

Test reactions can be read visually but are more accurately read using a urine reflectance analyzer. An example of an albumin creatinine reagent strip test is *Clinitek Microalbumin* manufactured by Siemens Healthcare GmbH. The strip measures albumin at 10, 30, 80, 150 mg/L and creatinine at 10, 50, 100, 200, 300 mg/dL. The test reaction takes 60 seconds. *Clinitek Status* urine analyzer is used to read the results and calculate the ACR. The results are printed out automatically by the built-in printer. The analyzer can be used to read a wide range of

Siemens' urine reagent strip tests (see Chemical testing of urine text).



Plate 3.35 *Clinitek Microalbumin* strips and analyzer.
Courtesy Siemens Healthcare www.healthcare.siemens.com

Quality control: Store the strips at 15–30 °C and do not use strips after their expiry date (strips are available 25/container). Albumin creatinine test strips are moisture-sensitive. Ensure reagent containers are tightly capped and protect the strips from direct sunlight. The use of liquid ready-to-use negative and positive controls is recommended whenever a new strip container is opened. Interference from some drugs may occur (refer to manufacturer's literature).

When *Clinitek Status* is first turned on it performs a system diagnostic check and ensures there is sufficient battery voltage to operate the analyzer (if powered by batteries). The analyzer is self-calibrating. Further performance checks are made each time a strip is read, including a humidity check. The clarity and colour of the sample are entered by the user. Patient information is entered using the key pad or barcode scanner provided.

Note: The results obtained when reading the strips visually will be close to but not necessarily the same as when strips are analyzer-read.

Performance: Albumin creatinine strip tests to determine ACR have been shown to have high sensitivity, specificity and negative predictive value when tested against laboratory quantitative tests.⁵

Determining ACR using DCA Vantage analyzer

The *DCA Vantage* analyzer (manufactured by Siemens Healthcare GmbH) measures urine albumin and creatinine using an immunological turbidimetric method* and calculates the ACR.

* **Principle of test:** Specific antibody binds with albumin in the presence of polyethylene glycol. The albumin-antibody

complex causes increased turbidity which is measured at absorbance 531 nm. Creatinine measurement is based on the Benedict-Behre reaction in which creatinine complexes with 3,5-dinitrobenzoic acid at high pH to form a coloured complex which is measured at 531 nm. Creatinine is quantified using an absorbance calibration curve. The ACR is calculated from the albumin and creatinine measurements.

The *DCA Vantage* analyzer is also used to measure glycated haemoglobin (HbA1c) which is described in Section 3.2. Using *DCA Vantage* to determine ACR takes about 7 minutes and requires a microalbumin/creatinine immunoassay reagent cartridge. The procedure is summarized as follows:

- 1 Scan the reagent cartridge barcode.
- 2 Fill the capillary tube provided with urine (0.4 µL). Wipe the outside of the tube.
- 3 Insert the capillary into the reagent cartridge and insert the cartridge into the cartridge compartment.
- 4 Pull the pull-tab from the reagent cartridge and close the compartment door. This starts the reaction. After 6–7 minutes the screen displays the test results. Print the results using the analyzer printer.
- 5 Remove and discard the reagent cartridge into a biohazard container.

Analyzer reporting range for albumin: 5–30 mg/L. For results greater than 300 mg/L, report as > 300 mg/L.

Analyzer reporting range for creatinine: 15–500 mg/dL. For results greater than 500 mg/dL, report as > 500 mg/dL.

Reporting ACR: The analyzer reportable range is 1–2000 mg/g. If outside the range, 'unable to calculate' will be displayed.

Quality control: The immunoassay cartridge requires storage at 2–8 °C (or up to 3 months at room temperature, 15–30 °C). Do not use cartridges beyond their expiry date. After opening a cartridge it must be used within 10 minutes. Urine microalbumin/creatinine control materials are available from the manufacturer (stable for 3 months when refrigerated at 2–8 °C).

The *DCA Vantage* performs optical, electronic, mechanical and reagent system checks during each assay. If an error occurs during a measurement, the system automatically reports an error message, preventing the reporting of incorrect patient results.

Interpreting ACR results⁶

The relationship of ACR to albuminuria categories in chronic kidney disease is as follows:

CATEGORY	ACR (mg/g)	TERMS
A1	< 30	Normal to mildly increased
A2	30–300	Moderately increased*
A3	> 300	Severely increased**

* Relative to young adult level. ACR 30–300 mg/g for > 3 months indicates chronic kidney disease.

** Including nephrotic syndrome (albumin excretion is usually > 2220 mg/24 h, ACR > 2220 mg/g).

Note: ACR < 30 mg/g refers to albumin excretion above the normal range but below the level detectable by tests that measure total protein. Ranges are different for patients with diabetes.

Urine albumin excretion can be temporarily raised by exercise, urinary tract infection and acute illness with fever. ACR results may be inaccurate in persons with decreased or increased muscle mass.

URINALYSIS

The production and composition of urine depend on glomerular filtration, tubular reabsorption and tubular secretion. Changes that can occur in the volume and appearance of urine in disease are as follows:

Volume of urine

The volume of urine excreted daily depends on fluid intake, diet, climate, and other physiological factors. It is usually between 1–2 litres per 24 hours. An increase in the volume of urine is called polyuria. It occurs in diabetes mellitus due to an increase in the osmolality of the filtrate preventing the normal reabsorption of water (osmotic diuresis). Polyuria also occurs when the secretion of the antidiuretic hormone is reduced, e.g. in diabetes insipidus.

A decrease in the volume of urine excreted is called oliguria. It occurs when the renal blood flow and, or, glomerular filtration rate is reduced. One of the causes of a reduced renal blood flow is low blood pressure (hypotension) caused for example by severe dehydration or cardiac failure. A fall in glomerular filtration rate occurs in acute glomerulonephritis (inflammation of the kidney glomeruli) and also in the early stages of acute tubular necrosis.

If severe oliguria progresses to a complete cessation of urine flow, this is called anuria and is usually due

to severe damage to the renal tubules (acute tubular necrosis). Acute tubular necrosis may follow any of the conditions which cause severe hypotension or may be due to a direct toxic effect on the tubules by drugs or following sepsis or an incompatible blood transfusion.

Appearance of urine

Normal freshly passed urine is clear and pale to dark yellow in colour. A dilute urine appears pale in colour and a concentrated one has a dark yellow appearance. The yellow colour is due to the pigments urochrome, urobilin, and porphyrins.

When normal urine has been allowed to stand for some time, a white phosphate deposit may form if the urine is alkaline or a pink uric acid deposit may form if the urine is highly acidic or concentrated. A 'mucus' cloud may also form if normal urine is left to stand.

The appearance of urine may be altered in many conditions including:

- Urinary tract infections in which the urine appears cloudy because it contains pus cells and bacteria.
- Urinary schistosomiasis in which the urine often appears red and cloudy because it contains blood (haematuria).
- Malaria haemoglobinuria (blackwater fever) and other conditions causing intravascular haemolysis in which the urine appears brown and cloudy because it contains free haemoglobin.
- Jaundice in which the urine may appear yellow-brown or green-brown because it contains bile pigments or increased amounts of urobilin (oxidized urobilinogen).
- Bancroftian filariasis in which the urine may appear milky-white because it contains chyle.

URINE MICROSCOPY

Preparation and examination of a wet preparation

- 1 Aseptically transfer about 10 mL of well mixed urine to a labelled conical tube.
- 2 Centrifuge at 500–1000 g for 5 minutes. Pour the supernatant fluid (by completely inverting the tube) into a second container not the original one. This can be used for biochemical tests to avoid contaminating the original urine which may need to be cultured (depending on the findings of the microscopical examination).

- 3 Remix the sediment by tapping the bottom of the tube. Transfer one drop of the *well-mixed* sediment to a slide and cover with cover glass.

Note: Do not discard the remaining sediment because this may be needed to prepare a Gram smear if WBCs and, or, bacteria are seen in the wet preparation.

- 4 Examine the preparation microscopically using the 10× and 40× objectives with the condenser iris *closed sufficiently* to give good contrast.

Report the following:

Bacteria (report only when the urine is *freshly* passed): Usually seen as rods, but sometimes cocci or streptococci. Bacteriuria is usually accompanied by pyuria (pus cells in urine).

Note: In a urinary infection, protein and nitrite are often found in the urine (see later text). With *E. coli* infections, the urine is markedly acid. An alkaline urine is found with *Proteus* infections.

White cells (pus cells): These are round, 10–15 µm in diameter, cells that contain granules as shown in Plate 3.36 on page 266. In urinary infections they are often found in clumps. In urine sediments, white blood cells (WBC) are usually reported as:

Few: Up to 10 WBCs/HPF (high power field, i.e. using 40× objective)

Moderate number: 11–40/HPF

Many: More than 40 WBC/HPF

Note: A few pus cells are normally excreted in urine. Pyuria is usually regarded as significant when moderate or many pus cells are present, (see previous text). Bacteriuria without pyuria may occur in diabetes, enteric fever, bacterial endocarditis, when urine contains many contaminating organisms, or when WBCs lyse in an alkaline urine, e.g. *Proteus* infection.

Pyuria with a sterile routine culture may be found with renal tuberculosis, gonococcal urethritis, *C. trachomatis* infection, and leptospirosis, or when a patient with urinary infection has been treated with antimicrobials.

Red cells: These are smaller and more refractile than white cells (see Plate 3.36). They have a definite outline and contain *no* granules. When the urine is isotonic, they have a ringed appearance. They are usually reported as few, moderate or many in number per high power field.

Note: When the urine is hypertonic, i.e. more concentrated than the fluid inside the red cells, fluid will be drawn out of the cells and they will appear smaller than normal and often crenated (spiky).

When haematuria is due to glomerulonephritis, the red cells often vary in size and shape (dysmorphic).

In sickle cell disease, sickled red cells can sometimes be seen in urine.

Haematuria (red cells in urine) may be found in urinary schistosomiasis (usually with proteinuria), bacterial infections, acute glomerulonephritis (inflammation of the glomeruli of the kidneys), sickle cell disease, leptospirosis, infective endocarditis, calculi (stones) in the urinary tract, malignancy of the urinary tract, and haemorrhagic conditions.

Note: The finding of red cells in the urine of women may be due to menstruation.

Casts: These can usually be seen with the 10× objective provided the condenser iris is *closed sufficiently* to give good contrast. They consist of solidified protein and are cylindrical in shape because they are formed in the kidney tubules. The following casts can be found in urine:

- Hyaline casts, which are colourless and empty (see Plate 3.37). They are associated with damage to the glomerular filter membrane. A few may be seen following strenuous exercise or during fever.
- Waxy casts, which are hyaline casts that have remained in the kidney tubules a long time. They are thicker and denser than hyaline casts, often appear indented or twisted, and may be yellow in colour. They usually indicate tubular damage and can sometimes be seen in renal failure.
- Cellular casts, which contain white cells or red cells (see Plate 3.39). Red cell casts appear orange red. They indicate haemorrhage into the renal tubules or glomerular bleeding. White cell casts are found when there is inflammation of the kidney pelvis or tubules. Yellow-brown pigmented casts may be seen in the urine of jaundiced patients.
- Granular casts, which contain irregular sized granules originating from degenerate cells and protein (see Plate 3.38). They are also associated with renal damage.

Epithelial cells: These are easily seen with the 10× objective. They are nucleated and vary in size and shape. They are usually reported as few, moderate, or many in number per low power (10× objective) field. It is normal to find a few epithelial cells in urine. When seen in large numbers, however, they usually indicate inflammation of the urinary tract or vaginal contamination of the sample.

Yeast cells: These can be differentiated from red cells by their oval shape and some of the yeasts

usually show single budding (see Plate 3.40). If in doubt, run a drop of dilute acetic acid under the cover glass. Red cells will be haemolyzed by the acid, but not yeast cells.

Note: Glove powder in urine also resembles yeasts. It can be distinguished by adding a drop of iodine (as used in Gram stain). Glove powder granules (starch), turn blue-black.

Yeast cells are usually reported as few, moderate, or many per HPF. They can be seen in the urine of women with vaginal candidiasis, and occasionally in samples from diabetics and those with immunosuppression.

Trichomonas vaginalis: Found in the urine of women with acute vaginitis (occasionally seen in the urine of men). The trichomonads are a little larger than white cells and are usually easily detected in fresh urine because they are motile. They move by flagella and an undulating membrane (see Plate 3.40 and also Section 2.13).

Eggs of S. haematobium: Recognized by their large size (about 145 × 55 μm) and spine at one end (see Plate 3.41). The urine will contain red cells and protein. Urinary schistosomiasis is described in Section 2.6.

Other parasites that may be found in urine

- Very occasionally the microfilariae of *Wuchereria bancrofti* can be found in urine. This happens when a urogenital lymphatic vessel ruptures. The urine appears milky-white or reddish-pink (chyle mixed with blood). The microfilariae are large (225–300 × 10 μm), motile, and sheathed. No nuclei are present in the tail (feature looked for in a Giemsa stained preparation). *W. bancrofti* microfilariae are shown in Section 2.4.
- Microfilariae of *Onchocerca volvulus* may be found in the urine in onchocerciasis, especially in heavy infections. The larvae are large (280–330 × 7 μm), unshathed, with a slightly enlarged head-end and a tail which is sharply pointed and contains no nuclei (see Section 2.4).
- Occasionally the eggs of *Enterobius vermicularis* are found in urine, especially from young girls when the eggs are washed off the external genitalia when urine is being passed.

Crystals

These have a characteristic refractile appearance. Normal urine contains many chemicals from which crystals can form, and therefore the finding of most crystals has little importance. Crystals should be looked for in fresh urine when calculi (stones) in the urinary tract are suspected.

Spermatozoa: Occasionally found in the urine of men, they can be easily recognized by their head and long thread-like tail. They may be motile in fresh urine.

Examination of a Gram stained smear

Prepare and examine a Gram stained smear of the urine when bacteria and, or white cells are seen in the wet preparation.

Examine the smear first with the 40× objective to see the distribution of material, and then with the oil immersion objective. Look especially for bacteria associated with urinary infections, especially Gram negative rods. Occasionally Gram positive cocci and streptococci may be seen.

Note: Usually only a single type of organism is present in uncomplicated acute urinary infections.

More than one type of organism is often seen in chronic and recurring infections. Vaginal contamination of the sample is indicated by a mixed bacterial flora (including Gram positive rods) and often the presence of epithelial cells.

***Neisseria gonorrhoeae* in urine**

In male patients with acute urethritis, it is often possible to make a presumptive diagnosis of gonorrhoea by finding Gram negative intracellular diplococci in pus cells passed in urine (see colour Plate 3.42).

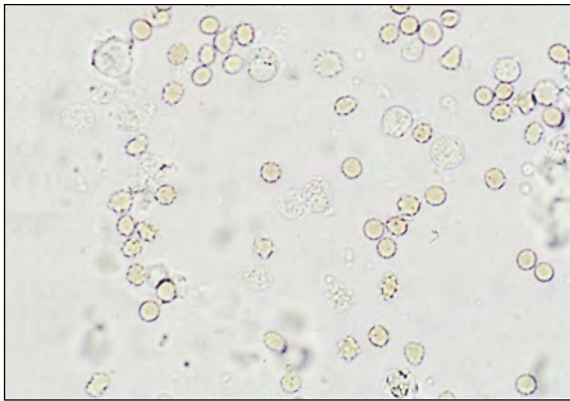
Urine microscopy

Plate 3.36 Urine from a patient with urinary tract infection. The larger indistinct colourless cells containing granules are pus cells (polymorphonuclear neutrophils). They are often seen in clumps. The smaller pale yellow cells are red blood cells. Some of the red cells are crenated. 40× objective.

Note: When bacteria are also seen, examine a Gram stained smear. This may show the presence of a single type of organism associated with urinary tract infections, e.g. Gram negative rods.

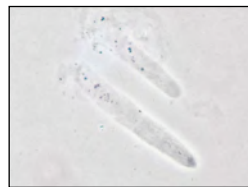


Plate 3.37 Hyaline casts: empty, colourless. May be seen when there is damage to the glomerular membrane.

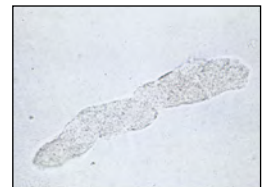


Plate 3.38 Granular cast, originating from degenerate cells and protein, associated with renal damage.

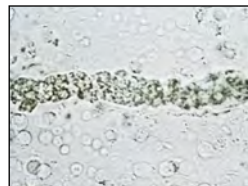


Plate 3.39 Cellular cast (may contain red cells or white cells). Frequently seen when there is glomerular bleeding or inflammation of the kidney pelvis or tubules.



Plate 3.40 Motile *Trichomonas vaginalis* trophozoites in fresh urine from a woman with trichomoniasis vaginitis. Yeast cells (groups of small round cells) are also present.

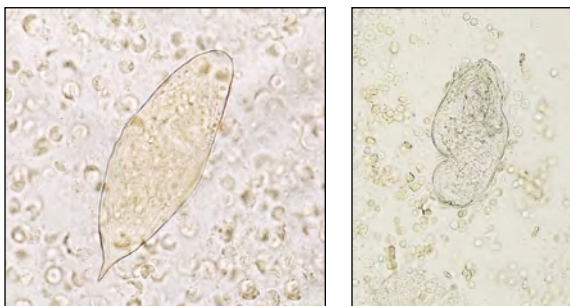


Plate 3.41 *Left:* Egg of *Schistosoma haematobium* in urine with red cells, measuring about 145 × 55 μm. It has a terminal spine. *Right:* Motile miracidium hatched from the egg.

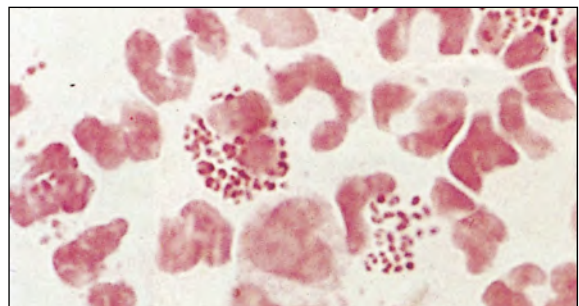


Plate 3.42 Gram stained centrifuged urine from a male patient with acute urethritis. Smear shows pus cells containing intracellular Gram negative diplococci, as seen with the 100× objective.

CHEMICAL TESTING OF URINE USING REAGENT TEST STRIPS

Urine reagents test strips are available to test for the following parameters:

- Glucose
- Bilirubin
- Ketones
- Specific Gravity
- Haemoglobin and whole blood
- pH
- Protein
- Urobilinogen
- Nitrite
- Leucocytes
- Microalbumen and creatinine

Urine test strips contain reagent impregnated test areas for the different parameters. The reagents react with the substances in the urine, producing colour changes in the test areas after 30–120 seconds (exact reaction time depending on the substance being tested). The number of test areas on a strip depends on the manufacturer.

Note: The following test reactions, test scales, limitations and interfering substances are those that apply to Siemens urine test strips. They are however applicable to the test strips produced by most other manufacturers.

Sample

Fresh 5–10 mL urine (not more than 2 hours old) is required, collected into a chemically clean, dry container, free from any stabilizer/preservative. If possible use first-voided morning urine as this will be the most concentrated, particularly when testing for nitrite. Protect the urine from direct sunlight, particularly if it is to be tested for bilirubin or urobilinogen which are unstable when exposed to light. Do not centrifuge the urine. Mix the urine well before testing. If the urine has been refrigerated, allow the sample to warm to room temperature before testing.

GLUCOSE

Glycosuria: Almost all the glucose which passes from the blood into the glomerular filtrate is normally reabsorbed back into the circulation by the kidney tubules. Usually less than 0.8 mmol/L is excreted in the urine. The term glycosuria refers to the presence of more than the usual amount of glucose in the urine.

Tests for glycosuria are usually requested to screen for diabetes mellitus.

Test scale

Negative, 100, 250, 500, 1000, >2000 mg/dL.
Sensitivity: 75–125 mg/dL glucose.

Limitations and interferences

Ketone bodies may reduce the sensitivity of the glucose strip.

Disinfectants such as bleach can cause a false positive reaction by direct oxidization of the chromogen.

Causes of glycosuria

- A rise in the concentration of blood glucose with the kidney tubules being unable to reabsorb the increased amount of glucose in the glomerular filtrate, e.g. in untreated diabetes mellitus.

Raised renal threshold for glucose

If the blood glucose level is high but the filtration rate is slow or the flow of blood to the kidneys is reduced as in heart failure, sodium depletion, or shock, there will be a rise in the renal threshold for glucose. Glucose will not appear in the urine until the blood glucose is well over 10 mmol/L. With diabetes mellitus the renal threshold for glucose is often raised, especially in elderly diabetic patients, those with cardiac failure, or those in diabetic coma with shock.

- A reduced rate of reabsorption of glucose by the kidney tubules as occurs in serious tubular damage or an inherited defect of tubular absorption causing a lowering of the glucose renal threshold. Glucose appears in the urine when the blood glucose level is well below 10 mmol/L (180 mg/dL).

Fanconi syndrome

When glucose, amino acids, phosphate, and other substances are excreted due to impaired reabsorption this is termed the Fanconi syndrome.

- An increase in the rate of glomerular filtration as may sometimes occur during pregnancy.

Important: Blood glucose should be measured whenever glucose is found in urine and the patient is not a known diabetic (see Section 3.2).

BILIRUBIN

Bilirubinuria: Bilirubin is not normally detected in urine. When found, the condition is referred to as bilirubinuria. Urine containing 8.4 $\mu\text{mol/L}$ or more of bilirubin has a characteristic yellow-brown colour (hepatocellular jaundice) or a yellow-green appearance (posthepatic jaundice).

Important: Urine samples require protection from direct sunlight to prevent oxidation of the bilirubin.

Test scale

Negative, + Small, ++ Moderate, +++ Large.
Sensitivity: 7–14 $\mu\text{mol/L}$ bilirubin.

Limitations and interferences

Indoxyl sulfate can produce a yellow-orange to red colour which can interfere with the interpretation of readings. Metabolites of etodolac may cause false positive or atypical test results. Bilirubin-derived bile pigments, rifampicin, and phenazopyridine may mask a bilirubin reaction.

Causes of bilirubinuria

Bilirubin can be found in the urine whenever there is an increase of conjugated bilirubin in the blood (see also Section 3.4). Bilirubinuria occurs therefore in posthepatic jaundice and also in hepatocellular jaundice when the blood usually contains both conjugated and unconjugated bilirubin.

Bilirubin is not found in the urine in prehepatic (haemolytic) jaundice or in other conditions in which the excess bilirubin in the blood is of the unconjugated type. In the early stages of viral hepatitis, bilirubinuria together with raised aminotransferase levels can be found before a patient becomes clinically jaundiced.

KETONES

Ketonuria: Acetoacetate, *beta*-hydroxybutyrate and acetone are collectively referred to as ketone bodies or simply ketones. The excretion of more than a trace of these substances in the urine is called ketonuria. Urine ketone tests detect acetoacetate and acetone. *Beta*-hydroxybutyrate is not detected.

Formation of ketones: The metabolism of glucose normally provides the body with its energy requirements. If, however, the intake of glucose is insufficient as in starvation, or glucose metabolism is defective due to a lack of insulin as occurs in untreated or uncontrolled diabetes, the body obtains its energy by breaking down fats. It is this increase in fat metabolism which leads to a build up of ketones in body tissues (ketosis). Ketones are toxic to the brain and if present in sufficiently high concentration in the blood they can contribute to the coma found in diabetic ketoacidosis. Ketonuria is always present.

Note: Urine samples for detecting ketones require testing as soon as possible to prevent the decomposition of acetoacetic acid.

Test scale

Negative, mg/dL: 5 Trace, 15 Small, 40 Moderate, 80 and 160 Large.
Sensitivity: 5–10 mg/dL acetoacetic acid.

Limitations and interferences

False positive reactions may occur with highly pigmented urine particularly samples that contain large amounts of L-dopa metabolites. Substances containing sulfhydryl groups may cause false positive results or an atypical colour reaction.

Causes of ketonuria

- Untreated diabetes. The finding of ketonuria in a diabetic patient being treated usually indicates an out-of-control state.
- Conditions of starvation when fat metabolism is increased.
- Eating a diet that is very low in carbohydrates.
- Severe dehydration following prolonged vomiting or diarrhoea.
- Glycogen storage disease.

SPECIFIC GRAVITY

Specific gravity: The test strip reacts to the concentration of ions in the urine. Non-ionic constituents such as urea, glucose or creatinine are not included. Test results can therefore differ from other techniques used to measure the relative mass density of urine. When tested using urine passed at the beginning of the day with the patient having taken no fluid for 10 hours, a specific gravity test may provide information on the concentrating and diluting ability of the kidneys.

Test scale

Sp G: 1,000 1,005 1,010 1,020 1,025 1,030.

Limitations and interferences

Highly buffered alkaline urines may cause low readings, while the presence of moderate quantities of protein (100–750 mg/dL) may cause elevated readings. The accuracy of specific gravity test strips is poor compared with urinometer and refractometer methods.

Changes in urine specific gravity

The reference interval for urine specific gravity in healthy adults is 1,010–1,035. Several factors contribute to test results including fluid intake, heavy perspiration, dehydration, presence of substances not normally found in urine such as glucose or protein and increased urine output, e.g. when taking diuretic drugs.

A consistently low urine specific gravity usually indicates poor tubular reabsorption or excessive fluid intake. The results of random specific gravity tests have little clinical significance. In general, the greater the volume of urine excreted the lower its specific gravity and lighter its colour.

BLOOD and HAEMOGLOBIN

Haemoglobinuria: The presence of free haemoglobin in urine is called haemoglobinuria. It occurs with severe intravascular haemolysis when the amount of haemoglobin being released into the plasma is more than can be taken up by haptoglobin (the plasma protein that binds free haemoglobin to prevent it being lost from the body). The renal threshold for free haemoglobin is 1.0–1.4 g/L.

Haematuria: The presence of intact red cells in urine is called haematuria. The blood test strip contains a separate test area with test scale for the detection of non-haemolyzed red cells. Differentiation between haemoglobinuria and haematuria is not possible when the urine visibly contains blood.

Note: The strip also detects myoglobin, however myoglobinuria is rare. It can be found in severe muscle crush injuries, strenuous muscle exercise, drug abuse and following some snake bites.

Test scale

Haemoglobin scale: Negative, Haemolyzed trace, + Small, ++ Moderate, +++ Large.

Non-haemolyzed (intact red cells) scale: Negative, Trace, Moderate.

Sensitivity: 0.015–0.062 mg/dL haemoglobin.

Limitations and interferences

Captopril may cause decreased reactivity. Oxidizing contaminants such as bleach can produce false positive results. Microbial peroxidase associated with urinary tract infection may cause a false positive reaction.

Causes of haemoglobinuria

Haemoglobinuria can occur with:

- Severe falciparum malaria.
- Typhoid fever.
- Glucose-6-phosphate dehydrogenase (G6PD) deficiency following the ingestion of certain drugs e.g. primaquine.
- *Escherichia coli* septicaemia.
- Incompatible blood transfusion.
- Snake bites that cause acute haemolysis.
- Sickle cell disease crisis.
- Thalassaemia.
- Viral haemorrhagic fevers.
- Severe burns.

Causes of haematuria

Causes include:

- Urinary schistosomiasis.
- Severe bacterial infection.
- Acute glomerulonephritis.
- Infective endocarditis.
- Sickle cell disease.
- Leptospirosis.
- Calculi (stones) in the urinary tract.
- Malignancy of the urinary tract.
- Anticoagulant therapy.

Note: Red cells found in the urine of women may be due to menstruation.

pH

Urine pH: A pH below 7 indicates an acidic urine and pH over 7, an alkaline urine. The pH of urine is determined by the body's metabolism and diet. A vegetarian diet usually gives an alkaline urine and an animal protein diet, an acidic urine. Changes in urine pH can occur when there is an acid-base imbalance.

Test scale

pH: 5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5.

Limitations and interferences

Urinary pathogens such as *Proteus* species that convert urea to ammonia can result in an alkaline urine (> pH 8.0). With *Escherichia coli* infection, the urine is acidic. When urine is left to stand it becomes alkaline. Vitamins and some drugs can also alter urine pH. Disinfectants and detergents may cause false results.

Changes in urine pH

The pH of morning urine is usually pH 5.0–6.0. During the day, the pH range is wider. An acidic urine occurs in conditions causing metabolic and respiratory acidosis. An alkaline urine may occur in conditions causing respiratory alkalosis.

PROTEIN

Proteinuria: Most plasma proteins are too large to pass through the glomeruli of the kidney. The small amount of protein which does filter through is normally reabsorbed back into the blood by the kidney tubules. Only trace amounts of protein (less than 50 mg per 24 h) can therefore be found in

normal urine. These amounts are insufficient for detection by routine laboratory tests.

When more than trace amounts of protein are found in urine this is termed proteinuria. The condition is often referred to as albuminuria because when there is glomerular damage most of the protein which passes through the glomerular filter is albumin.

Test scale

Negative, mg/dL: Trace, + 30, ++ 100, +++ 300, ++++ 2000 or more.

Strip sensitivity: Protein test strips are very sensitive, detecting as little as 10 mg/dL.

Limitations and interferences

False positive results may be obtained when the urine is contaminated with disinfectants which contain quaternary ammonium compounds or chlorhexidine. Strongly alkaline urine may give false positive and overstated test results. Urine containing visible blood, vaginal or urethral secretions may cause falsely elevated test results.

Causes of proteinuria

- Glomerular or tubular urinary disease.
Proteinuria accompanies acute glomerulonephritis and is due to increased permeability of the glomerular basement membrane. The degree of proteinuria reflects the severity of the condition and helps in assessing prognosis and response to treatment.
- HIV associated renal disease and treatment with nephrotoxic antiretroviral drugs.
- Pyogenic or tuberculous pyelonephritis.
- Severe lower urinary tract infection.
- Nephrotic syndrome which is a condition characterized by heavy proteinuria and oedema. The oedema is caused by a reduction in the colloid osmotic pressure due to a fall in the level of plasma albumin brought about when proteinuria rises to 5 or 10 g/L per day.
- Pre-eclampsia when there is moderate to marked proteinuria with raised blood pressure.
- Urinary schistosomiasis which is usually accompanied by both proteinuria and haematuria.
- Severe febrile illnesses including malaria.
- Occasionally in diabetes. Diabetic nephropathy may cause nephrotic syndrome.
- Hypertension.
- Accompanying haematuria.

Important: Whenever proteinuria is found, the urine should be examined for bacteria, pus cells, red cells, and casts.

