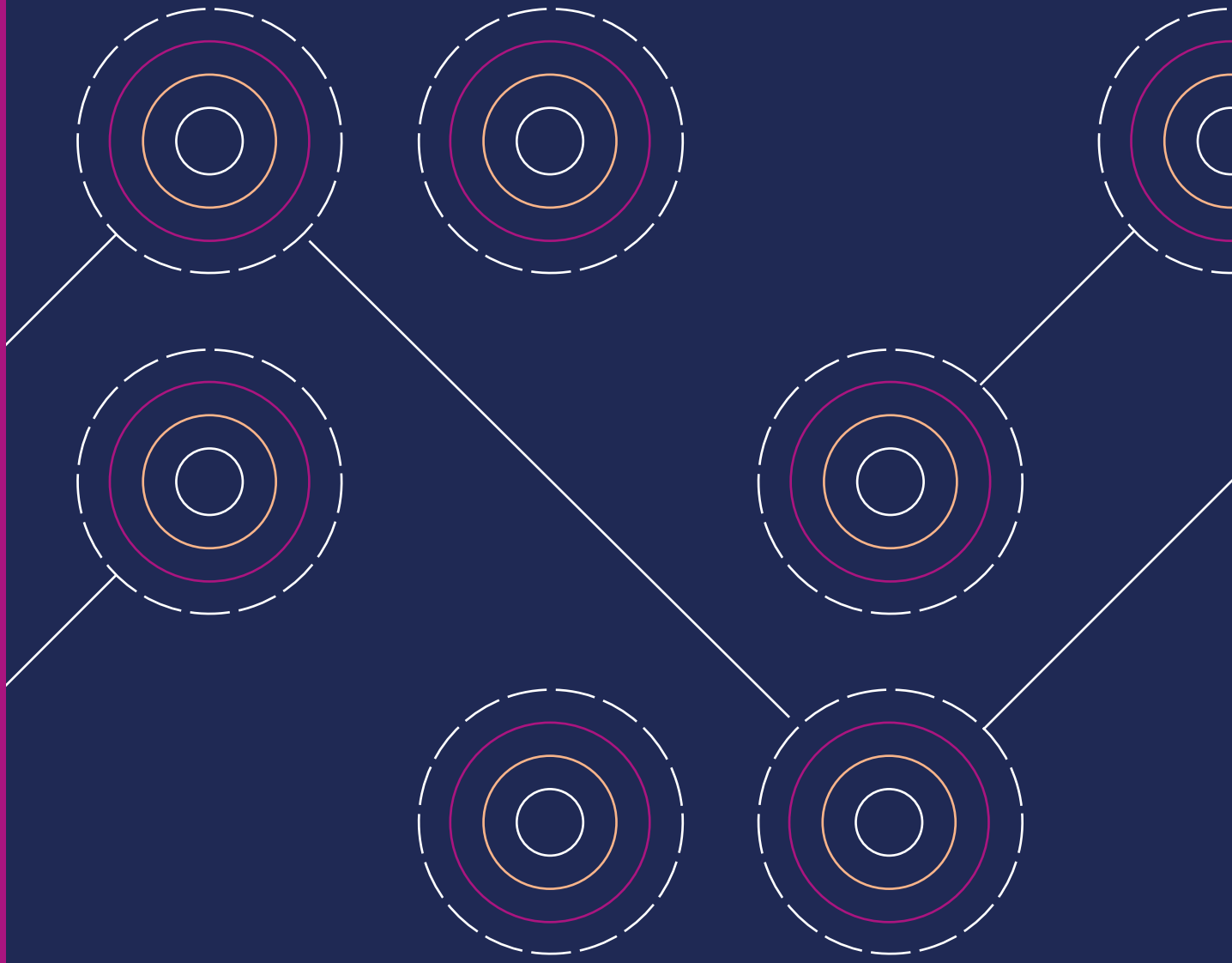
Laboratory and point-of-care diagnostic testing for sexually transmitted infections, including HIV



World Health Organization

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Contents

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Editor-in-Chief:

Magnus Unemo

Editors:

Michelle Cole

David Lewis

Francis Ndowa

Barbara Van Der Pol

Teodora Wi

Authors:

Chapter 1. Edward W. Hook III, Barbara Van Der Pol, Francis Ndowa and Teodora Wi

Chapter 2. Catherine Ison and Frances Keane

Chapter 3. Maurine Murtagh, Igor Toskin, Emma Harding-Esch and Charlotte Gaydos

Chapter 4. Susanne Jacobsson and David Whiley

Chapter 5. Michelle Cole and Rosanna Peeling

Chapter 6. Jørgen Skov Jensen, Catriona Bradshaw and Magnus Unemo

Chapter 7. Magnus Unemo, Daniel Golparian and Michelle Cole

Chapter 8. Barbara Van Der Pol and Magnus Unemo

Chapter 9. Barbara Van Der Pol, Yaw Adu-Sarkodie and Patricia Kissinger

Chapter 10. Catriona Bradshaw, Catherine Ison, Janet Wilson and Jörgen Skov Jensen

Chapter 11. Catriona Bradshaw, Catherine Ison, Janet Wilson and Jörgen Skov Jensen

Chapter 12. David Lewis and Anna Wald

Chapter 13. John R. Papp, Pingyu Zhou, Rosanna Peeling and Edward W. Hook III

Chapter 14. Michelle Cole and Olivia Peuchant

Chapter 15. David Lewis and Magnus Unemo

Chapter 16. David Lewis and Magnus Unemo

Chapter 17. Suzanne Marie Garland and Anna-Lise Williamson

Chapter 18. Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang

Annex 1. Sumudu Perera and Jo-Anne Dillon

Annex 2. Sumudu Perera and Jo-Anne Dillon

Abbreviations

Ab	antibody
ABC	avidin-biotin peroxidase complex
AE	acridinium ester
Ag	antigen
AMG	Aptima *Mycoplasma genitalium*
AMR	antimicrobial resistance
AMV RT	avian myeloblastosis virus reverse transcriptase
aPOC	antigen-based point-of-care
ART	antiretroviral therapy
ARV	antiretroviral
BD	Becton, Dickinson and Company
bDNA	branched-chain DNA
BV	bacterial vaginosis
CDC	Centers for Disease Control and Prevention (United States of America)
cDNA	complementary DNA
CDS	calibrated dichotomous sensitivity
CE	Conformité Européenne
CFU	colony-forming units
CIA	chemiluminescence immunoassay
CIN	cervical intraepithelial neoplasia
CLIA	Clinical Laboratory Improvement Amendments
CLSI	Clinical and Laboratory Standards Institute
CMT	cytosine DNA methyltransferase
CO_2	carbon dioxide
CPD	continuing professional development
CSF	cerebrospinal fluid
CST	community-state type
Ct	cycle threshold
CT	*Chlamydia trachomatis*
CTA	cysteine trypticase agar
CVA	cofactors-vitamins-amino acids

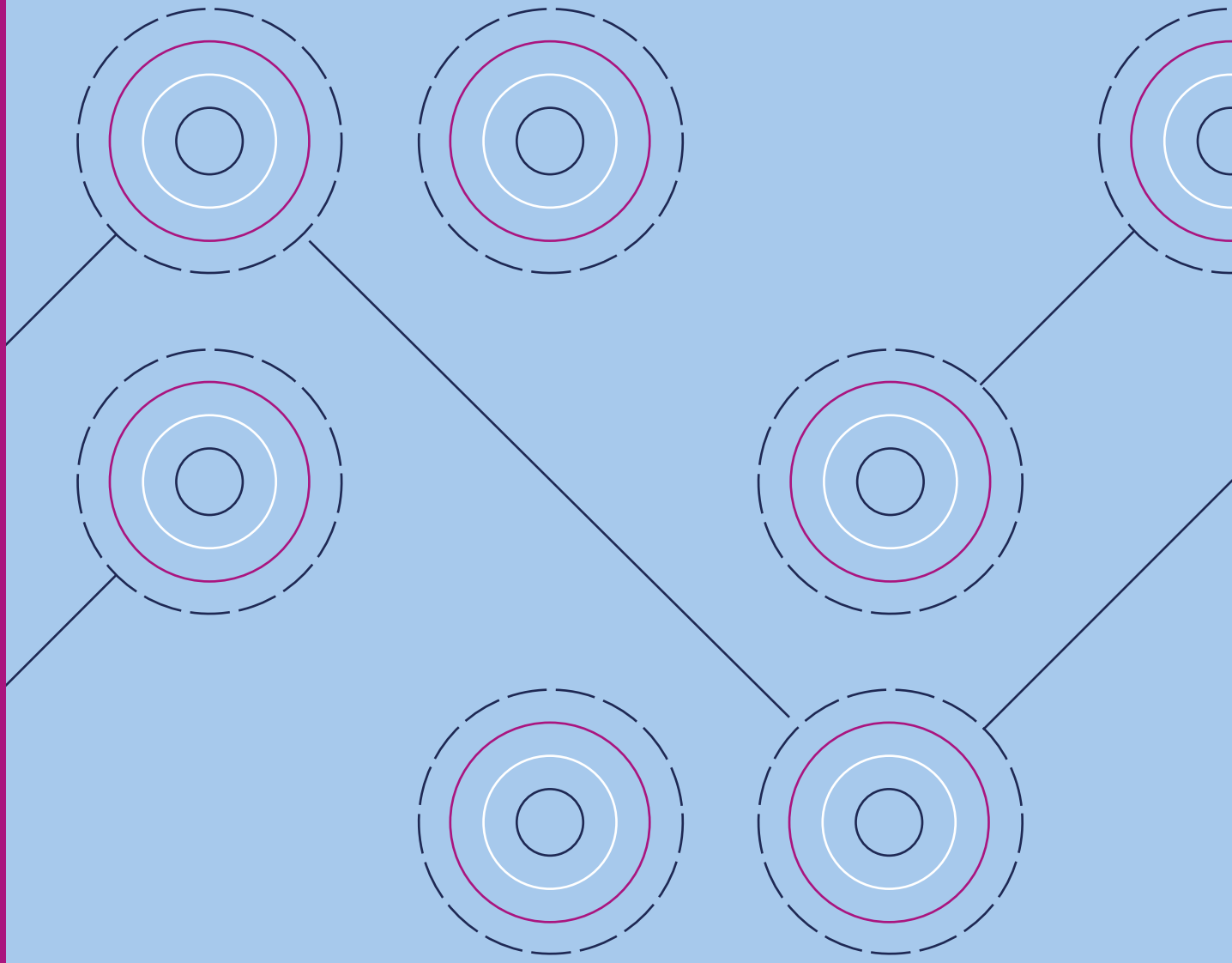
DBS	dried blood spot
DFA	direct fluorescent antibody
DFA	direct immunofluorescence assay
DGI	disseminated gonococcal infection
DKA	dual kinetic assay
DPO	dual priming oligonucleotides
dNTP	deoxyribonucleotide triphosphate
dsDNA	double-stranded DNA
ECDC	European Centre for Disease Prevention and Control
EGASP	Enhanced GASP
EIA	enzyme immunoassay
EID	early infant diagnosis
ELISA	enzyme-linked immunosorbent assay
EQA	external quality assessment
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Euro-GASP	European GASP
FDA	United States (U.S.) Food and Drug Administration
FI-nvCT	Finnish new variant of *Chlamydia trachomatis*
FITC	Fluorescein isothiocyanate
FRET	fluorescence resonance energy transfer
FTA-Abs	fluorescent treponemal antibody absorption
g	acceleration due to gravity
GASP	Gonococcal Antimicrobial Surveillance Programme
GC	gonococcus
gG	glycoprotein G
GLASS	Global Antimicrobial Resistance and Use Surveillance System
GUD	genital ulcer disease
HDA	helicase-dependent amplification
HBV	hepatitis B virus
HC	hybrid capture
HCV	hepatitis C virus
HIVDR	HIV drug resistance
HPA	hybridization protection assay
HPV	human papillomavirus
HR	high-risk
HSIL	high-grade squamous intraepithelial lesion
HSV	herpes simplex virus
IARC	International Agency for Research on Cancer
IF	immunofluorescence
IFA	immunofluorescence assay

IFU	instructions for use
IgA/IgG/IgM	immunoglobulin A, G, M
IHC	immunohistochemistry
IMDM	Iscove's modified Dulbecco's medium
IN	integrase
iNAAT	isothermal nucleic acid amplification test
IP	immunoperoxidase
IQA	internal quality assessment
IQC	internal quality control
ISO	International Organization for Standardization
IUSTI	International Union against STIs
IVD	in vitro diagnostic
JoRRP	junior onset form of RRP
KOH	potassium hydroxide
LAMP	loop-mediated isothermal amplification
LBC	liquid-based cytology
LDT	laboratory-developed tests
LGV	lymphogranuloma venereum
LIA	line immunoassay
LMIC	low- and middle-income country
LPS	lipopolysaccharide
LR	low-risk
LSIL	low-grade squamous intraepithelial lesion
MALDI–TOF	matrix-assisted laser desorption ionization time-of-flight
MG	*Mycoplasma genitalium*
MH	Mueller–Hinton
MHA-TP	microhemagglutination assay for *Treponema pallidum*
MIC	minimum inhibitory concentration
MRM	macrolide-resistance mutation
mRNA	messenger RNA
MS	mass spectrometry
MSM	men who have sex with men
MTM	modified Thayer–Martin medium
NAAT	nucleic acid amplification test
NAH	nucleic acid hybridization
NAP	nucleic acid probe
NASBA	nucleic acid sequence-based amplification
NCNGU	non-chlamydial NGU
NCTC	National Collection of Type Cultures
NEAR	nicking enzyme amplification reaction
NG	*Neisseria gonorrhoeae*

NGS	next generation sequencing
NGU	non-gonococcal urethritis
NIBSC	National Institute for Biological Standards and Control
NPV	negative predictive value
nvCT	new variant of *Chlamydia trachomatis*
OF	oral fluid
Pap	Papanicolaou
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PID	pelvic inflammatory disease
PIP	prolyliminopeptidase
PMNL	polymorphonuclear leukocyte
POC	point-of-care
POCT	point-of-care test
PPV	positive predictive value
PR	protease
PrEP	pre-exposure prophylaxis
QA	quality assurance
QC	quality control
QMS	quality management system
qPCR	quantitative real-time PCR
QRAM	quinolone resistance-associated mutation
RCT	randomized controlled trial
RDT	rapid diagnostic test
RNA	ribonucleic acid
RPR	rapid plasma reagin
rRNA	ribosomal RNA
RRP	recurrent respiratory papillomatosis
RT	reverse transcriptase
RT-PCR	reverse transcription-PCR
SARA	sexually acquired reactive arthritis
SDA	strand displacement amplification
SE-nvCT	Swedish new variant of *Chlamydia trachomatis*
SGLT2	sodium-glucose cotransporter 2
SNP	single nucleotide polymorphism
SOP	standard operating procedure
SPG	sucrose-phosphate-glutamate
spp.	species
STI	sexually transmitted infection
TAT	turnaround time
TC	target capture

TIF	tubal factor infertility
TMA	transcription-mediated amplification
TNA	threose nucleic acid
TOC	test-of-cure
TOCE	tagging oligonucleotide cleavage and extension
TP	*Treponema pallidum subspecies pallidum*
TPHA	*Treponema pallidum* haemagglutination assay
TPPA	*Treponema pallidum* passive particle agglutination assay
TRUST	toluidine red unheated serum test
TSB	tryptic soy broth
TV	*Trichomonas vaginalis*
UK NEQAS	United Kingdom National External Quality Assessment Service
UNAIDS	Joint United Nations Programme on HIV/AIDS
USA	United States of America
VDRL	Venereal Disease Research Laboratory
VIA	visual inspection with acetic acid
VL	viral load
VVC	vulvovaginal candidiasis
WB	western blot
WGS	whole-genome sequencing
WHO	World Health Organization
XDR	extensively drug resistant

Introduction



Introduction

Sexually transmitted infections (STIs), including those caused by the human immunodeficiency virus (HIV), remain an important focus area for global public health. This is due to the high morbidity associated with STIs, including the sequelae of reproductive tract infections, cervical pre-cancer and cancer, congenital syphilis, ectopic pregnancy and infertility, as well as HIV-related illness and death from advanced HIV disease. Public health strategies for STI control include promotion of safer sexual behaviour and provision of condoms (primary prevention), as well as early and effective management of patients with STIs, using either syndromic or etiological management approaches.

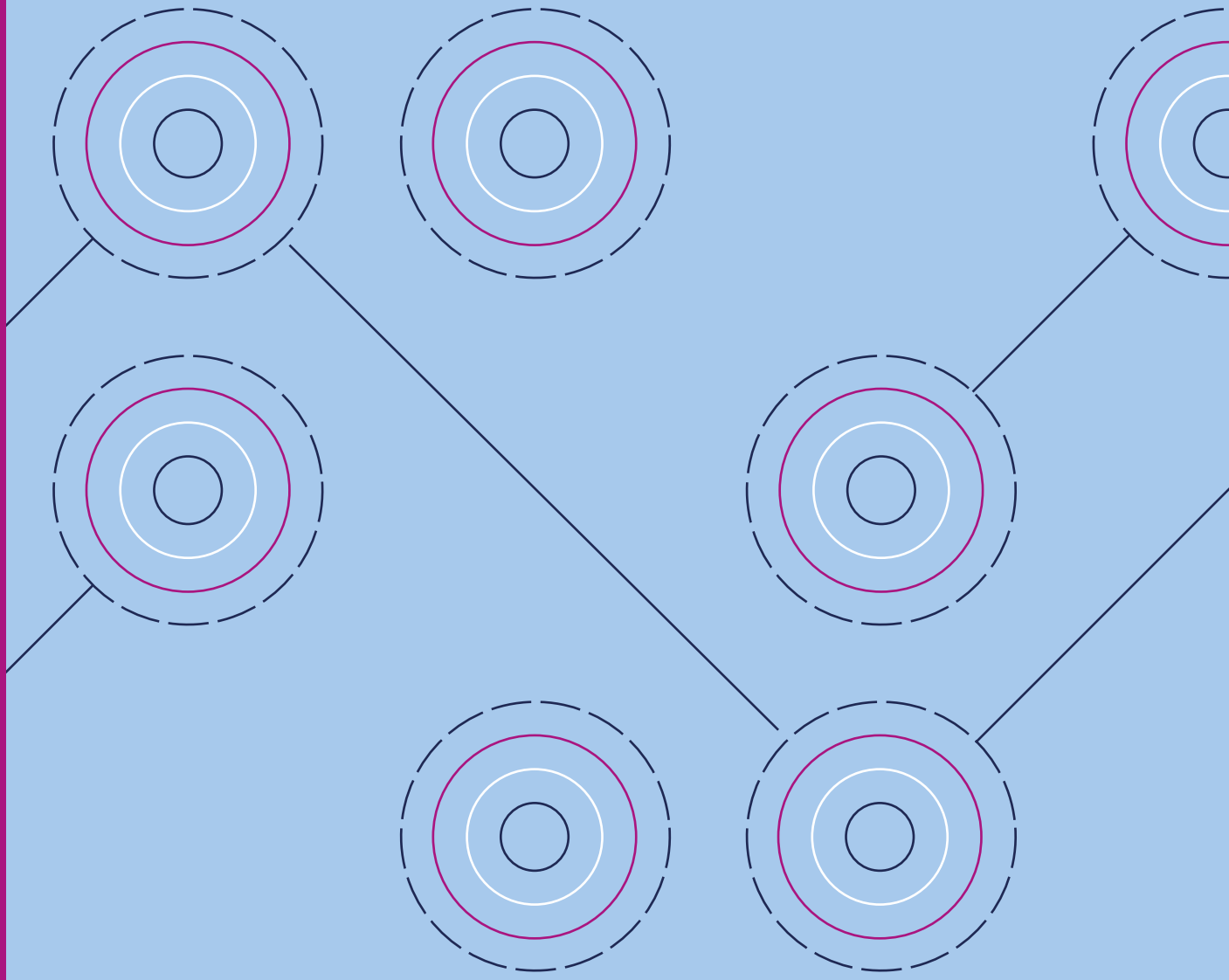
Laboratory diagnostics play an important role in STI case management, surveillance and research programmes in countries of all income levels. Laboratory tests are essential to screen for asymptomatic STIs as well as for diagnosis of symptomatic STIs. In settings where quality-assured STI laboratory diagnosis is available, same-day treatment of specific STIs should be based on results of laboratory tests. For management of symptomatic STIs where laboratory capacity is limited, the syndromic management approach can be applied. This approach allows accessible, affordable and effective management of individuals with STIs, using flowcharts (algorithms) that enable diagnosis of common STI syndromes and indicate country-specific appropriate treatments. Syndromic management also includes advice on the appropriate management of sexual partners, and places emphasis on the importance of same-visit HIV testing. The flowcharts should be based on current local etiological and antimicrobial susceptibility data provided through periodic laboratory-based surveys. However, the sensitivity and specificity of some of these flowcharts are inadequate to detect (and then appropriately treat) several STIs.

The World Health Organization (WHO) has published three previous versions of this manual, in 1989, 1999 and 2013, with the objective of providing a comprehensive guide to standard procedures for isolating, detecting and diagnosing STIs, for microbiologists and medical technologists. This manual was first conceived as a practical bench manual, tuned to the needs and capacities of laboratories at different levels within health-care systems across the world, and it proved to be very popular. An updated manual was published in 2013, but in the past decade there have been a number of key advances in diagnostic procedures, in particular with respect to nucleic acid amplification tests (NAATs) and rapid diagnostic tests (RDTs), also known as point-of-care (POC) tests, as well as antimicrobial susceptibility testing methodologies, quality management systems and WHO recommendations based on up-to-date evidence. For this updated manual, *Laboratory and point-of-care diagnostic testing for sexually transmitted infections, including HIV,* a number of international experts have extensively reviewed and updated the chapters of the manual.

This updated manual provides a basic understanding of the principles of laboratory and POC testing in the context of screening and diagnostic approaches, as well as antimicrobial susceptibility testing, as components of STI control. As with previous editions, this manual covers each disease in a separate chapter which provides detailed information on specimen collection, transport and laboratory testing. Two useful annexes covering media, reagents, diagnostic tests and stains (recipes) and laboratory supplies are included at the end of the manual. While this manual does cover the most important STI pathogens, it is not exhaustive, and the reader may need to consult other resources for more information, for example, with respect to national STI policies, antimicrobial susceptibility testing guidelines, medico-legal issues, and STI testing in minors.

It is envisaged that this updated manual will be informative to the primary target audience, which includes microbiologists, molecular biologists, medical technologists and scientists, and also to administrators, programme managers and medical staff, including doctors and nurses. The manual may also be a useful tool to assist in the procurement of the most appropriate diagnostic tests for specific settings, ideally in consultation with national and/or local expert advisory committees. The manual is furthermore a valuable resource for educators and for the students themselves, both within and outside of the laboratory environment. Finally, it is anticipated that the growth in diagnostic products and methodologies will continue in the next few years; therefore, it will be important for all readers to keep themselves up to date with the latest developments in the field.

Chapter 1. Choosing tests for sexually transmitted infections (STIs)



Chapter 1. Choosing tests for sexually transmitted infections (STIs)

Edward W. Hook III, Barbara Van Der Pol, Francis Ndowa and Teodora Wi

1.1 Introduction

More than 30 bacterial, viral and parasitic pathogens are transmissible through intimate contact. They constitute a group of infections referred to as sexually transmitted infections (STIs). The major pathogens, and their clinical manifestations, are listed in Table 1.1. Many other pathogens are, however, transmitted through intimate contact, as well as other routes. These potentially emerging STIs include *Shigella, Campylobacter, Neisseria meningitidis*, Zika virus and Ebola virus. Although some of these pathogens can be acquired through routes other than sexual transmission, epidemiologically, intimate contact is more important for their transmission from one person to another (Table 1.1). STIs may occur as localized infections at any site of intimate contact, including the oropharynx and rectum, as well as the genitals. STIs are also commonly asymptomatic. Most STIs are local, causing signs and symptoms at the site of infection. However, some STIs and their complications are systemic. Some may be transmitted to infants during pregnancy, childbirth or breastfeeding.

Laboratory and point-of-care (POC) tests are potentially powerful tools for the management and control of STIs, facilitating prevention of transmission of STIs and their subsequent sequelae. The large number of different STIs and the variety of potential tests for each one can make choosing the appropriate diagnostic test difficult. There is currently a wide variety of STI tests available, each with attributes and potential limitations that could affect how they might be used to enhance STI control. Further, in an era of limited resources, decision-making about which and how many STIs to invest in for testing, who to test, and which of the multiple available tests to use for a designated purpose can be challenging. Test selection should reflect a prioritization process that considers infection prevalence, their impact on both individuals and populations as a whole, test performance characteristics, cost of the tests, and the rationale for testing.

Table 1.1: Main sexually transmitted pathogens and the diseases they cause

Pathogen	Clinical manifestations and other associated diseases
Discharge-causing infections	
Neisseria gonorrhoeae	GONORRHOEA **Men:** urethral discharge (urethritis), epididymitis, orchitis, infertility **Women:** cervicitis, endometritis, salpingitis, pelvic inflammatory disease, infertility, preterm rupture of membranes, perihepatitis; commonly asymptomatic **Both sexes:** proctitis, pharyngitis **Neonates:** conjunctivitis
Chlamydia trachomatis	CHLAMYDIAL INFECTION **Men:** urethral discharge (nongonococcal urethritis), epididymitis, orchitis, infertility **Women:** cervicitis, endometritis, salpingitis, pelvic inflammatory disease, infertility, ectopic pregnancy, preterm rupture of membranes, perihepatitis; commonly asymptomatic **Both sexes:** proctitis, pharyngitis, Reiter's syndrome **Neonates:** conjunctivitis, pneumonia

Table 1.1 (continued): Main sexually transmitted pathogens and the diseases they cause

Pathogen	Clinical manifestations and other associated diseases
Trichomonas vaginalis	TRICHOMONIASIS **Men:** urethral discharge (nongonococcal urethritis); often asymptomatic **Women:** vaginosis with profuse, frothy vaginal discharge; preterm birth, low-birth-weight babies **Neonates:** low birth weight
Mycoplasma genitalium	**Men:** urethral discharge (nongonococcal urethritis) **Women:** cervicitis, endometritis, probably pelvic inflammatory disease; often asymptomatic
Lesion and genital ulcer-causing infections	
Chlamydia trachomatis (serovars L1–L3)	LYMPHOGRANULOMA VENEREUM **Both sexes:** ulcer, inguinal swelling (bubo), proctitis
Treponema pallidum	SYPHILIS **Both sexes:** primary ulcer (chancre) with local adenopathy, skin rashes, condylomata lata; bone, cardiovascular and neurological damage **Women:** pregnancy loss (abortion, stillbirth), premature delivery **Neonates:** stillbirth, congenital syphilis
Herpes simplex virus type 2 *Herpes simplex virus type 1 (less commonly)*	GENITAL HERPES **Both sexes:** anogenital vesicular lesions and ulcerations **Neonates:** neonatal herpes (often fatal)
Human papillomavirus	GENITAL WARTS **Men:** penile and anal warts; carcinoma of the penis **Women:** vulval, anal and cervical warts, cervical carcinoma, vulval carcinoma, anal carcinoma **Neonates:** laryngeal papilloma
Molluscum contagiosum virus	MOLLUSCUM CONTAGIOSUM **Both sexes:** genital or generalized umbilicated, firm skin nodules
Haemophilus ducreyi	CHANCROID **Both sexes:** painful genital ulcers; may be accompanied by bubo
Klebsiella (Calymmatobacterium) granulomatis	DONOVANOSIS (GRANULOMA INGUINALE) **Both sexes:** nodular swellings and ulcerative lesions of the inguinal and anogenital areas
Monkeypox virus (MPXV)	MPOX (MONKEYPOX) **Both sexes:** ulcerative rash at the site of intimate contact; proctitis
Systemic viral infections	
Human immunodeficiency virus (HIV)	ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS) **Both sexes:** HIV-related disease, AIDS
Hepatitis B virus	VIRAL HEPATITIS **Both sexes:** acute hepatitis, liver cirrhosis, liver cancer
Cytomegalovirus	CYTOMEGALOVIRUS INFECTION **Both sexes:** subclinical or nonspecific fever, diffuse lymph node swelling, liver disease, etc.
Kaposi sarcoma associated herpesvirus (human herpesvirus type 8)	KAPOSI SARCOMA **Both sexes:** aggressive type of cancer in immunosuppressed individuals
Fungal infections	
Candida albicans	CANDIDIASIS **Men:** superficial infection of the glans penis **Women:** vulvo-vaginitis with thick curd-like vaginal discharge, vulval itching or burning
Parasitic infestations	
Phthirus pubis	PUBIC LICE INFESTATION
Sarcoptes scabiei	SCABIES

Tests for STIs may be used for a variety of different purposes that, in turn, may affect the choice of tests. Reasons for testing include: evaluation and diagnosis of individuals experiencing signs and symptoms of a STI; screening of asymptomatic at-risk individuals; antimicrobial susceptibility testing; surveillance; validation of syndromic management algorithms; and quality assurance (QA). Some key elements of these terms are outlined below.

- **Diagnosis.** Symptoms of common STIs tend to be non-specific and typically have a variety of different potential causal agents that may require different treatments. Therefore, diagnostic testing is helpful for accurate diagnosis, test of cure or to evaluate the patient's response to treatment and to guide the management of sexual partners. Testing can also help with refinement or QA of syndromic management algorithms. When used for diagnosis, the time required for test results to become available to guide management should be considered when choosing a test. For symptomatic individuals, a POC test that allows for immediate or same-day diagnosis and treatment is preferred, to prevent the infected person from transmitting the infection to others, or from experiencing complications from infection, or being lost to follow-up in the interval between testing and notification of test results *(1–3)*. When diagnostic testing is performed as part of clinical services, it is useful to monitor the interval between testing and provision of treatment to patients who tested positive, if possible, to assess quality of care. Circumstances that may delay notification of individuals with infection may impact the choice of tests.
- **Screening.** Screening is an essential element of optimal STI management and control strategies that builds on the contributions of syndromic management and diagnostic testing. Although proportions vary, all STIs may be asymptomatic, or their symptoms may not be recognized by the person with the infection. Despite the absence of identified symptoms, individuals with asymptomatic infections may be at risk of transmitting them to others, as well as for developing complications of infection if they remain untreated. As a result, screening (i.e. testing of at-risk individuals without recognized signs or symptoms) will identify more people with the infection, reducing the risk of complications or transmission. As with testing for diagnosis, the time interval between testing and provision of treatment, as well as the proportion of screen-positive individuals that receive treatment are useful quality measures. The costs of screening must be balanced against the number of cases detected and the consequences of not screening. The prevalence of a particular STI should be known to be high in the population for which screening is implemented. Screening may be more cost-effective if it can be targeted to at-risk population subgroups. This targeting should be based on available surveillance data. The screening test must be inexpensive, easy to perform, acceptable to people, reliable and valid.
- **Determination of antimicrobial susceptibility.** For some STIs (*Neisseria gonorrhoeae* is a noteworthy example), the continuing development of antimicrobial resistance has periodically led to changes in recommended therapy. Systematic surveillance of antimicrobial susceptibility and/or testing of specific isolates for susceptibility to the antimicrobial agents used for therapy provide information that can be used to adjust treatment recommendations in anticipatory fashion, optimally before treatment failure becomes a problem. For most STIs, determination of antimicrobial susceptibility is best performed on living clinical isolates of cultured organisms and is typically better used to guide recommended therapy for populations, rather than for individual patient management. Molecular techniques for evaluation of antimicrobial susceptibility play a role in the management of individual patients with *Mycoplasma genitalium* and are an area of evolving technology, but are not yet in widespread use for additional STIs. For the most part, surveillance for antimicrobial resistance is best conducted by reference laboratories using specimens collected from a spectrum of geographically representative sites and with an adequate sample size of populations of interest.
- **Surveillance.** Surveillance is the systematic collection, collation and analysis of data to determine how common an infection is within a community or population. It is an essential element of planning for STI control efforts and to raise awareness among decision-makers to obtain the necessary support. STI laboratory testing supports documentation of the magnitude of the STI problem and to monitor trends. Investment in surveillance for uncommon infections or infections with little direct impact on public health may not be highly prioritized if resources are limited, if the infection is uncommon (e.g. chancroid), or if the associated morbidity is modest (e.g. pubic lice). Generally, the time needed to obtain a test result is not critical for surveillance purposes. In some instances, specimens collected for surveillance may also be used to monitor other clinically important factors, such as antimicrobial resistance (e.g. in *N. gonorrhoeae*).
- **Validation of syndromic management.** Syndromic diagnosis is a valuable element of STI control efforts, providing a rapid diagnostic assessment that can then be used to guide timely therapy for individuals with signs and symptoms of infection. In settings where syndromic diagnosis represents an element of STI management efforts, etiologies of syndromes should be determined, to inform the development of national syndromic management algorithms. In addition, periodic validation studies using gold standard laboratory tests among patients diagnosed and treated using syndromic management algorithms for STIs should be performed to ensure that syndromic diagnosis is succeeding in identifying

the infections targeted for intervention. In situations where syndromic diagnosis is not resulting in treatment of the targeted STI, efforts to evaluate the reasons for failure are warranted. Information gained through periodic use of testing to evaluate the quality of clinical services can be used for feedback and to develop steps to improve patient care.