Microalbuminuria

In poorly controlled diabetic patients the finding of albumin in the urine often indicates the development of renal disease (diabetic nephropathy). Only a very small amount of albumin is excreted, usually below that which can be detected by routine protein reagent strip tests.

Bence Jones protein in urine

Bence Jones protein is an abnormal low molecular weight globulin consisting of monoclonal free light chains of immunoglobulins. It may be found in the urine of patients with multiple myeloma which is a malignant disease of plasma cells, mainly affecting bone. If myeloma is suspected, urine protein electrophoresis is required to demonstrate Bence Jones protein, and serum electrophoresis to detect paraprotein (monoclonal immunoglobulin). Haematological investigations are also required, including a bone marrow examination.

Heat-precipitation test: This test is based on demonstrating a protein in urine which precipitates at 40–60 °C and redissolves upon cooling. The test lacks sensitivity and specificity and should not be used as a screening test for myeloma.

UROBILINOGEN

Urobilinogen in urine: It is normal to find small amounts of urobilinogen in urine from that which is reabsorbed from the intestine. The concentration of urobilinogen in the urine is therefore dependent on the amount of bilirubin being produced and entering the intestine and on the ability of the liver to excrete the urobilinogen. Urine is often tested for increases in urobilinogen when investigating haemolysis or liver disorders in which liver function is impaired.

Note: The test cannot be used to demonstrate the absence of urobilinogen in urine.

Test scale

mg/dL*: Normal, 0.2 and 1.0. Increased: 2.0, 4.0, 8.0.
* 1 mg/dL is approximately equal to 1 Ehrlich Unit/dL

Limitations and interferences

The test may react with interfering substances known to react with Ehrlich's reagent such as *p*-aminosalicylic acid and sulfonamides. Atypical colour reactions may be obtained in the presence of high concentrations of *p*-aminobenzoic acid. False negative results may be obtained if formalin is present.

Test reactivity increases with temperature. Optimum temperature is 22–26 °C. A momentary yellow coloration of the test area is indicative of large amounts of bilirubin but this does not affect the test reading.

Note: The test is not a reliable method for the detection of porphobilinogen.

Causes of increased urobilinogen

- Haemolytic disease when the amount of bilirubin being produced is increased leading to greater amounts of urobilinogen being formed.
- Paralytic ileus or enterocolitis when there is an increase in the production of urobilinogen in the intestine.
- Hepatocellular damage or hepatic congestion, resulting in less of the absorbed urobilinogen being excreted by the liver. The urobilinogen then passes into the general circulation leading to more being excreted by the kidneys.
- Cirrhosis of the liver.

Note: A chart summarizing the serum and urine findings in the different forms of jaundice can be found in Section 3.4.

Causes of a decrease or absence of urobilinogen

- Obstruction of bile ducts preventing the flow of bilirubin to the intestine for conversion to urobilinogen.
- Hepatocellular damage preventing the conjugation and excretion of bilirubin for conversion to urobilinogen.
- Absence or reduction of the normal intestinal bacterial flora (necessary to convert conjugated bilirubin to urobilinogen), leading to little or no urobilinogen being produced. This may occur in neonates or following intensive antimicrobial therapy.

Note: In viral hepatitis, urine urobilinogen is at first increased but as liver cell damage increases the small biliary ducts become obstructed leading to a reduction or even an absence of urobilinogen in the urine. In the recovery stages, urine urobilinogen again increases due to the bilirubin, now in excess, being able to pass through the biliary ducts into the intestine.

NITRITE

Nitrite in urine: Urine from a healthy person does not contain nitrite. The detection of nitrite in urine is a useful test in the investigation of urinary tract infections caused by nitrate-reducing bacteria, particularly when culture facilities are not available.

When first morning urine is tested, the nitrite reaction is positive in about 90% of nitrate-reducing bacterial urinary tract infections. When testing urine collected at other times, up to 70% of infections are detected when there is significant bacteriuria.

Note: Nitrite test strips deteriorate rapidly in humid conditions. Strips *must* be kept dry in a tightly closed container.

Test scale

Negative, Positive: Any degree of overall pink colour. The intensity of the colour reaction cannot be correlated to the severity of infection (it is a measure of nitrite concentration).

Limitations and interferences

A pink spot or pink edges to the test area should not be interpreted as a positive test result. A false negative test result may occur with shortened bladder incubation of the urine, presence of bacteria that do not reduce nitrate or insufficient dietary nitrate (a diet lacking vegetables).

A negative result can also occur when a patient is being treated with antimicrobials which reduce bacterial numbers. False positive results are rare. Drugs containing phenazopyridine can colour the test area pink-red.

Positive nitrite test

A positive test indicates a urinary infection caused by bacteria that produce nitrate reductase, an enzyme that reduces nitrate to nitrite.

Nitrate-reducing bacteria include:

<i>Escherichia coli</i>	<i>Aerobacter</i> species
<i>Proteus</i> species	<i>Citrobacter</i> species
<i>Klebsiella</i> species	<i>Salmonella</i> species

A negative test cannot rule out a urinary tract infection because the causative organism may be one that does not reduce nitrate to nitrite, e.g. *Enterococcus faecalis*, *Staphylococcus albus*, *Staphylococcus saprophyticus*, *Pseudomonas* species, or *Candida* species.

A negative reaction will occur if the nitrate-reducing bacteria are too few in the urine.

LEUCOCYTES

Leucocytes in urine: The presence of granulocyte leucocytes (polymorphonuclear neutrophils) in urine indicates inflammation of the urinary tract, the commonest cause of which is a urinary tract infection.

The test detects esterase activity in both active and lysed granulocytes. The reaction is not affected by bacteria or red cells in the urine.

Test scale

Negative, Trace, + Small, ++ Moderate, +++ Large. The sensitivity of the test is 5–15 white blood cells/HPF (high power microscope field).

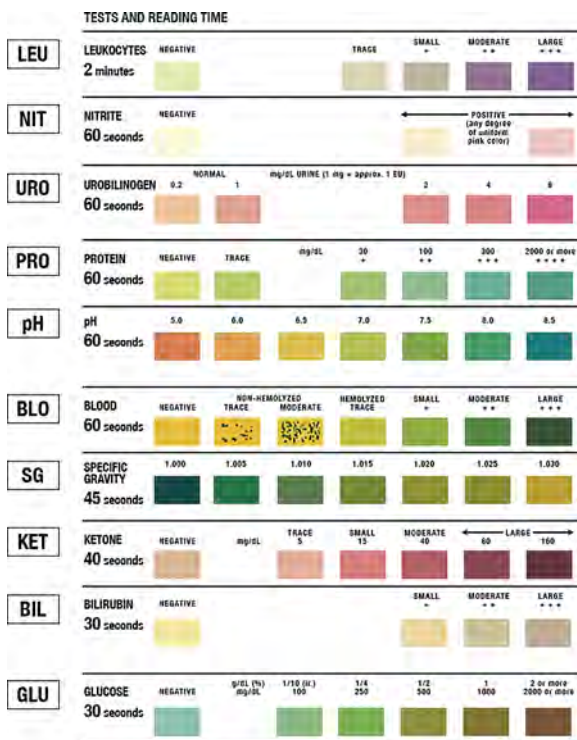
Limitations and interferences

Elevated glucose concentrations (>3 g/dL) and urinary protein in excess of 500 mg/dL may reduce the colour intensity of the reaction. The presence of cephalaxin (Keflex), cephalothin (Keflin) or high concentrations of oxalic acid may cause decreased test results. False negative results may be caused by the presence of boric acid or hydrochloric acid in the sample.

High levels of tetracycline may cause a false negative reaction. Medication with nitrofurantoin or antimicrobials containing imipenem, meropenem or clavulanic acid may cause false positive reactions. Positive results may occur when the sample is contaminated with vaginal discharge.

Positive leucocyte test

A positive leucocyte test indicates inflammation of the urinary tract, commonly caused by a bacterial or parasitic infection. The finding of leucocytes in a sterile routine urine culture may indicate infection with *M. tuberculosis* or the presence of a tumour.



Siemens *Multistix 10 SG* urine strip showing reading times for tests and test reactions.

Important: Do not use this Chart for reading test results because the colours are only approximate.

Quality control of urine reagent test strips

The reliability of urine test strips depends on using the correct urine sample as described previously, correct storage of strips, correct use and reading of test strips, and knowing the limitations of strips and substances that can affect test results. It is therefore essential to read carefully the instructions supplied with test strips by the manufacturer.

Correct storage of test strips

- Record the expiry date of the strips. Do not use strips beyond their expiry date or that show any discoloration of the test areas.
- Protect the strips from moisture, direct sunlight and excessive heat but do not refrigerate the strips.
- Do not remove the desiccant (drying agent) supplied either in the lid of the container or as a sachet in the strip container.
- Remove strips only as required. After removing a strip, replace the container top immediately and tightly to prevent moisture from the air entering. This is particularly important in humid climates. The reagents impregnating the strips deteriorate rapidly if they become damp.

Correct use of test strips

Read the manufacturer's instructions regarding the use, timing of reactions, recording of test results, and any substances interfering with test reactions. The following guidelines apply when reading test strips visually:

- Before removing a strip, become familiar with the the colour chart on the side of the container, noting when to read each test area and possible reactions.
- Do not contaminate a strip by touching the test areas with the fingers or by laying it down on the bench. Avoid using the strips in the presence of chemical fumes.
- Dip the strip *briefly* in the urine making sure that the test areas are fully immersed. Avoid prolonged contact with the urine because this may result in the reagents being dissolved out from the test areas.
- Remove excess urine from the strip by running the edge of the strip along the rim of the urine container.

- Read carefully the reactions in a good light at exactly the times stated by the manufacturer, particularly when using multiple test strips. Compare the reactions by holding the strip close to the colour chart on the container label, but avoid contaminating the container.
- When opening a new container of test strips, use a manufacturer’s control strip to check the strips are giving the correct reactions.

USING A REFLECTANCE ANALYZER TO READ URINE TEST STRIPS

In most district hospital laboratories, urine reagent strip tests are read using a urine reflectance analyzer which interprets the colour changes and prints out test results. Using a urine test reader eliminates those factors which can affect test results when tests are read visually, e.g. incorrect readings times for different test areas, individual reader interpretation of colours, transcription errors when recording test results manually and unsuitable lighting conditions when reading strips.

A urine strip analyzer reads only those strips for which it has been designed. It cannot read another manufacturer’s tests strips. An example of a urine strip reader is the *Clinitek Status* analyzer, manufactured by Siemens Healthcare for reading the following Siemens urine test strips, available in most tropical countries.

Siemens urine test strips	Test areas											
	Glucose	Bilirubin	Ketones	Specific Gravity	* Blood	pH	Protein	Urobilinogen	Nitrite	** Leucocytes	Micralbumin	Creatinine
MULTISTIX 10SG	■	■	■	■	■	■	■	■	■	■	■	■
MULTISTIX 8SG	■	■	■	■	■	■	■	■	■	■	■	■
MULTISTIX GP	■	■	■	■	■	■	■	■	■	■	■	■
LABSTIXGP	■	■	■	■	■	■	■	■	■	■	■	■
HEMA-COMBISTIX	■	■	■	■	■	■	■	■	■	■	■	■
URISTIX	■	■	■	■	■	■	■	■	■	■	■	■
KETOSTIX	■	■	■	■	■	■	■	■	■	■	■	■
MICROALBUSTIX	■	■	■	■	■	■	■	■	■	■	■	■
DIASTIX	■	■	■	■	■	■	■	■	■	■	■	■

*Haemoglobin and blood **Leucocyte esterase

Clinitek Status, in addition to reading routine urine tests strips, also reads mircoalbumin/creatinine ratio strips and Siemens hCG pregnancy test cassettes. It identifies strips automatically and checks for

common sample interferences. For those strips with a leucocyte test area, *Clinitek Status* also checks for strip exposure to moisture, avoiding incorrect test results.

The analyzer can be operated between a temperature of 22 °C and 26 °C, from mains electricity or from 6 AA batteries. It has a built-in printer, patient ID and connectivity facilities, and can store up to 200 test results with patient data. It is self-calibrating and easy to use with a touch screen and on-screen instructions.



Table for hCG pregnancy cassette

Table with channel for test strip

Plate 3.43 *Clinitek Status* urine analyzer with barcode reader.
Courtesy Siemens Healthcare.
www.healthcare.siemens.com

Procedure for using *Clinitek Status* analyzer

The following summarizes how to use *Clinitek Status*:

- 1 Turn on the analyzer. An automatic diagnostic system check will be performed.
- 2 Run a quality control check (QC) using the control material provided by the manufacturer.
- 3 Select the strip test by touching the “Strip test” box.
- 4 Using the keypad or barcode scanner, enter the patient’s information.
- 5 Enter or scan the strip lot number and expiry date.

- 6 Dip the reagent strip into the urine sample, wetting all test pads. Immediately remove the strip, dragging the edge of the strip against the rim of the urine container.
- 7 Press the green key at the time the strip is removed from the urine.
- 8 Blot the strip by touching its edge on a paper towel and place it in the channel of the test table, test pads facing upwards.
At the end of 8 seconds, the table automatically moves into the analyzer, an automatic calibration is performed and the test strip is analyzed.
- 9 While the strip is being analyzed, enter the colour and clarity of the sample as prompted by the "Select appearance" screen.
After about 60 seconds the screen displays the test results. The analyzer prints out the results.
- 10 Remove the strip from the analyzer and discard it into a biohazard waste container.
- 11 Clean the analyzer table as instructed by the manufacturer.

Quality control of *Clinitek Status*

Follow the manufacturer's instructions for analyzing QC materials before each test. The analyzer will not test patients' samples until the QC checks are successfully completed.

When first turned on, *Clinitek Status* performs a series of electronic and memory checks and ensures there is sufficient battery voltage to operate the analyzer (if powered by batteries). Each time a strip is read, the analyzer ensures the test table is positioned correctly, checks the electronic signals and takes reference readings.

Several error display codes are built in to the analyzer to alert the user of any problem affecting analyzer malfunction, failure of automatic calibration, print error, low battery, or strip errors including invalid bar code, misplaced strip or insufficient sample.

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- 5 **Eun-Hee Nah et al.** Comparison of urine albumin-to-creatinine ratio (ACR) between ACR strip test and quantitative test in prediabetes and diabetes. *Ann Lab Med*, 2017;37:28–33. <https://doi.org/10.3343/alm.2017371.28>
- 6 *Kidney International Organization Supplements*. Chapter 1: Definition and classification of CKD, 2013 3, 19–62; doi:10.1038/kisup.2012.64

FURTHER READING

- Dhanapriya J et al.** Acute kidney injury in tropical countries. *EMJ Nephrol*, 2017; 5(1):66–74.
Access by entering the title in Google.
- George C et al.** Chronic kidney disease in low-income to middle-income countries: the case for increased screening. *BMJ Glob Health*, 2017.
Access by entering the title in Google.

3.4 Investigating liver disease

Tests used in district hospital laboratories to differentiate acute hepatocellular disease, chronic liver disease and conditions causing cholestasis (bile flow obstruction), include the measurement of:

- Serum/plasma bilirubin
- Serum/plasma alanine aminotransferase activity
- Serum/plasma alkaline phosphatase activity
- Serum albumin

Also, testing urine for bilirubin and urobilinogen (described in Section 3.3).

Common causes of liver disease in tropical countries

- Viral hepatitis, particularly caused by hepatitis B virus (HBV) and hepatitis C virus (HCV).
In tropical countries, HBV and HCV infections are highly endemic and responsible for up to 80% of patients with cirrhosis and hepatocellular cancer with HBV being the main cause of end-stage liver disease.
- Hepatocellular disease caused by yellow fever virus, Epstein-Barr virus, dengue viruses, cytomegalovirus and haemorrhagic fever viruses, e.g. Ebola and Marburg.
- Hepatocellular cancer especially in areas where there is a high incidence of HCV and HBV and exposure to aflatoxin, a carcinogenic mycotoxin that contaminates crops.
- Cirrhosis of the liver caused by alcohol excess, dietary toxins, traditional herbal remedies and drugs.
- Liver cell damage caused by leptospirosis, typhoid and tuberculosis.
- Parasitic infections particularly malaria caused by *P. falciparum*, *P. vivax*, *P. knowlesi*. Also, hepatic amoebiasis, hepatosplenic schistosomiasis, visceral leishmaniasis, hepatic hydatid disease, and severe *Fasciola* and *Opisthorchis* infections.
- HIV coinfection with HBV, HCV, *M. tuberculosis* and opportunistic pathogens, and treatment of HIV disease with hepatotoxic antiretroviral drugs.

MEASURING SERUM/PLASMA BILIRUBIN

Analyte: Bilirubin is formed from the breakdown of erythrocytes and other haem-containing proteins such as myoglobin and cytochromes. The haem (iron porphyrin) of the haemoglobin molecule is separated from the globin and the haem is converted mainly in the spleen to biliverdin which is reduced to bilirubin. This bilirubin is referred to as *unconjugated (indirect) bilirubin*. It is not soluble in water and cannot be excreted in the urine.

It is transported in the blood to the liver where it is conjugated with glucuronic acid, forming bilirubin glucuronide (principally diglucuronide) which is water soluble. The conjugated bilirubin enters the bile canaliculi through the bile duct and passes in the bile into the intestine. In the intestine it is reduced by bacteria to various pigments and colourless chromogens most of which are excreted in the faeces as stercobilinogen. Small amounts of bilirubin are absorbed from the intestine, reach the liver and are re-excreted. A small amount passes into the kidneys and is excreted through urination.

Purpose of test: Measurement of serum/plasma bilirubin is performed to investigate the causes of liver disease and jaundice*. In jaundiced newborn infants it is important to monitor unconjugated bilirubin levels to prevent brain damage (kernicterus).

*Jaundice

Visible jaundice occurs when the concentration of bilirubin in the plasma rises to 40–50 $\mu\text{mol/L}$. The whites of the eyes appear yellow and the skin and body fluids also become pigmented.

Prehepatic jaundice

In prehepatic jaundice, more bilirubin is produced than the liver can metabolize, e.g. in severe haemolysis. The excess bilirubin which builds up in the plasma is mostly of the unconjugated type and is therefore not found in urine.

Hepatocellular jaundice

In hepatocellular (hepatic) jaundice, there is a build up of bilirubin in the plasma because it is not transported, conjugated, or excreted by the liver cells because they are damaged. The excess bilirubin is usually of both the unconjugated and conjugated type with bilirubin being found in the urine.

Posthepatic jaundice

In posthepatic jaundice, bilirubin builds up in the plasma because its flow is obstructed in the small bile channels or in the main bile duct. This can be caused by gall stones or a tumour obstructing or closing the biliary tract. The excess bilirubin is mostly of the conjugated type and is therefore found in urine. The term cholestasis is used to describe a failure of bile flow.

Note: Jaundiced patients often have both hepatocellular and obstructive features. It is usual to find

some obstruction when there is damage to liver cells and following obstruction there is usually some liver cell damage.

Sample: Haemolysis-free serum or plasma from EDTA or heparin anticoagulated blood can be used. The sample should be as fresh as possible (not more than 24 h old). The volume required depends on the analyzer used. Samples from newborn infants should be collected into a heparinized capillary, one end sealed and transported to the laboratory in a labelled Universal container for analysis as soon as possible.

Protect the sample from daylight and fluorescent light because bilirubin is photosensitive and rapidly destroyed by ultraviolet light. This is particularly important for neonatal samples.

Bilirubin is stable in serum or plasma protected from light, at 2–8 °C for up to 7 days.

Measuring bilirubin using a reagent test kit

Most semi-automatic analyzers measure total and direct (conjugated) bilirubin using a diazo based method. Indirect (unconjugated) bilirubin concentration is obtained by subtracting direct bilirubin from total bilirubin.

Various modifications of the diazo method are available depending on the indirect bilirubin solubilizing agents, accelerators and surfactants used in the reactions.

Jendrassik-Grof caffeine method

Total bilirubin is converted to azobilirubin by diazotized sulfanilic acid and the absorbance measured at 540 nm. Caffeine is added to solubilize the indirect (unconjugated) bilirubin. In the direct bilirubin method, no caffeine is added. The total bilirubin method is linear up to 340 µmol/L.

Walters-Gerard DMSO method

Total bilirubin is converted to azobilirubin by diazotized sulfanilic acid and the absorbance measured at 555 nm. Dimethylsulphoxide (DMSO) is incorporated to solubilize the indirect bilirubin. In the direct bilirubin method no DMSO is added. The total bilirubin method is linear up to 513 µmol/L.

2,4-DPD method

In the 2,4-dichlorophenyldiazonium (2,4-DPD) method, total bilirubin reacts with 2,4-DPD (which is used as the diazo reagent), to form azobilirubin in the presence of a surfactant to solubilize the indirect bilirubin. Increase in absorbance is measured at 546 nm. In the direct bilirubin method no surfactant is added. The total bilirubin method is linear up to 513 µmol/L.

Quality control

Haemolysis affects most bilirubin assays, decreasing bilirubin values. Haemolyzed samples should not be tested. Control sera are available from manufacturers covering normal and pathological bilirubin ranges. Reagents require storage at 2–8 °C in tightly

closed containers, protected from direct light and contamination.

Total bilirubin reference interval

Adults: 3–21 µmol/L

Newborns: 8–67 µmol/L*

*highest values in the first 3–5 days of life due to maternal blood influx at birth.

To convert µmol/L to mg%, multiply by 0.06.

To convert mg% to µmol/L, divide by 0.06

Reference intervals should be established locally.

Bilirubin reference intervals for some tropical countries

COUNTRY	umol/L total bilirubin
Ghana, adult	2.9–25.8 ^a
Kenya, adult	4.9–39.9 ^a
India, men < 40 y	6.6–20.0 ^b
India, women < 40 y	5.5–16.6 ^b

References: ^aMiri-Dashe T *et al.* Comprehensive reference ranges for haematology and clinical chemistry laboratory parameters derived from normal Nigerian adults. 2014, *PLoS ONE* 9(5):e93919.doi:10.1371/journal.pone.0093919

^bDosoo D K *et al.* Haematological and biochemical reference values for healthy adults in the middle belt of Ghana. 2012, *PLoS ONE* 7(4):e36308.doi10.1371/journal.pone.0036308

Increased bilirubin values

A rise in the level of bilirubin in the blood is called hyperbilirubinaemia. The main causes are as follows:

- Overproduction of bilirubin caused by an excessive breakdown of red cells (haemolytic jaundice). The bilirubin is of the unconjugated type. Haemolysis is due mainly to:
 - Severe malaria (*falciparum*, *vivax*, *knowlesi*).
 - Sickle cell disease haemolytic crisis.
 - Haemolysis associated with glucose-6-phosphate dehydrogenase deficiency and hereditary spherocytosis.
 - Sepsis
 - Antigen antibody reactions as in haemolytic disease of the newborn, autoimmune haemolytic anaemias, or following an incompatible blood transfusion.
 - Toxins from bacteria, snake venom, drugs, or herbal remedies.
- Liver cell damage in which there is usually an increase in both conjugated and unconjugated bilirubin (hepatocellular jaundice). The commonest causes are:
 - Hepatitis caused by hepatitis viruses and other viruses
 - Leptospirosis
 - Relapsing fever
 - Brucellosis

- Q fever
- Typhoid
- Chemicals, plant toxins e.g. aflatoxin and drugs e.g. hepatotoxic antiretroviral drugs, aspirin, paracetamol, some cardiac drugs.
- Metabolic disturbances in the liver involving defective conjugation, transport and, or, excretion of bilirubin. Examples include:
 - Type of neonatal jaundice, often referred to as ‘physiological jaundice’ (see following text).
 - Rare inherited disorders of conjugation such as Gilbert’s and Crigler-Najjar syndromes.
- Partial or complete stopping of the flow of bile through bile channels with a build up of conjugated bilirubin in the blood (jaundice). Cholestasis can be due to:
 - Obstruction of the extra-hepatic biliary ducts by gallstones, tumours (especially hepatomas and carcinoma of the pancreas), cholangitis (inflammation of the biliary ducts), or by helminths such as *Opisthorchis* and *Fasciola* species. Occasionally heavy *Ascaris* infections, especially in children, may result in blockage of the common bile duct.
 - Pressure on the small bile ducts as may occur in hepatitis or as a side effect of drugs.

Note: Mild to moderate hyperbilirubinaemia may also be found in association with any serious condition such as a terminal illness, or following major trauma, surgery, or blood transfusion.

Urine bilirubin and urobilinogen test results

	Bilirubin	Urobilinogen
Prehepatic jaundice	–	+
Hepatic jaundice	+ or trace	+
Posthepatic jaundice	+	–

Neonatal jaundice

‘Physiological’ jaundice may develop in a newborn baby if the conjugation mechanism of the infant is not fully developed. The jaundice begins to appear on the second day after birth and is of longer duration in premature infants.

Other common causes of neonatal jaundice in tropical countries are as follows:

- Glucose-6-phosphate dehydrogenase deficiency, especially in Papua New Guinea, Malaysia, Singapore and other parts of South-East Asia. Jaundice develops soon after birth.
- Infections, particularly septicaemia, congenital

syphilis, toxoplasmosis, and viral infections. Jaundice usually develops 3–4 days after birth.

- ABO haemolytic disease of the newborn. Jaundice occurs usually within 24 hours of birth.

In neonatal jaundice the level of unconjugated bilirubin is important. Levels in excess of 340 $\mu\text{mol/L}$ (20 mg/dL) in the normal child may result in unconjugated bilirubin being deposited in the basal ganglion of the brain, a condition known as kernicterus. This may cause permanent brain damage and therefore it is important to monitor unconjugated bilirubin levels. Even at levels lower than 340 $\mu\text{mol/L}$, the premature child is especially at risk as is the child with acidosis or low serum albumin levels.

A transcutaneous bilirubinometer can be used by ward staff on neonatal units to estimate and monitor hyperbilirubinaemia in jaundiced newborn infants. The device measures yellowness of subcutaneous tissue using a non-invasive procedure. It helps to identify infants at risk requiring further investigations including accurate measurement of bilirubin.

Neonatal bilirubin analyzers

Total bilirubin in jaundiced newborn infants can be measured accurately using a neonatal bilirubin analyzer. Recently developed analyzers measure bilirubin rapidly without the need for reagents, use small sample volumes and eliminate interference from haemolyzed and turbid samples by measuring at two wavelengths. Capillary blood is collected in a heparinized capillary, centrifuged, the capillary inserted in the analyzer and total bilirubin measured.

An example of a neonatal analyzer is *Easybil-P* manufactured by Micro Lab Instruments. It measures total bilirubin at wavelengths 460 nm and 500 nm over the range 0–680 $\mu\text{mol/L}$ (40 mg/dL). The analyzer has a built-in diagnostic check. Test results



Plate 3.44 *Easybil-P* neonatal bilirubin analyzer. Courtesy of Micro Lab Instruments. www.microlab.co.in

are displayed after 2 seconds and can be printed using the built-in printer. The analyzer can be connected to a computer through Wi-Fi.

A long-life LED is used as the light source. No prior warm up period is required. *Easybil-P* meter can also be connected to a 12V battery pack. Power consumption is 30W.

MEASURING ALT ACTIVITY

Analyte: Alanine aminotransferase (ALT) is an intracellular cytoplasmic enzyme with highest concentrations in the liver and kidney and lesser amounts in other body organs and tissues.

Purpose of test: ALT activity is measured to investigate liver cell damage caused by inflammation or necrosis, e.g. hepatitis, cirrhosis, overuse of alcohol and damage from hepatotoxic antiretroviral drugs. It is also used to monitor HBV and HCV chronic hepatitis.

ALT is often measured with aspartate aminotransferase (AST) activity. The level of both enzymes is increased in liver cell damage but ALT is the more specific liver enzyme and increased activity persists longer than AST activity. The activity ratio between ALT and AST is helpful in the investigation of fatty liver cell damage with an activity AST/ALT ratio of >2 indicating chronic alcohol overuse as the cause. Unlike raised AST activity that occurs with myocardial infarction, ALT activity is only slightly raised.

Sample: Haemolysis-free serum or plasma from a lithium heparinized sample can be used. If not tested on the day of collection, serum/plasma should be separated from the cells and the sample refrigerated at 2–8 °C. ALT activity is stable for 7 days. The volume required depends on the analyzer used.

Measuring ALT activity using a reagent test kit

Most semi-automatic analyzers measure ALT activity using an IFCC kinetic UV assay.

Principle of method: ALT catalyzes the reaction between L-alanine and 2-oxoglutarate. The pyruvate formed is reduced by nicotinamide adenine dinucleotide hydride (NADH) in a reaction catalyzed by lactate dehydrogenase (LDH) to form L-lactate and nicotinamide adenine dinucleotide (NAD⁺).

The rate of NADH oxidation is directly proportional to catalytic ALT activity which is obtained by measuring the decrease in absorbance at 340 nm.

The working reagent is stable for 21 days when stored at 2–8 °C. Most assays are linear up to at least 400 U/L.

Units: One international unit (IU) is the amount of enzyme that transforms 1 μmol of substrate per minute in standard conditions. Concentration is expressed in units per litre of sample (U/L).

Quality control

Care must be taken to follow the procedure exactly, particularly incubation temperature. A control serum must be run with each batch of tests. Control sera covering normal and pathological values are obtainable from manufacturers.

Haemolyzed samples should not be assayed. Highly lipaemic samples can interfere with the assay. Drugs such as deoxycycline hydrochloride, isoniazid, calcium dobesilate can cause artificially low test results. Furosemide can cause artificially high results. Refer to the manufacturer's literature for details of other drugs that may affect test results.

Reference interval for ALT activity

The following is a reference interval for ALT activity based on the kinetic UV IFCC assay method:

Men*: < 45 U/L Women*: < 34 U/L

*A non-gender value of <50 U/L is used by some laboratories.

Newborns: Up to 3 months, the reference interval for ALT activity in newborns can be significantly higher.

Raised levels of ALT activity

Causes include:

- Acute and chronic hepatitis, particularly HBV and HCV hepatitis.
- Toxic liver damage caused by alcohol excess, chemical toxins (e.g. lead poisoning), hepatotoxic antiretroviral drugs and other drugs. Paracetamol overdose causes greatly increased ALT activity levels.
- Acute kidney injury and diseases of skeletal muscle.

MEASURING ALP ACTIVITY

Analyte: Alkaline phosphatase (ALP) consists of a group of tissue-specific isoenzymes that hydrolyze phosphate esters at alkaline pH. ALP is present in cells throughout the body. Most plasma ALP activity originates from liver and bone cells and during pregnancy, also from placental ALP.

Purpose of test: Measurement of ALP activity is used to investigate hepatobiliary disease particularly

cholestasis and to diagnose bone diseases particularly osteomalacia (softening of bones commonly due to deficiency of vitamin D or calcium).