- **Quality assurance (QA)/improvement.** The large area of QA includes both quality control, which is performed to ensure that tests are being performed as designed; and quality assessment, which is performed to ensure that tests are being used appropriately and that all steps to manage the patients being tested are being carried out correctly. STI test accuracy may be impacted by a variety of factors, including variation in reagents, equipment function and technical proficiency. As a result, periodic testing of standardized specimens to assess laboratory proficiency and to ensure the accuracy of the test is recommended. Standardized specimens may be obtained from accrediting organizations, or generated within the laboratory. Evaluation of standardized specimens may identify a need for re-training or evaluation of the quality of individual components of the laboratory test.

1.2 Types of diagnostic tests

Scientific progress has provided a broad array of diagnostic tests for STIs. They vary greatly in terms of their level of complexity (i.e. the technical requirements for optimal test performance), the costs (both material- and labour-related) of performing them, and in their level of performance. Thus, each type of diagnostic test has its own strengths and shortcomings. As a result, in some settings, the most accurate test may not be the best one to use if it is so expensive that it cannot be used for large numbers of at-risk people, or if the test is so complex that results are not available in a timely fashion to guide patient management. In some instances, laboratory tests for STIs are available in a variety of formats and platforms, which influences the number of tests that can be performed in a given time period. Thus, test throughput (i.e. the number of tests completed in a given period of time) is also a consideration in test selection. In some settings, higher or lower volumes of tests will make some tests or test platforms preferable.

In general, diagnostic tests can be separated into at least three different types: direct detection, host response and metabolite detection. Direct detection of microorganisms themselves is the most obvious approach to STI diagnosis. This may be accomplished through the use of microscopy and appropriate staining or wet preparation to visualize pathogens. Culture, antigen detection, or nucleic acid detection using nucleic acid amplification tests (NAATs) are often more sensitive than microscopy, but they may have more complex technical requirements for optimal test performance, which will increase the interval between testing and the availability of test results (rapid POC tests help to overcome the latter limitation). Each of these approaches has its own strengths and weaknesses. Microscopy, particularly when performed in patients' presence, may provide immediate results to guide management decisions but it requires specialized equipment (the microscope), may require electrical power or special stain procedures, and its performance is dependent on specimen quality, as well as the training and experience of the microscopist. In contrast, other laboratory-based tests, such as culture or NAAT, may require special methods of specimen transport, and specialized equipment and procedures for optimal performance, thus delaying the availability of results for immediate management decisions. Highly accurate POC tests, particularly molecular tests that target DNA or RNA, are beginning to provide a clinical solution that offers the best of near-patient attributes and sensitivity, but come at a high purchase cost.

NAATs – whether laboratory-based or with attributes which would make them near-patient or POC tests – can be introduced judiciously to support the syndromic approach for the detection of STI pathogens. This is particularly useful in detecting those that perform poorly with the syndromic approach, such as cervical infections with *N. gonorrhoeae* and/or *Chlamydia trachomatis* in individuals presenting with vaginal discharge, as well as for detection of *M. genitalium* in men and women, where relevant *(3,4)*. In settings in which etiologies of urethral discharge in men are well characterized through periodic etiological studies, it may be more prudent to use the syndromic or presumptive treatment approach and reserve the use of NAATs or POC tests for those with recurrent urethritis, to ascertain the cause of persistence or aid in making a decision to embark on antimicrobial susceptibility testing. Another circumstance in which to consider the use of NAATs or POC tests is for screening of STIs, including HIV. Such a strategy is best guided by any available prevalence data or information on populations or settings at high risk of infection with STIs, including HIV.

For many important STIs (syphilis and HIV represent common examples), detection of the host response to infection (antibodies) represents a favoured diagnostic test. The strength of serological tests is that they may be useful, not only for diagnosis, but also for surveillance. All serological tests have occasional false-positive test results. The problem of false-positive serological tests can often be minimized by testing specimens that were positive on an initial screening serological test, using a second, unrelated confirmatory serologic test, which targets a different antigen. (The use of confirmatory tests is discussed in more detail in the sections on syphilis and HIV serological testing, in Chapters 13 and 18, respectively). Some serological tests may be able to differentiate recently acquired infections from more longstanding or previously treated infections through detection of immunoglobulin M (IgM) for recent infections. A shortcoming of serological diagnosis is that antibodies to an STI pathogen may persist long after successful treatment has been concluded. As such, serological testing of populations may be an indication

of total cumulative infection, rather than more recently acquired infections.

There are tests that detect microbial metabolites, such as materials altering the pH of genital secretions and biogenic amines. These tests are useful adjuncts for diagnostic purposes in some settings. An example of this is the importance of pH and whiff/amine testing in diagnosing bacterial vaginosis.

1.3 Test performance

Ultimately, the value of tests for STI detection also depends a great deal on their performance or validity of the test (Table 1.2). As measures of performance, calculations of sensitivity and specificity *(5)* – provided such calculations are performed using sufficiently large sample sizes – represent reliable estimates of the overall performance of tests. However, the predictive values (both positive and negative) of such tests can vary substantially between populations, depending on the prevalence of an infection in the community. Thus, tests that have substantial false-positive test rates and testing for relatively uncommon (i.e. low-prevalence) infections may have a low positive predictive value, despite having high sensitivity for detection of infection. In general, published peer-reviewed literature provides reliable estimates of test sensitivity and specificity. However, for any specific setting, local disease prevalence will partially determine the predictive value of laboratory tests. Thus, surveillance to determine local disease prevalence provides important data for choosing tests for STIs.

Although it is ideal to have a test that is both highly sensitive and specific, some tests may be sensitive, but less specific and vice versa. A balance has to be struck between the two. If an increase in sensitivity is preferred, in order to include all true positives and thereby reduce the number of missed treatments, this may lead to an increase in the number of false positives (i.e. decreased specificity). Making a decision on the appropriate test depends on the consequences of identifying false negatives and false positives. For infections (e.g. syphilis) where there are serious consequences to missing a diagnosis, it might be better to focus on high-sensitivity testing, and to accept the cost of false positives (which will result in overtreatment). Alternatively, an additional test can be performed that will increase specificity.

The ability to produce similar results for the same biological samples, the reliability of the test and the feasibility of performing the test are important considerations. Reliability increases with the validity of the laboratory procedure but also the ease of use, standardization of procedures and quality control procedures. The feasibility of performing the test will depend on the operational requirements of the test, such as space, clean water, a stable supply of electricity, and storage of specimens and reagents.

Table 1.2: Effect of sensitivity, specificity, and prevalence, using primary test only or supplementary test additionally, on positive predictive value

Population prevalence = 1%	A	B	C	D	E
Primary test Sensitivity/specificity	99/99	99/99.9	99/99	99.5/99.5	99.5/99.5
Supplementary/confirmatory test Sensitivity/specificity	ND	ND	99/99	ND	99.5/99.5
Number tested	1000	1000	11	1000	15
Negatives					
Total number	980	989	1	985	5
True negatives	980	989	1	985	5
False negatives	0	0	0	0	0
Positives					
Total	20	11	10	15	10
True positives	10	10	10	10	10
False positives	10	1	0	5	0
Positive predictive value (PPV)	50%	91%	100%	67%	100%

ND: not determined.

1.4 Laboratory types and roles

Although the traditional method of diagnosing STIs has been through laboratory analyses to determine the etiological agents, not all tests need to be performed in all laboratories for all purposes. Laboratory-based diagnosis of STIs tends to be expensive in terms of equipment, reagents, infrastructure and maintenance. Even more importantly – particularly in resource-constrained settings – the facilities where most patients with STIs are processed may have limited laboratory facilities *(2)*. To accommodate contexts in which laboratory facilities cannot be made available, the World Health Organization (WHO) developed guidelines on the use of the syndromic approach for the management of some STIs, and has advocated its use since the mid-1980s after field evaluations of its performance *(6)*. Subsequently, a number of field studies showed that the syndromic approach performs well in the management of men with symptomatic urethral discharge, and also in the management of men and women with bacterial genital ulcer disease *(7–11)*. This is not the case, however, for the diagnosis and management of cervical infection with *N. gonorrhoeae* or *C. trachomatis*, except in cases in which examination shows cervical mucopus, cervical erosions, and cervical friability, as well as a history of bleeding between menses and during sexual intercourse *(3,12,13)*. Nonetheless, syndromic diagnosis and treatment has the advantage of providing immediate care at the patient's first port of call for assessment and it is inexpensive; the patient, the service provider, or the state incur no direct laboratory costs. Using the syndromic approach also enables standardization of diagnosis, treatment, and reporting in a particular setting or situation. Patients with suspected STIs can be managed at outlets such as primary health-care facilities, family planning clinics and private practitioner consulting rooms, as well as in specialized STI clinics themselves *(14)*. Inevitably, the syndromic approach has the potential to lead to under- or over-diagnosis, resulting in missed or overtreatment of patients who may not be infected with any or some of the presumed causative organisms for the syndrome in question *(3,7,12,13)*.

Therefore, to support the syndromic approach to diagnosis, local clinical laboratories should be encouraged to perform the tests needed to facilitate clinical management of those with, or at risk for an STI *(3,4,15)*. In some settings where specimen transport is not problematic, economies of scale make specimen processing in central laboratories as timely and more efficient than testing at local sites. Thus, not all laboratories need to perform reference laboratory testing activities, such as antimicrobial susceptibility testing.

In settings where quality-assured molecular laboratory tests are available and same-day treatment is feasible, etiologic diagnoses for specific syndromes are recommended *(4)*.

The laboratory systems can be categorized into three levels, based on the levels of care and treatment services supported. However, it should be borne in mind that laboratory infrastructures and diagnostic capabilities vary widely between resource-constrained and industrialized countries. Consider the following categories as a general guide:

- Peripheral laboratories supporting the primary health care service level have limited laboratory equipment and minimally trained laboratory staff. These laboratories are designed to provide rapid on-site diagnosis of STIs. POC tests fit into this group as well as clinics with no laboratory capacity.
- Intermediate-level laboratories supporting the primary health care service laboratories and intermediate/district-level clinics and hospitals.
- Central-level laboratories supporting the tertiary health care facilities, including specialized reference STI clinics, as well as lower-level clinic laboratories and clinics.

This manual describes testing approaches ranging from basic and inexpensive, to sophisticated and expensive. Programme managers and laboratory experts, in collaboration with policy-makers, should determine the feasibility and utility of incorporating tests at the different levels of health-care facilities. The choices will also depend on whether different levels of the health-care facilities use the syndromic approach (basic or modified), the etiologic approach, or both.

1.5 Putting the pieces together

There is no single optimal test for the detection of agents causing STIs. For programmatic decision-making, the multiple STIs and impacted populations must be considered as a matrix to guide, not only which laboratory test is most appropriate for the community under consideration, but also what proportion of the total available resources (budget, personnel, etc.) should be allocated for each STI to be tested for (Table 1.3).

Table 1.3: Potential factors influencing choice of tests for STIs

1. Purpose of testing	
	• Diagnosis • Screening • Antimicrobial susceptibility testing • Surveillance • Quality assurance • Evaluation of syndromic diagnosis
2. Test-specific consideration	
	• Performance (sensitivity/specificity/positive and negative predictive values) • Specimen collection requirements including self-collection and transport requirements • Prevalence • Associated morbidity • Reliability – validity of the laboratory and ease of use • Feasibility – operational requirements • Resources – Financial – Personnel – Infrastructure (utilities, etc.) • Relative importance among other priorities

Decisions regarding the choice of tests must be conducted in the context of the prevalence of the infection under consideration, the impact of the infection on the community, the resources available to support testing and treatment, and the prioritization of that infection within the context of other STIs. Further, in choosing diagnostic tests, factors such as complexity, time to test results, and cost are also essential considerations. Mathematical modelling has clearly demonstrated that, in situations and settings in which test results are made available at the time of initial patient evaluation and where patients may be delayed in receiving treatment if they do not receive it at the time of evaluation, less sensitive tests provided at POC may actually increase the number of people treated and reduce the complications of infection when compared with more sensitive tests that require more time to make the test results available *(2)*. The acceptability of the test to the person is also an essential consideration. It has been shown that self-sampling and self-testing increases acceptability *(16)*. However, it will depend on the biological sample requested to determine whether self-sampling is possible.

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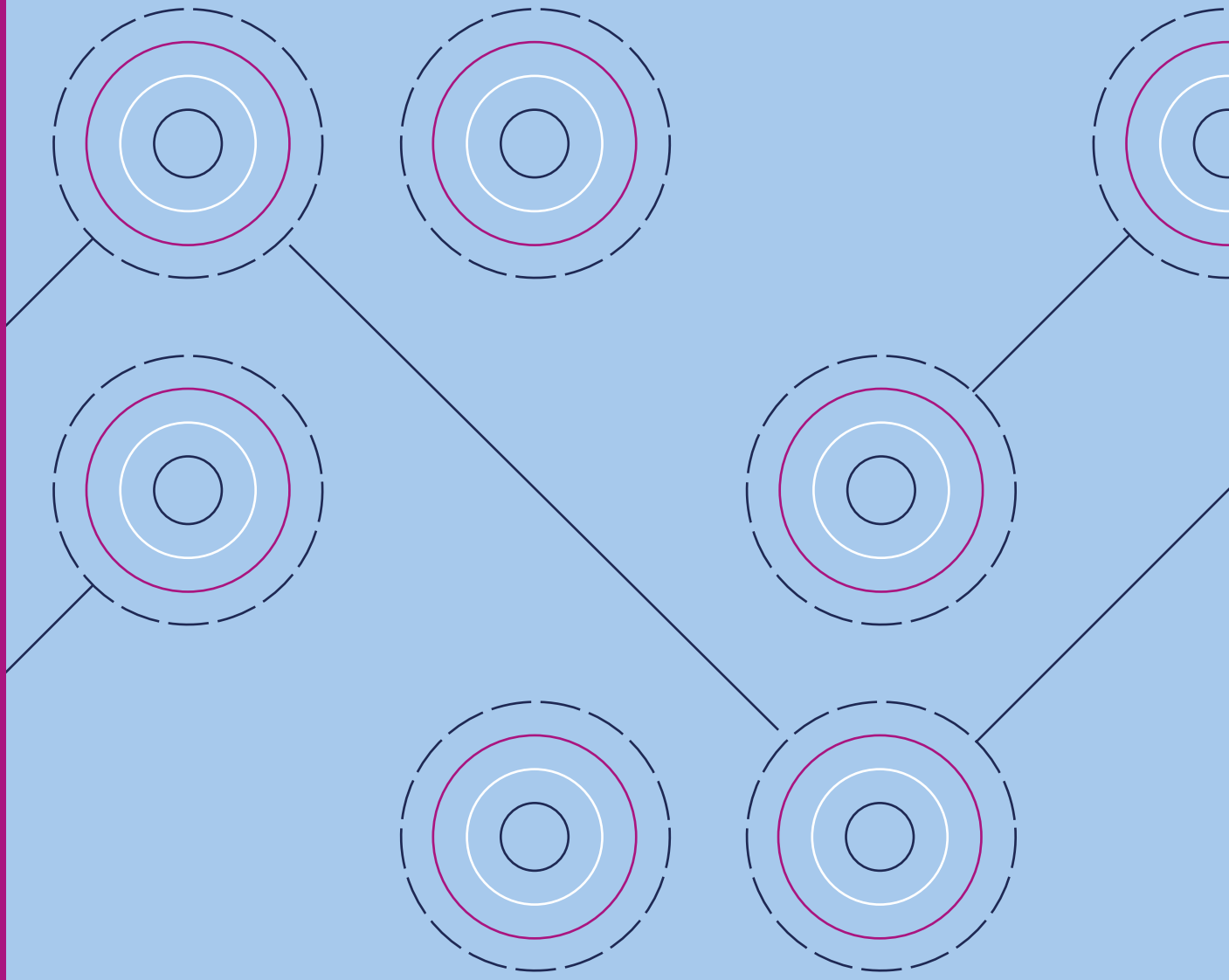
Chapter 2. Microscopy and principles of staining



Chapter 2. Microscopy and principles of staining

Catherine Ison and Frances Keane

2.1 Introduction

Microscopy for STIs and other genital conditions provides a simple, rapid and inexpensive test that can be used in close proximity to the patient *(1)*. Sensitive and specific, microscopy can give a presumptive diagnosis that guides treatment. Where appropriate, it can also facilitate notification of the patient's sexual partners at the time of consultation. This helps to break the chain of infection transmission. Interpretation of the microscopic image is a skill that requires training, including a good working knowledge of the microscope *(1)*.