Sample: Haemolysis-free serum or plasma from a lithium heparinized sample. (An EDTA sample must not be used.) Separate the plasma or serum as soon as possible after collection and test on the day of collection. ALP activity is stable in serum or plasma at 2–8 °C for 7 days. The volume required depends on the analyzer used.

Measuring ALP using a reagent test kit

Most semi-automatic analyzers measure ALP activity using a kinetic *p*-nitrophenylphosphate (*p*-NPP) IFCC method.

Principle of method: In the presence of magnesium and zinc ions and 2-amino-2-methyl-1-propanol (AMP), ALP catalyzes the hydrolysis of *p*-NPP to give *p*-nitrophenol and inorganic phosphate. The *p*-nitrophenol released is directly proportional to ALP activity and can be obtained by measuring the rate of absorbance increase at 404 nm.

The *p*-NPP substrate is light sensitive and must be protected from direct light. The working reagent (AMP buffer and substrate) is stable for 21 days when stored at 2–8 °C. Most assays are linear up to 1400 U/L.

Units: One international unit (IU) is the amount of enzyme that transforms 1 μmol of substrate per minute, in standard conditions. The concentration is expressed in units per litre of sample (U/L).

Quality control

Haemolyzed samples should not be assayed due to the high concentration of ALP in red cells. A quality control serum should be run with each batch of tests (available from the test kit manufacturer). The assay procedure must be followed exactly, particularly the incubation temperature.

Reference interval for ALP activity

The following is a reference interval for ALP activity based on the IFCC method:

Men*: 40–129 U/L Women*: 35–104 U/L
Children: Up to 350 U/L

*A non-gender value of 30–130 U/L is used by some laboratories.

Note: Reference intervals should be established locally.

Increases in ALP activity

Causes include:

- Liver disease particularly cholestasis and also hepatitis, and liver cell damage caused by alcohol overuse, drugs and toxins.

- Bone disorders including osteomalacia, rickets, Paget's disease and metastatic carcinoma.
- Hodgkin's disease, congestive heart failure and intestinal disorders including ulcerative colitis.

MEASURING SERUM ALBUMIN

Analyte: Albumin is produced entirely in the liver and constitutes about 60% of total serum protein. It is important in regulating the flow of water between the plasma and tissue fluid by its effect on plasma colloid osmotic pressure. When albumin is significantly reduced, the plasma osmotic pressure is insufficient to draw water from the tissue spaces back into the plasma. This leads to a build-up of fluid in the tissues, referred to as oedema.

Albumin also has important binding and transport functions. It binds substances including calcium, bilirubin, fatty acids, urate, hormones, and magnesium, and also drugs such as penicillin, salicylates, sulphonamides, and barbiturates. Albumin diffuses easily through damaged membranes and is filtered out by the kidneys more than most globulins because its molecules are smaller.

Purpose of test: Serum albumin is mainly measured to investigate liver disease, protein energy malnutrition, disorders of water balance, nephrotic syndrome, and protein-losing gastrointestinal diseases.

Sample: Haemolysis free serum is required, collected with the minimum of venous stasis. Serum levels can be up to 3g/L lower when a patient is lying down. The volume of serum will depend on the analyzer used. Separate the serum and test on the day of collection. Albumin is stable for 6 days at 20 °C, 4 weeks at 4–8 °C and 1 year at –20 °C.

Measuring albumin using a reagent test kit

Most analyzers measure serum albumin by a dye-binding method using the indicator bromocresol green (BCG) or bromocresol purple (BCP).

Principle of method: The indicator reagent binds to albumin producing a colour change which is measured at 630 nm for BCG and 600 nm for BCP. The BCP reagent changes colour at a pH of 5.2–6.8, slightly higher than the BCG reagent (changes colour at pH 3.8–5.4). This reduces interferences in the assay, making the BCP method slightly more specific. The BCG assay is more affected by acute phase proteins.

The colour is stable for 1 hour and the assay is linear up to at least 60 g/L. Both dye-binding methods underestimate albumin levels at low concentration, i.e. below 25 g/L.

Quality control

A control serum must be run with each batch of tests and whenever a new test kit is opened. Most manufacturers provide control sera covering normal and pathological ranges. The indicator reagent and albumin calibrator are stable until the expiry date when stored tightly closed, protected from light and contamination and stored at 2–8 °C.

Haemolysis affects test results. The assay is not affected by bilirubin up to 500 µmol/L and triglycerides up to 11 mmol/L. Some drugs interfere with test reactions (see manufacturer's literature).

Reference interval for serum albumin

30–45 g/L for ambulatory adults

26–36 g/L for newborns, rising to adult values by 4 y

Note: Reference intervals should be established locally.

Albumin reference intervals for some tropical countries

COUNTRY	g/L
Ghana, adult	33–50 ^a
Kenya, adult	36–48 ^a
India, men, <40 y	40–52 ^b
women, . <40 y	37–49 ^b

References: ^a **Dosoo D K et al.** Haematological and biochemical reference values for healthy adults in the middle belt of Ghana. 2012, *PLoS ONE* 7(4): e36308. Doi:10.1371/journal.pone.0036308. Publication also covers other countries.

^b **Sairam S et al.** Haematological and biochemical parameters in apparently healthy Indian population: Defining reference intervals. July 2014, *Indian J Clinical Biochemistry*. Doi: 10.1007/s.12291-013-0365-5

Increased serum albumin

Serum levels are rarely raised except in diarrhoea or prolonged vomiting.

Decreased serum albumin

Hypoalbuminaemia occurs whenever there is increased plasma volume (e.g. in pregnancy). Pathological causes include:

- Low protein intake as in protein energy malnutrition.
- Malabsorption as in chronic pancreatitis, coeliac disease, and sprue.
- Loss of albumin in urine as in nephrotic syndrome, from the skin following severe burns, or from the bowel as in ulcerative colitis and other forms of protein-losing gastroenteropathy.

- Liver disease associated with reduction in the production of albumin (total protein levels may be within the reference interval because globulin levels rise). Several parasitic infections can also cause a reduction in albumin production.
- Increase in the body's need for protein, e.g. as a result of infection, malignant disease, following surgery or serious tissue damage when protein is required for energy and repair.
- In HIV disease, hypoalbuminaemia is common and may be due to several of the above causes, e.g. malnutrition, malabsorption, high protein demands.

FURTHER READING

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Cainelli F. Liver disease in developing countries. *World J Hepatol*, 2012 Mar 27;4(3): 66–67. doi: 10.4254/wjh.v4.i3.66

Debes J D et al. Knowledge of hepatitis B transmission risks among health workers in Tanzania. *Am J Trop Med Hyg*, 2016 May 4;94(5): 1100–1102. doi 10.4269/ajtmh.15-0797

Mukherjee P S et al. Etiology and mode of presentation of chronic liver disease in India: A multi-centric study. *PLoS ONE*, 2017 129100:e0187033 <https://doi.org/10.1371/journal.pone.0187033>

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Appendix I

**Preparation of
stains and reagents**

Acetone-alcohol decolorizer**No. 1***To make 1 litre:*

Acetone.....	500 mL
Ethanol or methanol, absolute.....	475 mL
Distilled water	25 mL

- Mix the distilled water with the absolute ethanol (ethyl alcohol) or methanol (methyl alcohol). * Transfer the solution to a screw-cap bottle of 1 litre capacity.

*Technical grade is adequate.

Caution: Ethanol and methanol are highly flammable, therefore use well away from an open flame.

- Measure the acetone, and add immediately to the alcohol solution. Mix well.

Caution: Acetone is a highly flammable chemical that vapourizes rapidly, therefore use it well away from an open flame.

- Initial, date and label the bottle, and mark it *Highly Flammable*. Store in a safe place at room temperature. The reagent is stable indefinitely.

For use: Transfer a small amount of the reagent to a dispensing container that can be closed when not in use.

Acid alcohol, 0.5% v/v**No. 2***To make 1 litre:*

Ethanol or methanol, absolute.....	995 mL
Hydrochloric acid, concentrated	5 mL

- Fill a 1 litre cylinder to the 995 mL mark with the ethanol or methanol.

Caution: Ethanol and methanol are highly flammable. Handle well away from an open flame.

- Slowly add 5 mL of concentrated hydrochloric acid.

Caution: Concentrated hydrochloric acid is a corrosive chemical with an injurious vapour. Handle it with care in a well-ventilated room.

- Transfer the solution to a reagent bottle and mix well.
- Initial, date and label the bottle and mark it *Flammable*. Store at room temperature in a safe place. The reagent is stable indefinitely.

For use: Transfer a small volume of the reagent to a dispensing container that can be closed when not in use.

Acid alcohol, 1% v/v**No. 3**

This is a 1% v/v hydrochloric acid solution in 70% v/v alcohol.

Prepare from the 3% v/v solution by mixing 10 mL of Reagent No. 4 with 20 mL of water, or make as follows;

To make 1 litre:

Ethanol or methanol, absolute.....	693 mL
Distilled water	297 mL
Hydrochloric acid, concentrated	10 mL

- Fill a 1 litre cylinder to the 693 mL mark with absolute ethanol (ethyl alcohol) or methanol (methyl alcohol). * Add water to the 990 mL mark.

*Technical grade is adequate.

Caution: Ethanol and methanol are highly flammable, therefore use these chemicals well away from an open flame.

- Add 10 mL of concentrated hydrochloric acid, i.e. to the 1 litre mark.

Caution: Concentrated hydrochloric acid is a corrosive chemical with an injurious vapour, therefore handle it with great care in a well ventilated room.

- Transfer the solution to a screw-cap bottle, and mix well.
- Initial, date and label the bottle and mark it *Flammable*. Store at room temperature in a safe place. The reagent is stable indefinitely.

For use: Transfer a small amount of the reagent to a dispensing container that can be closed when not in use.

Acid alcohol, 3% v/v**No. 4**

This is a 3% hydrochloric acid solution in 70% alcohol.

To make 1 litre:

Ethanol or methanol, absolute.....	680 mL
Distilled water	290 mL
Hydrochloric acid, concentrated	30 mL

- Measure the ethanol or methanol and transfer to a reagent bottle.

Caution: Ethanol and methanol are highly flammable. Handle well away from an open flame.

- Measure the water, add to the bottle and mix.

- Slowly add 30 mL of concentrated hydrochloric acid and mix well.

Caution: Concentrated hydrochloric acid is a corrosive chemical with an injurious vapour. Handle it with care in a well-ventilated room.

- Initial, date and label the bottle and mark it *Flammable*. Store at room temperature in a safe place. The reagent is stable indefinitely.

For use: Transfer a volume of the reagent to a dispensing container that can be closed when not in use.

Alkaline peptone water **No. 5**
To make about 50 bottles:

Peptone 5 g
 Sodium chloride 5 g
 Distilled water 500 mL

- Dissolve the peptone and sodium chloride in the water.
- Adjust the reaction of the medium to pH 8.6–9.0 using 1 mol/L sodium hydroxide.
- Dispense the medium in 10 mL amounts in screw-cap bottles.
- Sterilize by autoclaving (with caps loosened) at 121 °C for 15 minutes. Tighten the bottle caps after the medium has cooled.
- Date the medium and give it a batch number. Initial and label the bottles and record on each the expiry date of the medium (2 years from preparation).
- Store in a cool dark place with the bottle caps screwed tightly to prevent a change in pH.

Shelf-life: Up to 2 years providing there is no change in the volume or appearance of the medium to suggest contamination.

pH of medium: This should be within the range pH 8.6–9.0 at room temperature.

Inoculation

For use as an enrichment medium: Inoculate with 3–4 loopfuls of faecal sample and incubate at 35–37 °C for 5–8 hours. Subculture several loopfuls (from the surface) on to a plate of alkaline (pH 8.6) nutrient agar or preferably TCBS agar.

For use as a transport medium: Inoculate with about 1 mL of fluid sample or with a rectal swab. The inoculated medium should reach the laboratory within 10 hours (*Proteus* species will grow eventually in alkaline peptone water).

Amies transport medium **No. 6**

This medium is best prepared from ready to use dehydrated powder, available from most suppliers of culture media.

Contents: Charcoal, sodium chloride, phosphate buffer, potassium chloride, sodium thioglycollate, calcium chloride, magnesium chloride, agar.

The medium is usually used at a concentration of 2 grams in every 100 mL distilled water (concentration may vary depending on manufacturer).

- Prepare as instructed by the manufacturer. Dispense the well-mixed medium in screw-cap Bijou bottles, filling each container. Sterilize by autoclaving (with bottle caps loosened) at 121 °C for 15 minutes.
- When the medium has cooled, tighten the bottle caps. During cooling, invert the bottles to ensure an even distribution of the charcoal.
- Date the medium and give it a batch number. Initial and label the bottles and record on each the expiring date of the medium (9 months from preparation).
- Store the medium in a cool place away from direct light with the bottle tops tightly screwed.
Shelf-life: Up to 9 months providing there is no change in the *volume or appearance* of the medium to suggest contamination, or an alteration of its pH.

pH of medium: This should be within the range pH 7.1–7.3 at room temperature.

Inoculation

Clinic and ward staff should be advised to check the expiry date before using the medium. Collect the sample on a sterile cotton wool swab and immerse it in the medium, cutting off the swab stick to allow the bottle top to be replaced tightly. Protect the swab from direct light and heat. It should reach the laboratory within 24 hours.

Ammonium chloride, 0.87% w/v **No. 7**
To make 1 litre:

Ammonium chloride 8.7 g
 Distilled water to 1 litre

- Weigh the chemical and transfer it to a 1 litre capacity container, previously marked to hold 1 litre.

Caution: Ammonium chloride is a harmful chemical. Do not ingest or inhale the chemical. Wear eye protection.

- Add about half the volume of water and mix to dissolve the chemical. Make up to the 1 litre mark and mix.
- Initial, date and label the bottle and store at room temperature. Renew if the reagent becomes cloudy.

Auramine 1g/L **No. 8**
(0.1%)

A working 0.1% auramine reagent is best prepared from stock auramine and phenol reagents.

Stock auramine 1% reagent

To make 1 litre:

Auramine 0.....10 g
Ethanol or methanol.....1 000 mL

- Weigh the auramine and transfer to a brown bottle.
- Add about half the volume of alcohol and mix until the auramine is completely dissolved.

Caution: Ethanol and methanol are highly flammable. Handle well away from an open flame.

- Add the remainder of alcohol and mix well.
- Initial, date and label the bottle "Stock 1% auramine". Store in the dark at room temperature. The reagent is stable for up to 12 months.

Stock phenol 3% reagent

To make 1 litre:

Phenol crystals.....30 g
Distilled water.....1 000 mL

- Weigh the phenol in a beaker.
Caution: Phenol is a highly corrosive, toxic hygroscopic chemical. Handle it with *great care*. Avoid spilling any of the corrosive chemical on the balance pan.
- Transfer to a reagent bottle and add the water. Mix well until the phenol is completely dissolved.
- Initial, date and label "Stock 3% phenol". Store at room temperature. The reagent is stable for up to 12 months.

Working 0.1% auramine reagent

To make 500 mL:

Stock auramine 1% reagent.....25 mL
Stock phenol 3% reagent.....225 mL

- Transfer 25 mL of the stock auramine reagent to a brown bottle.
- Add 225 mL of the stock phenol reagent and mix well.
- Initial, date and label the bottle "Working 0.1% auramine". Store in the dark at room temperature. The working reagent can be used for up to 2 months.

For use: Filter a volume of the working reagent into a dispensing container that can be closed when not in use.

Buffered saline, pH 7.2

No. 9

Sodium chloride.....8.5 g
di-Sodium hydrogen phosphate.....1.91 g
Potassium *di*-hydrogen phosphate.....0.38 g
Distilled water.....up to 1 litre

- Weigh the chemicals and transfer to a 1 litre volumetric flask.
- Half fill the flask with distilled water and mix to dissolve the chemicals.
- Make up to the 1 litre mark with distilled water and mix well. Check the pH is 7.2.
- Transfer to a reagent bottle. Initial, date and label the bottle. Store at room temperature. The reagent is stable for several months.

**Buffered water pH 6.4, pH 6.8,
pH 7.0, pH 7.2, pH 8.0**

No. 10

These reagents can be conveniently prepared from stock phosphate buffer solutions.

Stock phosphate solution A

Sodium dihydrogen phosphate,.....27.6 g
1-hydrate (NaH₂PO₄·H₂O)
Distilled water.....to 1 litre

- Accurately weigh the chemical and transfer it to a 1 litre volumetric flask.
- Half fill the flask with water, and mix to dissolve the chemical. Make up to the 1 litre mark with distilled water, and mix well. Transfer to a leak-proof bottle.
- Initial, date and label the bottle 'Stock phosphate solution A'. Store in a cool place or

preferably at 2–8 °C. The solution is stable for several months.

Stock phosphate solution B

di-Sodium hydrogen phosphate, 28.39 g anhydrous (Na₂HPO₄)
Distilled water to 1 litre

Prepare as described above for solution A. Initial, date and label the bottle 'Stock phosphate solution B'. Store in a cool place or preferably at 2–8 °C. The solution is stable for several months.

Prepare 1 litre pH 6.4, pH 6.8, pH 7.0, pH 7.2, pH 8.0 as follows:

Measure the stock solutions and mix with the distilled water. Check the pH using narrow range pH papers or a pH meter. Store in a leak proof container in a cool place.

pH	A Stock mL	B Stock mL	Distilled Water mL
6.4	367.5	132.5	500
6.8	255	245	500
7.0	195	305	500
7.2	140	360	500
8.0	26.5	473.5	500

Alternative preparation of buffered water reagents using Na₂HPO₄ and KH₂PO₄ chemicals

■ *To make 1 litre pH 6.8 buffered water:*
di-Sodium hydrogen phosphate. 0.47 g anhydrous (Na₂HPO₄)
Potassium *di*-hydrogen phosphate. 0.46 g anhydrous (KH₂PO₄)
Distilled water to 1 litre
Weigh the chemicals and dissolve completely in the water. Check the pH. Store in a leak proof labelled container in a cool place.

■ *To make 1 litre pH 7.0 buffered water:*
di-Sodium hydrogen phosphate. 0.58 g anhydrous (Na₂HPO₄)
Potassium *di*-hydrogen phosphate. 0.35 g anhydrous (KH₂PO₄)
Distilled water to 1 litre
Weigh the chemicals and dissolve completely in the water. Check the pH. Store in a leak proof labelled container in a cool place.

Burrow's stain

No. 11

To make 100 mL:

Thionin 0.02 g
Ethanol, absolute (absolute alcohol) 3 mL
Acetic acid, glacial 3 mL
Distilled water 94 mL

1 Weigh the thionin* and transfer it to a leak-proof bottle of 100 mL capacity.

* If an accurate balance is not available to weigh the 0.02 g (20 mg) of thionin, transfer a small amount of the powdered stain to a small tube and dip the end of a wet swab stick in the powder. This will give approximately 20 mg.

2 Measure the ethanol and acetic acid and add these to the bottle. Mix until the thionin is completely dissolved.

Caution: Ethanol is highly flammable and glacial acetic acid is corrosive, flammable, and has an irritating vapour, therefore handle these chemicals with care, well away from any open flame, in a well ventilated room.

3 Add the water and mix well.

4 Initial, date and label the bottle and store preferably at 2–8 °C. Renew every 3 months.

Carbol fuchsin, 10g/L

No. 12

(1%)

To make 1 litre:

Basic fuchsin. 10 g
Ethanol or methanol, absolute. 100 mL
Phenol 50 g
Distilled water 900 mL

1 Weigh the basic fuchsin and transfer it to a reagent bottle (of at least 1.5 litre capacity).

2 Add the ethanol or methanol and mix until the basic fuchsin is completely dissolved.

Caution: Ethanol and methanol are highly flammable. Handle well away from an open flame.

3 Weigh the phenol in a large beaker. Add some of the water and stir to dissolve the chemical. Transfer to the bottle of stain and mix well.

Caution: Phenol is a highly corrosive toxic, hygroscopic chemical. Handle it with *great care*. Avoid spilling any of the chemical on the balance pan.

4 Add the remainder of the water and mix well.

5 Initial, date and label the bottle. Store at room temperature. The stain is stable indefinitely.

For use: Filter a volume of the stain into a dispensing container that can be closed when not in use.

Cary-Blair transport medium **No. 13**

This medium is best prepared from ready to use dehydrated powder available from most suppliers of culture media.

Contents: Sodium thioglycollate, *di*-sodium hydrogen phosphate, sodium chloride, agar, calcium chloride.

The medium is usually used at a concentration of 1.3 g in every 100 mL distilled water (concentration may vary depending on manufacturer).

- 1 Prepare as instructed by the manufacturer. Dispense the medium in 7 mL amounts in screw-cap bottles of 9 mL capacity (large size Bijou bottles).
- 2 Sterilize by steaming (with caps loosened) for 15 minutes. When cool, tighten the bottle caps.
- 3 Initial, date and label the medium and give it a batch number. Record the expiry date (6 months from preparation) on each bottle.
- 4 Store in a cool dark place with the bottle tops screwed tightly.

Shelf-life: Up to about 18 months providing there is no change in the appearance of the medium to suggest contamination or alteration of pH.

pH of medium: This should be within the range pH 8.3–8.5 at room temperature.

Inoculation

Immerse a swab of the faecal sample in the medium, cutting off the swab stick to allow the bottle top to be replaced tightly. Protect the swab from direct light and excessive heat.

Salmonella, Shigella, Vibrio, and Y. enterocolitica survive well in Cary-Blair medium for at least 48 hours (several days for *Salmonella, Shigella, Vibrio* species) and *Campylobacter* species for up to 6 hours. It is also a good transport medium for *Y. pestis*.

Citric acid-Tween solution **No. 14**

To make 1 litre:

Citric acid	12.91 g
<i>di</i> -Sodium hydrogen phosphate	27.61 g
hydrated (Na ₂ HPO ₄ ·12H ₂ O)	
Tween 80	50 mL
Merthiolate	0.1 g
Distilled water	to 1 litre

- 1 Weigh the citric acid and *di*-sodium hydrogen phosphate and transfer these to a 1 litre volumetric flask.
- 2 Half fill the flask with warm water and mix until the chemicals are completely dissolved.
- 3 Add 50 mL of Tween 80 and make up to the 1 litre mark with water.
- 4 Transfer to a leak-proof bottle. Add 0.1 g merthiolate (preservative) and mix gently but well.
- 5 Initial, date and label the bottle and store at room temperature. The solution is stable for several months.

Crystal violet Gram stain **No. 15**

To make 1 litre:

Crystal violet	20 g
Ammonium oxalate	9 g
Ethanol or methanol, absolute	95 mL
Distilled water	to 1 litre

- 1 Weigh the crystal violet on a piece of clean paper (preweighed). Transfer to a brown bottle premarked to hold 1 litre.
- 2 Add the absolute ethanol or methanol (technical grade is suitable) and mix until the dye is completely dissolved.

Caution: Ethanol and methanol are highly flammable, therefore use these chemicals well away from an open flame.

- 3 Weigh the ammonium oxalate and dissolve in about 200 mL of distilled water. Add to the stain. Make up to the 1 litre mark with distilled water, and mix well.

Caution: Ammonium oxalate is a toxic chemical, therefore handle it with care.

- 4 Initial, date and label the bottle, and store it at room temperature. The stain is stable for several months.

For use: Filter a small amount of the stain into a dropper bottle or other stain dispensing container.

Dobell's iodine for faecal preparations No. 16

To make about 100 mL:

Potassium iodide	4 g
Iodine	2 g
Distilled water	100 mL

- 1 Weigh the potassium iodide and dissolve *completely* in about 50 mL of the water.
- 2 Weigh the iodine and add to the potassium iodide solution. Mix well to dissolve.

Caution: Iodine is injurious to health if inhaled or allowed to come in contact with the eyes, therefore handle with care in a well ventilated room.

- 3 Add the remainder of the water, and mix. Transfer to a brown bottle.
- 4 Initial, date and label the bottle, and mark it *Toxic* and *Corrosive*. Store in the dark at room temperature. The reagent is stable for several months.

For use: Transfer to a small brown bottle with a cap into which a dropper can be inserted.

EDTA (sequestrene) anticoagulant No. 17

Obtain ready-prepared anticoagulated bottles, or prepare as follows:

Di-potassium ethylene-	2.5 g
diamine-tetra-acetic acid	
Distilled water	25 mL

- 1 Weigh the chemical, and transfer it to a small glass bottle.
- 2 Measure 25 mL of water, add to the chemical, and mix to dissolve. Initial, date and label the bottle.
- 3 For use, pipette 0.04 mL of the reagent into small bottles marked to hold 2.5 mL of blood.
- 4 Place the small bottles without tops, on a warm bench for the anticoagulant to dry. Protect from dust and flies.
- 5 When dry, replace the bottle tops, and store ready for use.

Eosin stain, 5 g/L (0.5% w/v) No. 18

To make about 100 mL:

Eosin powder	0.5 g
Distilled water	100 mL

- 1 Weigh the eosin on a clean piece of paper (pre-weighed), and transfer the powder to a leak-proof, brown bottle of 100 mL capacity.
- 2 Add 100 mL of water, and mix to dissolve the stain.
- 3 Initial, date and label the bottle, and store it at room temperature. The stain is stable indefinitely.

For use: Transfer a small amount of the stain to a bottle with a cap into which a dropper can be inserted.

Field stain A No. 19

To make 500 mL:

Field stain A powder*	10 g
Distilled water (hot)	500 mL

* Obtain from HD Supplies, Merck/BDH Chemicals, or other reliable supplier.

- 1 Weigh the powder on a piece of clean paper (pre-weighed), and transfer it to a large Pyrex beaker or high density polyethylene reagent bottle.
- 2 Measure the water and heat to boiling.
- 3 Add the hot water to the stain and mix to dissolve the powder.
- 4 When cool, filter the stain into a storage bottle. Add 0.5 g sodium azide and mix.
- 5 Initial, date and label the bottle and store it at room temperature. The stain is stable indefinitely.

Field stain B No. 20

To make 500 mL:

Field stain powder B*	10 g
Distilled water (hot)	500 mL

* Obtain from HD Supplies, Merck/BDH Chemicals, or other reliable supplier.

- 1 Weigh the powder on a piece of clean paper (pre-weighed), and transfer it to a large Pyrex beaker or high density polyethylene reagent bottle.
- 2 Measure the water and heat to boiling.
- 3 Add the hot water to the stain and mix to dissolve the powder.
- 4 When cool, filter the stain into a storage bottle. Add 0.5 g sodium azide and mix.
- 5 Initial, date and label the bottle and store it at room temperature. The stain is stable indefinitely.

Formol saline, 10% v/v **No. 21**

To make 500 mL:

Physiological saline 450 mL
(Reagent No. 34)

Formaldehyde solution, concentrated 50 mL

- 1 Measure the physiological saline and transfer it to a leak-proof bottle.
- 2 Measure the formaldehyde solution and add to the saline. Mix well.

Caution: Concentrated formaldehyde solution is a toxic chemical with a vapour that is irritating to the eyes and mucous membranes, therefore handle it with great care in a well ventilated room.

- 3 Initial, date and label the bottle and store at room temperature in a safe place. The reagent is stable indefinitely.

Giemsa stain **No. 22**

Purchase ready-made or prepare using the following formula.

To make about 500 mL:

Giemsa powder 3.8 g

Glycerol (glycerine), Laboratory 250 mL
quality

Methanol (methyl alcohol) 250 mL

- 1 Weigh the Giemsa on a piece of clean paper (preweighed), and transfer to a *dry* brown bottle of 500 mL capacity which contains a few glass beads.

Note: Giemsa stain will be spoilt if water enters the stock solution during its preparation or storage.

- 2 Using a *dry* cylinder, measure the methanol, and add to the stain. Mix well.

Caution: Methanol is toxic and highly flammable, therefore handle it with care and use well away from an open flame.

- 3 Using the same cylinder, measure the glycerol, and add to the stain. Mix well.
- 4 Place the bottle of stain in a water bath at 50–60 °C, or if not available at 37 °C, for up to 2 hours to help the stain to dissolve. Mix well at intervals.
- 5 Initial, date and label the bottle, and mark it *Flammable and Toxic*. Store at room temperature in the dark. If kept well-stoppered, the stain is stable for several months.

For use: Filter a small amount of the stain into a dry stain dispensing container.

Iodine, Dobell's for faecal preparations **No. 23**

See Reagent No. 16

Lactophenol solution **No. 24**

To make about 110 mL:

Phenol 25 g

Lactic acid 25 mL

Glycerol 50 mL

Distilled water 25 mL

- 1 Rapidly weigh the phenol in a preweighed beaker.
- 2 Measure the water and add to the phenol. Mix to dissolve the phenol. Transfer to a leak-proof brown bottle.

Caution: Phenol is a highly corrosive, toxic, and hygroscopic chemical, therefore handle it with great care. To avoid damaging the balance pan, remove the beaker when adding or subtracting the chemical. Make sure the stock bottle of phenol is tightly stoppered after use.

- 3 Measure the lactic acid and glycerol and add to the bottle. Cap the bottle and mix well.
- 4 Initial, date and label the bottle and mark it *Toxic* and *Corrosive*. Store at room temperature in a safe place. The reagent is stable for many months.

Leishman stain **No. 25**

To make about 400 mL:

Leishman stain powder* 0.6 g

Methanol (methyl alcohol)** 400 mL

*Purchase from a reliable source, e.g. Merck/BDH.

**The methanol *must* be water-free.

- 1 Weigh the Leishman powder and transfer it to a completely *dry* brown bottle. Add a few dry glass beads (to assist in dissolving the dye).
- 2 Using a *dry* cylinder, measure the methanol and add this to the stain. Mix well at intervals until the dye is completely dissolved. Warming the solution in a 37 °C water bath will help the dye to dissolve.

Caution: Methanol is toxic and highly flammable, therefore handle it with care well away from an open flame.

- 3 Initial, date and label the bottle and mark it *Flammable* and *Toxic*. Store it at room temperature in the dark. When kept tightly stoppered, the stain is stable for several weeks. Moisture *must not* be allowed to enter the stain.

For use: Filter 50–100 mL of the stain into a stain dispensing container which can be closed when not in use.

Lugol's iodine solution**No. 26***To make 1 litre:*

Potassium iodide20 g
 Iodine10 g
 Distilled waterto 1 litre

- 1 Weigh the potassium iodide, and transfer to a brown bottle premarked to hold 1 litre.
- 2 Add about a quarter of the volume of water, and mix until the potassium iodide is *completely* dissolved.
- 3 Weigh the iodine, and add to the potassium iodide solution. Mix until the iodine is dissolved.