The key to good microscopy is to choose the most appropriate microscope for your purposes and to have it set up correctly. The choice of microscope will depend on whether there is a requirement to examine wet preparations or Gram-stained smears (for which a bright-field transmitted light microscope is sufficient), or to detect treponemes to aid the diagnosis of syphilis (for which a dark-field microscope or attachments will be required). If there is a need to demonstrate the interpretation of images, a dual or triple head, or a video facility may be considered. Practical requirements, such as the space required, electricity supply, appropriate seating (see section 2.3) and cost of maintenance should also be considered.

2.2 Preparation of smears for microscopy

A good-quality specimen from the appropriate site, taken with a suitable swab or loop is an essential requirement for good microscopy technique.

It is important that the slide is clean and the specimen is placed on the correct side if it is frosted on one side. For wet preparations, sufficient sample should be placed onto the slide, with a drop of saline if necessary, and a coverslip placed carefully on the top. There should be sufficient liquid to spread under the coverslip only, and the sample should be examined immediately under low magnification without oil immersion. For smears requiring Gram staining, the specimen should be rolled evenly across the slide and fixed, either by heat or alcohol, stained well, and examine under oil immersion. If sampling for symptomatic urethritis in a man, he should hold his urine for at least two hours before the specimen is taken. When sampling women, it is preferable to avoid lubricant for speculum examinations, as this can make interpretation of microscopy findings difficult.

2.3 Bright-field transmitted light microscopy

Before setting up the microscope, a comfortable seating position should be adopted, with the back being supported and the eyepiece at eye level. The microscopist should have a good knowledge of the individual components of the microscope, which are shown in Fig. 2.1. Key terms are defined in Box 2.1. The microscope should be kept clean and covered when not in use, and serviced regularly. It is good practice to rack the stage away from the lens, and to use the lowest power that gives the best image.

Fig. 2.1: Components of a light transmission microscope

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Box 2.1: Key terms relevant to light microscopy

- **Magnification** is the number of times the length, breadth or diameter (but not the area) of an object is multiplied.
  - **Useful magnification** means that the image is sharp and fine detail is revealed, usually at a maximum of 1000×.
  - **Empty magnification** is seen at a certain power, where image sharpness is lost and no further detail is revealed.

Magnification = power of eyepiece × power of objective × power of inclined head.

  - It is often better to use a lower magnification to see object detail clearly and give a larger field of view.
- **Resolution** is the ability to reveal closely adjacent structural details as separate and distinct.
- **Definition** is the capacity of a lens to render a clear, distinct outline of the object image.
- **Field diaphragm** protects the object from excess heat and light that is not necessary for image formation.
- **Condenser or aperture diaphragm** contributes to the resolution and contrast of the image. Do not use for light brightness control.
- **Condenser** focuses light on the object. Modern instruments have a "flip-out" or auxiliary lens for use with objectives of 20× and greater to avoid continual resetting of the optics.

Procedure for setting up the light microscope:

1. Switch on the microscope.
2. Rack the stage down, place slide on the stage.
3. Swing in the 10× objective lens.
4. Looking at the stage, rack stage up (thinking about the working distance).
5. Adjust intraocular distance and focus on specimen.
6. Close the field diaphragm and open condenser fully.
7. Move the condenser up or down until the edge is sharp.
8. Using centring screws, centre the image.
9. Open field diaphragm (take care of light intensity).
10. Readjust light intensity for comfortable viewing.
11. Close condenser diaphragm slowly until the image sharpens and the glare disappears.
12. Readjust light intensity if required.

A demonstration of setting up a bright-field microscope can be seen at BSIGmicro *(1)*. Box 2.2 lists potential problems and their causes.

Box 2.2: Problems that may occur with light microscopy, and possible causes of the problems

- Object fuzzy and unable to get clear focus
  - oil on the lens, which should be cleaned with a recommended solvent
  - oil may be behind the lens; may need to contact the manufacturer
  - the condenser lens may have oil on it
  - slide may have been wet before oil was applied
  - different batches of oil may have been mixed together
  - coverslip may be too thick
  - slide is not spread sufficiently and is too thick
  - the correct side of the slide may not be in use
  - coverslip may be dirty with oil or finger marks
  - dried oil on the slide.
- Bubbles moving across the slide
  - different batches of oil may have been mixed
  - water still on slide when oil was applied
  - air bubbles may have formed when the oil was applied.
- Uneven illumination
  - microscope not set up correctly; condenser not centred
  - lens turret may be aligned incorrectly.
- Extraneous particles appear
  - dust may be present on eyepiece, lens, slide or coverslip, which should be removed carefully.

2.4 Typical microscopy findings in bright-field microscopy

2.4.1 Gram-stained samples

Microscopy of Gram-stained samples will, for the most part, involve male urethral, and female cervical and vaginal samples.

i. Male urethral samples

These can be examined for evidence of urethritis and, if present, additional features to distinguish between gonococcal and non-gonococcal urethritis. Some of the key features to look for are male epithelial cells (narrow Gram-negative ovoid cells with a single nucleus) and polymorphonuclear leucocytes (PMNLs; circular Gram-negative cells containing multilobulated nuclei). The presence of five or more of these cells over five or more high-power fields is diagnostic of urethritis. If urethritis is confirmed, then the microscopist should also look across all areas of the slide for small Gram-negative intracellular diplococci, the presence of which are strongly suggestive of gonorrhoea infection. The presence of Gram-negative extracellular diplococci is often described as suspicious "pairs" and need to be interpreted in the context of the clinical picture.

ii. Female cervical and vaginal samples

Cervical samples can also be examined for PMNLs and evidence of gonorrhoea, although there are no set criteria for the number of PMNLs per high-power field required to diagnose cervicitis. A female cervical sample is much less sensitive than a male urethral sample from a symptomatic man in the diagnosis of gonorrhoea. Gram-stained vaginal samples can be used to diagnose bacterial vaginosis (BV) and *Candida* infection. Vaginal epithelial cells, which are large polygonal cells with single nucleus (sometimes referred to as looking like fried eggs) are usually observed. Most laboratories use a scoring system based on the presence or absence of lactobacilli and other micro-organisms to diagnose BV; examples of which are the Ison/Hay criteria and Nugent's scoring system. The presence of yeast cells and pseudo-hyphae are diagnostic of *Candida* infection, but the particular subtype of *Candida* spp. cannot be determined by microscopy alone.

2.4.2 "Wet mount" vaginal preparations

"Wet mount" vaginal preparations can be used to diagnose BV as part of the Amsel criteria. The clue cells described are vaginal epithelial cells covered with many BV-associated organisms. Yeast cells and pseudo-hyphae can also be visualized on vaginal "wet mount" preparations, but the use of vaginal "wet mount" preparations would be generally regarded as slightly less accurate in the diagnosis of BV and *Candida* than a vaginal Gram-stained sample. The "wet mount" preparation is also routinely used to look for the motile flagellate protozoae *Trichomonas vaginalis*. Phase-contrast, bright-field or dark-ground microscopy can be used. Blood cells can often be seen on both cervical and vaginal Gram-stained samples and vaginal "wet mount" preparations.

Examples of the appearance of *Neisseria gonorrhoeae*, BV (including "clue cells", epithelial cells studded with bacteria), *T. vaginalis,* etc., can be found in the corresponding chapters of this manual.

2.5 Fluorescence microscopy

Fluorescence microscopy can be used to help visualize some bacteria and viruses that are not easily visible by bright-field microscopy, following staining by a specific antibody attached to a fluorochrome, immunofluorescence. It can be useful for the detection for microbial agents such as *Treponema pallidum, Chlamydia trachomatis,* and herpes simplex virus, or to aid identification using a species-specific antibody, such as for *N. gonorrhoeae*. Fluorescence microscopy requires training to provide expertise to interpret images, particularly in distinguishing artificial background fluorescence from specific fluorescence. The microscope is best kept and used in a room that can be darkened.

In fluorescence microscopy, the sample is stained using a fluorochrome and then illuminated with light of a wavelength that excites the fluorochrome in the sample; this is captured by the objective lens. For general purposes, for STIs, this can be achieved with an attachment to a transmitted light microscope, which houses a lamp (i.e. mercury-vapour or tungsten-halogen) and has two filters. One is an illumination (or excitation) filter, which ensures the illumination is near monochromatic and at the correct wavelength; and the second is an emission (or barrier) filter, which ensures none of the excitation light source reaches the detector.

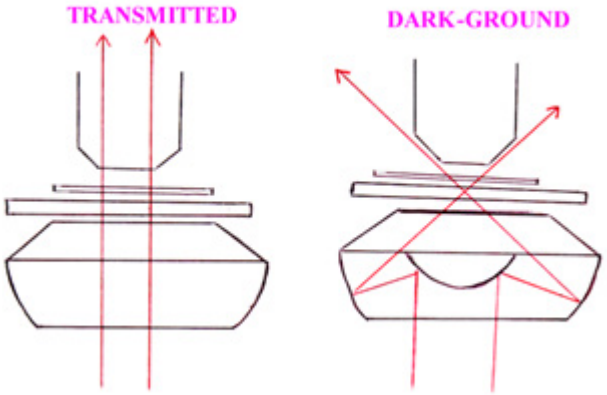
The following problems can occur with fluorescence microscopy:

- image is not visible or is darker than expected if the microscope is set up incorrectly or the shutter knob is closed;
- specimen is emitting secondary or autofluorescence caused by non-specific staining of the specimen or a dirty objective lens;
- the image is illuminated unevenly due to the components of the microscope not being properly aligned;
- there is excessive glare in the eyepiece because the correct filters are not present;
- rapid bleaching of the specimen when being viewed.

2.6 Dark-field microscopy

Dark-field (or dark-ground) microscopy differs from bright-field (transmitted light) microscopy in that only light rays striking organisms or particles at an oblique angle enter the microscope objective, giving rise to bright, white luminescent bodies against a black background (Fig. 2.2). Dark-field microscopy for the detection of *T. pallidum* to diagnose syphilis has to be performed by well-trained and experienced personnel, who are able to adjust the microscope correctly and to differentiate *T. pallidum* from non-pathogenic treponemes and other spiral organisms commonly found on genital and anal mucous membranes. The treponemes appear as coiled organisms that display characteristic movement, including angulation and corkscrew motility. As the oral cavity is often colonized by spirochaetes other than treponemes, dark-field examination of material from oral lesions is not recommended.

Fig. 2.2: Comparison of light path between a transmitted light microscope and a dark-field (dark-ground) microscope



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Procedure for use of the dark-field microscope

i. Make a thin preparation (see Chapter 13 for details) using a thoroughly cleaned slide and coverslip. Too much material will negate the effect.

ii. Rack the condenser down and place a drop of oil on top of the lens. Take care to avoid trapping air bubbles.

iii. Place the slide on the stage and slowly rack up the condenser until the oil just touches the slide. A brief flash of light will be seen. Take care not to allow bubbles to form.

iv. Focus on the object with a low power objective (10×). If the condenser is correctly focused, a small point of light will illuminate the object against a dark background. If a hollow ring of light is seen, the condenser needs to be adjusted.

v. With the centring screws, adjust the condenser until the point of light is in the middle of the field.

vi. Place a drop of oil on the coverslip and focus the object with the oil-immersion lens. If an objective with an iris diaphragm is used, slight adjustment may be necessary. If the objectives are not parcentral, then centring of the condenser may be required.

vii. Dark-field microscopy is best conducted in a darkened room.

A demonstration of setting up a dark-field microscope can be seen at BSIGmicro *(1)*.

The following problems may occur with dark-field microscopy:

- slides or coverslips may be too thick
- preparation has too many air bubbles
- condenser may not be correctly focused or centred
- lighting may not be sufficiently bright
- field of view is too small (open field diaphragm)
- centre of the field of view is dark (the slide is too thick or the condenser is in the wrong position).

2.7 Phase contrast microscopy

Phase contrast microscopy is seldom available or used, but it has some advantages when examining living, unstained specimens, because they have the same refractive index as mounting fluid and internal detail may be difficult to see. Unstained objects may be considered to have the properties of a diffraction grating. Light passing through different parts of the object is affected and its phase slightly changed, but this is not discernible to the eye. However, by optical means these phase differences may be made visible, as light and dark areas.

It is necessary to have the following:

- a special condenser carrying a series of annular diaphragms, the annulus size varying with the numerical aperture of the objective used;
- special phase objectives containing a phase plate (this plate consists of a disk of glass with a circular trough etched in it and of such depth that light passing through this area has a phase difference of one quarter of a wavelength compared with the rest of the plate).

Procedure for use of the phase contrast microscope

i. Prepare a thin, not too crowded, specimen slide and swing in the objective (objectives are labelled Ph, Phaco or similar).

ii. Rotate the appropriate annulus for that objective into position in the condenser.

iii. Check that the annulus and phase plate are in the correct position using the telescope supplied with the system.

iv. Remove an eyepiece a nd insert the telescope. Images of the annulus and phase plate should be coincident. If not, adjust with the condenser centring screws.

v. Replace the eyepiece and examine the specimen.

vi. All powers of objective may be used, provided they are fitted with a phase plate and that there is an appropriate annulus in the condenser.

The following problems can occur with phase contrast microscopy:

- condenser and objective not matched by Ph code;
- phase plate is not aligned so it is necessary to check using the telescope;
- the field diaphragm is not focused on the specimen surface – this should be solved by moving the condenser up or down.

2.8 Gram stain

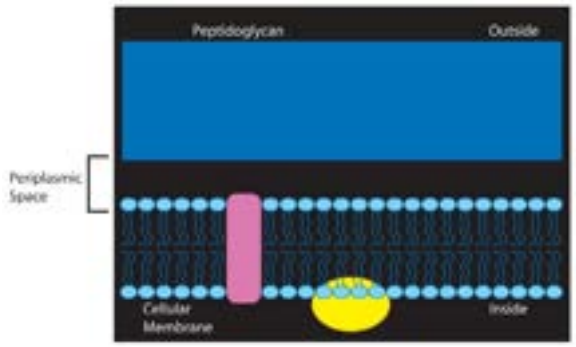
This stain was first described in 1884 by Gram and demonstrates the morphology of bacteria and fungi. It divides bacteria into two main groups: those that appear Gram-positive (purple in colour) or those that appear Gram-negative (red or pink in colour, depending on the type of counterstain used). Each of these then can be further divided into cocci (spheres) or bacilli (rods), resulting in four groups. Many bacteria that appear very distinctive, such as *N. gonorrhoeae*, are Gram-negative diplococci, but it is important to remember that the Gram stain reaction only gives a presumptive identification. Other identification methods are necessary to classify any bacteria. Fungi appear as Gram-positive.