Caution: Iodine is injurious to health if inhaled or allowed to come in contact with the eyes, therefore handle it with care in a well ventilated room.

- 4 Make up to the 1 litre mark with distilled water, and mix well. Initial, date and label the bottle, and mark it *Toxic*. Store it in a dark place at room temperature. Renew the solution if its colour fades.

For use: Transfer a small amount of the reagent to a brown dispensing bottle.

Malachite green, 5 g/l (0.5% w/v)**No. 27***To make 1 litre:*

Malachite green5 g
 Distilled waterto 1 litre

- 1 Weigh the malachite green on a piece of clean paper (preweighed), and transfer it to a bottle of 1 litre capacity.
- 2 Measure the water, and add about a quarter of it to the bottle. Mix until the dye is completely dissolved.
- 3 Add the remainder of the water and mix well.
- 4 Initial, date and label the bottle and store it at room temperature. The stain is stable for several months.

For use: Transfer a small amount of the stain to a dropper bottle or other stain dispensing container.

Methylene blue-saline, 1% w/v**No. 28***To make 100 mL:*

Physiological saline (Reagent No. 34) ... 100 mL
 Methylene blue 1.0 g

- 1 Weigh the methylene blue and transfer it to a leak-proof bottle.
- 2 Measure the saline and add to the dye. Mix until the methylene blue is completely dissolved.
- 3 Initial, date and label the bottle and store it at room temperature. Discard the stain if it becomes contaminated.

For use: Filter a small amount of the stain into a dropper bottle.

Methylene blue, 1g/L**No. 29****(0.1%)***To make 1 litre:*

Methylene blue powder1 g
 Distilled water1 000 mL

- 1 Weigh the powder and transfer it to a brown bottle.
- 2 Add about half the water and mix until the powder is completely dissolved.
- 3 Add the remainder of the water and mix well,
- 4 Initial, date and label the bottle. Store in the dark at room temperature. The reagent is stable for up to 12 months.

For use: Filter a volume of the stain into a dispensing container that can be closed when not in use.

Methylene blue, 3 g/L**No. 30****(0.3%)***To make 1 litre:*

Methylene blue powder3 g
 Distilled water1 000 g

- 1 Weigh the powder and transfer to a brown bottle.
- 2 Add about half the water and mix well until the powder is completely dissolved.
- 3 Add the remainder of the water and mix well.
- 4 Initial, date and label the bottle. Store in the dark at room temperature. The reagent is stable for up to 12 months.

For use: Filter a volume of the stain into a dispensing container that can be closed when not in use.

Neutral red, 1 g/l (0.1% w/v) No. 31*To make 1 litre:*

Neutral red 1 g
 Distilled water 1 litre

- 1 Weigh the neutral red on a piece of clean paper (preweighed), and transfer it to a bottle of 1 litre capacity.
- 2 Add about a quarter of the volume of water, and mix until the dye is completely dissolved.
- 3 Add the remainder of the water, and mix well.
- 4 Initial, date and label the bottle and store at room temperature. The stain is stable for several months.

For use: Transfer a small amount of the stain to a dropper bottle or other stain dispensing container.

Nigrosin stain No. 32*To make about 120 mL:*

Acetic acid, glacial 10 mL
 Methanol, absolute 50 mL
 Nigrosin, saturated aqueous solution* 10 mL
 Distilled water 50 mL

* Prepare by dissolving sufficient water-soluble nigrosin in about 12 mL of warm distilled water until no more can be dissolved. Filter 10 mL.

- 1 Measure the water and transfer it to a leak-proof brown bottle.
- 2 Measure the acetic acid and methanol and add these to the water.

Caution: Glacial acetic acid is a corrosive and flammable chemical with an irritating vapour and methanol is highly flammable. Therefore handle these chemicals with care, well away from any open flame and in a well ventilated room.

- 3 Add the nigrosin stain and mix well. Initial, date and label the bottle and store it at room temperature. The reagent is stable for many months.

Phenol, saturated solution No. 33*To make about 30 mL:*

Phenol crystals 2 g
 Distilled water 30 mL

- 1 Weigh the phenol in a beaker (preweighed). Add the water, and stir to dissolve the chemical. Transfer to a leak-proof bottle and mix well.

Caution: Phenol is a highly corrosive, toxic, and hygroscopic chemical, therefore handle it with great care. To avoid damaging the balance pan, always remove the beaker when adding or subtracting the chemical. Make sure the stock bottle of phenol is tightly stoppered after use.

- 2 Initial, date and label the bottle, and mark it *Corrosive*. Store at room temperature in a safe place. The reagent is stable indefinitely.

Physiological saline, 8.5 g/L (0.85% w/v) No. 34*To make 1 litre:*

Sodium chloride 8.5 g
 Distilled water to 1 litre

- 1 Weigh the sodium chloride, and transfer it to a leak-proof bottle premarked to hold 1 litre.
- 2 Add distilled water to the 1 litre mark, and mix until the salt is fully dissolved.
- 3 Initial, date and label the bottle, and store it at room temperature. The reagent is stable for several months. Discard if it becomes contaminated.

Potassium permanganate, 5 g/L (0.5%) No. 35*To make 1 litre:*

Potassium permanganate 5 g
 Distilled water 1 000 g

- 1 Weigh the potassium permanganate and transfer to a brown bottle.
- 2 Add about half the water and mix to dissolve the chemical. Add the remainder of the water and mix well.
- 3 Initial, date and label the bottle. Store in the dark at room temperature. The reagent is stable for up to 12 months.

Note: The reagent should be bright purple. If it appears brick-red (oxidized), discard it and make a new reagent.

For use: Transfer a small volume of the reagent into a dispensing container that can be closed when not in use.

Saponin-saline solution**No. 36**

This is a 1% w/v solution of saponin in physiological saline.

To make about 500 mL:

Saponin (white, pure grade) 5 g
Physiological saline (Reagent No. 34) ... 500 mL

- 1 Weigh the saponin and transfer this to a leak-proof bottle.

Caution: Saponin powder is a harmful chemical, therefore handle it with great care. Do not inhale or ingest the powder.

- 2 Measure the saline and add about half of it to the bottle. Mix gently (by swirling) to dissolve the chemical (standing the bottle in hot water will help the saponin to dissolve).
- 3 Add the remainder of the saline and mix gently but well (avoid excess frothing).
- 4 Initial, date and label the bottle and store it preferably at 2–8 °C. The reagent is stable for several months.

For use: Transfer a small amount to a dropper bottle.

Sargeaunt stain**No. 37**

To make 100 mL:

Malachite green 0.2 g
Glacial acetic acid 3 mL
Ethanol, 95% v/v 3 mL
Distilled water 94 mL

- 1 Weigh the malachite green and transfer it to a container of 100 mL capacity.
- 2 Measure the ethanol and glacial acetic acid and add to the stain. Mix until the stain is fully dissolved.

Caution: The ethanol reagent is flammable, therefore use well away from any open flame. Glacial acetic acid is a corrosive chemical with an irritating vapour, therefore use in a well ventilated area.

- 3 Add the water and mix well. Initial, date and label the bottle and store at room temperature. The stain is stable indefinitely.

Sodium citrate anticoagulant, 38g/L**No. 38**

To make about 100 mL:

tri-Sodium citrate 3.8 g
Distilled water 100 mL

- 1 Weigh the sodium citrate and transfer it to a leak-proof bottle.
- 2 Add 100 mL of water and mix to dissolve the chemical.

- 3 Initial, date and label the bottle and store preferably at 2–8 °C. Renew every 6 weeks or sooner if it becomes contaminated (cloudy).

Sodium citrate anticoagulant, 32 g/l**No. 39**

To make 100 mL

tri-Sodium citrate, dihydrate. 3.2 g
Distilled water to 100 mL

- 1 Weigh the chemical and transfer it to a container pre-marked to hold 100 mL (or to a 100 mL volumetric flask).
- 2 Add about half the water and mix until the chemical is completely dissolved.
- 3 Add water to the 100 mL mark and mix well.
- 4 Initial, date and label the container and store it at 2–8 °C. Discard the reagent if it becomes contaminated (turbid appearance).

Sodium citrate, 6% w/v**No. 40**

To make about 100 mL:

tri-Sodium citrate 6.0 g
Distilled water 100 mL

Prepare as described for Reagent No. 38.

Sulphuric acid, 25% v/v**No. 41**

To make 1 litre:

Concentrated sulphuric acid.....250 mL
Distilled water.....750 mL

Note: Battery acid (20% sulphuric acid) which is available from most petrol stations, can also be used.

- 1 Measure the water and add to a reagent bottle.
- 2 With great care, measure the concentrated sulphuric acid and slowly add it to the water. Mix well.

Caution: Concentrated sulphuric acid is a highly corrosive chemical with a toxic vapour.

ALWAYS ADD THE ACID TO THE WATER.

If water is added to acid in a glass bottle, the acid will shatter the glass.

- 3 Initial, date and label the bottle and mark it *Corrosive*. Store at room temperature. The reagent is stable indefinitely.

For use: With care, transfer a volume of the reagent to a dispensing container that can be closed when not in use.

Stock phenol 3% reagent
(see Reagent No. 8)

No. 42

Toluidine blue diluting fluid

No. 43

*To make 100 mL:*Toluidine blue* 0.1 g
Sodium chloride, 8.5 g/L** 100 mL

*Available from Merck/BDH, order No. 34077 4Y (25 g).

**See Reagent No. 34.

- 1 Weigh the toluidine blue on a piece of clean paper (preweighed). Transfer it to a bottle of 100 mL capacity.
- 2 Add about half the saline solution, and mix to dissolve the dye. Add the remainder of the water, and mix well. Initial, date and label the bottle, and store it at 2–8 °C.

For use: Filter a small amount of the stain into a brown bottle with a cap into which a dropper can be inserted.

Trichloroacetic acid, 50 g/L (5% w/v)

No. 44

*To make 100 mL:*Trichloroacetic acid (TCA) 5 g
Distilled water to 100 mL

- 1 Weigh the TCA in a beaker (preweighed). Add about 30 mL of the water, and stir to dissolve the TCA.

Caution: TCA is a strongly corrosive and deliquescent chemical with an irritating vapour, therefore handle it with care in a well ventilated room. Make sure the stock bottle of chemical is tightly stoppered after use.

- 2 Transfer to a 100 mL volumetric flask. Make up to the mark with distilled water, and mix well.
- 3 Transfer to a leak-proof bottle. Initial, date and label, and mark it *Corrosive*. Store at room temperature. The reagent is stable indefinitely.

WBC diluting fluid

No. 45

*To make 100 mL:*Acetic acid, glacial 2 mL
Distilled water 98 mL
Gentian violet, 1% w/v* 2 mL

*Prepare by dissolving 0.1 g gentian violet in 10 mL distilled water and filter.

- 1 Fill a 100 mL cylinder to the 98 mL mark with distilled water.
- 2 Add 2 mL concentrated (glacial) acetic acid and mix.
Caution: Glacial acetic acid is a corrosive chemical with an irritating vapour, therefore handle it with care and in a well ventilated room. Do *not* mouth-pipette (use a pipette filler).
- 3 Add the gentian violet solution and mix. Transfer to a storage bottle and label, initial and date. Store in the dark at room temperature.

Wright's stain

No. 46

*To make about 400 mL:*Wright stain powder* 1.0 g
Methanol (methyl alcohol)** 400 mL

*Available from Merck/BDH, code 340804U.

**The methanol must be water-free.

- 1 Weigh the Wright powder and transfer it to a *dry* brown bottle. Add a few glass beads (to assist in dissolving the dye).
- 2 Using a *dry* cylinder, measure the methanol and add this to the stain. Mix well at intervals until the powder is completely dissolved. Warming the solution in a 37 °C water bath will help the dye to dissolve.
Caution: Methanol is toxic and highly flammable, therefore handle it with care well away from an open flame.
- 3 Initial, date and label the bottle and mark it *Flammable* and *Toxic*. Store it at room temperature in the dark. When kept tightly stoppered, the stain is stable for several weeks. Moisture must *not* be allowed to enter the stain.

For use: Allow 3–5 days before using freshly made stain to allow time for the stain to 'ripen'. Filter 50–100 mL of the stain into a stain dispensing container which can be closed when not in use.

Zinc sulphate solution, 33% w/v **No. 47**

To make approximately 500 mL:

Zinc sulphate 165 g
Distilled water 500 mL

- 1 Weigh the zinc sulphate and transfer it to a leak-proof bottle of about 1 litre capacity.
 - 2 Measure the water and add it to the chemical.
 - 3 Stopper the bottle, and mix well.
 - 4 Stand the bottle in a container of hot water to dissolve the zinc sulphate. Mix until the chemical is completely dissolved.
 - 5 Allow the solution to cool to room temperature.
 - 6 Using a hydrometer, check the relative density of the solution. If the density is not within 1.180–1.200, add more chemical or water to bring the solution within the correct density range.
 - 7 Initial, date and label the bottle and store at room temperature. When stored with the bottle top tightly stoppered, the solution is stable for several weeks. The relative density should be checked periodically.
-

FURTHER INFORMATION

The following information can be found in sub-unit 2.6 Part 1 DLPTC* Guidelines for preparing stains and chemical reagents.

* Can be accessed on-line by entering the title in Google and click on PDF entry <https://medicallabtechno.weebly.com>
Scroll left to locate subunit 2.6 and click to display text.

- Avoiding errors in preparing stains and reagents.
 - Preparing accurate solutions.
 - Calculating correctly when preparing mol/L solutions.
 - Formulas for converting a percentage solution to a mol/L solution and a normal solution into a mol/L solution.
 - Formula for diluting solutions and body fluids.
 - Properties of commonly used acids and bases.
 - Purchasing chemicals and reagents.
-

Appendix II

**Checklist for monitoring QMS
Training curriculum**

Suggested Checklist for Monitoring QMS in District Laboratories

The following basic Checklist can be used during a district laboratory supervisory support visit. It should reflect the work of the laboratory, staff skills and available resources of the laboratory being visited. A supervisory support visit gives the opportunity for laboratory staff to increase their knowledge, discuss problems, get help with SOPs and other quality issues and improve the quality of their service to patients.

The Checklist can be divided into the following sections:

- Laboratory staff
- Laboratory facilities and security
- Safety assessment
- Equipment
- Supplies
- Tests
- Quality assurance
- Communications with laboratory users
- Records

	YES	NO	COMMENTS
LABORATORY STAFF			
● Are staff numbers and their skills matched to healthcare needs and workload?			
● Has the competence of staff been assessed?			
● Is there a duty roster for staff?			
● Is the work of trainees and new members of staff supervised adequately?			
● Are working conditions safe and acceptable?			
● Are contracts of employment being met?			
● Are communications good between laboratory and clinical staff?			
● Is CPD provided?			
● Are there opportunities for career promotion and advancement?			
● OTHER			
LABORATORY FACILITIES AND SECURITY			
● Is the main testing area separate from sample reception and outpatient blood collection?			
● Is there a staff room separate from the working area with toilet and hand washing facilities nearby (separate from patient facilities)?			
● Is the water supply adequate and quality satisfactory?			
● Is the waste disposal system adequate and safe?			
● Are there power supply problems: <ul style="list-style-type: none"> – frequent power cuts? 			

	YES	NO	COMMENTS
– is the laboratory electric circuit overloaded?			
– are too many adaptors and extension leads being used?			
● Is lighting in the laboratory satisfactory?			
● Is ventilation adequate with airflow directed away from test areas?			
● Are work stations and equipment placements appropriate?			
● Is bench space and storage facilities adequate?			
● Is the laboratory clean and tidy?			
● Are benches and floor free from unnecessary items?			
● Are windows secure, sun blinds and wire mesh screens in good condition?			
● Are doors in good repair and door locks secure?			
● Is the laboratory in need of repair or repainting?			
● OTHER			
SAFETY ASSESSMENT			
● Is infectious and other laboratory waste handled safely:			
– “Sharps” placed in secure waste containers?			
– Waste containers not overfilled?			
– Disinfectants used correctly and changed daily?			
– TST control strips used when autoclaving?			
● Are there signs showing the banning of eating, drinking, smoking, or chewing gum in the laboratory?			
● Is the Biohazard symbol displayed on the laboratory door?			
● Is food and drink being stored in the laboratory refrigerator?			
● Are staff pipetting and dispensing samples and reagents safely?			
● Is hand washing satisfactory?			
● Is protective clothing worn, removed and left in the laboratory when leaving, and decontaminated before being laundered?			
● Are protective gloves being worn?			
● When required are eye goggles and a face visor being worn?			

	YES	NO	COMMENTS
● Is an eyewash bottle provided?			
● Is the First Aid kit complete and accessible?			
● Are staff trained in emergency First Aid?			
● Are flammable, toxic, harmful, corrosive and explosive chemicals, labelled, handled and stored correctly?			
● Are flammable chemicals kept well away from open flames?			
● Do staff know what to do in the event of a fire and are fire exit routes clearly marked?			
● Are fire drills performed regularly?			
● Does the laboratory have essential fire-fighting equipment:			
– buckets of sand or earth?			
– fire extinguisher (s)?			
– fire blanket?			
● Are all laboratory-related accidents documented in a <i>Laboratory Accident Book</i> ?			
● OTHER			
EQUIPMENT			
● Is there an equipment inventory?			
● Are major items of equipment in working order:			
– Microscope?			
– Analyzers?			
– Haemoglobin meter?			
– Centrifuge?			
– Incubator?			
– Water bath or heat block?			
– Water still and water filter?			
– Autoclave?			
– Balance?			
– Mixer?			
– Pipetting and dispensing equipment?			
– Refrigerator?			
– Incinerator?			
● Are SOPs available for all equipment?			

	YES	NO	COMMENTS
● Are maintenance and servicing documented?			
● Can equipment be repaired locally?			
● For automated equipment, is back-up manual equipment available?			
● Are voltage stabilisers fitted to electrical equipment?			
● Are essential equipment consumables in stock and easily available?			
● Has new equipment been installed correctly and calibrated?			
● Is new equipment validated before being put into routine use?			
● Are trainees and new members of staff trained how to use and maintain equipment correctly?			
● Is equipment inspected regularly for corrosion, rodent and insect damage?			
● Has non-functional obsolete equipment been removed from test areas and disposed of appropriately?			
● OTHER			
SUPPLIES			
● Are inventory records matching stock levels?			
● Are supplies:			
– overstocked?			
– understocked?			
● Is sufficient and correct information supplied when ordering supplies?			
● Are supplies ordered in good time?			
● Are there supply problems:			
– shortfalls?			
– incorrect supplies sent?			
– transported incorrectly?			
● Are supplies logged and checked upon arrival?			
● Are expiry dates recorded?			
● Is there more than one supplier for essential consumables?			
● Is the performance of new test kits checked?			
● Is expired stock disposed of correctly?			
● OTHER			

	YES	NO	COMMENTS
TESTS			
● Are tests meeting clinical and public health needs?			
● Are samples collected and labelled correctly?			
● Have sample collecting instructions been issued to clinical and nursing staff?			
● Is there a clear procedure for rejecting samples?			
● Are test reports verified before being issued?			
● Is helpful information provided with test reports?			
● When asked, are staff able to discuss test results with clinical staff?			
● Is there a procedure detailing the action to take when a test result is seriously abnormal or not matching clinical findings?			
● Are test kits stored and used correctly?			
● Are test turnaround times being met?			
● Are the dates of sample referrals and the return of test results recorded?			
● Are SOPs available and followed for all tests?			
Assessing analytical performance			
Technical performance can be assessed by the Supervisor observing how staff carry out and control tests, e.g sickle cell test, HIV test, measurement of haemoglobin, blood glucose and other analytes.			
Assessing microscopy			
The quality of microscopy can be assessed by the Supervisor:			
– Re-checking (validating) a selection of previously reported slides, e.g. blood films for malaria parasites, sputum smears for AFB, blood films for WBC and RBC morphology.			
– Observing how faecal samples are examined microscopically and reported.			
– Observing how urine samples are examined microscopically and reported.			
Assessing chemical urinalysis			
Chemical testing can be assessed by the Supervisor observing how urine reagent strip tests are used and stored.			
Assessing blood grouping and compatibility testing			
Blood transfusion techniques can be assessed by the Supervisor observing how staff carry out blood grouping and compatibility testing by emergency and standard slide and tube methods using appropriate controls.			
● OTHER			

	YES	NO	COMMENTS
QUALITY ASSURANCE			
INTERNAL QUALITY CONTROL			
● Are quality control sera and control charts used for quantitative chemical tests?			
● Are control slides used to control staining reactions?			
● Are control slides used to check microscopes?			
● Are internal audits used?			
● Is the temperature of the refrigerator, incubator, water bath checked daily and recorded?			
EQA (PROFICIENCY TESTING)			
● Are EQA samples tested and reported with the minimum of delay?			
● Are EQA samples tested in the same way as patient samples?			
● Are the recommendations from the EQA Organizing Centre followed and checked?			
● Are EQA samples stored and used in training?			
QUALITY INDICATORS			
● Are Quality Indicators used, documenting the number of times:			
– samples are rejected?			
– test turnaround times have been missed?			
– tests have not been performed due to equipment malfunction?			
– tests have not been performed due to unavailability of a reagent or test kit?			
– errors have been detected?			
– cultures have been contaminated?			
– laboratory -related accidents have occurred?			
● OTHER			
COMMUNICATIONS WITH LABORATORY USERS			
● Have clinicians and nursing staff been issued with a <i>Laboratory User Handbook</i> detailing:			
– laboratory working hours?			
– how to collect, store and transport samples?			
– test turn around times?			
– arrangements for testing out of hours samples?			
● Are clinicians notified when a test is unavailable?			

	YES	NO	COMMENTS
● Are all complaints documented and resolved with the minimum of delay?			
● Are communications with users of the laboratory courteous and helpful?			
● Are outpatient test waiting times monitored and kept as short as possible?			
● Is patient confidentiality respected?			
● Are regular meetings held with clinical staff and suggested improvements implemented?			
● OTHER			
RECORDS			
● Does the <i>Laboratory Quality Manual</i> contain all the laboratory policies, processes and procedures?			
● Are records kept of:			
– request forms?			
– worksheets?			
– quality control charts?			
– EQA reports?			
– audit findings?			
– user surveys?			
– user complaints?			
– equipment calibration?			
– equipment servicing?			
– clinical meetings?			
● Is there a reliable system for storing and tracking patient data and test results?			
● When using a computerized system:			
– is there a back-up system in place should there be a power failure or computer malfunction?			
– are error codes being interpreted correctly?			
– are there secure passwords to prevent unauthorized use?			
● Is the confidentiality of patient data and test results secure?			
● Are monthly reports of laboratory activities sent to district health management and public health authorities?			
● OTHER			

CHANGES AND RECOMMENDATIONS REPORT

Depending on the Checklist findings, the Supervisor and laboratory staff:

- Should make those changes which are required to comply with patient requirements, standard working procedures and safety.
- Recommend improvements which can be implemented within an agreed time frame to improve the quality and reliability of laboratory service, to be checked at the next visit.

The Supervisor should demonstrate any changes in techniques which are required, carry out whenever possible equipment repairs, suggest ways of resolving difficulties and provide a report of the visit with agreed changes and recommendations. The report of the visit should be signed by the Supervisor and laboratory staff.

TRAINING CURRICULUM FOR DISTRICT LABORATORY PERSONNEL

Developing a training curriculum involves more than making a list of the topics to be taught and learned. This is what is meant by a syllabus. A curriculum is needed because it details:

- The learning objectives of a course, i.e. the tasks the student must learn.
- Facts, skills, and attitudes laboratory officers need to learn to do their tasks competently and provide a quality service.
- Methods used to help students learn.
- Time and place where the students will learn, i.e. timetable.
- The resources available for training, e.g. teachers, workrooms, laboratories, equipment, learning materials, books, internet.
- Methods used to assess students.

Designing the curriculum

Designing a competency-based training curriculum for district laboratory personnel involves:

- 1 Performing a situation analysis, i.e. studying the work and working environment of district laboratory staff and making a list of the technical and management tasks that make up the job.
- 2 Performing a task analysis to identify those facts, skill, and attitudes that are *essential* to perform each task.
- 3 Deciding what training is required after considering the educational background and qualifications of students.
- 4 Writing the learning (educational) objectives.
- 5 Selecting the most appropriate ways to help students learn.
- 6 Organizing the learning.
- 7 Assessing whether students can perform their tasks competently, i.e. whether the learning objectives have been achieved.
- 8 Evaluating and modifying the course as required.

Note: Each of these stages requires careful planning.

1 Situation analysis

The first and most important requirement in designing a competency-based curriculum for district laboratory personnel is for those in charge of training, and the district laboratory coordinator, to visit as many as possible of the laboratories in which trainees will work after qualifying:

- getting to know the working situation and understanding the problems laboratory staff experience.
- identifying accurately the technical and management tasks and communication skills needed.
- discussing with laboratory staff and laboratory users, which tests are required, what type of service is needed, and what resources are available.
- assessing how long the training programme should be.

Consultations will also need to be held with the district health management team. The information obtained will determine what district laboratory students should learn.

Working situation in district laboratories

- District laboratory officers work closely with the community and need to have a clear understanding of the clinical and public health functions of district laboratories, i.e. an awareness of individual and community health needs.
- Many test requests will be from outpatients with results needed as soon as possible, i.e. during the patient's visit.
- District laboratories may not have a regular water supply or stable mains electricity and laboratory equipment service engineers are rarely available.
- Community-based laboratories need to be as self-reliant as possible.
- District laboratory officers are responsible for health and safety in their place of work, the efficient management of resources, and the quality of laboratory service provided.

Only by knowing the job, working environment, and responsibilities of district laboratory staff, can the learning objectives of a training programme be identified. The list of technical and management

tasks produced at the end of the situation analysis is the list of learning objectives for the course.

2 Task analysis

This is best carried out by observing and noting in detail how a competent district laboratory officer performs the tasks identified in the situation analysis. For each task tutors will need to decide the facts, skills, and attitudes that are essential for the task to be performed competently, intelligently, and safely.

What facts are essential?

A particular fact should be considered essential information if it is needed by the student:

- to understand why a test is required, e.g. clinical and public health indications for a test request.
- to perform and control a test in a certain way, i.e. follow a standard operating procedure (SOP).
- to perform a task safely.
- to perform a task efficiently without waste.
- to calculate, report, and record a test result correctly.
- to understand the meaning of a test result.

When knowing a fact does not influence the way a task is performed, it usually means such information is not essential. The teaching of irrelevant information should be avoided, particularly if it reduces the time a student may have for performing skills. Useful and interesting to learn facts can be added at a later stage, if time permits, and when *basic competency has been achieved*.

The teaching of non-essential facts by tutors to fill time or impress students indicates poor planning of a training programme and a misunderstanding by tutors of competency-based learning objectives. Any unplanned spare time that arises in a training session, e.g. due to a power failure, is better spent in using realistic problem-solving exercises to assess whether trainees can apply what they have learned (district laboratory practice is not short of problems that trainees will need to know how to address).

What skills are essential?

Identifying accurately which skills are necessary to perform different tasks is of utmost importance in designing a job related curriculum. Many tasks will involve similar skills, others will require specific skills as follows:

- Basic skills such as:
 - use and care of a microscope and other equipment.
 - accurate and precise pipetting, dispensing, and measuring fluids.
 - weighing and handling chemicals.
 - preparing and staining slide preparations.
 - safety skills and personal hygiene.
- Specific technical skills which require a longer period to learn and more personal tuition such as:
 - reporting microscopical examinations.
 - reporting cultures.
 - interpreting water-testing results.
- Numerical and accountancy skills such as:
 - use of SI units in laboratory work.
 - calculating test results from formulae.
 - working out dilutions and percentages.
 - using quality control charts.
 - calculating volumes and preparing calibrators, stains and reagents.
 - estimating laboratory operating costs.
 - keeping basic accounts and preparing a budget.
- Management, problem-solving, and decision-making skills, such as:
 - organizing and coordinating work activities and staff duties.
 - monitoring standard operating procedures.
 - requisitioning supplies and keeping an inventory.
 - monitoring health and safety regulations.
 - helping to formulate laboratory policies.
 - implementing quality management essentials.
 - monitoring decontamination and cleaning procedures.
 - maintaining patient confidentiality and the security of patient information and reports.
 - arranging equipment maintenance.
 - assisting in epidemiological surveys.
 - managing problems such as work overload, incorrect requesting of laboratory tests and collection of samples, urgent test requests, delayed test reports, laboratory errors, staff disagreements, problems due to equipment failure, shortages of reagents, monitoring working conditions, and managing laboratory accidents.

- Communication skills such as:
 - communicating appropriately with patients, patient’s relatives, clinicians, professional colleagues, and ward staff.
 - using the internet, a mobile phone, and other channels of communication.
 - communicating test reports, collating laboratory data, and using tables and charts.
 - presenting information at meetings and participating in discussions.
 - using the laboratory in health education.
 - reporting problems affecting work performance and staff welfare.

Important: Skills need to be learned in the context of both routine and emergency working conditions.

What attitudes are essential?

Attitudes and personal qualities influence the way an individual thinks, behaves, and works. They can also affect the performance of others. Having the correct attitudes and qualities for the job are important if district laboratory practice is to be accepted and respected by the community and used effectively in healthcare.

The following are among the attitudes and qualities most frequently identified in laboratory task analyses:

- Responsibility, reliability, and a professional approach.
- Thoroughness with attention to detail.
- Alertness.
- Coordinated approach to the work with correct attitudes to accuracy and precision.
- Honesty and integrity with an ability to act appropriately when errors are made.
- Correct attitudes towards patients, relatives of patients, fellow workers and those requesting laboratory tests. These include respect, thoughtfulness, patience, kindness, approachability, and helpfulness.
- Correct attitudes to health, safety, and security.
- Neatness of work and appearance.

Important: Students need to learn the correct approach to different working conditions, e.g. ability to perform reliably and consistently when working alone or the need to work quickly, competently, and calmly under emergency conditions, heavy workload, or when unforeseen situations develop such as a power failure or equipment breakdown.

3 Deciding what training is required

Deciding what needs to be taught will depend on what the students enrolling for the course know and can do. Most laboratory training programmes accept as students those with a proven knowledge of basic mathematics, science and biology, and ability to understand, communicate, and write in the language of the course.

Depending therefore on the educational background and experiences of the students, tutors may be able to reduce the time spent learning some of the basic facts and skills. It must be remembered, however, that passing school examinations does not guarantee that students will possess the adequate skills or be able to apply their knowledge and experience in medical laboratory work.