2.8.1 Principle of the Gram stain

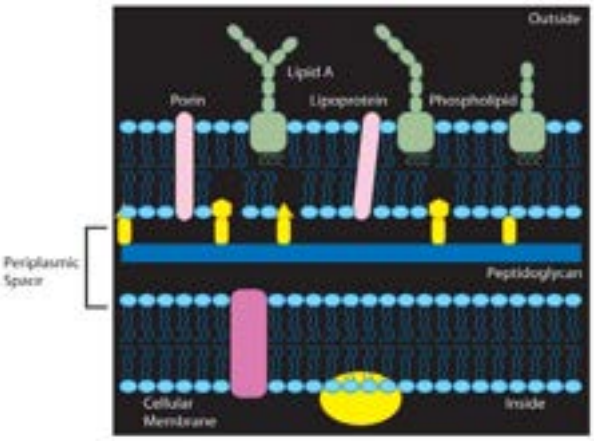
The Gram stain reflects differences in the bacterial cell wall structure in that Gram-positive bacteria have large amounts of peptidoglycan in the cell wall, which retains the purple stain (Fig. 2.3A), whereas Gram-negative bacteria have a complex cell wall with less peptidoglycan, which does not retain the purple stain, but takes up the pink counterstain (Fig. 2.3B).

Fig. 2.3: Cell wall structure of Gram-positive and Gram-negative bacteria

A. Gram-positive cell wall structure



B. Gram-negative cell wall structure



Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

2.8.2 Method of use

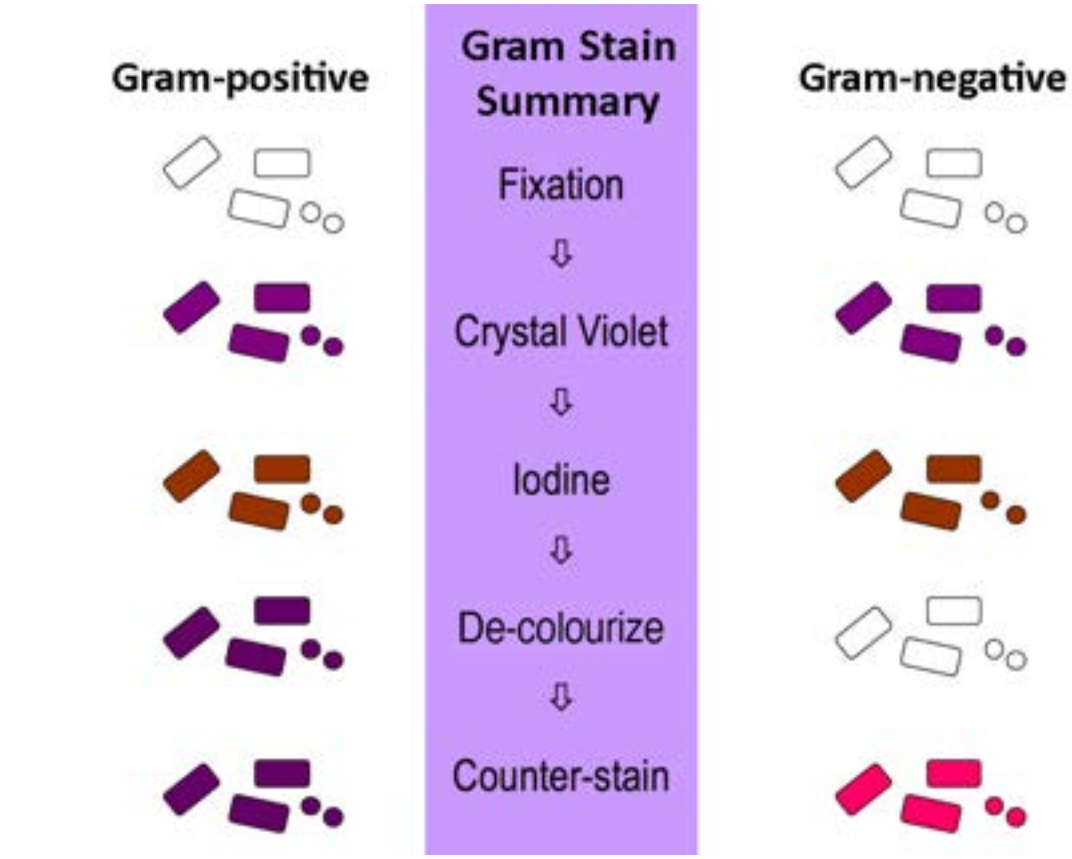
A fixed smear is flooded with crystal violet for 30 seconds, during which all bacteria take up this stain (Fig. 2.4). The smear then is gently washed in tap water and flooded with Lugol's iodine for a further 30 seconds. This acts as a mordant and fixes the purple stain in the cell membrane.

After washing with water, a decolourizer is used to remove excess stain; it is at this stage that the crystal violet/iodine complex is washed out of the cell membrane of Gram-negative bacteria, as there is insufficient peptidoglycan to retain it. A number of decolourizers are used, ranging from alcohol, which is slow and gentle, to acetone, which is harsh and fast. The time the decolourizer is left on the smear varies from a few minutes for alcohol to just a few seconds for acetone. It is advisable to hold the smear over the sink and to allow the decolourizer to run over the smear. The purple complex will run out of the smear and, as soon as this stops, the smear should be washed with water. It is easy at this stage to over-decolourize the smear, particularly if acetone is used; many find a mixture of acetone and alcohol (at a ratio of 1 : 1) a good combination, as the speed of the acetone is slightly tempered by the alcohol.

After decolourization, the smear is flooded with a counterstain for approximately 1 minute and it is at this stage that Gram-negative bacteria take up the pink stain. There are, again, a number of counterstains used: safranin, neutral red, and carbol fuchsin. Safranin and neutral red are good stains for smears that contain polymorphs, as these give good definition of the cell structure, whereas carbol fuchsin is preferable for bacteria.

The individual times used for each of the stains can vary, but the absolute times used are not crucial. However, it is important to remember that, while the primary stain (crystal violet) acts almost instantly, counterstaining is a slower process and requires more time. It is advisable to leave the counterstain on for twice the time of that for the primary stain. Washing with water between the addition of each stain is not essential and tends to be a personal preference, but it is necessary to remove the decolourizer by flooding with water, as described above.

Fig. 2.4: Reaction of Gram-positive and Gram-negative bacteria to the different stages of the Gram stain



Source: Reprinted with permission from the British Association for Sexual Health and HIV (BASHH).

2.8.3 Problems and remedies

Stains that are well-prepared and stored correctly are essential for producing a well-stained smear. The individual stains are available commercially and it is now relatively unusual for these to be prepared from the powder. However, even when stains are purchased, either ready for use or as concentrated stain to be diluted for use, it is important to store the stains correctly. Stains should be stored in clean bottles and, if the bottles are reusable, any residual stain should be discarded and then rinsed with water before refilling. Inappropriately stored stains can result in stain deposit, either over the entire smear or adhering to epithelial cells, which could be confused with "clue cells".

The smear needs to be of an appropriate thickness; a smear that is too thick will retain the stain and prevent differentiation between Gram-negative and -positive organisms, and a smear that is too thin and has insufficient material may not give a true representation of the sample.

The decolourizing step is the most crucial in the Gram stain, and over- or under-decolourization will result in bacteria being categorized incorrectly as either Gram-negative or Gram-positive, respectively.

2.9 Methylene blue stain

The use of methylene blue as a single stain is very useful in settings where there are either inadequate facilities or resources for using the Gram stain. It will show the morphology of both bacteria and host cells, but lacks the differentiation obtained with the Gram stain. Its primary use is for staining smears for the presumptive diagnosis of gonorrhoea, and gives a distinctive picture that has a high predictive value in high-risk patients.

2.10 Giemsa stain

Giemsa stain is a differential stain used for the diagnosis of donovanosis. It is a mixture of methylene blue, eosin and azure B, and is available commercially. A smear is prepared, fixed in methanol for 30 seconds, immersed in freshly prepared 5% Giemsa stain for 20–30 minutes, washed and allowed to dry.

2.11 Immunofluorescence

Immunofluorescence is a technique that uses the highly specific binding of an antibody to its antigen to label specific proteins or other molecules within the cell. A sample is treated with a primary antibody, specific for the molecule of interest. A fluorophore can be conjugated directly to the primary antibody. Alternatively, a secondary antibody conjugated to a fluorophore, which binds specifically to the first antibody, can be used.

2.12 Health and safety

There should be a person nominated to be responsible for health and safety in each workplace, either clinic or laboratory. The staining methods should be described in standard operating procedures, which should be regularly updated, and read and understood by all staff.

The following specific issues need to be addressed:

- no eating, smoking, or drinking in a laboratory area;
- provide good ventilation in the area used for staining to avoid inhalation of fumes and fire hazards;
- wear gloves and appropriate laboratory coats when handling the individual reagents used for staining, as these are known to be toxic; some Gram stains are carcinogenic;
- store stains carefully to prevent spillage;
- take care when disposing of slides and coverslips from infected patients;
- store flammable material in a specific cupboard and keep away from heat sources.

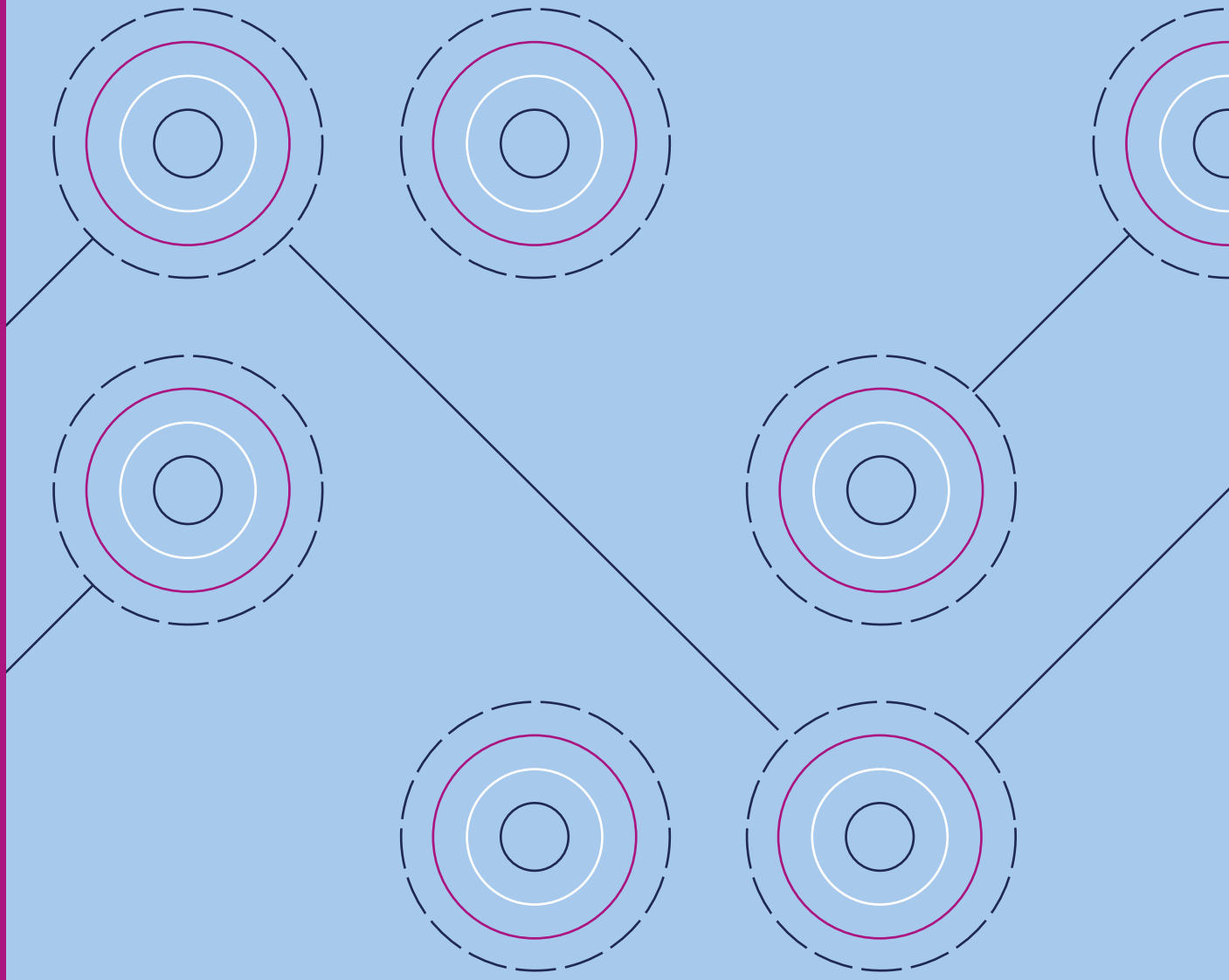
2.13 Quality assurance

Quality assurance (QA) programmes for any procedure are designed to ensure that the correct result is obtained and the best outcome for the patient is achieved (see Chapter 5). However, QA protocols for microscopy are not as well developed or commonly used in clinics, compared with laboratories. National programmes are not available, to our knowledge, and competency assessments are often organized at a local level. This is discussed in more detail on the BSIGmicro website or app *(1)*.

2.14 Reference

1. BSIG Micro [website and mobile application]. British Association for Sexual Health and HIV (BASHH); 2021 (https://bsigmicro.org/, accessed 24 May 2023).

Chapter 3. Point-of-care (POC) tests and test principles



Chapter 3. Point-of-care (POC) tests and test principles

Maurine Murtagh, Igor Toskin, Emma Harding-Esch and Charlotte Gaydos

3.1 Introduction

Diagnostics have been undervalued in public health. However, the WHO "triple billion" targets include improved access to diagnostics for primary health care, as a strategy to provide universal health coverage for 1 billion more people *(1)*. With some exceptions, diagnostics for STIs are laboratory-based platforms, which typically require a strong infrastructure and well-trained technicians. In addition, test turnaround time (TAT) is often long, requiring patients to return to the clinic for results. This, in turn, can lead to significant loss to follow-up. In low- and middle-income countries (LMICs), these requirements limit diagnostic access and delivery. Therefore, in order to achieve the "triple billion" goals, moving testing closer to the health service user, i.e. providing testing at or near the point of care (POC) for patients, will be essential.

The need to implement POC testing in LMICs is especially critical because, while WHO recommends the use of syndromic management, whereby patients with symptoms of STIs are managed for all the major causes of their particular syndrome, many STIs, especially in extragenital sites, are asymptomatic *(2,3)*. In light of this, recent research publications have called for the development, implementation and subsequent widespread use of POC tests (also known as POCTs) for STIs *(2,4–6)*, as it promises significantly improved performance compared with syndromic management. It also reduces overtreatment with antimicrobials, thereby decreasing the selection for antimicrobial resistance (AMR) among STI pathogens and bystander commensal or non-STI pathogenic bacterial species *(6)*. Compared with traditional laboratory diagnosis, POC tests may lower testing costs for health service users and health-care systems, reduce waiting times, speed up and increase accurate management, and improve patient follow-up. Therefore, simple, affordable, rapid tests that can be performed at POC are needed, not only to increase the specificity of syndromic management and reduce overtreatment of STIs, but also to screen for asymptomatic STIs *(2,7–9)*. POC tests for STIs also offer the opportunity to initiate immediate patient counselling. POC tests have also been shown to be cost-effective compared with alternatives such as syndromic management and laboratory diagnosis *(10–13)*.

3.2 Definition and characteristics of an ideal point-of-care (POC) test

A consensus definition proposed for a provider-based POC test is a "test to support clinical decision making, which is performed: (i) by qualified staff nearby the patient; (ii) during or very close to the time of consultation; (iii) to help the patient and clinician to decide upon the most appropriate approach; and (iv) for which results should be known at the time of clinical decision making" *(14,15)*. This definition can accommodate POC tests utilizing various technologies and in a variety of formats – for example, lateral flow (or vertical flow) tests (these are similar to pregnancy tests in format and are hereafter referred to as rapid diagnostic tests [RDTs], see section 3.4), or molecular tests (see section 3.5) – so long as they permit patient treatment decisions to be made at the time of patient encounter.

In order to meet this definition, regardless of its format, a POC test should have certain characteristics. In 2003, the acronym, "ASSURED", was coined to describe the ideal criteria for a POC test to be used at all levels of a health-care system. This requires POC tests to be affordable, sensitive, specific, user-friendly, robust and rapid, equipment-free and deliverable to end-users *(16)*. In recent years, two additional criteria for an ideal POC test have been proposed: real-time connectivity (R) and ease of specimen collection (E). This has led to the definition of a new acronym, the "REASSURED" criteria (see Box 3.1) *(6)*.