4 Writing the learning objectives

Well written learning objectives are dependent on performing an accurate situation analysis and a careful task analysis to identify the tasks that are needed and the facts, skills, and attitudes a student needs to learn. Learning objectives state what a course aims to achieve and the standard of work required. Some teachers prefer the term learning goals. Learning objectives are important because they determine:

- what is taught.
- how the teaching is done.
- how students are tested.

Learning objectives must be achievable, observable, and above all relevant to the job that students are being trained to do.

5 Selecting appropriate ways to help students learn their objectives

When helping students to learn, tutors need to remember that students learn best by doing. ‘What I hear I forget, what I see I remember, WHAT I DO, I KNOW. Tutors should teach theory, practice, and attitudes together because this reflects the working situation.

Active learning

This helps to prevent theory overload and students simply listening to lectures and learning very little. Active learning is always more interesting and helps

students to remember better and more quickly achieve competence in performing their tasks. Active learning teaching methods include:

- Practising skills following demonstrations.
- Problem-solving and decision-making exercises which will help students to apply what they have learned and cope with difficulties in their job.
- Question and answer sessions and discussions.
- Role play exercises which are particularly helpful when teaching attitudes and skills.
- Project work to encourage students to learn by their own efforts.
- Active use of effective visual aids and the internet.

Dividing a class into groups and involving each group in a different learning method, not only gives students the opportunity to learn a task more comprehensively but also helps to ensure all students receive 'hands on' experience when equipment is in short supply, e.g. microscopes.

It is particularly important to give laboratory students exercises that will help them to work economically and efficiently with good coordination, accuracy, and precision. Students must be taught how to work systematically with attention to detail so that they can follow standard operating procedures.

Other ways to help students learn

Besides making learning active, other ways in which tutors can help students learn include:

- providing feedback to students by frequent testing.
- teaching clearly by making sure students can hear what is said and see what is written and demonstrated.
- using simple language, incorporating easy to understand examples, and using good visual aids.
- checking that all students can perform the necessary tasks.
- reviewing whether students have remembered what they have been taught.
- allowing enough free time for individual study and project work.
- using a variety of teaching methods.
- tutors showing that they care whether their students learn.

Note: Teaching methods are well described in the WHO book *Teaching for better learning*, 1992, 2nd

ed. Publication can be accessed electronically by entering the title in Google.

6 Organizing the learning

This stage involves:

- Identifying appropriate locations for the teaching and learning. Lecturing in a classroom about laboratory techniques will not help students to learn or become competent in their tasks. 70–80% of the teaching for district laboratory students should be practical active learning.
- Deciding on how long teaching sessions and study periods should be and the time of day when learning should take place (particularly important in tropical countries).
- Deciding the sequence of learning, taking note of what has been learned previously upon which the learning of a new knowledge or skill can be based. When initial tuition is carried out in a training centre with practical work performed in the working laboratory, it is particularly important for tutors to teach a technique before students are expected to perform it in the laboratory.
- Identifying appropriate resources and facilities to use during teaching sessions and study periods to motivate students to learn.

Note: Whenever possible teaching aids should be prepared nationally. Manuals, charts, posters, bench aids, videos and computerized learning programmes that are produced outside the country should be evaluated carefully by laboratory tutors for their appropriateness and effectiveness. Consideration will need to be given to the educational background of trainees and resources available.

Importance of work experience during training

During their training, students must spend sufficient time in a district laboratory as similar as possible to the one in which they will work after qualifying, e.g. health centre laboratory or district hospital laboratory. Only in the working situation can students:

- appreciate the importance of laboratory practice in healthcare.
- practice their Code of Professional Conduct.
- gain work experience under supervision.
- learn to work in a coordinated way, meeting target turnaround times for test results.
- experience working under pressure and how to manage difficult situations.

- learn what action to take if a mistake is made.
- learn to respond appropriately when tests are requested urgently.
- develop communication skills and the correct approach to patients, patient's relatives, fellow workers, clinicians, and other health staff.

Note: Adequate work experience during training is also necessary so that tutors, supervisors, and the district laboratory coordinator can assess the suitability of students for district medical laboratory work. On-site job experience is usually best carried out towards the end of training when students can benefit most from their experience and still have the opportunity to return to their training centre for further tuition if required. Tutors *must* ensure that the time a student spends in the working laboratory is adequately supervised and assessed.

7 Assessing students

Testing students is required for the following reasons:

- To assess whether students have learned what is needed and can perform their tasks to the standard required for qualification and registration.
- To help students learn.
- To assess whether the learning objectives of the course have been achieved and what changes to the curriculum are indicated.
- To provide tutors with feedback to help them improve their teaching methods.
- To provide employing health authorities with the assurance that a student who has been assessed as competent and holds a *Certificate of Qualification* is fit to work in a district laboratory.

To meet these requirements the method(s) selected for assessing students must be:

- effective and appropriate to the task and standard of performance being assessed, e.g. practical skills require testing in a practical way.
- workable, affordable, and achievable for tutors and students.
- sufficiently informative, precise and objective to be of help to students and tutors and avoid significant differences in marking between tutors.
- acceptable to the health authority and those in charge of the training and registration of laboratory personnel.

Assessment methods for laboratory students

Traditional methods of assessing laboratory students by using written, practical, and oral examinations at the end of the training course have been largely replaced by methods which test learning objectives more appropriately and are less stressful to students.

Poor assessment of laboratory students by written, practical, and oral examinations

From surveys reported by McMinn, it was estimated that:

- 80% of all written questions tested only recall of information. Very few questions tested the ability of the student to use the information to solve problems.
- 67% of question papers, sampled less than 20% of the syllabus.
- 90% of practical examinations tested only 15% of the practical skills expected of trained technical officers.
- 37% of the questions on written examinations asked students to describe how to perform specific practical skills.

Oral examinations were equally poor at assessing the performance of students with 78% of questions simply testing recall information.

Effective methods of assessing the performance of laboratory students include:

- continuous, or frequent, testing.
- use of logbooks.

Continuous assessment

This involves testing the facts, skills, and attitudes of students throughout their training. When qualification is dependent on the results of these tests, continuous assessment makes students work harder. Guidance is provided both to students and tutors. Students are shown throughout the course what standard is expected.

Use of logbooks

This helps to personalize the learning for students and makes sure students are able to carry out *all* the required tasks competently. Each student is given a logbook which lists all the technical *and* management tasks which require assessment. When the student is ready, the tutor or head of the department in which the training is taking place, records in the logbook when a student is able to perform a particular task.

Assessment of tests should include how well a method is performed, the correctness of the result, how results are presented and interpreted, and the attitudes of the student to the patient and their work. Assessment of many of the management tasks can take the form of a problem-solving exercise. If the student fails the assessment, he

or she is able to try again at a later stage after discussion with the tutor.

The logbook system helps to identify at an early stage any learning difficulties or inadequate teaching. The tasks in the assessment logbook should form the basis of a Certificate of Qualification.

Certificate of Qualification for district laboratory personnel

The *Certificate of Qualification* awarded to successful trainees at the end of their basic training must state *exactly what the person is qualified to do*. It should list the tests, test methods, and the management tasks the person is qualified to perform. This type of certificate is of far greater value to the student and employers than one which simply states that a person is qualified as a district laboratory officer with a certain grade.

The Certificate should show the name and address of the Training School (qualifying centre), duration of training, and date of qualification. It must be signed by the person in charge of training.

Important: Newly qualified staff require supervision and support in the workplace if they are successfully to put into practice what they have learned, remain motivated and continue professional development.

8 Evaluating and modifying the course

A training programme will develop as experience is gained in using it. With experience the good points and less good features of the course will become apparent. Almost always the teaching methods and the ways students learn can be improved and often also the learning locations, sequence of learning, resources used, and assessment methods.

Evaluating the quality of a training programme involves reviewing the learning patterns and performances of students, the teaching effectiveness of tutors, and how well the curriculum is achieving its learning objectives.

Encouragement to tutors

In a competency-based training curriculum, try to avoid introducing too much theory in a non-integrated way. This will only lead to students 'not seeing the wood for the trees'. Spend more time making sure students can do tests correctly and will be able to work reliably, efficiently and safely. This is what will benefit patients and the health service and lead to job satisfaction, motivation, better use and resourcing of district laboratories and increased financing of laboratory training programmes.

REFERENCES

McMinn. *Design of basic training for laboratory technicians.* Developing Country Proceedings 17th Congress International Association of Medical Laboratory Technologists. Stockholm, 1986, pp. 176–188.

ACKNOWLEDGEMENT

Reproduced with amendments from Part 1 2nd ed update *District Laboratory Practice in Tropical Countries*, copublished Cambridge University Press & Tropical Health Technology.

Appendix III

SOP formats

Chemical hazards information

SI Unit tables

Suggested format for Test SOP

TITLE	SOP NUMBER
Date prepared	Version number
Date of commencement	Prepared by
Date for review	Authorized by
Staff able to perform test:	Staff signatures
Unsupervised	
Supervised	
Abbreviations and terms used in SOP	
.....	
HEADINGS	WHAT TO WRITE UNDER HEADINGS
Purpose	Main reason(s) for performing test.
Background	State briefly the principle of the test and technology used.
Sample	State how to collect it, temperature requirements, volume needed, use of any anticoagulant, preservative/stabilizer, and time within which the sample should reach the laboratory and be tested, registration checks.
Equipment	List items of equipment needed to perform the test, e.g. microscope, analyzer, centrifuge, incubator, water-bath, dispenser, pipettes (refer to relevant Equipment SOPs).
Reagents/stains	List reagents/stains needed to perform the test. For the preparation of each, refer to the relevant Stain SOP or Reagent SOP.
Procedure	Describe in numerical sequence how to perform the test. For quantitative tests, include details of calibration, use of a graph or factor, and calculations.
Quality control	List the controls used in the test and their source(s) e.g. positive and negative controls for serological tests, control sera for clinical chemistry tests, control blood films for malaria microscopy.
Procedure notes	Summarize the factors that can affect test results, e.g. sample not well mixed, clots in an anticoagulated sample, slide smear too thick for staining, sides of a cuvette scratched or dirty, air bubbles in the solution, whether sample affected by antimicrobials, herbal remedies or other interfering substances.
Reporting, interpretation	State how to report test, detailing units, reference intervals for quantitative tests, action to take when a result is unexpected or seriously abnormal, anticipated test turnaround time, and comments that should accompany the report. When indicated, suggest further tests to perform.
Safety	Include safe handling of samples and reagents, safe pipetting and dispensing, sample disposal and decontamination procedures (refer to the relevant SOPs).
Sources of information, references	List the references and sources of information contained in the SOP, e.g. National guidelines, publication(s), manufacturers' literature.
Other related documents	List other relevant SOPs and documents.

Suggested format for Stain Preparation Test SOP

TITLE	SOP NUMBER
Date prepared	Version number
Date of commencement	Prepared by
Date for review	Authorized by
Staff able to prepare the stain:	Staff signatures
Unsupervised	
Supervised	
Abbreviations and terms used in SOP	
.....	
HEADINGS	WHAT TO WRITE UNDER HEADINGS
Purpose	State the reason(s) for using the stain.
Background	Detail the source(s) of dry and liquid stains, e.g. government stores or manufacturer/supplier.
	Provide ordering details, i.e. correct name, ordering codes/numbers.
Equipment	List the equipment required to prepare the stain, e.g. balance, water-bath, glassware. Refer to relevant Equipment SOPs.
Procedure	Describe how to prepare the stain: <ul style="list-style-type: none"> – from a dry product, – from a working stock solution of stain, detailing the calculations. <i>Detailed instructions on the preparation of individual stains</i> can be found in Appendix 1.
Quality control	Describe the slide preparations to be used to check the performance of the stain before it is put into routine use.
Labelling	Detail the information required to label the stain, e.g. stain concentration, date of preparation, date of validation, stability (shelf-life) of stock and working stains, name or initials of person preparing the stain.
Storage	State how to store the stain, e.g. use of an opaque container to protect the stain from direct light. All stains require storage away from direct sunlight and dry stains must be kept in airtight containers.
Procedure notes	List the factors that affect the preparation of the stain, e.g. quality of stain, accuracy of calculations, pH of water, need for water-free absolute methanol when preparing alcohol-based Romanowsky stains.
Safety	List any associated hazard, e.g. use of chemicals or solvents that are flammable, toxic, corrosive or irritant, precautions to take when using concentrated acids.
Sources of information	List the source(s) of the information used to prepare the stain, e.g. publication, leaflet supplied with the stain, calculation tables.

Suggested format for Reagent Preparation SOP

TITLE	SOP NUMBER
Date prepared	Version number
Date of commencement	Prepared by
Date for review	Authorized by
Staff able to prepare the reagent:	Staff signatures
Unsupervised	
Supervised	
Abbreviations and terms used in SOP	
.....	
HEADINGS	WHAT TO WRITE UNDER HEADINGS
Purpose	State the reason(s) for using the reagent.
Background	State the sources of ready-made reagents and constituents of laboratory-made reagents, e.g. government stores or purchased from a manufacturer/supplier. Provide ordering details, e.g. the <i>correct and complete name</i> of the reagent or chemical. For chemicals, also provide the <i>full chemical formula</i> .
Equipment	List the equipment needed to prepare the reagent, e.g. analytical balance, water-still, volumetric glassware, cylinders, pipettes. Refer to the relevant Equipment SOPs.
Procedure	Describe in numerical sequence how to prepare the reagent. <i>Detailed instructions on the preparation of individual reagents</i> can be found in Appendix I.
Quality control	Describe how to check the performance of the newly made reagent.
Labelling	For a laboratory-made reagent, state its full name, concentration, date of preparation, shelf-life, name or initials of person preparing the reagent, any associated hazard, e.g. whether the reagent is flammable, toxic, corrosive, irritant (see page 314).
Storage	Detail how to store the reagent, e.g. in an airtight, clear or opaque container, in a well-ventilated, easily accessible, secure location, away from direct sunlight.
Procedure notes	List the factors that affect the preparation of the reagent, e.g. quality of water, purity of chemicals, accuracy of weighing.
Safety	List any hazards associated with preparing the reagent, e.g. handling of chemicals, use of strong acids.
Sources of information	List the sources of information used to prepare the reagent, e.g. manufacturer's chemical catalogue, other publication, <i>Safety Manual</i> , chemical tables.

Suggested format for Equipment SOP

TITLE	SOP NUMBER
Date prepared	Version number
Date of commencement	Prepared by
Date for review	Authorized by
Staff able to use the equipment:	Staff signatures
Unsupervised	
Supervised	
Abbreviations and terms used in SOP	
.....	
HEADINGS	WHAT TO WRITE UNDER HEADINGS
Purpose	State the reason for using the equipment.
Background	State briefly how the equipment works. It may be useful to include diagrams from the <i>Equipment User Manual</i> .
Specifications	Detail the model, serial number, manufacturer, supplier, date of purchase, warranty conditions (if still applicable), power requirements.
Parts and essential consumables	List the recommended replacement parts and essential consumables, source(s) of supply, ordering details and number of each to hold in stock.
Placement	Detail the manufacturer's recommendations for placement of the equipment, e.g. need for adequate space and ventilation, level secure bench, away from moisture, heat, dust, and direct sunlight. List local requirements, e.g. placing equipment near to a power point and protecting it from corrosion and damage caused by rodents and insects.
Procedure	Describe how to calibrate and use the equipment as instructed by the manufacturer.
Trouble-shooting	Detail the manufacturer's recommendations for identifying and resolving faults and how to replace defective components. Provide information on the person/ company to contact for repair when the equipment cannot be repaired locally.
Maintenance	Describe how to clean and maintain the equipment as instructed by the manufacturer.
Service contract for major equipment	Detail the frequency of servicing as recommended by the manufacturer and provide contact details for the authorized service engineer.
Safety	List the safety precautions needed to avoid equipment-related accidents, and action to take when an accident occurs, e.g. when tubes break in a centrifuge (see Section 1.4).
Procedure notes	Detail factors that can affect the use of equipment, e.g. need for a voltage stabiliser, not positioning microscopes near to centrifuges.
Sources of information	Detail manufacturer's information, publications, other sources.

CHEMICAL HAZARDS

For commonly used chemicals

Extremely flammable

- Acetone
- Diethylamine
- Ether, diethyl



Highly flammable

- Acetone-alcohol
- Acid alcohol
- Alcoholic stains: Giemsa, Leishman, Wrights, May Grunwald
- Ethanol and alcohol reagents
- Methanol
- Methylated spirit
- Toluene



Oxidizing (fire-promoting)

- Calcium hypochlorite
- Chromic acid
- Hydrogen peroxide (strong)
- Nitric acid
- Perchlorates
- Potassium permanganate
- Sodium nitrite



Corrosive

- Acetic acid, glacial
- Ammonia solution
- Calcium hypochlorite
- Chromic acid
- Fouchet's reagent
- Hydrochloric acid
- Nitric acid
- Phenol
- *o*-Phosphoric acid
- Potassium hydroxide
- Silver nitrate
- Sodium hydroxide
- Sodium hypochlorite
- Sulphuric acid
- Thymol
- Trichloroacetic acid



Harmful

- Aminophenazone
- Ammonium oxalate
- Auramine phenol stain
- Barium chloride
- Benedicts reagent
- Benzoic acid
- Carbol fuchsin stain
- Cetylpyridinium chloride
- Chloroform
- Copper II sulphate
- Dobell's iodine
- Ethylenediaminetetraacetic acid (EDTA)
- Ethylene glycol (Ethanediol)
- Formol saline
- Glycerol jelly
- Glutaraldehyde
- Iodine
- Iron III chloride (Ferric chloride)
- Lugol's iodine
- Methylated spirit
- Naphthylamine
- *p*-Nitrophenyl
- Oxalic acid
- Potassium oxalate
- Potassium permanganate
- Salicylic acid
- Saponin
- Sodium deoxycholate
- Sodium dithionite
- Sodium dodecyl sulphate
- Sodium tetraborate
- Sodium tungstate
- Stains (powder form):
 - acridine orange
 - auramine O
 - brilliant cresyl blue
 - crystal violet
 - Giemsa
 - Harris's haematoxylin
 - malachite green
 - May Grunwald
 - methylene blue
 - neutral red
 - toluidine blue



- Sulphanilic acid
- Thiourea
- Toluene
- Xylene

Toxic

- Cadmium sulphate
- Diphenylamine
- Formaldehyde solution
- Lactophenol
- Mercury
- Mercury II chloride (Mercuric chloride)
- Methanol
- Phenol
- Picric acid
- Potassium cyanide
- Sodium azide
- Sodium fluoride
- Sodium nitrite
- Sodium nitrosopentacyanoferrate III (Sodium nitroprusside)
- Thiomersal
- Thiosemicarbazide
- *o*-Tolidine
- *o*-Toluidine



Irritant

- Acetic acid, glacial
- Ammonia solution
- Calcium chloride
- Formaldehyde solution
- Hydrogen peroxide
- Lactic acid
- Naphthylethylenediamine dihydrochloride
- Potassium dichromate
- Sodium carbonate
- Sulphosalicylic acid
- Zinc sulphate













Explosive














- Picric acid (when dry)
- Sodium azide



























Hazardous chemicals, safety precautions and storage














Important: *Not all hazardous chemicals are included in this list. Always read carefully the storage instructions and safety information printed on the container label.* The source of the information in this chart is the BDH/Merck *Laboratory Supplies Catalogue*.







CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
Acetic acid, glacial	 Corrosive, causing severe burns.	Protect eyes, skin, clothing, and equipment. Avoid breathing fumes. Use in well ventilated area. Keep away from oxidizers, particularly nitric acid, chromic acid, peroxides and permanganates.
	 Irritant vapour	
	 Flammable, with flash point 40°C.	
Acetone	 <i>Extremely</i> flammable and volatile with flash point -18°C.	Keep away from sources of ignition, chloroform, chromic acid, sulphuric acid, nitric acid and other oxidizers. Avoid breathing fumes. Use in well ventilated area. Protect eyes.
Alcohol	See Ethanol	
Ammonia solution	 Corrosive	Protect eyes, skin, clothing, and equipment. Place cloth over cap before removing it or preferably use in a fume cupboard. Keep away from mercury and halogens such as chlorine and iodine.
	 Irritant vapour	
Barium chloride	 Harmful if ingested or inhaled.	Protect against ingestion, inhalation, and skin contact.
Benzoic acid	 Harmful if ingested. Irritating to the eyes.	Protect against ingestion, skin and eye contact.
Calcium hypochlorite bleaching powder	 Corrosive Contact with acids liberates toxic gas. Oxidizing.	Protect eyes, skin, clothing and equipment. Keep away from acids, flammable chemicals, and combustible materials.
Chlorine solutions	See Sodium hypochlorite solution.	
Chloroform	 Harmful if ingested or inhaled. Irritating to the skin. Suspected carcinogen. Volatile with vapour that is anaesthetic. Toxic product formed if heated. Attacks plastics and rubber.	Avoid breathing vapour. Use in a well ventilated area or preferably in a fume cupboard. Protect eyes and skin. Keep away from acetone.

CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
Chromic acid (reagent)	 Corrosive	Protect eyes, skin, clothing, and equipment. Keep away from combustible materials, acetone and other flammable chemicals.
	 Oxidizing	
Diethylamine	 <i>Extremely</i> flammable with flash point -39°C .	Protect eyes. Avoid breathing fumes. Use in a well ventilated area or preferably in a fume cupboard. Keep away from sources of ignition and oxidizers. Do not empty into drains.
	 Irritant to eyes and respiratory system.	
Diphenylamine	 Toxic if ingested, inhaled, or in contact with skin.	Wear appropriate protection. Wash immediately any affected area.
Ethanol, absolute	 <i>Highly</i> flammable and volatile with flash point 13°C . Harmful if ingested.	Keep away from sources of ignition, silver nitrate, and oxidizers.
Ethanolamine	 Harmful if inhaled. Irritant to eyes, respiratory system, and skin. Also corrosive	Protect skin and eyes. Use in a well ventilated area. Keep away from oxidizers.
Ether, diethyl	 <i>Extremely</i> flammable, with flashpoint -40°C . Volatile with anaesthetic vapour. May form explosive peroxides when exposed to light.	Keep away from sources of ignition, oxidizers, iodine and chlorine. Do not refrigerate. Store in opaque container, not plastic unless polypropylene. Do not use cap with rubber liner. Use in well ventilated area or preferably in a fume cupboard.
Ethylene glycol (Ethanediol)	 Harmful if ingested.	Protect against ingestion and skin contact.
Formaldehyde solution	 Toxic if ingested, inhaled, or in contact with skin. Causes burns and can cause dermatitis. Probable carcinogen.	Wear appropriate protection. Use in well ventilated area or preferably in a fume cupboard. Wash immediately any affected area. Keep away from hydrochloric acid and oxidizers. Do not store below 21°C . (Keep at $21-25^{\circ}\text{C}$).
	 Irritating and unpleasant odour.	
Giemsa stock stain (alcoholic stain)	 <i>Highly</i> flammable with flash point 12°C .	Keep away from sources of ignition. Avoid inhaling fumes and contact with skin.
Glutaraldehyde	 Harmful if ingested. Irritating to skin and respiratory system. May cause dermatitis. Can damage eyes.	Wear appropriate protection. Use in well ventilated area. Keep away from oxidizers.

CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
Hydrochloric acid, concentrated	 Corrosive, causing severe burns. Irritating to respiratory system. Unpleasant corrosive fumes. Releases toxic fumes in fires.	Protect skin, eyes, clothing, and equipment. Do not breathe fumes. Use in well ventilated area or preferably in a fume cupboard. Keep away from alkalis, chromic acid, potassium permanganate.
Hydrogen peroxide solution	 Corrosive  Irritating to eyes, respiratory system, and skin. Stronger solutions are oxidizing.	Protect eyes, skin, clothing, and equipment. Store in opaque non-metallic container. Keep out of direct sunlight in a cool dark place away from flammable chemicals and combustible materials.
Iodine	 Harmful if ingested, inhaled, or in contact with skin. Irritating to eyes and respiratory system. Evolves toxic fumes in fires.	Avoid skin and eye contact, and breathing in vapour. Store iodine containing reagents in opaque container. Do not use caps with rubber liners. Reacts violently with metals, acetylene, and ammonia.
Iron 111 chloride (Ferric chloride)	 Harmful if ingested. Irritating to the skin. Can cause eye damage.	Protect against ingestion, skin and eye contact.
Leishman stock stain	As for Giemsa stain.	
Mercury	 Toxic by inhalation. May cause dermatitis. Attacks lead piping and soldered joints. Evolves toxic fumes in fires.	Ventilate area if mercury escapes from broken thermometer (discard in deep covered pit). Avoid skin contact. Keep away from ammonia, bromine, azides, ethylene oxide.
Mercury 11 chloride (Mercuric chloride)	 Very toxic if ingested. Toxic in contact with skin and can cause burns.	Protect against ingestion and contact with skin. Minimize use. Wash hands immediately after use. Keep in locked cupboard.
Mercury 11 nitrate (Mercuric nitrate)	 Very toxic if ingested, inhaled, or in contact with skin.	Wear appropriate protection. Minimize use. Wash hands immediately after use. Keep in locked cupboard.
Methanol (Methyl alcohol)	 Highly flammable with flashpoint 12°C. Volatile and hygroscopic.  Toxic if ingested or inhaled. Can cause dermatitis and damage optic nerve and central nervous system.	Keep away from sources of ignition, sodium hypochlorite, nitric acid, chloroform, hydrogen peroxide. Avoid breathing vapour. Protect skin and eyes. Use in a well ventilated area or preferably in a fume cupboard.
Nitric acid, concentrated (fuming)	 Corrosive, causing severe burns. Has irritating and harmful vapour.  Oxidizing	Protect skin, eyes, clothing, and equipment. Avoid breathing fumes. Preferably use in a fume cupboard. Use an eyewash bottle immediately if acid enters eye. Keep away from alkalis, metals, acetic acid, chromic acid, reducing agents, flammable chemicals and combustible materials.

CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
Phenol	 Toxic in contact with skin or if ingested or inhaled. Corrosive and hygroscopic. Oxidizes and turns pink on exposure to light.	Protect skin, eyes, clothing, and equipment. Wash immediately any affected area. Keep container tightly closed. Store away from oxidizers.
o-Phosphoric acid	 Corrosive Evolves toxic fumes in fires.	Protect skin, eyes, clothing, and equipment.
Picric acid, (solid)	 Toxic if ingested, inhaled, or in contact with skin. Evolves toxic fumes in fires.  Explosive when dry. Can form highly explosive salts with many metals and explosive calcium picrate when in contact with concrete.	Wear appropriate protection. Ensure chemical is always covered with water. Do not leave chemical to dry in pipes. Keep away from reducing agents. <i>Note:</i> If the water on top of the chemical has evaporated, submerge the container in a bucket of water overnight. Loosen cap carefully and fill to the top with water (wear gloves and eyeshields).
Potassium cyanide	 Very toxic if ingested, inhaled, or in contact with skin. Contact with acid liberates very toxic gas.	Wear appropriate protection. Wash immediately any affected area. Keep in a locked cupboard away from acids. Do not empty into drains.
Potassium dichromate	 Irritating to the eyes, respiratory system, and skin. May cause dermatitis.	Protect eyes and skin. Avoid breathing dust.
Potassium hydroxide, pellets and solutions	 Corrosive Pellets are deliquescent.	Protect skin, eyes, clothing, and equipment. Store away from acids. Do not use reagent bottles with glass stoppers.
Potassium oxalate	 Harmful in contact with skin and if ingested.	Avoid contact with skin and eyes.
Potassium permanganate	 Oxidizing  Harmful if ingested.	Keep away from combustible materials, flammable chemicals, glycerol, ethylene glycol, sulphuric acid.
Salicylic acid	 Harmful if ingested. Irritating to skin and respiratory system. Can damage the eyes.	Wear appropriate protection and avoid breathing in chemical.
Saponin powder	 Harmful if ingested and in contact with skin.	Wear appropriate protection and avoid inhaling powder.

CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
Silver nitrate	 Corrosive and irritating to the eyes and skin. May ignite combustible materials.	Protect skin, eyes, clothing, and equipment. Keep away from ammonia solution, ethanol, charcoal, and combustible materials. Causes brown staining.
Sodium azide	 Very toxic if ingested, inhaled, or by skin contact. Mutagen. Contact with acids liberates toxic gas. Toxic gas liberated in fires.  Violent reaction occurs if water is added to heated solid chemical. Reacts with copper, lead, mercury to form explosive metal azide salts.	Avoid contact by wearing appropriate clothing. Do not inhale dust. Wash immediately any affected area. Keep in a locked cupboard away from acids and metals. Do not flush down copper or lead drainage systems.
Sodium dithionite	 Harmful if ingested. Contact with acid liberates toxic gas. May cause fire.	Protect from skin contact. Keep away from acids, flammable chemicals and combustible material.
Sodium fluoride	 Toxic if ingested. Irritating to eyes and skin. Liberates toxic gas in contact with acids.	Protect skin and eyes. Keep away from acids.
Sodium hydroxide, pellets and solutions	 Corrosive. Causes severe internal injury if ingested. Deliquescent (pellets). Heat evolved when mixed with water.	Protect skin and eyes, clothing, and equipment. Store in dry place, away from acids, methanol, and chloroform. Do not use reagent bottles with ground glass stoppers.
Sodium hypochlorite solution	 Corrosive and toxic if ingested or inhaled. Irritating vapour. May evolve toxic fumes in fires. Liberates toxic gas in contact with acids.	Protect skin, eyes, clothing, and equipment. Use in well ventilated area. Keep away from acids, methanol and oxidizers.
Sodium nitroprusside*	 Toxic if ingested. *sodium nitrosopentacyanoferrate III	Do not breathe dust.
Sodium nitrite	 Toxic if ingested.  Oxidizing	Protect against skin contact. Keep away from combustible materials and flammable chemicals.
Sulphanilic acid	 Harmful if ingested, inhaled, or in contact with skin or eyes.	Avoid contact with eyes and skin. Wear appropriate protection.
Sulphosalicylic acid	 Irritating to eyes and skin.	Protect eyes and skin. Use in well ventilated area.
Sulphuric acid	 Corrosive, causing severe burns. Toxic vapour.	Protect skin, eyes, clothing, and equipment. Avoid inhaling fumes. <i>Never add water to conc. sulphuric acid.</i> Keep in dry place away from alkalis, potassium permanganate, perchlorates, chlorates and flammable substances.

CHEMICAL	MAIN HAZARD	SAFETY PRECAUTIONS AND STORAGE
o-Tolidine	 Toxic and harmful if ingested. Carcinogen.	Minimize exposure. Wear appropriate protection.
Toluene	 Highly flammable, with flash point 4°C. Volatile.  Harmful if ingested, inhaled, or by skin contact. May cause dermatitis. Vapour can be irritating to the eyes.	Keep away from sources of ignition. Wear appropriate protective clothing. Keep away from oxidizers and do not empty into drains.
o-Toluidine solution	 Toxic if ingested or inhaled. Carcinogen. Irritating to the eyes. Reacts violently with acids.	Minimize exposure and wear appropriate protection. Keep away from acids.
Trichloroacetic acid	 Corrosive and hygroscopic. Unpleasant odour that is irritating to the eyes. May evolve toxic fumes in fires.	Protect skin, eyes, clothing, and equipment.
Wrights stain	As for Giemsa stain.	
Xylene	 Harmful if inhaled or in contact with skin. May cause dermatitis. Flammable with flashpoint 25°C.	Protect from skin contact and use in well ventilated area. Do not keep in plastic containers unless polypropylene. Do not use caps with rubber liners.

ACKNOWLEDGEMENT

Reproduced with amendments from Part 1 2nd ed update *District Laboratory Practice in Tropical Countries*, copublished Cambridge University Press & Tropical Health Technology.

SI UNIT TABLES

SI unit prefixes commonly used in laboratory work include:

Prefix	Symbol	Function
femto	f	10^{-15}
pico	p	10^{-12}
nano	n	10^{-9}
micro	μ	10^{-6}
milli	m	10^{-3}
centi	c	10^{-2}
deci	d	10^{-1}
kilo	k	10^3

SI derived units

SI derived unit	Symbol	Quantity measure
square metre	m^2	area
cubic metre	m^3	volume
metre per second	m/s	speed

Weight

1 kg	=	10^3 g (1000 g)
1 mg	=	10^{-3} g (0.001 g)
1 μ g	=	10^{-6} g (0.000 001 g)
1 kg	=	1000 g
1 g	=	1000 mg
1 g	=	1 000 000 μ g
1 mg	=	0.001 g
1 mg	=	1000 μ g
1 kg	=	2.205 lb

Amount of substance

1 mmol	=	10^{-3} mol (0.001 mol)
1 μ mol	=	10^{-6} mol (0.000 001 mol)
1 mol	=	1000 mmol
1 mol	=	1 000 000 μ mol
1 mmol	=	1000 μ mol
1 mmol	=	1 000 000 nmol
1 μ mol	=	1000 nmol

Pressure

Approx. conversion: $\frac{\text{mm Hg} \times 2}{15} = \text{kPa}$

lb/sq inch \times 6.895 = kPa

Length

1 dm	=	10^{-1} m (0.1 m)
1 cm	=	10^{-2} m (0.01 m)
1 mm	=	10^{-3} m (0.001 m)
1 μ m	=	10^{-6} m (0.000 001 m)
1 km	=	1000 m (10^3 m)
1 m	=	10 dm
1 m	=	100 cm
1 m	=	1000 mm
1 m	=	1 000 000 μ m
1 m	=	1 000 000 000 nm
1 cm	=	10 mm
1 cm	=	10 000 μ m
1 mm	=	1000 μ m
1 m	=	3.281 feet
1 km	=	0.62137 mile
1 inch	=	2.54 cm

Volume

1 dL	=	10^{-1} (0.1 L)
1 mL	=	10^{-3} (0.001 L)
1 μ L	=	10^{-6} (0.000 001 L or 1 mm^3)
1 L	=	10 dL
1 L	=	1000 mL
1 L	=	1 000 000 μ L
1 dL	=	100 cm^3 , formerly 100 mL
1 mL	=	1000 μ L
1 pint	=	0.568 L
1 L	=	1.760 pints
1 L	=	0.22 gallons

Temperature conversion

To convert $^{\circ}\text{C}$ to $^{\circ}\text{F}$:
multiply by 9, divide by 5, and add 32.

To convert $^{\circ}\text{F}$ to $^{\circ}\text{C}$:
subtract 32, multiply by 5, and divide by 9.

0 $^{\circ}\text{C}$	=	32 $^{\circ}\text{F}$
10 $^{\circ}\text{C}$	=	52 $^{\circ}\text{F}$
20 $^{\circ}\text{C}$	=	68 $^{\circ}\text{F}$
30 $^{\circ}\text{C}$	=	86 $^{\circ}\text{F}$
36.9 $^{\circ}\text{C}$	=	98.4 $^{\circ}\text{F}$
40 $^{\circ}\text{C}$	=	104 $^{\circ}\text{F}$
50 $^{\circ}\text{C}$	=	122 $^{\circ}\text{F}$
100 $^{\circ}\text{C}$	=	212 $^{\circ}\text{F}$

Appendix IV

**Collection and transport of samples
Laboratory request form**

Collection of samples

The reliability and accuracy of test results are directly affected by the quality of the sample received by the laboratory and the length of time between sample collection and processing. Managing the quality of samples involves:

- Obtaining the correct sample.
- Reporting abnormal sample appearances.
- Collecting samples at the best time.
- Managing urgent samples.
- Using the correct collecting procedure and sample container.
- Managing HIGH RISK samples.
- Transporting samples correctly.
- Reasons for rejecting samples.
- Information which needs to accompany samples.

CORRECT SAMPLE

It is essential to obtain the correct sample for the test being requested.

Examples

- Sputum not saliva is required when testing for *M. tuberculosis* and other respiratory pathogens.
- An endocervical swab and not a vaginal swab is required when isolating *N. gonorrhoeae* from female patients.
- A fresh dysenteric sample is required when testing for *E. histolytica* amoebae.
- A blood-free skin snip should be obtained when testing for *O. volvulus* microfilariae.
- Thick blood films of the correct thickness are required when testing for malaria parasites.

Select those parts of a sample that are most likely to contain pathogens, e.g. blood and mucus in faecal samples or caseous material in sputum samples.

Several samples may need to be tested when pathogens are likely to be few in number, e.g. *P. malariae*, *P. ovale* in blood samples, acid fast bacilli (AFB) in sputum, trypanosomes in cerebrospinal fluid (CSF), borreliae in blood samples.

Note: Handle with special care those samples which are more difficult to obtain and demanding for patients, such as CSF, pleural fluid, bone marrow, lymph gland fluid, aspirates from ulcers, skin snips and biopsies.

REPORTING APPEARANCE

Note and record any abnormal sample appearance. The following are examples of abnormal appearances and their possible significance:

- Cloudiness in CSF or in a fresh urine sample, usually indicates bacterial infection.
- Urine containing whole blood, possibly indicates the presence of *S. haematobium* eggs.
- Black faeces may indicate the presence of altered blood due to gastrointestinal bleeding.
- Faecal sample that contains blood and mucus, possibly indicative of amoebic dysentery, shigellosis, intestinal schistosomiasis.
- “Rice-water” faecal sample, usually indicative of cholera.
- Dark red brown-black serum, indicative of intravascular haemolysis caused by, e.g. severe falciparum malaria, sickle cell disease crisis, G6PD deficiency, or an incompatible blood transfusion.
- A lipaemic (fatty) serum is associated with raised triglycerides (above 3.4 mmol/L).
- A deep yellow (icteric) serum indicates a patient is jaundiced.
- A serum sample that is abnormally viscous (thick) or turbid may contain paraproteins.
- A serum that becomes markedly turbid after being refrigerated may contain cryoglobulins or cold agglutinins.
- A blood sample that contains a high concentration of red cells from which little serum or plasma can be obtained indicates severe dehydration or a blood disorder.

COLLECT AT THE CORRECT TIME

Samples for pathogen detection are best collected when pathogen numbers are likely to be at their highest and before a patient is treated with drugs such as antimicrobials or antimalarials.

Urine and sputum samples are best collected soon after a patient wakes when pathogens have multiplied over several hours. Other samples are

best collected during times of fever such as malaria blood films or blood to detect borreliae, or when a patient's temperature is beginning to rise, e.g. blood cultures.

The best time to collect blood samples to detect microfilariae depends on species periodicity and geographical location, e.g. blood to detect *L. loa* is best collected between 10.00–15.00h, and for *W. bancrofti* and *Brugia* spp, between 20.00–04.00h.

Fasting samples are required for the measurement of some chemical analytes. To minimize any alteration in the concentration of chemical substances due to the ingestion of food or daytime variation, most blood samples are best collected at the beginning of the day before a patient takes food.

URGENT SAMPLES

All samples should reach the laboratory as soon as possible after collection. For some samples, timing is critical due to the severity of a patient's condition or to ensure the viability of pathogens. Samples that require attention with the minimum of delay include:

- blood samples for urgent compatibility testing, measurement of blood glucose, sodium and potassium, or haemoglobin (in an anaemic child),
- CSF for the detection of trypanosomes or bacteria that cause meningitis,
- blood cultures to diagnose septicaemia,
- malaria blood films particularly from children and pregnant women,
- dysenteric and “rice-water” faecal samples,
- wet preparations such as aspirates or exudates,
- samples to investigate a disease outbreak.

CORRECT PROCEDURE CORRECT CONTAINER

The correct collection of samples depends on:

- those responsible for the collection of samples following standardized procedures,
- patients receiving clear instructions on how to collect a sample correctly and safely using the container provided.

A strictly aseptic technique must be followed when collecting samples for culture from sites that are normally sterile, e.g. blood, CSF, effusions. Contaminating organisms in a sample can lead to difficulties when interpreting cultures and delays in issuing reports.

When collecting venous blood it is important to use a technique which avoids haemolysis of the sample.

Avoiding haemolysis

The haemolysis (rupture) of red cells can be a serious source of unreliable test results. If red cells are haemolyzed, substances from the cells are released into the serum or plasma leading to a false increase in the concentration of analytes, e.g. potassium. Haemolysis also interferes with many chemical reactions.

When using a vacuum-tube blood collection system, haemolysis is minimized. When using a syringe and needle, haemolysis can be avoided by:

- Not using a needle with too fine a bore.
- Not withdrawing the blood too rapidly or moving the needle once it is in the vein.
- Removing the needle from the syringe before dispensing the blood into the sample container. Allow the blood to run gently down the inside wall of the container.
- Adding the correct amount of blood to anticoagulant. Do not shake the blood but gently mix it with the anticoagulant.
- Allowing sufficient time for blood to clot and clot retraction to take place. Red cells are easily haemolyzed by the rough use of an applicator stick to dislodge a clot.

Haemolysis can also be caused by storing whole blood samples next to the freezing compartment of a refrigerator or centrifuging blood samples for longer than is necessary.

Use of Vacutainers (Vacuum blood containers)

Single use *Vacutainers* containing a range of anticoagulants and collecting different volumes of blood are being used increasingly to collect blood samples due to their safety and ease of use. A *Vacutainer* uses a double ended needle. In use, the *Vacutainer* tube is inserted into its holder, its rubber cap is punctured by the inner needle and the vacuum in the tube pulls blood into the tube through the needle inserted in the patient's vein. Several blood samples can be collected using a single venepuncture and the risks from blood transfer are avoided. They are however expensive. *Vacutainers* containing a clot activator and gel for serum separation are also available to obtain serum rapidly, e.g. for emergency compatibility testing.

Order of collecting samples when using vacuum-tubes

The order in which samples are collected is important to minimize contamination of sterile samples and avoid incorrect test results from anticoagulant carry-over between tubes. First collect sterile samples (e.g. blood cultures), followed by a sample in a gel separator/clot inactivator tube, sodium citrate tube, lithium heparin tube, EDTA tube and lastly, fluoride containing tube. *NEVER* pour blood from one vacuum tube to another.

Clotted blood samples: Vacuum-tubes are available with a clot activator (cap coloured yellow) or without a clot activator (cap coloured brick-red). When not using vacuum-tubes, blood samples for serum are best collected into glass tubes. Avoid using plastic tubes because blood takes much longer to clot and clot retraction is poor.

Sample containers

Containers for collecting samples must be clean, dry, leak-proof and easy to open (avoid using snap-closing containers). A sterile container is required when samples are to be cultured. For faeces and sputum, containers need not be sterile. For these samples, plastic containers are preferable as these can be disposed of easily. Glass containers should be used when collecting blood for serum.

The following anticoagulants, preservatives, stabilizers, and transport media may be used in containers:

- Ethylenediamine tetra-acetic acid (EDTA) which is used for measuring haemoglobin and other analytes requiring whole blood, also for lipids, lipoproteins and red cell enzyme analysis. It is used when making blood films because it preserves cell morphology. The cap of vacuum-tubes is coloured pink-mauve. EDTA containers can also be prepared in the laboratory (Reagent No. 17).
- Lithium heparin which is a general anticoagulant for clinical chemistry tests because it does not interfere with most chemical reactions. It is used particularly when measuring electrolytes and enzymes and to obtain plasma for urgent tests (excluding glucose). The cap of vacuum-tubes containing lithium heparin is coloured green. Tubes are available with or without a gel barrier.
- Citrated sodium fluoride* which is used occasionally when measuring blood glucose to prevent glycolysis when it is not possible to separate the plasma from cells within 30 minutes of collecting the blood.

* Fluoride oxalate by itself has been shown to be a poor inhibitor of glycolysis, failing to prevent glycolysis for the first 3–4 hours after blood is collected. Citrated sodium fluoride is an effective inhibitor of glycolysis, e.g. *Terumo Venosafe Glycemia* tubes, *Sarstedt S-Monovette* and *GlucoExact* tubes.

Note: The author has not been able to locate a formula for preparing citrated sodium fluoride.

- Sodium citrate 32g/L, an anticoagulant used in blood coagulation tests. Obtain ready-prepared containers or prepare as described in Reagent No. 39.

Note: Blood samples collected into anticoagulant require gently inversion about six times to prevent clotting.

- Cary-Blair transport medium, used to maintain the viability of faecal pathogens such as *Salmonella*, *Shigella*, and *Campylobacter* species and *V. cholerae*, *Y. enterocolitica* for up to 48h (6h for *Campylobacter*) and also *Y. pestis* in throat swabs and sputum. Obtain as ready-prepared medium or prepare as described in Reagent No. 13.

Note: If bubonic or septicaemic plague is suspected, lymph gland aspirates or blood should be inoculated into blood culture bottles, e.g. containing tryptone soya broth, kept at 25–30 °C to reach the Referral laboratory within 24 hours. Follow safety procedures for HIGH RISK samples.

Alkaline peptone water can also be used for *V. cholerae* (see following text).

- Alkaline peptone water, used as an enrichment and transport medium for *V. cholerae* in faecal samples (to reach the laboratory within 10h). Obtain as ready-prepared medium or prepare as described in Reagent No. 5.
- Amies transport medium, an efficient medium for transporting urethral, endocervical and vaginal swabs to ensure the survival of *N. gonorrhoeae*. Samples transported in a cool box should reach the laboratory within 24 h. Obtain as ready-prepared medium or prepare as described in Reagent No. 6.

Note: If anthrax is suspected, Amies transport medium can also be used to maintain the viability of *B. anthracis* (swabs of exudate, sputum, CSF, faeces). Follow safety procedures for HIGH RISK samples.

- Trans-isolate medium (TIM), used as a transport medium to maintain the viability of pathogens in CSF. Obtain ready-prepared medium from a referral laboratory.
- Alcohol-fixative, used to fix cervical smears for Papanicolaou staining to be examined for malignant cells. Obtain as ready to use *Cytofix*.
- Formol saline, used as a tissue fixative. Prepare as described in Reagent No. 21.

Note: Preservatives that contain formaldehyde solution such as formol saline and merthiolate iodone formaldehyde (MIF) must not be used for microbiology samples because formaldehyde kills living organisms.

HIGH RISK SAMPLES

A sample is regarded as HIGH RISK (danger of infection) when it is known or suspected to contain highly infectious pathogens based on National guidelines.

Both the sample and the request form should be labelled HIGH RISK or marked with a designated Biohazard label, indicating that the sample should be handled and processed with extra care, e.g. use of protective gloves, protective gown, eye protection and face mask and when available, a biological safety cabinet.

HIGH RISK samples include:

- sputum which may contain *M. tuberculosis*,
- faeces which may contain *V. cholerae*, *S. Typhi*, *E. coli* 0157 or *S. dysenteriae*,
- fluid from ulcers, pustules or discharging lesions that may contain anthrax bacilli, treponemes, *Leishmania* amastigotes or *Y. pestis*,
- blood samples that may contain HIV, hepatitis B and C viruses, trypanosomes, *Brucella* species, *E. granulosus*, *Histoplasma* species, and filoviruses causing haemorrhagic fever (requiring special protective measures as directed by health authorities).

Immediately after collection, HIGH RISK samples should be labelled and sealed inside a resealable plastic bag or in a container with a tight-fitting lid. The request form must not be placed in with the sample. Use triple packaging to transport samples (see following text).

Important: District laboratories should follow the procedures provided by the district communicable diseases officer and Public Health Reference Laboratory for the management of HIGH RISK samples.

Transporting samples

Use a triple packaging system for the safe and secure transport of samples:

- 1 Check the sample is correctly labelled, the container is tightly closed and the cap is *not leaking*. Wrap each container in sufficient absorbent material to absorb the sample should the container break. The wrapped samples are referred to as the primary container.
- 2 Place the wrapped samples in a plastic carton or resealable bag, i.e. secondary container. Ensure there is sufficient packaging material around the samples to prevent them moving in the carton or bag. Place the request forms in a resealable plastic bag.
- 3 Place the secondary container and request forms in a rigid, leak-proof plastic box with tight fitting lid, i.e. the tertiary container. For items requiring cold transportation, place the tertiary container in a cool box or polystyrene carton containing ice packs. Whole blood samples must not be frozen.
- 4 Label the tertiary container with the name and address of the receiving and sending laboratory. Attach a "Handle with Care – Pathological Sample" label and a Biohazard Infectious Substance label (see page 32).
- 5 Use the fastest and most reliable system for transporting the samples.
- 6 Keep a record of the samples despatched and record the date when reports are received back.

Transporting samples using local postal services

The postal system is appropriate for sending samples such as formol saline preserved biopsies or fixed smears to a histology laboratory. Always follow National postal regulations which apply to the mailing of *Biological infectious substances*.

Transporting samples by air, sea or rail

Strict regulations apply to the transporting of infectious samples by these routes both nationally and internationally. The regulations are based on the category of substances being transported. Category A contains infectious substances

capable of causing permanent disability or life-threatening or fatal diseases to humans and animals. Category A infectious substances relevant to humans are labelled UN 2814. Category B includes infectious substances not included in Category A and are labelled UN 3373.

Full details of the regulations covering package marking, and transportation of both categories can be found in the WHO publication *Guidance on regulations for the transport of infectious substances, 2015–2016*. WHO reference number WHO/HSE/GCR 2015.2. It can be downloaded from the WHO website www.who.int Use the Search facility to locate the publication.

RECOMMENDED READING

Guidelines on Specimen Collection, Storage and Transport. 2nd ed 2019, Materu S, Dena R, Odhiambo N, Carter J. Amref International University, Nairobi, Kenya

E-mail: enquiry@amref.ac.ke

The Guidelines focus on the investigation of bacterial, viral and parasitic diseases, and outbreak situations, for which samples need special care. Samples include blood, stool, urine, sputum, body fluids including CSF, swabs, biopsies, cytological smears, food and water.

LABORATORY REQUEST FORM

The laboratory request form should be dated and provide the following information:

- Patient's full name, age, and gender.
- Address or village of patient
- Inpatient or outpatient registration number.
- Relevant clinical information regarding patient's condition and suspected diagnosis.
- Details of drugs or local medicines taken by or administered to the patient before visiting the health unit or hospital, and medication administered by the health unit or hospital prior to collecting the sample, e.g. antimicrobials, antimalarial drugs.
- Specific test(s) required.
- Sample provided, date and time collected.
- Origin of request if from an outreach health centre or maternity unit.
- Name and signature of the requesting officer, community health worker, or midwife to whom the report should be sent.

Urgent tests: Only those tests should be requested urgently that are required for the immediate care of a patient or to manage a serious public health situation.

Note: The sample container must be clearly labelled with the patient's name, registration number, date and time of collection and initials of the person who has collected the sample.

Laboratory Request Form for samples collected during a disease outbreak

The following information is required:

- Patient's full name, age, gender and registration number.
- Village and location, places visited in the previous 2 weeks, details of contacts.
- Description and duration of symptoms and suspected diagnosis.
- Details of vaccinations and any antimicrobials taken.
- Results of previous tests.
- Investigations required.
- Sample (s) provided, date and time of collection, name of person authorising and collecting the sample and to whom test results should be sent.
- Any other information requested by the Public Health Laboratory and District Communicable Diseases Officer.

Checking samples

Before samples reach the laboratory, those responsible for collecting samples must check that every sample is labelled clearly with the patient's name and registration number and the name and number agree with what are written on the request form. Clerical mistakes can lead to serious errors and cause delays in the testing of samples and subsequent treatment of patients.

A sample should be rejected by the laboratory if:

- It is unlabelled or the identity on the form does not match that on the sample.
- It is not correct for the test being requested or it has been collected in the wrong container, e.g. containing an incorrect anticoagulant.
- The sample container is leaking or there is evidence of contamination.
- A blood sample appears haemolyzed or an anticoagulated sample contains clots.
- There is too long a delay in the sample reaching the laboratory or it has not been transported correctly.

When a sample is rejected the requesting officer should be notified at the earliest opportunity with the reason for rejection and arrangements made for a repeat sample.

COLLECTION AND DISPATCH OF SAMPLES FOR SOME CLINICAL CHEMISTRY TESTS

The following chart gives information regarding the collection of samples and stability of substances for biochemical analysis. Although guidelines are also provided regarding the referral and dispatch of samples, each laboratory must obtain detailed instructions from its own Referral Laboratory. If special sample containers are required, these should be obtained from the Referral Laboratory. Where an automated analyzer is used, follow the manufacturer's instructions regarding the collection of individual samples.

Notes

- 'Room temperature' refers to 20–28 °C.
- The term 'kept cool' refers to the transportation of samples in an insulated flask or box that contains a freezer pack or ice cubes.

Sterile container: When a blood sample is likely to take longer than 24 h to reach its destination, to reduce the risk of bacterial contamination, collect the sample into a sterile container. Aseptically transfer the plasma or serum to a sterile tube or bottle for transport.

Note: *Vacuutainer* tubes are sterile.

BLOOD SAMPLES

Test	Sample for referral	Container	Stability
Alanine aminotransferase (ALT)	3–5 mL clotted blood. Haemolysis interferes with test. <i>Referral:</i> Send 1 mL serum, kept cool, to reach destination within 24 h.	Dry container.	<i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Albumin	2 mL clotted blood. Avoid venous stasis. Haemolysis interferes with test. <i>Referral:</i> Send 0.5 mL serum to reach destination within 72 h	Dry container.	<i>Sterile serum:</i> Stable up to 4 weeks at 4–8 °C and 1 year at –20 °C.
Alcohol (Ethanol)	Contact Referral Laboratory for information on sample required.	Container from Referral Laboratory	Contact Referral Laboratory for stability information.
Alkaline phosphatase	see Phosphatase, alkaline		
<i>alpha</i> Amylase	3–5 mL clotted blood. <i>Referral:</i> Send 1–2 mL serum kept cool, to reach destination within 24 h.	Dry container.	<i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Aspartate aminotransferase (AST)	3–5 mL anticoagulated blood. Haemolysis interferes with test.	Preferably lithium heparin container	<i>Sterile plasma:</i> Stable up to 7 days at 2–8 °C.

BLOOD SAMPLES

Test	Sample for referral	Container	Stability
Bicarbonate	see Carbon dioxide		
Bilirubin	3–5 mL clotted blood. <i>Infants:</i> 1–2 mL anticoagulated Haemolysis interferes with test. <i>Referral:</i> Send 0.5–1.0 mL serum or plasma, kept cool and protected from light, to reach destination within 6 h.	Dry container. <i>Infants:</i> Lithium heparin or EDTA container.	Protect from light. <i>Sterile serum/plasma:</i> Stable up to 4 days in the dark at 2–8 °C.
Calcium, total	1–2 mL clotted blood. Collect fasting blood and avoid venous stasis. Patient must not be receiving EDTA therapy. <i>Referral:</i> Send 0.5 mL serum kept cool, to reach destination within 48 h.	Dry container which must be chemically clean if not purchased from a manufacturer.	<i>Sterile serum:</i> Stable up to 3 weeks at 2–8 °C.
Carbon dioxide (biocarbonate) Blood pH PCO ₂	5 mL anticoagulated blood. Perform venepuncture with great care. Avoid introducing air bubbles into sample.	Lithium heparin.	Very poor stability. Plasma must be separated from cells and analyzed as soon as possible (within 2 hours).
Cholesterol, total	3–5 mL clotted blood. <i>Also:</i> 2.5 mL of blood into EDTA. <i>Referral:</i> Send 0.5–1.0 mL serum and 2.5 mL EDTA blood to reach destination within 72 h.	Dry container.	Separate serum from cells as soon as possible. <i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Cholesterol, HDL	3–5 mL clotted blood	Dry container	As above
Cholinesterase	1–2 mL clotted blood. <i>Referral:</i> Send 0.2–0.5 mL serum to reach destination within 24 h.	Dry container.	<i>Sterile serum:</i> Stable for 7 days at 2–8 °C or for 6 months if stored frozen.
Creatinine	2–3 mL clotted blood. Haemolysis interferes with test (depending on method). <i>Referral:</i> Send 0.5–1.0 mL serum kept cool, to reach destination within 18 h.	Dry container.	Stable in serum at 2–8 °C up to 24 h. <i>Sterile serum:</i> Stable for up to 7 days at 2–8 °C.

BLOOD SAMPLES

Test	Sample for referral	Container	Stability
Electrolytes: Sodium Potassium	5–7 mL clotted blood or 5 mL anticoagulated blood (not EDTA). Haemolyzed blood cannot be used. Do not collect blood from an arm receiving an IV infusion. <i>Referral:</i> Send about 1 mL serum or plasma, kept cool, to reach destination within 12 h.	Dry container or lithium heparin container.	Separate serum/plasma from cells within 1 hour. Do not refrigerate sample before removing serum or plasma. Stable in serum or plasma at 2–8 °C up to 24 h.
Glucose	1 mL anticoagulated blood (separate plasma from cells within 30 minutes). <i>Referral:</i> Send 0.5 mL plasma from citrated sodium fluoride blood, kept cool, to reach destination within 36 h.	Citrated sodium fluoride (see page 325).	<i>Sterile plasma</i> from citrated sodium fluoride blood, stable at 2–8 °C for 7 days.
HbA1c	See Section 3.2		
Iron Total iron-binding capacity (TIBC)	10–12 mL clotted blood. Collect at the beginning of the day. Haemolyzed samples cannot be used. <i>Referral:</i> Send at least 4 mL serum, kept cool, to reach destination within 18 h.	Dry preferably plastic container. Must be chemically clean if not purchased from a manufacturer.	<i>Sterile serum:</i> Stable for up to 3 weeks at 2–8 °C.
Lactate dehydrogenase (LDH)	3–5 mL anticoagulated blood. Blood which is haemolyzed cannot be used. <i>Referral:</i> Send 1–2 mL plasma to reach destination within 48 h.	Lithium heparin.	Unstable in whole blood. <i>Sterile plasma:</i> Stable for up to 4 days at 2–8 °C.
Phosphatase, alkaline	3–5 mL anticoagulated blood. Haemolysis interferes with test. <i>Referral:</i> Send 1 mL plasma to reach destination within 24 h.	Lithium heparin.	<i>Sterile plasma:</i> Stable for up to 7 days at 2–8 °C.
Phosphate	3–5 mL anticoagulated blood. Haemolyzed blood cannot be used. <i>Referral:</i> Send 1 mL plasma to reach destination within 24 h.	Lithium heparin.	Unstable in whole blood. <i>Sterile plasma:</i> Stable up to 4 days at 2–8 °C.

BLOOD SAMPLES

Test	Sample for referral	Container	Stability
Potassium	see Electrolytes		
Protein, total	2 mL clotted blood. Avoid venous stasis. Haemolysis interferes with test. <i>Referral:</i> Send 0.5 mL serum to reach destination within 24 h.	Dry container.	<i>Sterile serum:</i> Stable up to 4 weeks at 4–8 °C and 1 year at –20 °C.
Protein, electrophoresis: Paraproteins	3–5 mL clotted blood. <i>Referral:</i> Send 1 mL serum, kept cool, to reach destination within 8 h.	Dry container.	<i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Sodium	see Electrolytes		
Thyroxine (T ₄) and TSH	5 mL clotted blood.	Dry container.	<i>Sterile serum:</i> Stable up to 3 days at 2–8 °C.
Triglycerides (Triacylglycerols)	3–5 mL clotted blood. Also: 2.5 mL of blood into EDTA. <i>Referral:</i> Send 1 mL serum and 2.5 mL EDTA blood, kept cool, to reach destination within 48 h.	Dry container.	As for Cholesterol.
Urate (Uric acid)	5 mL clotted blood. <i>Referral:</i> Send 1–2 mL serum to reach destination within 72 h.	Dry container.	Protect from daylight. <i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Urea	1–2 mL clotted blood. Plasma from EDTA blood can also be used. <i>Referral:</i> Send 0.2–0.5 mL serum to reach destination within 48 h.	Dry container.	<i>Sterile serum:</i> Stable up to 7 days at 2–8 °C.
Bence Jones protein	10–20 mL midstream first morning urine. <i>Referral:</i> For protein electrophoresis, send urine (and serum sample) kept cool, to reach destination within 6 h.	Dry container.	Stable for up to 1 month at 2–8 °C.

URINE SAMPLES			
Test	Sample for referral	Container	Stability
Albumin (microalbumin)	5–10 mL	Dry container.	Stable for up to 7 days at 2–8 °C.
Bilirubin	5–10 mL midstream urine.	Dry container.	Stable for a few hours at room temperature if protected from light.
Calcium	24 hour urine. <i>Referral:</i> Measure volume of urine, mix, and send 5 mL aliquot, kept cool, to reach destination within 6 h.	Large bottle containing 20 mL HCl, 6 mol/L.	Stable for up to 4 days at 2–8 °C.
Creatinine	5–10 mL urine.	Dry container.	Stable for up to 4 days at 2–8 °C.
Glucose	5–10 mL midstream urine.	Dry container.	Test as soon as possible after collection.
Haemoglobin	5–10 mL midstream urine. <i>Referral:</i> Send urine, kept cool, to reach destination within 6 h.	Dry container.	Stable at room temperature for a few hours.
Ketones	5–10 mL midstream urine.	Dry container.	Test as soon as possible after collection.
Morphine and other opium-containing compounds	20–30 mL urine. <i>Referral:</i> Send urine, kept cool to reach destination within 6 h.	Dry container.	Contact Referral Laboratory for stability information
pH	3–5 mL midstream urine.	Dry container.	Test as soon as possible after collection.
Phosphate	24 hour urine.	Large bottle containing 20 mL HCl, 6 mol/L.	As for amino acids.
Protein, semi-quantitative:	10–20 mL midstream urine.	Dry container.	Stable for up to 7 days at 2–8 °C.
quantitative:	24 hour urine. <i>Referral:</i> Send 50 mL well-mixed aliquot, kept cool to reach destination within 6 h.	Large dry container.	Measure volume of urine.
Urobilinogen	5–10 mL midstream urine.	Dry container.	Test as soon as possible after collection.