Box 3.1: The REASSURED criteria for the ideal point-of-care (POC) diagnostic test

R = Real-time connectivity
E = Ease of specimen collection
A = Affordable
S = Sensitive
S = Specific
U = User-friendly (simple to perform in a few steps with minimal training)
R = Robust and rapid (can be stored at room temperature and results available in < 30 minutes)
E = Equipment-free or minimal equipment that can be solar- or battery-powered
D = Deliverable to those who need them

POC tests that meet the REASSURED criteria could increase access to testing and identification of STIs in a single health service encounter in LMICs, as well as in high-income countries. They could be used at all levels of the health-care system, while contributing to the improvement of STI surveillance through POC test electronic or cloud connectivity.

3.3 Availability and impact of point-of-care (POC) tests

Although there are no STI tests currently available for use at or near the POC that meet all of the REASSURED criteria, a number do meet the original ASSURED criteria. These include RDTs for syphilis and dual rapid HIV/syphilis RDTs, which exhibit adequate performance and acceptability. Similarly, rapid, accurate, relatively low-cost and equipment-free RDTs for *Trichomonas vaginalis* are also available *(17)*. However, for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, RDTs suffer from low diagnostic accuracy, particularly low sensitivity. RDTs are also not adequate for diagnosing *Mycoplasma genitalium*, human papillomavirus (HPV), or herpes simplex virus (HSV) type 1 (HSV-1) and type 2 (HSV-2).

In circumstances where RDTs for STIs exhibit acceptable performance, their use is recommended in LMICs with respect to syndromic management *(5)*. However, molecular POC tests are preferred. Molecular POC tests for *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, *M. genitalium* and other STIs overcome the performance limitations of RDTs while maintaining rapidity and offering real-time connectivity. However, they are not yet equipment-free and are generally too expensive for routine implementation in most LMICs.

Where POC tests that meet the ASSURED criteria have been implemented, they have demonstrably improved patient management and public health. The individual criteria are of varying importance, depending on the context of implementation *(10,17–19)*. These benefits include:

- reduced time to notification of results: POC test TAT enables patients to receive test results faster, including during the initial clinical visit;[1]
- faster (and appropriate) treatment delivery: receiving test results in an initial clinical visit means patients can be treated immediately, thus reducing loss to follow-up; asymptomatic infections can be treated; and presumptive treatment can be avoided with detected specific causative organism treated, thus reducing overtreatment with antibiotics;
- the opportunity for immediate patient counselling to be initiated: individuals with positive test results can receive appropriate counselling that is tailored to the specific infection and the patient's situation;
- improved partner treatment/tracing and reduction of transmission; having a specific diagnosis at the time of the initial visit can facilitate partner notification discussions, leading to improved partner treatment;
- reduced onward transmission and progression of disease; faster and appropriate treatment can help break the chain of transmission between partners and prevent disease progression;
- improved patient acceptability: patients and health workers find POC testing clinical pathways acceptable, and the ability to know the test result at the initial clinic visit desirable; and
- cost-effectiveness; the ability to diagnose and treat the patient in the same clinic visit avoids the need for a second visit for treatment and partner notification discussions, saving both the patient and the health worker time and money; and reducing transmission, which in turn reduces the number of individuals needing of diagnosis and treatment, and successful treatment of infections saves the costs associated with managing the reproductive health sequelae to which the infection would have progressed.

The increased focus of manufacturers on developing STI POC tests in recent years has resulted in a reasonably strong pipeline for platforms that are suitable for use in LMICs, as evidenced by the current landscape of STI

[1] For the purposes of this chapter, TAT of a POC test means the time interval between placing the sample onto or into the test device (i.e. a strip, cassette, cartridge or instrument) and a result being reported on the test device.

POC diagnostics *(20)*. However, continued support for pipeline technologies is required. This includes technical guidance, financial support, ongoing advocacy and political will *(6)*.

3.4 Principles of rapid diagnostic test (RDT) technologies

3.4.1 Lateral flow tests (LFTs) for STIs

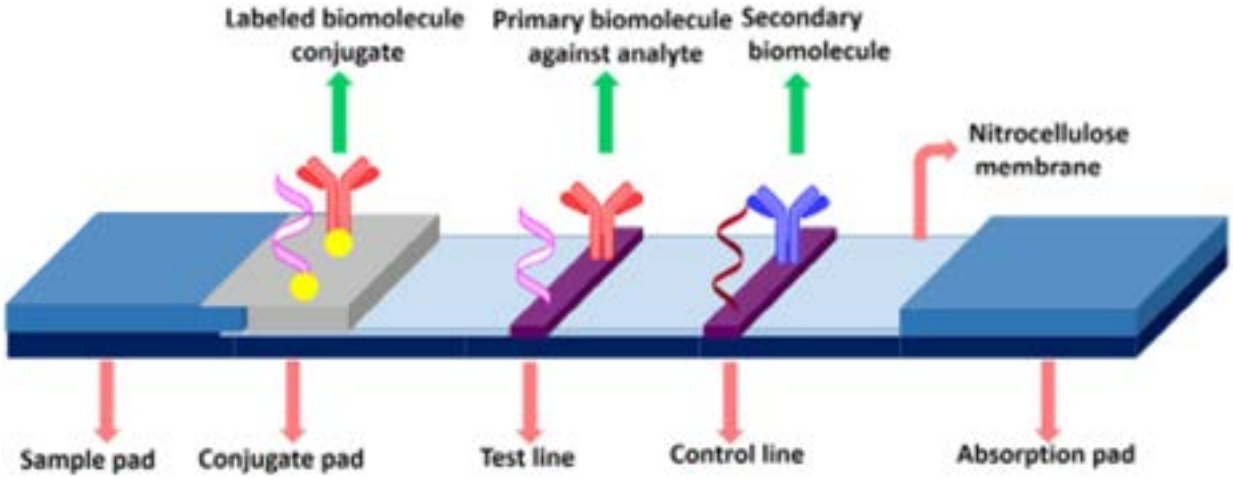
Most RDTs for the detection of STIs are in lateral flow (immunochromatographic) format.[2] Such LFTs generally consist of a plastic device that contains a composite assay strip, with a reagent/sample pad at one end and an absorption pad at the other.

Fig. 3.1 shows the construction of an antibody-based LFT. The centre of the strip contains two or more lines bound in a nitrocellulose membrane. One or more of the test lines consists of a infection-specific antibody (Ab) and one line is used as a control. The same test format can be used for antigen (Ag) testing, with the substitution of an infection-specific Ag for the Ab. The reagent pad contains dried conjugate consisting of colloidal gold (Au)-labeled Abs (or Ags) specific to each test.

To perform the test, the specimen (e.g. fingerstick whole blood, urine, vaginal secretion) is dropped onto the specimen pad and a few drops of buffer are added. The Abs flow through the conjugate pad and combine with colloidal gold-labelled Ag and form a gold Ag-Ab complex. As the complex crosses the test line, it combines with the Ag immobilized on the test line and, when sufficient labelled Ag accumulates, the dye labels will become visible to the naked eye as a narrow red line (the test line). Finally, free conjugate combines with the Ab immobilized on the control line, turning it red.

If a specimen does not contain any infection-specific Abs, it flows through the conjugate pad and the test line without turning the line red. The free conjugate that flows through with the specimen will just turn the control line red. If a specimen did not migrate from the specimen well, no test or control line will appear, indicating an invalid as say, as illustrated in Fig. 3.2.

Fig. 3.1: Schematic of an antibody-based lateral flow test (LFT) and its components



Source: Bahadır and Sezgintürk, 2016 *(21)*. © 2016, reprinted with permission from Elsevier.

Fig. 3.2: Possible lateral flow test (LFT) results

[2] It should be noted, however, that some assays use a vertical flow (immunofiltration) format that is generally contained in a cassette. Fluids (i.e. sample, reagents, wash solutions) are passed through a reactive, porous membrane into an absorbent pad. The target analyte is captured in the membrane.

Test results from these LFTs can generally be read visually after 10–30 minutes, depending on the test. It should be noted that most LFTs are read with the naked eye, which means that the interpretation of the results is subjective, and can be highly variable (leading to false-positive and false-negative results), especially in settings where training and supervision may be inadequate.

One potential tool that can be used to standardize RDT interpretation and to support quality assurance is a diagnostic test reader. Such readers can capture an image of the RDT window and store the test result, which can then be retrieved through a USB port, or uploaded via Wi-Fi or cellular networks. Readers also permit transmission of test results to a cloud or central database, allowing tests to be reviewed remotely, for quality control purposes. These capabilities enable strengthening of in-country test surveillance and, by linking the data to supply chain management software, could also be used to improve supply chain management *(22)*.

It should also be noted that LFTs can be multiplexed, either with multiple analytes on the same test strip (with a single sample inlet) or with several strips on the same cassette (with the same number of sample inlets as the number of strips) *(23)*.

3.4.2 RDTs for HIV and syphilis

i. HIV screening tests

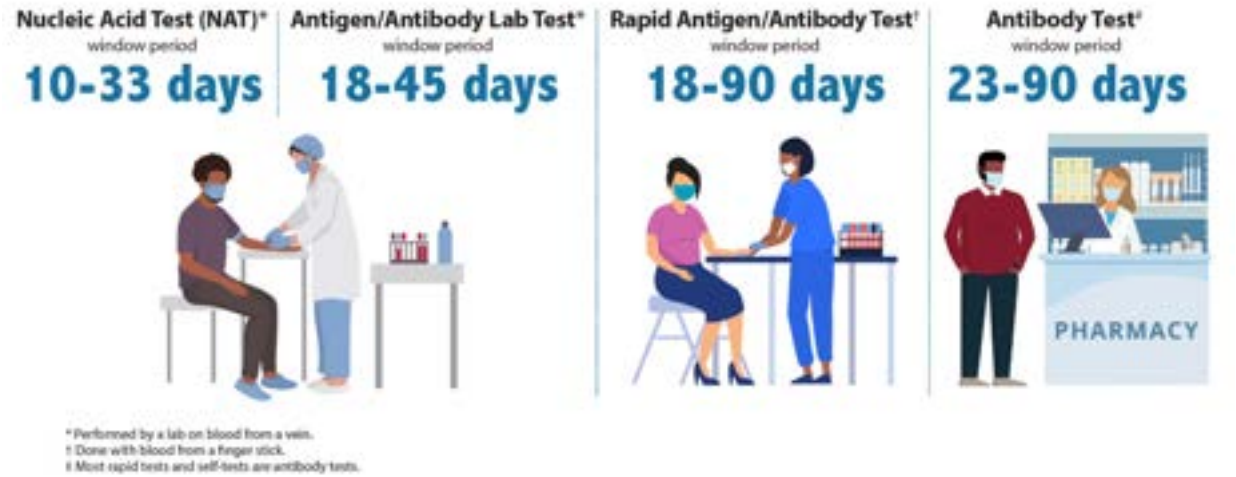
For the most part, HIV RDT screening tests are Ab assays. They are commonly used for the routine diagnosis of patients older than 18 months.

Because of the persistence of maternal Abs in infants under 18 months of age, however, such Ab tests cannot be used to accurately screen infants for HIV. Instead, DNA or RNA testing (i.e. virological testing) should be used to determine the HIV status of infants in this age group. Such early infant diagnosis (EID) is most commonly performed using molecular tests, which are discussed later in this chapter.

With respect to all HIV screening tests, it is important to keep in mind the time between HIV exposure and when a test can detect HIV in the body, which is known as the "window period". Fig. 3.3 shows how the window period varies with the type of HIV test and the type of specimen used. Currently, HIV Ab RDTs can usually detect HIV 23–90 days following exposure. A combined HIV Ag/Ab RDT can detect HIV 18–90 days following exposure using fingerstick blood; a lab-based HIV Ag/Ab test can detect HIV 18–45 days following exposure using venous blood. Molecular tests, in particular nucleic acid amplification tests (NAATs), shorten the window period to 10–33 days *(24)*. For additional information on HIV screening tests, see Chapter 18.

First-generation HIV tests (i.e. enzyme-linked immunosorbent assay [ELISA] and chemiluminescence methods) detected immunoglobin G (IgG) antibody to HIV-1 only; they only became positive 6–12 weeks after infection *(25)*. Second-generation tests, including RDTs, added glycopeptides (recombinant Ags), specifically HIV-1 p24 Ag. Third-generation tests added immunoglobin M (IgM) detection to the assay. Finally, fourth-generation tests simultaneously detect p24 Ag (HIV-1 Ag), as well as HIV-1/HIV-2 Ab *(25)*.

Fig. 3.3: Window period for HIV tests



Source: Adapted from the Centers for Disease Control and Prevention (CDC), 2022 *(24)*.

There are numerous commercially available RDTs for detecting HIV. Most use either fingerstick capillary blood or oral fluid as the sample, but can also take plasma, serum or venous blood. The tests vary in design, TAT and stability of results. Tests can be read 1–20 minutes after the sample has been applied to the test, with most taking 10–20 minutes. The stability of test results for most assays, known as the read window, is short. The shelf life of the tests also varies, with most lasting 15–30 months.

Currently, 21 HIV RDTs, most of which are third-generation tests, are WHO prequalified for professional use only. These tests are to be performed by health workers, and are intended to be used as part of an algorithm wherein multiple reactive results are needed to confirm HIV infection. Currently, there are also six HIV RDTs that are WHO prequalified for self-testing. In self-testing, the user collects a specimen, performs the test and interprets the result themselves. Of these, only the OraQuick HIV Self Test uses oral fluid; the others take fingerstick capillary blood samples. The tests also vary in the number of steps involved, TAT (which ranges from 1–20 minutes) and shelf life (which ranges from 15–30 months). Both types of WHO prequalified HIV RDTs are listed in Box 3.2.[3]

Box 3.2: WHO prequalified (PQ) HIV rapid diagnostic tests (RDTs)

PQ for professional use only (to be performed by health workers):

ABON HIV 1/2/O Tri-Line HIV Rapid Test (ABON Biopharm [Hangzhou] Co. Ltd, China)
Bioline HIV-1/2 3.0 (Abbott Diagnostics, USA)
Determine HIV Early Detect (Abbott Diagnostics, USA)
Determine HIV-1/2 (Abbott Diagnostics, USA)
Diagnostic kit for HIV (1+2) antibody (colloidal gold) v2 (Shanghai Kehua Bio-Engineering, China)
DPP HIV-1/2 Assay (Chembio Diagnostic Systems, USA)
First Response HIV 1-2-O Card Test v2.0 (Premier Medical, India)
Geenius HIV 1/2 Confirmatory Assay (Bio-Rad Laboratories, France)
Genie Fast HIV 1/2 (Bio-Rad Laboratories, France)
HIV 1/2 STAT-PAK (Chembio Diagnostics, USA)
HIV 1/2 STAT-PAK Dipstick (Chembio Diagnostics, USA)
INSTI HIV-1/HIV-2 Antibody Test (bioLytical Laboratories, Canada)
MERISCREEN HIV 1-2 WB (Meril Diagnostics Pvt. Ltd., India)
ONE STEP Anti-HIV (1&2) Test (INTEC Products, Inc., China)
One Step HIV 1/2 Whole Blood/Serum/Plasma Test (Guangzhou Wondfo Biotech Co., China)
OraQuick HIV 1/2 Rapid Antibody Test (OraSure Technologies, Inc., USA)
Rapid Test for Antibody to HIV (Colloidal Gold Device) (Beijing Wantai Biological Pharmacy Enterprise Co. Ltd, China)
STANDARD Q HIV 1/2 Ab 3-Line Test (SD Biosensor, Inc., Republic of Korea)
SURE CHECK HIV 1/2 Assay (Chembio Diagnostics, USA)
TrinScreen HIV (Trinity Biotech Manufacturing, Ireland)
Uni-Gold HIV Test (Trinity Biotech Manufacturing, Ireland)

PQ for self-testing (using a self-collected specimen):

Check-NOW HIV (Abbott Diagnostics, USA)
INSTI HIV Self Test (bioLytical Laboratories, Canada)
Mylan HIV Self Test (Atomo Diagnostics Pvt. Ltd, Australia)
OraQuick HIV Self Test (OraSure Technologies, Inc., USA)
SURE CHECK (Chembio Diagnostics, USA)
Wondfo HIV Self-Test (Guangzhou Wondfo Biotech Co., China)

[3] The regularly updated list of WHO prequalified in vitro diagnostic products can be found at: https://extranet.who.int/pqweb/vitro-diagnostics/vitro-diagnostics-lists. In addition, WHO has a toolkit to optimize HIV testing algorithms, which is a good source of information for the professional-use tests, available at: https://www.who.int/tools/optimizing-hiv-testing-algorithms-toolkit. The toolkit contains numerous specifics, including sensitivity and specificity of the tests, as well as price and operational characteristics, and is kept up to date *(26)*.