FAECAL SAMPLES

Occult blood See Section 3.2

CEREBROSPINAL FLUID

Glucose	1 mL CSF	Dry container (sterile, if CSF for culture).	Examine with the minimum of delay.
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Protein, total	1 mL CSF	Dry container (sterile, if CSF for culture).	Examine with the minimum of delay.
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Note: If only a small CSF sample is available, this should be used for Gram staining, cell counting and culture.

Postal regulations for transport of samples

In most countries there are regulations regarding the sending of pathological samples through the post. Each laboratory should therefore obtain a copy of these postal regulations from its local post office. The regulations usually state that:

- A sample must be sent using triple packaging (see page 326).
- All sample packages must be clearly addressed and marked with the words: 'HANDLE WITH CARE – PATHOLOGICAL SAMPLE'. Attach a Biohazard label.
- Strict regulations apply when sending samples by air, sea or rail, see previous text.

Appendix V

**Examination of faeces for parasites
Faecal concentration techniques**

Examination of faeces for parasites

Reasons for examining faecal samples for parasites

The examination of faecal samples for parasites is requested:

- To identify the parasitic causes of blood and mucus in faeces and differentiate amoebic dysentery from bacillary dysentery.
- To identify intestinal parasitic infections that require treatment, i.e. those associated with serious illhealth, persistent diarrhoea, weight loss, intestinal malabsorption and the impairment of development and nutrition in children.
- To identify chronic significant infections that if not treated can lead to serious complications developing later in life, e.g. intestinal schistosomiasis leading to portal hypertension, or chronic *O. viverrini* infection leading to cancer of the bile duct.
- To detect serious hookworm infection in patients with iron deficiency anaemia, especially in a child or pregnant woman.

Heavy worm infections

Although hookworms and most other nematodes, flukes and tapeworms do not multiply in humans, moderate and heavy infections can occur when a person becomes infected with a heavy parasitic load and reinfected frequently. Symptoms in children tend to be more severe and may occur with lower worm loads, particularly when there is undernutrition or co-existing illhealth.

- To assist in the surveillance and control of local parasitic infections caused by geohelminths (soil transmitted nematodes), and helminths transmitted by the ingestion of infected meat, freshwater fish, crabs, or shell-fish.

Importance of infection rates

In the control and management of parasitic helminth infections, it is important to know prevalence rates (proportion of those infected with a parasite at any one time), ages, and groups of those infected. When prevalence of infection is low, the mean worm burden is usually low and heavy infections are rare, whereas with high prevalence rates (e.g. over 50%) the worm burden increases and with it the severity of infection. A knowledge of prevalence rates of geohelminths provides information on the degree of faecal contamination of the environment.

Collection of faecal samples for parasitic examination

For clinical purposes, a fresh faecal sample is required. It should be uncontaminated with urine and collected into a suitable size, clean, dry, leak-proof container. The container need not be sterile but must be free of all traces of antiseptics and disinfectants. A large teaspoon amount of faeces is adequate or about 10 mL of a fluid sample. Several samples collected on alternate days may be required to detect parasites that are excreted intermittently, e.g. *Giardia*.

Important: Dysenteric and watery samples must reach the laboratory as soon as possible after being passed (within 15 minutes) otherwise motile parasites such as *E. histolytica* and *G. intestinalis* trophozoites may not be detected. Other samples should reach the laboratory within 2 hours of being collected. Samples must be labelled correctly and accompanied by a correctly completed request form.

Public health faecal samples. The Public Health Laboratory should provide the district laboratory with containers and detailed instructions on the method of collecting samples. The collection technique will depend on the parasite being investigated, the method that will be used to examine the faeces and whether any fixative or preservative is required.

Caution: Faeces like other samples received in the laboratory, must be handled with care to avoid acquiring infection from infectious parasites, bacteria, or viruses. Faeces may contain:

- infective forms of parasites such as *S. stercoralis*, *E. vermicularis*, *T. solium*, *G. intestinalis*, *E. histolytica*, or *Cryptosporidium*,
- bacteria such as *V. cholerae*, *Shigella* or *Salmonella* species,
- viruses including hepatitis viruses, HIV, viral haemorrhagic fever viruses, and rotaviruses.

ROUTINE EXAMINATION OF FAECES FOR PARASITES

Routinely faecal samples are examined in district laboratories by direct technique. This involves:

- Reporting the appearance of the sample and any parasitic worms or tapeworm segments.
- Examining the sample microscopically for:
 - motile parasites such as the larvae of *S. stercoralis* and trophozoites of *E. histolytica*, *G. intestinalis*, and more rarely, *B. coli*,
 - helminth eggs,
 - cysts and oocysts of intestinal protozoa.

Reporting the appearance of faecal samples

Report the following:

- Colour of the sample and consistency, i.e. whether formed, semiformed, unformed, watery.
- Presence of blood, mucus, and/or, pus. If blood is present note whether this is mixed in the faeces. If only on the surface this indicates rectal or anal bleeding.
- Whether the sample contains worms, e.g. *A. lumbricoides* (large roundworm), *E. vermicularis* (threadworm) or tapeworm segments, e.g. *T. solium*, *T. saginata*, *T. asiatica*.

Blood and mucus in faeces

Blood and mucus may be found in faeces from patients with amoebic dysentery, intestinal schistosomiasis, invasive balantidiasis (rare infection), and in severe *T. trichiura* infections. Other non-parasitic conditions in which blood and mucus may be found include bacillary dysentery, *Escherichia coli* dysentery, *Campylobacter* enteritis, ulcerative colitis, intestinal tumour, and haemorrhoids.

Presence of pus

This can be found when there is inflammation of the intestinal tract. Many pus cells can be found in faecal samples from patients with bacillary dysentery. They can also be found in amoebic dysentery but are less numerous.

Pale coloured samples

Pale coloured and frothy samples (containing fat) can be found in giardiasis and other infections associated with intestinal malabsorption. Pale coloured faeces (lacking stercobilinogen) are also excreted by patients with obstructive jaundice.

Microscopical examination of faecal samples

Examine immediately those samples containing blood and mucus and those that are unformed because these may contain motile trophozoites of *E. histolytica* or *G. intestinalis*.

Examination of dysenteric and unformed samples

- 1 Using a wire loop or piece of stick, place a small amount of sample, to include blood and mucus on one end of a slide. Without adding saline, cover with a cover glass and using a tissue, press gently on the cover glass to make a thin preparation.
- 2 Place a drop of eosin stain (Reagent No. 18) on the other end of the slide. Mix a small amount of the sample with the eosin and cover with a cover glass.

Value of eosin: Eosin does not stain living trophozoites but provides a pink background which can make them easier to see.

- 3 Examine immediately the preparations microscopically, first using the 10× objective with the condenser iris *closed sufficiently* to give good contrast. Use the 40× objective to identify motile trophozoites, e.g. *E. histolytica* amoebae or *G. intestinalis* flagellates (see Section 2.17).

Note: Samples containing the eggs of *Schistosoma* species and *T. trichiura* and the trophozoites of *B. coli* can also contain blood and mucus.

Examination of semi-formed and formed faeces

- 1 Place a drop of fresh physiological saline (Reagent No 34) on one end of a slide and a drop of Dobell's iodine (Reagent No 16) on the other end.

To avoid contaminating the fingers and stage of the microscope, do not use too large a drop of saline or iodine.

- 2 Using a wire loop or piece of stick, mix a small amount of sample, about 2 mg, (matchstick head amount) with the saline and a similar amount with the iodine. Make smooth thin preparations. Cover each preparation with a cover glass.

Important: Sample from different areas in and on the sample or preferably mix the faeces before sampling to distribute evenly any parasites in the sample. Do not use too much sample otherwise the preparations will be too thick, making it difficult to detect and identify parasites.

- 3 Examine systematically the entire saline preparation for larvae, ciliates, helminth eggs, cysts and oocysts. Use the 10× objective with the condenser iris *closed sufficiently* to give good contrast.

Use the 40× objective to assist in the detection and identification of eggs, cysts and oocysts. Always examine several microscope fields with this objective before reporting 'No parasites found'.

- 4 Use the iodine preparation to assist in the identification of cysts (see Section 2.17).
- 5 Report the number of larvae and each species of egg found in the entire saline preparation as follows:

Scanty	1–3 per preparation
Few	4–10 per preparation
Moderate number	11–20 per preparation
Many	21–40 per preparation
Very many	over 40 per preparation

Note: Plate App. 1 shows the relative sizes of helminth eggs and a *Strongyloides* larva in a saline preparation as seen in a microscope field viewed with the 10× objective and 10× widefield eyepiece. Plate App. 2

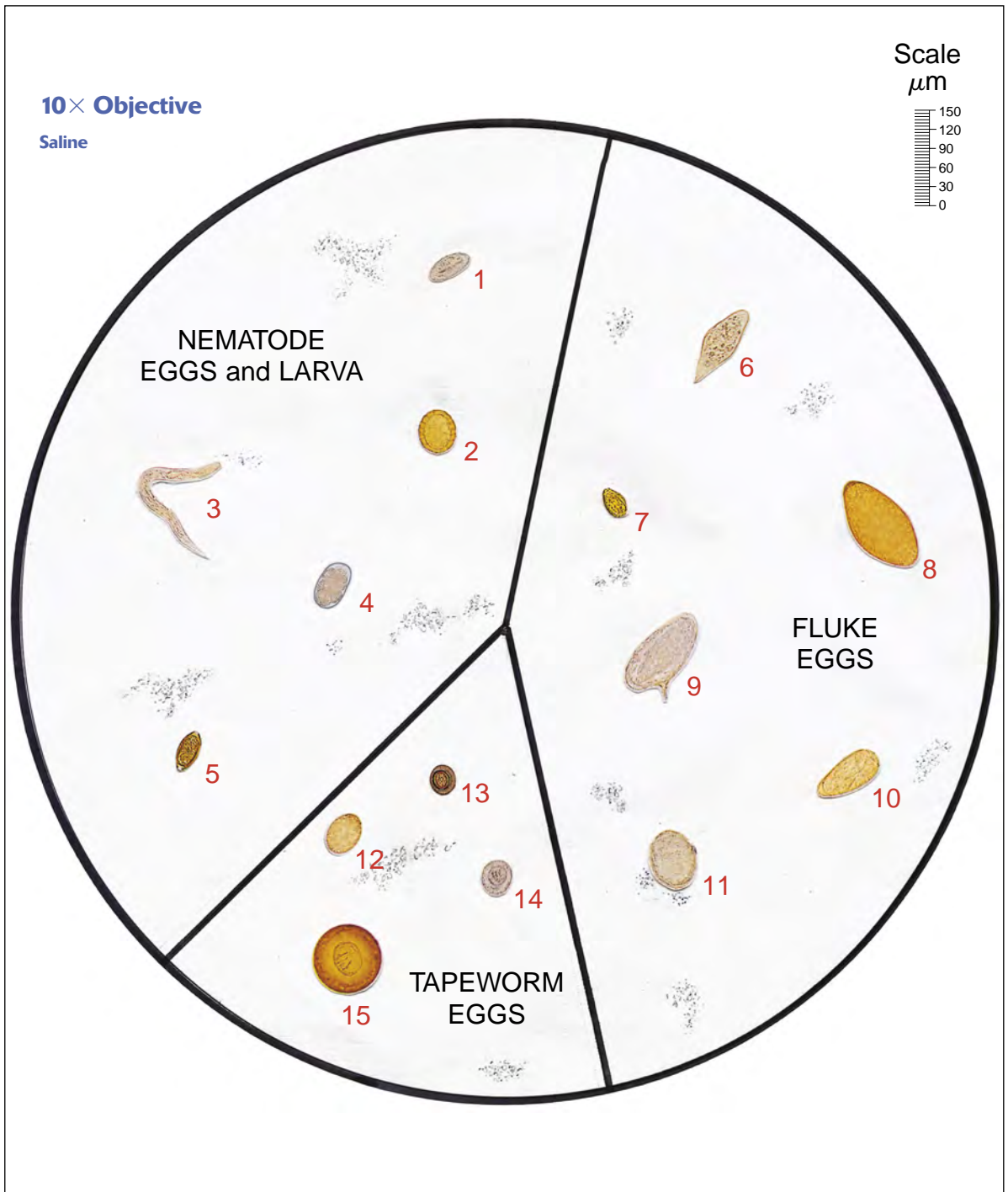


Plate App. 1 Relative sizes of helminth eggs as seen in microscope field using the 10× objective (with 10× eye-pieces). Eggs are as seen in a saline preparation.

1. *E. vermicularis*, 2. *A. lumbricoides*, 3. *S. stercoralis* larva (motile), 4. Hookworm, 5. *T. trichiura*, 6. *S. intercalatum*, 7. *C. sinensis*, 8. *Fasciola* sp, 9. *S. mansoni*, 10. *Paragonimus* sp, 11. *S. japonicum*, 12. *D. latum*, 13. *Taenia* sp, 14. *H. nana*, 15. *H. diminuta*.

shows the relative sizes of protozoan trophozoites, cysts, and oocysts in saline and iodine when viewed with the 40× objective and 10× eyepiece.

Identification of larvae: In a fresh faecal sample, *S. stercoralis* is the only larva that will be found. It can be easily detected in a saline preparation by its motility and large size. It is described in Section 2.5. If the sample is not fresh, *S. stercoralis* will require differentiation from hookworm larvae (see Section 2.5).

Identification of helminth eggs: Eggs are recognized by their:

- size,
- colour (colourless, pale yellow, brown),
- morphological features.

Note: Some helminths also have a limited geographical distribution.

Identification of intestinal flagellates: The trophozoite of *G. intestinalis* and its differentiation from non-pathogenic flagellates that can be found in faeces is described in Section 2.17.

Identification of cysts and oocysts: In saline and eosin preparations, protozoan cysts can be recognized as refractile bodies (shine brightly when focused). Cysts can be identified by their shape, size, nuclei, and inclusions as seen in an iodine preparation (see Section 2.17).

Iodine stains nuclei and glycogen but not chromatoid bodies. Burrows stain or Sargeant's stain can be used to stain chromatoid bodies (see 2.17).

The cysts of *E. histolytica*/*E. dispar*/*E. moshkovskii* and those of *G. intestinalis* and their differentiation from non-pathogenic or other species are described in 2.17. Identification of the oocysts of *C. belli*, *Cryptosporidium*, and *C. cayetanensis* are described in 2.17.

Faecal leucocytes: The presence of pus cells in faeces is mainly associated with inflammatory diarrhoea caused by bacteria.

Non-parasitic structures found in faeces

Care must be taken not to report as parasites those structures that can be normally found in faeces such as muscle fibres, vegetable fibres, starch cells (stain blue-black with iodine), pollen grains, fatty acid crystals, soaps, spores, yeasts, and hairs (see opposite).

Large numbers of fat globules may be seen in faeces when there is malabsorption. Charcot Leyden crystals (breakdown products of eosinophils) can sometimes be seen in faeces (also in sputum) in parasitic infections. They appear as slender crystals with pointed ends, about 30–40 mm in length as shown opposite.



Hair



Vegetable fibres



Charcot Leyden crystal



Starch cell



Yeast cells and spores



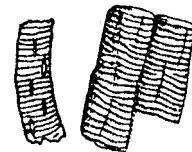
Air Bubbles



Pollen grains and fungal spores



Fat globules



Muscle fibres

Structures found in faeces that required differentiation from parasites.

FAECAL CONCENTRATION TECHNIQUES

The direct examination of faeces is usually adequate to detect significant helminth infections. Important exceptions are *Schistosoma* species because only a few eggs are usually produced even in moderate and severe infections, therefore a concentration technique should be performed when intestinal schistosomiasis is suspected and no eggs are found by direct examination.

Concentration techniques may also be required:

- To detect *Strongyloides* larvae, the eggs of *Taenia* species, cysts of *G. intestinalis*, and to make it easier to detect small parasites, e.g. small fluke eggs.

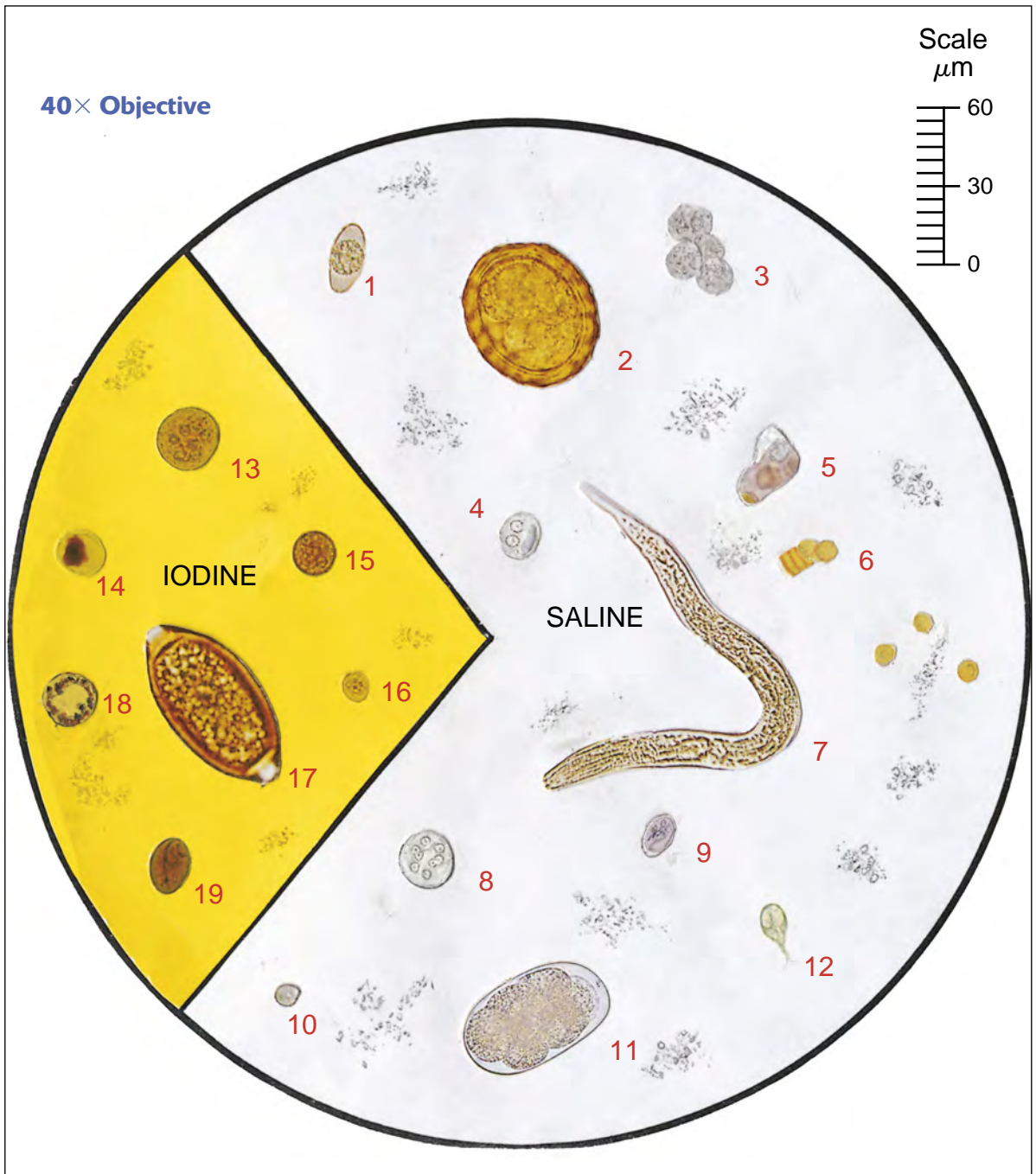


Plate App. 2 Relative sizes of trophozoites and cysts of intestinal protozoa, common nematode eggs and larva of *Strongyloides* as seen in microscope field using the 40× objective (with 10× eyepieces).

Saline preparation: 1. *C. belli* oocyst, 2. *A. lumbricoides* egg, 3. Leucocytes, 4. *E. histolytica*/*E. dispar*/*E. moshkovskii* cyst, 5. *E. histolytica* trophozoite (motile), 6. Red cells, 7. *S. stercoralis* larva (motile), 8. *E. coli* cyst (mature), 9. *G. intestinalis* cyst, 10. *C. mesnili* cyst, 11. Hookworm egg, 12. *G. intestinalis* trophozoite (motile).

Iodine preparation: 13. *E. coli* cyst, 14. *I. buetschlii* cyst, 15. *E. histolytica*/*E. dispar*/*E. moshkovskii* cyst, 16. *E. nana* cyst, 17. *T. trichiura* egg, 18. *Blastocystis hominis*, 19. *G. intestinalis* cyst.

- To check whether treatment has been successful.
- To quantify intestinal parasites.
- To assist in eradication programmes.

The following techniques are commonly used to concentrate faecal parasites in district laboratories:

- Sedimentation techniques in which parasites are sedimented by gravity or centrifugal force, e.g. formol ether/ethyl acetate concentration method which is the most frequently used technique because it concentrates a wide range of parasites with minimum damage to their morphology.
- Flotation (also spelt floatation) techniques in which parasites are concentrated by being floated in solutions of high specific gravity, i.e. solutions that are denser than the parasites being concentrated. Examples include the zinc sulphate method, saturated sodium chloride method and the mini-FLOTAC technique.

For certain parasites and situations, flotation techniques are recommended and can be easily performed in the field with the minimum of equipment, providing adequate health and safety measures are taken.

Note: The techniques that are recommended for the different parasites are shown below.

Faecal concentration techniques

	Formol ether, ethyl acetate	Sat. NaCl ^a	Zn SO ₄ ^b	Mini- FLOTAC
CYSTS				
<i>G. intestinalis</i>	•	–	•	–
<i>E. histolytica</i> / <i>E. dispar</i> / <i>E. moshkovskii</i>	•	–	•	–
OOCYSTS				
<i>C. belli</i>	• ^c	–	–	–
<i>Cryptosporidium</i>	• ^c	–	–	–
<i>C. cayetanensis</i>	• ^c	–	–	–
EGGS				
<i>A. lumbricoides</i>	•	•	–	•
Hookworm	•	•	–	•
<i>T. trichiura</i>	•	–	•	•
<i>Schistosoma</i> species	• ^d	–	–	•
<i>Taenia</i> species	•	–	–	•
<i>C. sinensis</i>	•	–	•	•
<i>O. viverrini</i>	•	–	•	•
LARVAE				
<i>Strongyloides</i>	• ^e	–	–	–

NOTES

- Saturated sodium chloride, specific gravity 1.200.
- Zinc sulphate, 33% v/v, specific gravity 1.180.
- Use formol ether/ethyl acetate oocyst concentration technique.
- Kato-Katz technique can also be used.
- Water emergence technique in which larvae remain motile is also recommended (see Section 2.5).

Formol ether/ethyl acetate concentration technique

This can be used to concentrate most cysts and helminth eggs in fresh and preserved faeces. The use of formalin minimizes the risk of infection from faecal pathogens. To reduce the hazard from using highly flammable diethyl ether, the use of ethyl acetate is recommended. Formol ethyl acetate concentration has been shown to give the best recovery of parasites.¹ When concentrating oocysts, an additional centrifugation stage is recommended.

Principle

Faeces are emulsified in formol water, the suspension is strained to remove large faecal particles, ether or ethyl acetate is added and the suspension is mixed and centrifuged. Cysts, oocysts, eggs and larvae are fixed and sedimented and the faecal debris is separated in a layer between the ether and formol water. Faecal fat is dissolved in the ether or when ethyl acetate is used, the non-ionic detergent Triton-X 100 is added which results in a less dense sediment.

Required

- Formol water, 10% v/v *
* Prepare by mixing 50 mL of strong formaldehyde solution with 450 mL distilled or deionised water.
- Diethyl ether or ethyl acetate
- Triton-X 100, 0.1% v/v if using ethyl acetate *
* Prepare by adding 1mL Triton X 100 to 1 litre of formol water.
- Sieve (strainer) 400–450 µm pore size*
* Using a sieve of higher pore size results in a more dense sediment making microscopical examination difficult.

Procedure

The following is the Ridley technique with recently proposed recommendations for standardization of the procedure.¹

- Using a rod or stick, emulsify an estimated 1 g (pea-size) of faeces in about 4 mL of 10% formol water contained in a screw-cap bottle or tube.

Note: Include in the sample, faeces from the surface and several places in the sample.

- Add a further 3–4 mL of 10% v/v formol water, cap the bottle, and mix well by shaking.
- Sieve the emulsified faeces, collecting the sieved suspension in a beaker.
- Transfer the suspension to a conical (centrifuge) tube made of strong glass, copolymer, or polypropylene. Add 3–4 mL of diethyl ether or ethyl acetate. If using ethyl acetate, add 2 drops of 0.1% Triton-X 100.

Caution: Ether is highly flammable and ethyl acetate is flammable, therefore use well away from an open flame, e.g. flame from a Bunsen burner, or spirit lamp. Ether vapour is anaesthetic, therefore make sure the laboratory is well-ventilated.

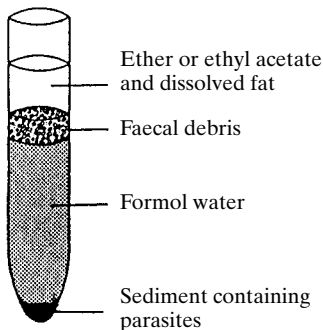
- 5 Stopper* the tube and mix for 1 minute or use a Vortex mixer for about 15 seconds.

* Do not use a rubber bung or a cap with a rubber liner because ether attacks rubber.

- 6 With a tissue or piece of cloth wrapped around the top of the tube, loosen the stopper (considerable pressure will have built up inside the tube).

- 7 Centrifuge immediately at RCF $1\,000\times g$ (3 000 rpm) for 3 minutes.

After centrifuging, the parasites will have sedimented to the bottom of the tube and the faecal debris will have collected in a layer between the ether or ethyl acetate and formol water as shown below.



Formal ether or ethyl acetate concentration technique, after centrifugation.

- 8 Using a stick or the stem of a plastic bulb pipette, loosen the layer of faecal debris from the side of the tube and invert the tube to discard the ether or ethyl acetate, faecal debris, and formol water. The sediment will remain.
- 9 Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Add 3 drops of saline solution. Tap the bottom of the tube to resuspend and mix the sediment. Transfer the sediment to a slide and cover with a cover glass.
- 10 Examine the preparation microscopically using the $10\times$ objective with the condenser iris *closed sufficiently* to give good contrast. Use the $40\times$ objective to examine small cysts and eggs. To assist in the identification of cysts, run a small drop of iodine under the cover glass.

Although the motility of *Strongyloides* larvae will not be seen, the non-motile larvae can be easily recognized.

- 11 If required, count the number of each species of egg in the entire preparation. This will give the approximate number per gram of faeces.

Formol ether or ethyl acetate oocyst concentration technique

Follow steps 1 to 6 of the previous method. Continue as follows:

- 7 Centrifuge immediately at low speed, i.e. RCF $300\text{--}400\times g$ (about 1 000 rpm) for 1 minute.
- 8 Using a plastic bulb pipette or Pasteur pipette, carefully remove the entire column of fluid below the faecal debris and ether or ethyl acetate and transfer this to another centrifuge tube.
- 9 Add formol water to make the volume up to 10–15 mL. Centrifuge at RCF $1\,000\times g$ (3 000 rpm) for 5–10 minutes.
- 10 Remove the supernatant. Tap the bottom of the tube to resuspend and mix the sediment. Transfer the sediment to a slide and examine for oocysts using the $40\times$ objective.

Note: *Cryptosporidium* oocysts are best identified in a modified Ziehl-Neelsen stained smear as described in Section 2.17.

Commercially available faecal parasite concentration kits

Single use faecal parasite kits provide a closed system for concentrating faecal parasites by the formol ethyl acetate technique, eliminating the open tube and filtering process. All the equipment and reagents needed are provided in the kits.

Zinc sulphate flotation technique

The zinc sulphate technique is recommended for concentrating the cysts of *G. intestinalis* and *E. histolytica*/*E. dispar*/*E. moshkovskii*, and the eggs of *T. trichiura*, *C. sinensis*, and *O. viverrini*.

Other nematode eggs are concentrated less well. The technique is not suitable for concentrating eggs or cysts in fatty faeces. Adequate safety precautions should be taken because faecal pathogens are not killed by zinc sulphate.

Principle

A zinc sulphate solution is used which has a specific gravity (relative density) of 1.180–1.200. Faeces are emulsified in the solution and the suspension is left undisturbed for the eggs and cysts to float to the surface. They are collected on a cover glass.

Required

- Zinc sulphate solution, 33% w/v Reagent No 47, specific gravity, 1.180–1.200.

Use a hydrometer to check that the specific gravity of the solution is correct. Adjust with distilled water or more chemical if required.

- Test tube (without a lip) of about 15 mL capacity which has a completely smooth rim.
- Strainer (nylon coffee or tea strainer is suitable).

Procedure

- 1 Fill the tube about one quarter full with the zinc sulphate solution. Add an estimated 1 gram of faeces (or 2 mL if a fluid sample). Using a rod or stick, emulsify the sample in the solution.
- 2 Fill the tube with the zinc sulphate solution and mix well. Strain the faecal suspension to remove large faecal particles.
- 3 Return the suspension to the tube. Stand the tube in a completely vertical position in a rack.
- 4 Using a plastic bulb pipette or Pasteur pipette, add further solution to ensure the tube is filled to the brim.
- 5 Carefully place a clean (grease-free) cover glass on top of the tube. Avoid trapping any air bubbles.
- 6 Leave undisturbed for 30–45 minutes to give time for the cysts and eggs to float.

Note: Do not leave longer because the cysts can become distorted and the eggs will begin to sink.

- 7 Carefully lift the cover glass from the tube by a straight pull upwards. Place the cover glass face downwards on a slide.

Caution: Wear protective gloves. Mature *E. histolytica* and *G. intestinalis* cysts are infective when passed in faeces.

- 8 Examine microscopically the entire preparation using the 10× objective with the condenser iris closed sufficiently to give good contrast. Use the 40× objective, and run a drop of iodine under the cover glass, to identify the cysts.
- 9 Count the number of eggs to give the approximate number per gram of faeces.

Note: Parasites can also be recovered from the surface of the flotation fluid after centrifuging. If however, a centrifuge is available, the safer formol ether or ethyl acetate technique is recommended for concentrating eggs and infective cysts from faeces.

Saturated sodium chloride flotation technique

The saturated sodium chloride technique is a useful and inexpensive method of concentrating hookworm or *Ascaris* eggs, e.g. in field surveys.

Preparation of saturated sodium chloride solution

Stir sodium chloride (e.g. table salt) into hot clean distilled or boiled filtered water until no more can be dissolved. Add a few more grams of the salt so that a layer of the undissolved salt remains in the bottom of the stock container. Mix well and leave the undissolved salt to sediment.

When cool, filter some of the solution and use a hydrometer to check that the specific gravity is 1.200.

Immediately before use, filter the amount of solution required from the stock bottle and recheck the specific gravity.

Procedure

The technique is the same as that described for the zinc sulphate flotation technique except that a saturated solution of sodium chloride is used and a flat bottomed vial (50 mm tall and 20 mm wide) is used instead of a tube. A glass slide is used to recover the eggs instead of a cover glass. Eggs can be recovered after about 20 minutes.

Stoll's technique for counting helminth eggs

- 1 Weigh 3 g of faeces in a screw-cap container. Add 42 mL of water.* If the faeces is formed, use sodium hydroxide 0.1 mol/L (0.1N) solution instead of water.

* **Formol water:** The use of 10% formol water is recommended as this will kill faecal pathogens, making the technique safer.

- 2 Using a rod, break up the faeces and mix it with the water. Cap the container and shake hard to complete the mixing.
- 3 Without delay, transfer 0.15 mL of the suspension and transfer this to a slide. Cover with a long cover glass or if unavailable with two square cover glasses side by side.
- 4 Examine systematically the entire preparation, using the 10× objective with the condenser iris reduced to give good contrast.

Include in the count any eggs lying outside the edges of the cover glass because these are also contained in the 0.15 mL sample.

- 5 Multiply the number of eggs counted by 100 to give an estimate of the number of eggs per gram of faeces.

Note: If the faeces is not formed, the following additional calculation is recommended to give the approximate number of eggs per gram of faeces:

Fluid sample	multiply by 5
Unformed watery sample	multiply by 4
Unformed soft sample	multiply by 3
Semiformed sample	multiply by 2

Kato-Katz technique

In recent years several modified Kato techniques have been developed for the semi-concentration and quantitative estimation of schistosome eggs in

faeces. Such techniques have been shown to be of value in schistosomiasis epidemiology and control work, particularly in areas of moderate to high transmission.

In the Kato-Katz technique faeces are pressed through a mesh screen to remove large particles. A portion of the sieved sample is then transferred to the hole of a template on a slide. After filling the hole, the template is removed and the remaining sample is covered with a piece of cellophane soaked in glycerol (glycerine) malachite green solution. The glycerol 'clears' the faecal material from around the eggs. The malachite green makes the eggs easier to see. The eggs are then counted and the number calculated per gram (g) of faeces.

Note: The following Kato-Katz technique is that recommended by WHO.³

Required

Glycerol-malachite green solution

Prepare as follows:

- 1 Dissolve 1 g malachite green crystals in 100mL distilled water. Transfer to a dark bottle and label 1% malachite green stock solution.
- 2 Make a working solution of glycerol-malachite green by mixing 100mL glycerol with 100 mL distilled water. Add 1 mL malachite green stock solution and mix well. Store in a dark bottle.

Note: If preferred, 0.3% methylene blue can be used instead of 1% malachite green.

- Nylon, plastic or stainless steel screen of 60–105 mesh
- Plastic or cardboard template
- Cellophane (40–50 µm thick) strips measuring 25 × 30 mm or 25 × 35 mm
- Absorbent tissue
- Scrap paper, e.g newspaper
- Wooden applicator stick or plastic spatula

Procedure

- 1 Soak the pieces of cellophane in the glycerol-malachite green solution (or glycerol-methylene blue solution) for at least 24 h before use.
- 2 Wearing protective gloves, transfer about 0.5 g faeces on to a piece of scrap paper. Press the screen on top of the faeces.
- 3 Using an applicator stick or spatula, scrape across the screen to sieve the faeces.
- 4 Place the template on a slide and fill completely

the hole with the sieved faeces, avoiding air bubbles and overfilling.

- 5 Remove carefully the template, taking care not to remove any of the faeces.
- 6 Using forceps, cover the faecal sample with a glycerol-malachite green (or glycerol-methylene blue solution) soaked strip.
Use absorbent paper to wipe clean the upper surface of the cellophane.
- 7 Place a clean slide on top of the preparation and carefully press downwards to spread evenly the faecal sample.
- 8 Leave the preparation until the faecal matter has cleared (usually takes 1–2 h).

If looking also for hookworm eggs, examine the preparation after 30 minutes.

- 9 Examine systematically the entire preparation microscopically for schistosome eggs. Use the 10x objective with the condenser iris closed sufficiently to give good contrast.

Constant focusing is necessary to detect the eggs. Report any other helminth egg present.

- 10 Multiply the number of eggs counted by 24* to give the number of eggs per gram of faeces.

* This figure applies if using a standard 41.7 mg template. If using another size, amend the figure to give eggs/gram.

Mini-FLOTAC

This recently developed flotation test kit detects and quantifies helminth eggs in faeces.

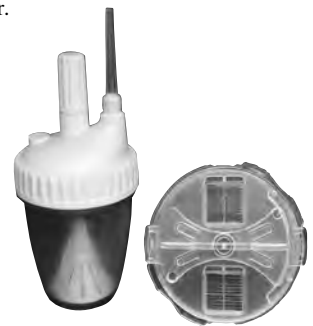
Mini-FLOTAC Test kit

Left: Fill-FLOTAC container.

This is used to emulsify faeces in 5% formalin, mix with the flotation solutions, and sieve the suspension.

Right: Mini-FLOTAC reading disc containing two flotation chambers (each holding 1 mL) and reading grids.

Note: Both items are reusable with careful washing.



Two flotation solutions are used: FS2, a saturated sodium chloride solution of s.g. 1.200 for the recovery of nematode and cestode eggs and FS7 zinc sulphate solution of s.g. 1.350 for the recovery of trematode eggs. Ten minutes are required for

the eggs to float. A plastic microscope adaptor is supplied with the kit for holding the reading disc on the microscope stage.

Several Mini-FLOTAC videos (YouTubes) are available on the internet demonstrating how to use the Mini-FLOTAC kit. Details of the technique can be found on the internet, by entering in Google: Standard Operating Procedures for the Mini-FLOTAC, 2016.

Field studies have shown the Mini-FLOTAC to be a sensitive technique for the recovery of helminth eggs.² Further evaluations are expected.

Availability of Mini-FLOTAC equipment

The Fill-FLOTAC container, Mini-FLOTAC reading disc and microscope adaptor are available from the Dept of Veterinary Medicine of Animal Productions, University of Naples. Details of cost and ordering information can be found on the website: www.parassitologia.unina.it/flotac/mini-flotac/how-to-get/

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ACKNOWLEDGEMENT

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Appendix VI

**Staining thin blood films
Counting WBCs manually**

Staining thin blood films

In district laboratories, thin blood films are usually stained manually using Leishman or Wright's stain. These stains are examples of alcohol containing Romanowsky stains which stain blood cells differentially.

Romanowsky stains

These stains contain eosin Y which is an acidic anionic dye and azure B and other thiazine dyes (derived from the oxidation, or polychroming, of methylene blue) which are basic cationic dyes. When diluted in buffered water, ionization occurs. Eosin stains the basic components of blood cells, e.g. haemoglobin stains pink-red, and the granules of eosinophils stain orange-red. Azure B and other methylene blue derived dyes, stain the acidic components of cells. Nucleic acids and nucleoprotein stain various shades of mauve-purple and violet, the granules of basophils stain dark blue-violet, and the cytoplasm of monocytes and lymphocytes stains blue or blue-grey. The staining reactions of Romanowsky stains are pH dependent which is why the stains are diluted in buffered water of specific pH.

Note: There is some variation between batches of most Romanowsky stains due to the different thiazines and impurities they contain. A highly purified Romanowsky stain has been developed which contains only azure B and eosin Y. It is recommended by ICSH for standardized Romanowsky staining but it is expensive and not needed for routine work.

Leishman staining technique

Many of the difficulties in reporting blood films, particularly red cell morphology, are due to variable staining. It is important therefore for laboratories to use a reproducible standardized staining technique.

Reagents

- Leishman stain* Reagent No. 25
*For daily use, store the stain in an amber container, e.g. TK dropper bottle, which can be closed in between use to prevent moisture entering the stain. Make sure the stain is kept in a cool place (not refrigerated) and never left in direct sunlight. Bright light and heat will cause the stain to deteriorate rapidly. Keep the stock stain in a tightly stoppered light opaque (e.g. amber) container in a cool dark place. Renew every 3 months or more often if indicated. Allow 3–5 days before using freshly made stain (to obtain optimum colour reaction).
- pH 6.8 buffered water* Reagent No. 10
*Renew if the water becomes cloudy. When refilling the dispensing container, always check that the pH is correct, e.g. by using narrow range pH papers.

Procedure

- 1 Cover the blood film with undiluted stain but do not flood the slide. If using a dropper bottle count the number of drops required to cover the film.

Note: The undiluted stain not only acts as a fixative but also partially stains the smear. This stage is required to obtain the best possible staining results.

- 2 Add twice the volume of pH 6.8 buffered water (i.e. twice the number of drops as stain). The diluted stain should not overflow. Ensure the water is well mixed with the stain by blowing on the diluted stain or mixing the stain and water using a plastic bulb pipette. Allow to stain for 10 minutes (time may require adjusting).

Note: Diluting the stain in buffered water brings about full staining of the blood cells. The exact staining time to use should be decided when a new batch of stain is prepared.

- 3 Wash off the stain with pH 6.8 buffered water. Do *not* tip off the stain, because this will leave a fine deposit covering the film. Wipe the back of the slide clean and stand it in a draining rack for the smear to dry. The blood film should appear neither too pink nor too blue (check results microscopically).

Quality control

- When a new batch of stain is prepared, decide the best staining time to use, e.g. stain films made from the same blood at different times, e.g. 5, 7, 10, 12, 15 minutes. Compare the results with a stained control blood film.
- Check the pH of newly prepared buffered water and re-check it at weekly intervals. The pH of the buffered water used to dilute the stain must be correct. It is mainly responsible for the staining reactions.
- Maintain consistency in the staining procedure by following exactly a standard operating procedure (SOP). The staining procedure should be checked at the beginning of each week.

External quality assessment: Whenever possible the Regional or Central Haematology Reference Laboratory should send fixed blood films to district laboratories for staining and reporting (films and report to be returned to the Reference Laboratory), and control stained blood films for reporting and comparing with films being stained in the district laboratory. These control stained films should be retained by the district laboratory (protect control films from light).

Staining results

Red cells Pink-red
Nucleus of cells Purple-violet

Cytoplasm

Neutrophils, eosinophils Pale pink
Large lymphocytes Clear blue
Small lymphocytes Darker clear blue
Monocytes Grey-blue

Granules

Eosinophils Orange-red
Neutrophils Mauve-purple
Toxic granules Dark violet
Basophils Dark blue-violet

Platelets Purple-blue

Inclusions

Malaria pigment (in WBCs) Brown-black

Note: The appearances of Leishman stained blood cells are illustrated in Section 3.1.

Wright's staining technique

Wright's stain is similar to Leishman stain*

*A different polychroming technique is used in the production of Wright's stain and nuclear staining is usually paler than when using Leishman stain.

Reagents

■ Wright's stain* Reagent No. 46

*Storage guidelines as previously described for Leishman stain, also apply to Wright's stain. Staining properties of Wright's stain also deteriorate rapidly when the stain absorbs moisture or is stored at high temperatures or in bright sunlight. Wright's stain should also be renewed every 3 months and left 3–5 days before being used.

■ pH 6.8 buffered water Reagent No. 10

Note: Some users of Wright's stain prefer to use pH 6.4 buffered water (see Reagent No. 10)

Procedure

The method of staining thin blood films using Wright's stain is the same as that described previously for Leishman stain except:

- The *same* volume of buffered water as stain is used (not twice the volume).
- The recommended staining time is 5 minutes but this should be decided locally and re-checked when a new batch of stain is prepared.

Quality control: This is the same as described for Leishman stain.

Staining results: Similar to those described previously for Leishman stain. When pH 6.4 buffered water is used, more acidic (pink) colour reactions will be seen.

Counting WBCs manually

Value of test: A white blood cell (WBC) count is used to investigate HIV/AIDS, infections and unexplained fever, and to monitor treatments which can cause leukopenia. In most situations when a total WBC count is requested it is usual to perform also a differential WBC count as described in Section 3.1.

Principle of test

Whole blood is diluted 1 in 20 in an acid reagent which haemolyzes the red cells (not the nucleus of nucleated red cells), leaving the white cells to be counted. White cells are counted microscopically using an Improved Neubauer ruled counting chamber (haemocytometer) and the number of WBCs per litre of blood calculated.

Note: When after examining a stained blood film, many nucleated red cells are present (more than 10%), the WBC count should be corrected (see end of *Procedure*).

Electronic counting of WBCs: This is described in Section 3.1.

Blood sample: EDTA anticoagulated blood or capillary blood can be used for counting white cells. Heparin or sodium citrate anticoagulated blood must not be used. The count should be performed within 6 hours of collecting the blood.

Equipment

■ Counting chamber (haemocytometer)

The counting chamber recommended for cell counts is a metallized surface ('Bright-line') double cell Improved Neubauer ruled chamber.

Non-metallized haemocytometers: While these are less expensive, they are not recommended. It is more difficult to count WBCs reliably using this type of chamber because the background rulings and cells are not as easily seen. Non-metallized chambers are also more difficult to fill.

The rulings of an Improved Neubauer ruled chamber are shown in Fig. App. 3. The chamber grid has an area of 9 mm² and the depth of the chamber (space between cover glass and grid) is 0.1 mm.

Bürker ruled counting chamber

This chamber is occasionally found in laboratories and may be the only one available for counting cells. To count white cells using a Bürker Chamber, the four large corner squares are used (4 mm²) and the same calculation as described for the Improved Neubauer ruled chamber is used.

■ Counting chamber cover glasses

Special optically plane cover glasses of defined thickness (designed for use with haemocytometers) are required. Other cover glasses must *not* be used. Manufacturers of counting chambers provide two cover glasses with each chamber. The laboratory should always keep in stock spare cover glasses.

■ Pipettes/calibrated capillaries and safe filling device

A 20 µL (0.02 mL, 20 cumm) micropipette e.g. white shellback type, or calibrated capillary is required to measure blood samples. A safe pipette/capillary filler should be used to aspirate and dispense the blood. This can be a simple bulb filler or device as shown in Section 1.4.

Note: Safe systems for measuring and dispensing blood samples and diluting fluid are described in Section 1.4.

■ Hand counter

To count white cells accurately, a simple inexpensive mechanical hand tally counter is required. Turning the knob on the side returns the counter to zero after each count.

Reagent

WBC diluting fluid*

Reagent No. 45

*This is a weak acid solution to which gentian violet is added which stains the nucleus of white cells.

Procedure

1 Measure 0.38 mL of diluting fluid and dispense it into a small container or tube.

2 Add 20 µL (0.02 mL, 20 cumm) of *well-mixed* EDTA anticoagulated venous blood or free-flowing capillary blood and mix.

Important: The volume of blood used in the test must be correct.

3 Assemble the counting chamber:

- Make sure the central grid areas of the chamber and the special haemocytometer cover glass are completely clean and dry.

- Slide the cover glass into position over the grid areas and press down on each side until rainbow colours (Newton's rings) are seen. Prior moistening of the chamber surface on each side of the grid areas will help the cover glass to adhere to the chamber.

4 Re-mix the diluted blood sample. Using a capillary, Pasteur pipette, or plastic bulb pipette held at an angle of about 45°, fill one of the grids of the chamber with the sample, taking care not to overfill the area.

Important: The chamber must be refilled if the sample overfills into the channel beyond the grid or an air bubble forms in the grid area.

5 Leave the chamber undisturbed for 2 minutes to allow time for the white cells to settle.

Note: To prevent drying of the fluid, place the chamber in a petri dish or plastic container on dampened tissue or blotting paper and cover with a lid.

6 Dry the underside of the chamber and place it on the microscope stage.

Using the 10× objective with the condenser iris *closed sufficiently* to give good contrast, focus the rulings of the chamber and white cells. Focus the cells until they appear as small black dots.

7 Count the cells in the four large corner squares of the chamber marked W1, W2, W3, W4 in Fig.

App. 3 (total area of 4 mm²). Include in the count cells lying on the lines of two sides of each large square as shown in Fig. App. 4.

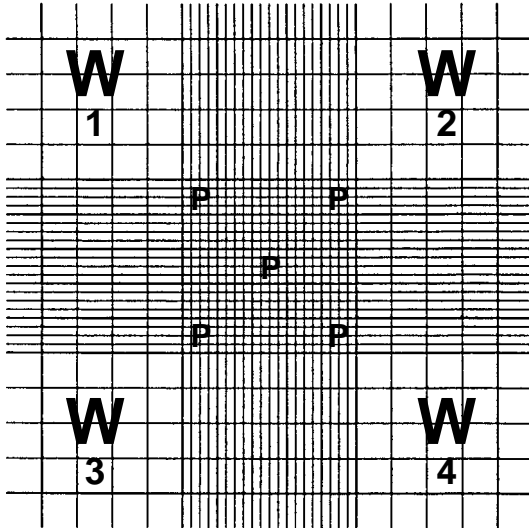


Fig. App. 3 Improved Neubauer ruled counting chamber. The four large squares marked W1, W2, W3, W4 are used for counting WBCs and the five small squares marked P are used for counting platelets manually.

- 8 Report the number of white cells per litre of blood using the following simple calculation:
- Divide the total number of cells counted by 2.
 - Divide the number obtained by 10.

The number obtained $\times 10^9$ is the white cell count.

Example

Cells counted in 4 squares = 84

$84 \div 2 = 42$

$42 \div 10 = 4.2$

WBC count = 4.2 cells $\times 10^9/L$

WBC calculation details

$$\text{WBC count (per litre)} = \frac{\text{Cells counted} \times 20^* \times 10^6}{4^{\ddagger} \times 0.1^{\S}}$$

where* = 1 in 20 dilution of blood, ‡ = 4 mm² area counted, § = 0.1 mm depth of chamber.

- 9 After performing the count, before the sample dries, dismantle the chamber, wash and dry it. Store it with the cover glass in a safe place.

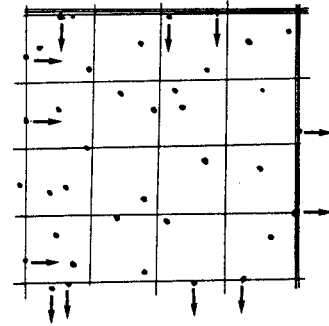


Fig. App. 4 Cells lying on two sides of the square are included in the count.

Counts higher than $50.0 \times 10^9/L$

When a count is higher than $50.0/10^9/L$, repeat the count using 0.76 mL of diluting fluid and 20 μL of blood. Multiply the result by 2.

Always examine a stained thin blood film.

Counts lower than $2.0 \times 10^9/L$

When a count is lower than $2.0 \times 10^9/L$, repeat the count using 0.38 mL of diluting fluid and 40 μL of blood. Divide the result by 2.

Correcting a WBC count when there are many nucleated RBCs

When more than 10 nucleated red blood cells (RBCs) per 100 WBC are present in the blood film, correct the WBC count as follows:

$$\text{Corrected WBC count} = \frac{\text{Uncorrected WBC count} \times 100}{\text{Nucleated RBCs}^* + 100}$$

*Number of nucleated RBCs per 100 WBC as seen in stained blood film.

Quality control of WBC counts

- Whenever possible perform WBC counts in duplicate. The difference between the two counts (as a percentage of the mean) should not be more than 20%.

How to calculate the % difference between two counts

- 1 Record the number of cells counted in Count 1 and Count 2.
- 2 Calculate:
 - the difference in the number of cells counted between the two counts.
 - the mean of the two counts
- 3 Calculate the difference of the two counts as a percentage of the mean.

Example

Cells counted in Count 1 = 88, Count 2 = 76

- Difference in numbers of cells between the two counts: $88 - 76 = 12$
- Mean of Count 1 and Count 2: $\frac{88 + 76}{2} = 82$
- Difference of the two counts as a % of the mean: $\frac{12 \times 100}{82} = 14.6\%$

Note: When the difference between the two counts is more than 20%, repeat the counts.

- Check that the diluting fluid is free from particles which could be mistaken for WBCs. To do this, fill a counting chamber with a sample of the diluting fluid and examine the grid areas microscopically using the 10× objective with *greatly reduced* condenser iris. If the fluid contains particles resembling WBCs, filter it and recheck or discard the fluid and prepare fresh diluting fluid.
- When examining the blood film, check that there is no major discrepancy between the total white cell count and white cells seen in the blood film.

External quality assessment

Whenever possible the Regional or Central Haematology Laboratory should send control blood samples to district laboratories for analysis and reporting.

Sources of error in manual WBC counts

- Incorrect measurement of blood due to poor technique or using a wet or chipped pipette.
- When using anticoagulated blood, not mixing the blood sufficiently or not checking the sample for clots.
- Inadequate mixing of blood with diluting fluid.
- Not checking whether the chamber and cover glass are completely clean.
- Not using a haemocytometer cover glass.
- Over-filling a counting chamber or counting cells when the sample contains air-bubbles.
- Not allowing sufficient time (2 minutes) for the cells to settle in the chamber.
- Using too intense a light source or not reducing the iris diaphragm sufficiently to give good contrast (poor focusing and difficulty in seeing clearly the cells and rulings are common when using non-metallized haemocytometers, see previous text).

- Not completing counting of the cells before the sample begins to dry in the chamber.
- Counting too few cells (see previous text). Precision increases with the number of cells counted.
- Not correcting a count when the sample contains many nucleated RBCs (see previous text).

Interpretation of WBC counts

Reference ranges for white cell counts vary with age with higher counts being found in children. There are also gender differences with higher total WBC and neutrophil counts being found in women of child-bearing age and during pregnancy. Counts also vary in different populations with lower total WBC and neutrophil counts being found in Africans and people of African descent.

*WBC reference range**

*These are guideline figures which should be checked locally.

Children at 1 y	6.0–18.0 × 10 ⁹ /L
Children 4–7 y	5.0–15.0 × 10 ⁹ /L
Adults	4.0–10.0 × 10 ⁹ /L
Adults of African origin	2.6–8.3 × 10 ⁹ /L
Pregnant women	Up to 15 × 10 ⁹ /L

Note: The causes of raised and reduced total white blood cell counts are described in Section 3.1.

ACKNOWLEDGEMENT

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Use and care of a microscope

Microscope with built-in illumination

The following instructions apply to microscopes fitted with an Abbe condenser and illumination system similar to that shown opposite.

- 1 Turn the lamp brightness control to its lowest setting. Switch on the microscope. Increase the lamp brightness to about three quarters of its power.
- 2 Bring the 10× objective into place.
- 3 Make sure the underside of the sample slide and surface of the stage are *completely dry and clean*.
- 4 Place the sample on the stage in the slide holder. Focus the sample with the 10× objective:

- While looking from the side (not down the eyepieces), turn the coarse focusing control to bring the sample close to the objective i.e. about 5 mm from the objective.
- Looking down through the eyepieces, bring the sample into focus by slowly turning the coarse focusing control in the opposite direction to increase the distance between the sample and objective.
- Use the fine focusing control to obtain a sharp image (this will not be the best image because the condenser has yet to be focused and the illumination adjusted).

- 5 Focus the condenser (should be within 1 mm of its topmost position) and leave it in this position for all objectives.

6 *Examine the sample with the 10× objective.*

Obtain the best image by:

- closing the iris about two thirds,
- adjusting the lamp brightness control to give good illumination with the minimum of glare.

- 7 Use the mechanical stage to examine the sample systematically.

8 Focus the sample with the 40× objective:

Carefully revolve the nosepiece to bring the 40× objective into place. It will locate very close to the sample. Providing the objectives are parfocal (in focus one with another), only slight focusing with the fine focusing control should be necessary to bring the sample into sharp focus.

9 *Examine the sample with the 40× objective.*

Obtain the best image by:

- opening the iris more,
- increasing the illumination.

Difficulty in obtaining a clear image with the 40× objective

If the image cannot be focused with the 40× objective or is blurred, the objective lens probably has oil on it or has become contaminated from a wet preparation. Revolve the nosepiece and carefully clean the lens.

10 Focus the sample with the 100× objective:

Move the 40× objective to the side, place a drop of oil on the sample and bring the 100× objective into position.

11 *Examine the sample with the 100× objective:*

Obtain the best image by:

- opening the iris fully,
- increasing the illumination.

Difficulty in obtaining a clear image with the 100× objective

If no image can be focused, check that the oil has been added to the *smear side* of the slide.

An image may appear blurred when a new type of oil is being used and all the previous oil has not been removed from the objective.

Safety precautions when using a microscope with built-in illumination

- Use the microscope with the correct voltage supply. Read the manufacturer's literature.
- Make sure the microscope is earthed via the mains plug. Use fuses of the correct rating in the mains plug and in the microscope fuse housing (read the manufacturer's literature).
- Always switch off and unplug the microscope from the mains:
 - at the end of the day's work,
 - when there is a storm with lightning,
 - when performing any cleaning and maintenance, e.g. changing a lamp, replacing a fuse.
- Locate the microscope away from any source of water and out of direct sunlight.
- Regularly check the electrical cable for signs of wear and loose connections in the mains plug.
- If there is any electrical problem consult a qualified electrician.

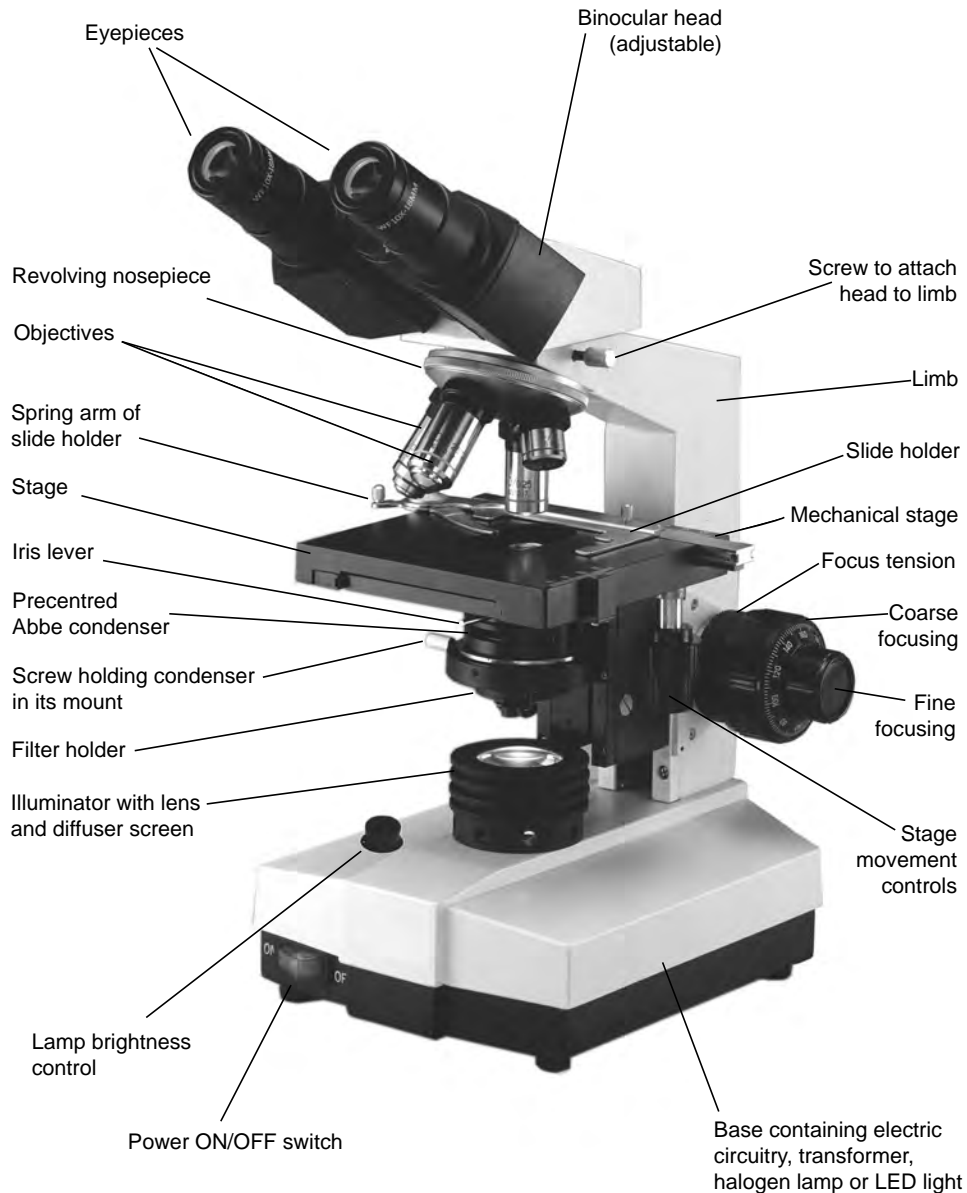
In hot humid conditions

If the microscope is not to be used the next day, protect the lenses and other components from fungal growth by sealing the microscope in an airtight plastic bag with a container of dry (blue) self-indicating silica gel. About 100 g is adequate. The silica gel will remove moisture from the air inside the bag, providing a less humid environment. After use the silica gel will appear pink and require drying in an oven or in a container over a burner. When dry the silica gel will appear blue (active) again and should be stored immediately in an airtight container ready for reuse.

Do not place the microscope in a box with a lamp unless the box is sufficiently well ventilated otherwise heat from the lamp may damage the microscope.

Strengthening Microscopy

- Prepare samples correctly using standardized techniques and preparations of the correct thickness.
- Focus and scan first with a lower power before using a higher magnification.
- *Focus continuously* to avoid organisms and features in different planes being missed or misidentified.
- Use good quality non-drying immersion oil. Do not mix oils.



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The book has been produced to assist those working at the laboratory bench, those training laboratory personnel and those studying tropical medicine. It describes the important role of district laboratories in point-of-care testing to achieve the targets of the “Good health and well-being” goal of the UN 2030 Agenda for Sustainable Development:

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