In addition to WHO prequalification, some HIV RDT assays have U.S. Food and Drug Administration (FDA) approval, including, but not limited to, the DPP HIV-1/2 Assay and HIV 1/2 STAT-PAK (both from Chembio Diagnostics), Determine HIV 1/2 (Abbott Laboratories), INSTI HIV-1/HIV-2 Antibody Test (bioLytical Laboratories), and the OraQuick HIV Self-Test (OraSure Technologies).[4] Of particular note, the Determine HIV 1/2 Ag/Ab Combo Test (Abbott Laboratories) – which is currently the only fourth-generation HIV RDT available – is FDA approved. There are also many commercialized HIV RDTs that are Conformité Européenne (CE)-marked in vitro diagnostic (IVD) medical devices (CE-IVDs), having been approved for use in the European Economic Area (EEA).[5]

HIV RDTs have been extensively evaluated. In a systematic review and meta-analysis, Kingwara et al. evaluated the operational characteristics and performance of HIV RDTs from 2012 to 2020. The analysis included 26 studies for diagnostic accuracy. The authors found that the average RDT sensitivity for diagnostic accuracy was 99% (95% confidence interval (CI) = 0.99–100), while specificity was 100% (95% CI = 99–100) *(28)*. The analysis also included 15 qualitative studies that evaluated the acceptability, ease of use, and cost-effectiveness of RDTs. In this regard, most of the studies demonstrated a high level of acceptability of RDTs, and several studies indicated that cost remains a barrier to accessing HIV RDT testing. TAT is a significant factor influencing test preference *(28)*.

ii. Syphilis screening tests

There are many RDTs for syphilis screening that detect *Treponema pallidum subspecies pallidum* (TP), which is the bacterium that causes syphilis (see also Chapter 13). These include, but are not limited to, the five CE-IVD-marked RDTs listed in Table 3.1 along with their operational characteristics.

Because of the persistence of TP Abs (i.e. treponemal Abs), these TP RDTs cannot distinguish between active and past-treated infections. In LMICs, where many people do not have access to laboratory-based non-treponemal tests (e.g. rapid plasma reagin [RPR]) to confirm active syphilis, pregnant women who are found to be seropositive with a TP RDT are treated for syphilis in order to prevent transmission of the infection. As indicated by Jafari et al., with respect to syphilis, "This is now accepted practice as the risk of over-treatment due to biological false positives that are not syphilis in origin is more acceptable than the risk of non-treatment of syphilis" *(29)*.

Table 3.1: Operational characteristics of select CE-IVD-marked rapid diagnostic tests (RDTs) for syphilis screening

RDT (manufacturer)	Specimen	Volume of whole blood or other specimen	Time to result (minutes)	Storage temperature (°C)	Test type
Alere Determine Syphilis TP (Abbott Laboratories, USA)	Whole blood (fingerstick), plasma or serum	50 µL	15 minutes (up to 24 hours)	2–30 °C	Lateral flow strip
Reveal TP (Syphilis) (MedMira Laboratories, Canada)	Whole blood (venous or fingerstick), plasma or serum	35 µL	Should be read immediately after test procedure is complete	2–30 °C	Cassette
SD Syphilis 3.0 (SD Bioline/Abbott Laboratories, Republic of Korea)	Whole blood (venous or fingerstick), plasma or serum	20 µL (whole blood) 10 µL (plasma or serum)	5–20 minutes	2–30 °C	Cassette enclosed test card
Syphicheck WB (The Tulip Group/ Qualpro, India)	Whole blood (venous or fingerstick), plasma or serum	25 µL	15 minutes	4–30 °C	Cassette enclosed test card
Visitect Syphilis (Omega Diagnostics, United Kingdom)	Whole blood (venous or fingerstick), plasma or serum	50 µL	30 minutes	4–30 °C	Cassette enclosed test card

[4] For a complete list of U.S. FDA-approved HIV tests, see https://www.fda.gov/vaccines-blood-biologics/complete-list-donor-screening-assays-infectious-agents-and-hiv-diagnostic-assays#Anti-HIV-1/2%20Assays%20(detect%20antibodies%20to%20Human%20Immunodeficiency%20Virus%20types%201%20and%202.

[5] For a regularly updated list of tests that are CE-IVD marked, see Global Fund, 2023 *(27)*.

Table 3.2: Meta-analysis data on performance of select treponemal rapid diagnostic tests (RDTs) for syphilis

RDT (manufacturer)	Sample	Parameters	Assuming imperfect reference standards (95% CrI)	Storage temperature (°C)
Alere Determine (Abbott Laboratories)	Serum	Sensitivity Specificity	90.04% (80.45–95.21%) 94.15% (89.26–97.66%)	2–30 °C
	Whole blood	Sensitivity Specificity	86.32% (77.26–91.70%) 95.85% (92.42–97.74%)	
SD Syphilis 3.0 (SD Bioline, Abbott Laboratories)	Serum	Sensitivity Specificity	87.06% (75.67–94.50%) 95.85% (89.89–99.53%)	2–30 °C
	Whole blood	Sensitivity Specificity	84.50% (78.81–92.61%) 97.95% (92.54–99.33%)	
Syphicheck WB (The Tulip Group, QualPro)	Serum	Sensitivity Specificity	74.48% (56.85–88.44%) 99.14% (96.37–100.0%)	2–30 °C
	Whole blood	Sensitivity Specificity	74.47% (63.94–82.13%) 99.58% (98.91–99.96%)	
Visitect Syphilis (Omega Diagnostics)	Serum	Sensitivity Specificity	85.13% (72.83–92.57%) 96.45% (91.92–99.29%)	4–30 °C
	Whole blood	Sensitivity Specificity	74.26% (53.62–83.68%) 99.43% (98.22–99.98%)	

CrI: credible interval; RDT: rapid diagnostic test.

Source: Jafari et al., 2013 *(29)*.

Another concern about TP RDTs is their performance. A recent meta-analysis on the performance of the tests summarized in Table 3.2, however, demonstrates that reported sensitivity and specificity estimates are relatively comparable to laboratory-based tests, i.e. despite somewhat lower sensitivity *(29)*. As there is no gold standard for TP assays, in this review, adjustments were made for imperfect reference standards using the Bayesian Hierarchical Summary Receiver Operating Characteristic Curve method. The result is point estimates of sensitivity and specificity for each test, using serum and whole blood, around a 95% credible interval (as opposed to a confidence interval), as shown in Table 3.2.

In addition to syphilis RDTs using whole blood, several companies, including OraSure Technologies and SD Bioline are developing oral fluid tests for syphilis alone, which in early evaluations have proven to have adequate sensitivity and specificity *(30)*.

3.4.3 Multiplex RDTs for syphilis and HIV/syphilis

Because syphilis RDT mono tests perform well, there is generally no current need for additional tests in this category. However, there are several important areas of need for new dual syphilis tests, preferably in the form of RDTs, in resource-limited settings. One of these is for a combination treponemal/non-treponemal test that can be used to diagnose and confirm active syphilis at POC, in places where traditional laboratory-based testing may not be available. Another need is for dual HIV/syphilis tests.

i. Non-treponemal/treponemal rapid diagnostic syphilis test

Chembio Diagnostics has developed the first dual non-treponemal/treponemal syphilis test for use at POC. The assay is CE-marked and uses a unique combination of protein A and anti-human IgM antibody, which are conjugated to colloidal gold particles. It also employs a recombinant antigen to TP and synthetic non-treponemal antigens, separately bound to the membrane solid phase. The result is an assay that permits the simultaneous, yet separate, detection of both markers.

The DPP Syphilis Screen & Confirm Assay requires a sample amount of only 10 µl of whole blood (fingerstick or venipuncture), and tests can be stored at 2–30 °C. The TAT for the test is 15–20 minutes. There are several peer-reviewed publications about the performance of the DPP assay, including a meta-analysis by Marks et al. *(31)*. Each of the studies found that the test performed adequately against laboratory-based reference tests *(32–34)* and concluded that the DPP Syphilis Screen & Confirm Assay could be useful for supporting the diagnosis of syphilis in primary health care facilities, in resource-limited settings.

Table 3.3: Performance of four commercially available combined HIV/syphilis tests

RDT (manufacturer)	Sample	Parameters	Performance (95% CI) HIV antibody	Performance (95% CI) TP antibody
DPP HIV-Syphilis Assay (Chembio Diagnostics Systems, Inc.)	Serum/plasma	Sensitivity	98.9% (93.6–99.9%)	95.3% (87.9–98.5%)
			99.6% (98.8–99.9%)	97.0% (95.5–98.0%)
			100% (98.2–100%)	86.5% (81–90.9%)
		Specificity	98.1% (88.6–99.9%)	100% (92.9–100%)
			97.9% (96.7–98.7%)	99.6% (98.9–99.9%)
			97.5% (94.3–99.2%)	100% (98.2–100%)
INSTI Multiplex HIV-1/HIV-2/Syphilis Antibody Test (bioLytical Laboratories, Inc.)	Serum/plasma	Sensitivity	NA	NA
			NA	NA
			99.5% (97.2–100%)	81.0% (74.9–86.2%)
		Specificity	NA	NA
			NA	NA
			93.5% (89.1–96.5%)	99.0% (96.4–99.9%)
Multiplo Rapid TP/HIV Antibody Test (MedMira, Inc.)	Serum/plasma	Sensitivity	97.9% (92.0–99.6%)	94.1% (86.3–97.8%)
			99.5% (99.4–99.8%)	94.2% (92.3–95.7%)
			99.5% (97.2–100%)	73.5% (66.8–79.5%)
		Specificity	94.2% (83.1–98.5%)	96.9% (88.2–99.5%)
			98.3% (97.2–99.0%)	99.1% (98.2–99.6%)
			99.5% (97.2–100%)	99.5% (97.2–100%)
SD Bioline HIV/Syphilis Duo Rapid Test (Standard Diagnostics/ Abbott)	Serum/plasma	Sensitivity	97.9% (92.0–99.6%)	93.0% (84.8–97.1%)
			99.0% (98.8–99.9%)	99.6% (95.0–97.7%)
			100% (98.2–100%)	87.0% (81.5–91.3%)
		Specificity	100% (91.5–100%)	100% (92.9–100%)
			99.0% (98.0–99.5%)	99.1% (98.2–99.6%)
			99.5% (97.2–100%)	99.5% (97.2–100%)

CI: confidence interval; NA: not available; RDT: rapid diagnostic test; TP: treponemal.

ii. HIV/syphilis dual tests

There is a significant need in LMICs for combination tests to screen for syphilis and HIV for certain target populations, including men who have sex with men (MSM), sex workers and pregnant women. Perhaps the most urgent need is for a dual test to help eliminate mother-to-child transmission (MTCT) of HIV and syphilis, which is a significant cause of death in infants and young children globally each year *(35)*. WHO recommends the use of dual HIV/syphilis RDTs at the POC as the first test to screen pregnant women as part of antenatal care *(36)*.

There are three WHO prequalified combined HIV/syphilis assays available:

- First Response HIV 1+2/syphilis (Premier Medical Corporation)
- SD Bioline HIV/syphilis Duo (Abbott Laboratories) and
- STANDARD Q HIV/Syphilis Combo (SD Biosensor).

There are also at least four additional combo assays: DPP HIV-Syphilis (Chembio Diagnostics), INSTI Multiplex HIV-1/HIV-2/Syphilis Antibody Test (bioLytical Laboratories), Multiplo Rapid TP/HIV Antibody Test (MedMira Laboratories), and OnSite HIV/Syphilis (CTK Biotech).

Of these seven tests, four of them – that is, the DPP HIV-Syphilis, the INSTI Multiplex HIV-1/HIV-2/Syphilis Antibody Test, Multiplo Rapid TP/HIV Antibody Test and the SD Bioline HIV/syphilis Duo – have been the subject of published, independent laboratory evaluations. Results from three such studies are summarized in Table 3.3.

In each case, the first performance line shows results from a laboratory evaluation in the United States of America (USA) by Humphries et al. *(37)*. The second performance line shows results from a laboratory evaluation at three sites; two in Nigeria and one in China by Yin et al. *(38)*. The third line shows results for a laboratory evaluation at the Institute of Tropical Medicine in Antwerp, Belgium by Van den Heuvel et al. *(39)*.

In addition to the studies described above, Gliddon et al. have conducted a systematic review and meta-analysis of studies evaluating the performance and operational characteristics of combined RDTs for HIV and syphilis *(40)*. The overall findings of the authors are that the studies indicate that the dual tests demonstrated high sensitivity with respect to HIV, and somewhat lower, but adequate, sensitivity with respect to syphilis *(40)*. As well as examining the literature with respect to the diagnostic accuracy of the combined HIV/syphilis tests, the authors evaluated the findings of the studies in terms of their cost-effectiveness, feasibility, acceptability and ease of interpretation of results. Here, the authors found that the studies indicated that combined HIV/syphilis tests are acceptable to clients, feasible for implementation in antenatal care centres and cost-effective *(40)*.

3.4.4 Singleplex or multiplex RDTs for *C. trachomatis, N. gonorrhoeae* and *T. vaginalis*

i. Screening tests for *C. trachomatis* and *N. gonorrhoeae*[6]

There are several Ag detection-based RDTs for *C. trachomatis* available that are easy to use and relatively inexpensive. These include:

- ACON Chlamydia (ACON Laboratories)
- aQcare Chlamydia TRF kit (Medisensor)
- BioRapid Chlamydia Ag Test (Biokit S.A.)
- Chlamydia Rapid Test SAS (Diagnostics for the Real World [DRW])
- Clearview Chlamydia (Abbot Laboratories)
- Chlamydia test card (Ultimed Products, GmbH)
- HandiLab-C (HandiLab)
- QuickVue (QuidelOrtho)

In a systematic review of these assays, Kelly et al. found that, although *C. trachomatis* antigen detection RDTs exhibited high specificity (97–100%) across all specimen types, pooled sensitivity was much lower, as shown in Table 3.4 *(41)*.

With respect to *N. gonorrhoeae*, few Ag detection-based immunoassays designed for use at POC are available. Four of these have been evaluated. These are:

- ACON CT/NG Duo (ACON Laboratories)
- ACON NG Plate (ACON Laboratories)
- BioStar Optical ImmunoAssay gonorrhoea (Thermo Fisher Scientific)[7]
- One Step Gonorrhea RapiCard InstaTest (Cortez Diagnostics).

The performance results of these tests are summarized in Table 3.5.

Similar to the *C. trachomatis* studies discussed above, the ACON Duo and ACON NG tests had sensitivities below 25% *(43)*. While the One Step Gonorrhea RapiCard InstaTest had overall sensitivity of just over 30%, its sensitivity in women was considerably lower, at 7.1%, versus SDA *(44)*.

Many researchers have noted that, although current lateral flow/RDT diagnostic tests for *C. trachomatis* and *N. gonorrhoeae* often have specificities above 90%, sensitivities are often 50% or lower and, as such, they do not perform well enough to be used as screening tests; improved assays are required *(10,38–43)*. This need is particularly acute with respect to women, where the syndromic approach to managing STIs is inadequate *(45)*. In particular, NAAT-based platforms (including those that are capable of AMR testing) for use at, or near to POC are needed.

ii. Screening tests for *T. vaginalis*

There is at least one Ag detection-based RDT available for *T. vaginalis*. This is the OSOM Trichomonas Test (Sekisui Diagnostics), which studies have shown performed reasonably well when compared with wet mount and culture *(10,48,49)*. The test, for which Clinical Laboratory Improvement Amendments (CLIA) have been waived by the FDA, has a TAT of 10 minutes.

3.5 Point-of-care (POC) molecular assays

Molecular amplification testing methods, which detect specific sequences of DNA or RNA, have substantially changed the microbiological diagnosis of pathogens over the last two decades. In many cases they have become the gold standard or test of choice for particular pathogens, including *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium* and HSV-1/HSV-2.[8] In particular, NAATs are widely used in clinical laboratory diagnostics, including for the detection of STIs. However, these laboratory-based systems not only require highly trained staff, but also require continuous power, clean running water and favourable operating conditions (e.g. temperature and humidity control). These requirements have been obstacles to NAAT-based testing, especially in LMICs, where long delays in returning sample results from laboratories to patients has led to loss to follow-up *(19,50)*.

What is needed, therefore, particularly in LMICs, are molecular amplification tests for STIs that meet the REASSURED criteria, the results of which can be delivered in a single patient clinical visit *(6)*. In recent years, a number of NAAT-based POC and near-POC diagnostic platforms have become available, some of which meet a number of the REASSURED criteria.[9]

[6] It should be noted that WHO does not currently prequalify assays for *C. trachomatis, N. gonorrhoeae* or *T. vaginalis*.

[7] The BioStar Optical ImmunoAssay gonorrhoea is no longer commercially available.

[8] Note that the discussion of molecular testing in this chapter focuses on the testing methods most often used in POC test platforms. See Chapter 4 of this manual for a description of additional molecular testing principles and methods.

[9] For additional information on these and other platforms for STIs, see: WHO, 2023 *(20)*.

Table 3.4: Pooled performance of point-of-care (POC) antigen detection assays for *Chlamydia trachomatis*

Specimen type	Number of studies; participants	Sensitivity (95% CI)	Specificity (95% CI)
Cervical swab	8; 4588	53.1% (34.7–70.8)	98.9% (98.0–99.4)
Vaginal swab	10; 6252	36.6% (22.9–52.9)	96.9% (94.0–98.4)
Male urine	5; 2568	62.5% (43.2–78.5)	98.0% (95.1–99.0)

CI; confidence interval.

Source: Adapted from Kelly et al., 2017 *(41)*.

Table 3.5: Performance of four antigen-based point-of-care (POC) tests for *Neisseria gonorrhoeae*

POC test (manufacturer)	Specimen type	Reference test	Participants	Sensitivity (95% CI)	Specificity (95% CI)
ACON CT/NG Duo Test (ACON Laboratories)	Endocervical swab	cobas AMPLICOR Analyzer CT/NG assay (Roche)	491 sexually active females aged 14–49, asymptomatic	12.5% (0–41.7)	99.8% (99.3–100)
ACON NG individual test (ACON Laboratories)	Endocervical swab	cobas AMPLICOR Analyzer CT/NG assay (Roche)	773 sexually active females aged 14–49, asymptomatic	Not quantifiable (no true positives)	97.2% (96–98.5)
BioStar Optical ImmunoAssay (Thermo Fisher Scientific)	Urine	Aptima Combo 2 assay (Hologic)	52 men, aged 18+, attending sexual health clinic	100% (57–100)	98% (89–100)
BioStar Optical ImmunoAssay (Thermo Fisher Scientific)	Urine	Microscopy	33 men, aged 18+, attending sexual health clinic	100% (51–100)	93% (78–98)
BioStar Optical ImmunoAssay (Thermo Fisher Scientific)	Urine	Culture	32 men, aged 18+, attending sexual health clinic	100% (51–100)	93% (77–98)
One Step Gonorrhea RapiCard InstaTest (Cortez Diagnostics)	Women: endocervical swab Men: urethral swab	BD ProbeTec SDA (Becton Dickinson) Culture	138 overall (86 women, 52 men)	SDA: 33.3% (20.4–49.4) Culture: 32.4% (18.9–49.7)	SDA: 97.9% (91.9–99.5) Culture: 96% (89.8–98.5)

CI: confidence interval; CT: *Chlamydia trachomatis*; NG: *Neisseria gonorrhoeae*; SDA: strand displacement amplification.

Source: de Cortina et al., 2016 *(10)* and Abbai et al., 2015 *(42)*.

3.5.1 NAAT technology

At the most basic level, a NAAT-based assay consists of three steps: sample preparation, nucleic acid amplification and detection. In order to move NAAT-based testing from the laboratory to POC or near-POC, it is necessary to simplify these processes, improve the speed at which they can be done, and lower their cost, all while maintaining test accuracy.

i. Sample preparation

Sample preparation has proven to be a challenging problem, with regard to molecular testing at or near the POC. The body fluids needed for STI testing, for example, can be complex, and include blood and urine, as well as vaginal and rectal secretions. Each of these may have a significant number of cells, proteins, DNA and other materials, collectively referred to as a "matrix", which are not the target biomarker required for the assay. This matrix must be minimized via sample preparation, which can be done in laboratories by repetitively filtering and washing the sample in buffers and chemicals using manual or robotic pipetting techniques. It has proven to be difficult to simplify and miniaturize these processes in diagnostic technologies for use at POC *(51)*.

Microfluidic approaches to sample preparation, including mechanical, magnetic, electrokinetic and chemical techniques, have been developed *(51, 52)*. These systems manipulate small (10^{-9} to 10^{-18} L) amounts of fluids in micrometer-scale channels or chambers, which makes it possible to simplify complex assay protocols, dramatically reduce sample volumes, reduce reagent costs and process batches of samples in parallel *(53)*. In general, the POC/near-POC molecular diagnostic platforms available for STI testing use either pressure-driven or centrifugal microfluidic approaches for sample preparation. Although some platforms still use macrofluidic approaches (e.g. syringes and plungers), the newest application of microfluidic test techniques are preferred, especially for sample preparation.

ii. Amplification

Among the available NAAT technologies described in Chapter 4, polymerase chain reaction (PCR)-based technology, which was first introduced in 1983, is the most commonly utilized *(23)*. Over the years, there have been variations and improvements on basic PCR-based NAATs, including, importantly, real-time and multiplex PCR. Real-time PCR arguably has had the greatest impact on detecting and identifying human pathogens in clinical microbiology laboratories *(54)*. This method monitors the quantity of amplicons over time (after each cycle), rather than at the end-point of the reaction, thereby often leading to faster results than end-point PCR.

In addition, real-time PCR can either be qualitative (indicating the presence or absence of a sequence) or quantitative (giving a copy number). Quantitative real-time PCR can quantitate the "infectious burden" of a disease, thereby providing a better understanding of the disease state, establishing the prognosis of certain infections, and monitoring the effectiveness of antibiotic or antiretroviral therapy (e.g. quantifying HIV viral load to determine therapeutic efficacy and monitoring disease progression) *(55)*.

Also of note is the development of multiplex PCR testing, which combines a number of primer pairs into a single PCR, to simultaneously detect several pathogens at once (such as *C. trachomatis* and *N. gonorrhoeae*), which can make testing more cost-effective, as platforms can be used for a variety of infectious diseases, including STIs. Multiplex PCR does, however, have limitations. For example, mixing primer pairs can cause interference in the amplification process, decreasing test performance (e.g. sensitivity) for individual infections *(55)*. Optimizing multiplex PCR conditions can be challenging, and the choice of pathogens to include should be carefully considered *(55)*. A number of different PCR-based instruments and detection probe formats are available commercially, including those for use at or near POC *(56)*. Many of these are real-time systems and some are capable of multiplexing.

In recent years, amplification techniques have turned to isothermal methods of nucleic acid amplification, or iNAATs, which eliminate the need for the rapid thermal cycling required by PCR-based techniques, and can be more specific due to non-temperature dependence of the reactions *(57)*. They can also be combined with detection technologies, such as fluorescent-probe-independent methods, which eliminate the need for sophisticated optics. The most common iNAAT methods for which POC/near-POC commercial platforms are available or in the pipeline are described briefly below *(58)*.

a. Nucleic acid sequence-based amplification (NASBA)

Unlike PCR-based testing, NASBA amplifies RNA rather than DNA and it is performed at 41 °C. Facilitated by three enzymes, NASBA uses reverse transcriptase (RT) replication mechanisms to produce a modified complementary DNA molecule (cDNA) from an RNA template, which is then rapidly amplified into RNA amplicons. In other words, it effectively imitates in vivo retroviral replication mechanisms to produce RNA amplicons from the RNA template *(59,60)*. NASBA offers the advantage of speed over RT-PCR, amplifying a nucleic acid sequence to > 10^9 in approximately 90 minutes *(23)*.

b. Loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA)

LAMP amplifies DNA under isothermal conditions. It generally uses four specifically designed primers to recognize six different areas of the DNA target combined with strand displacement activity. In brief, unlike PCR, which uses heat to denature and anneal primers to the target sequence, LAMP relies on complex binding kinetics and physical proximity of the target sequences and primers in a loop in order to generate a single-strand template without the need for heat denaturation *(60,61)*. High levels of amplicons can be generated at 60–65 °C and are generally achieved in less than an hour; additionally, LAMP has the advantage of demonstrating high specificity and sensitivity equivalent or better than PCR *(23)*.

HDA uses thermostable helicase enzymes to effect DNA strand separation. Once separated, single-stranded DNA binding proteins stabilize the single strands to allow binding of the PCR primers. DNA polymerase extends the primers, and the newly synthesized DNA duplexes serve as templates that are then hybridized by sequence-specific primers for further amplification cycles *(55,61,62)*. Exponential amplification can be achieved at a single amplification temperature (60–65 °C), usually in 60–90 minutes *(56)*.

c. Recombinase polymerase amplification (RPA)

RPA, which is generally performed at 37–42 °C, partners isothermal recombinase-driven primer targeting of template material with strand-displacement DNA synthesis *(23)*. This assay uses recombinases that are capable of pairing oligonucleotide primers with homologous sequences in duplex DNA *(63)*. Without the need to split double-stranded DNA before amplification, this assay can achieve exponential amplification from just a few target copies.

In addition to the isothermal methods of nucleic acid amplification described above, there are other methods described in the literature. These include:

- nicking enzyme amplification reaction (NEAR)
- signal-mediated amplification of RNA technology (SMART)
- rolling circle amplification (RCA)
- isothermal multiple displacement amplification (IMDA)
- single primer isothermal amplification (SPIA)
- circular helicase-dependent amplification (cHDA) *(57)*.

d. Detection

Most currently available near-patient testing platforms use optical detection methods, in particular, real-time fluorescence (see also Chapter 4). Some platforms use label-free technologies, which employ optics-based biosensors to convert biological binding responses into signals without using fluorescence or other detection labels *(23)*. Because label-free methods do not require washing steps and the addition of reagents, they have the potential to provide a simpler and more rapid measurement procedure than label-based methods (e.g. fluorescence) *(64)*.

3.5.2 Commercially available molecular platforms/assays for STIs

There are a number of commercially available integrated NAAT-based platforms for near-patient testing that have assays for some of the following STIs: *C. trachomatis* and *N. gonorrhoeae* (separately or combined), as well as *T. vaginalis, M. genitalium*, HSV-1 and HSV-2, and HIV (in particular, for EID and viral load testing). These include:

- the ARIES Systems (Luminex)
- EasyNAT System (Ustar Biotechnologies)
- GeneXpert system (Cepheid)
- HG Swift (Hibergene Diagnostics)
- io Diagnostic System (binx health, inc.)
- m-PIMA (Abbott Laboratories)
- SAMBA II (DRW)
- Sexual Health Test (Visby Medical, Inc.)
- Solana (QuidelOrtho Corporation)
- the Truelab Real Time micro PCR system (Molbio).

Some of these systems are suitable for use at primary health care centres; others are more suitable for use at district hospital level or above. They are referred to as level-1 and level-2 facilities, respectively. The technologies employed and the available tests are set out in Table 3.6.[10]

3.5.3 Pipeline molecular platforms/assays for STIs

In addition to the commercially available molecular platforms/assays for STIs described above, a number of NAAT/iNAAT-based POC/near-POC systems for STIs are currently in pre-commercial or late-stage development.

[10] For additional information, see WHO, 2023 *(20)*.

These include:

- cobas Liat (Roche Molecular Diagnostics)
- DASH (Minute Molecular)
- GeneXpert Xpress (Cepheid)
- ID NOW (Abbott Laboratories)
- NINAAT (Selfdiagnostics)
- Novel Dx (Novel Microdevices)
- Q-POC (QuantuMDx Group)
- Savanna (QuidelOrtho Corporation)
- Talis One (Talis Biomedical).

The technologies employed and the tests in development for these platforms are set out in Table 3.7.[10]

3.6 Future use of point-of-care (POC) testing in clinical care

It has been recognized that better diagnostics for primary health care, including improved diagnostics for STIs, will be important in providing universal health coverage for 1 billion more individuals. To achieve this goal, moving testing closer to the health service-user is essential. However, most diagnostics for STIs are still performed in the laboratory in LMICs. Adoption of POC testing in clinical care also needs to be considered from the perspectives of all stakeholders, including the patient, in order to achieve value *(18,65)*.

With the exception of RDTs for the serodiagnosis of HIV, syphilis, HIV/syphilis and TV, few commercially available tests meet the majority of the REASSURED criteria. NAAT/iNAAT-based tests offer improved performance over Ab and Ag detection for patient management, but most platforms are available only at central reference laboratories in LMICs. This severely limits access to STI testing, particularly for *C. trachomatis, N. gonorrhoeae, M. genitalium*, HPV and HSV-1 and -2.

There are now at least 10 commercially available integrated NAAT-based platforms for near-patient testing for *C. trachomatis* and *N. gonorrhoeae* (separately or combined), as well as *T. vaginalis, M. genitalium*, HSV-1 and -2 and HIV. A good number of these are FDA cleared and/or CE-IVD marked. More such tests for use at POC are in the pipeline. Collectively, these platforms have the potential to improve STI testing in LMICs, thereby enhancing the public health response to the STI global epidemic.

It should be noted that AMR is problematic, in particular with respect to two of the pathogens covered in this chapter: *N. gonorrhoeae* and *M. genitalium*. *N. gonorrhoeae* has developed resistance to the last remaining options for treatment of gonorrhoea; that is, the third-generation extended-spectrum cephalosporin ceftriaxone. As a result, WHO considers *N. gonorrhoeae* to be a priority organism for AMR monitoring in the Global Antimicrobial Resistance and Use Surveillance System (GLASS)[11] and for drug development *(66,67)*. Similarly, *M. genitalium* has demonstrated rapidly increasing resistance to the first-line macrolides, in particular, azithromycin, and there are reports of multidrug resistance to both first-line and second-line treatments; that is, macrolides and fluoroquinolones, respectively *(68)*. Therefore, molecular prediction of antimicrobial susceptibility testing/resistance in *N. gonorrhoeae* would be very valuable testing *(69,70)*, and it is important to examine *M. genitalium*-positive samples for macrolide-resistance prior to initiation of treatment of *M. genitalium*-positive cases *(68,71)*. There is the potential to test tor AMR mutations for *N. gonorrhoeae* and *M. genitalium* using POC tests that are in the pipeline and development in this area needs to be encouraged and supported *(70,72)*.

In order to make the most of these tests and test platforms in LMICs, there must be continuing efforts to design platforms for use at primary health care facilities and to lower their cost. In other words, tests need to meet the REASSURED criteria. Ongoing efforts with respect to health system strengthening, regulatory harmonization and procurement are also needed. In addition, the use/integration of currently available POC tests requires sustainable, well funded training and supervision programmes, as well as appropriate external quality assurance and quality control programmes. It is only when all of these elements come together that new POC tests for STIs will be well positioned to achieve the desired level of uptake and impact in global health.

[11] For further information, see: https://www.who.int/initiatives/glass

Table 3.6: Commercially available point-of-care (POC) or near-POC platforms for STIs

Platform (manufacturer)	System type; setting	Sample preparation; TAT	Amplification technology	Detection technology	Fluidic handling	Available assays regulatory status	Pipeline assays
ARIES and ARIES M1 (Luminex)	Multiplex; Level 2	Integrated in test cassette; 2 hours	Real-time PCR	Real-time fluorescence	Rotary valves	HSV 1&2 (CE-IVD/FDA)	NA
EasyNAT (Ustar Biotechnologies)	Multiplex; Level 2	Integrated; ~50 minutes	iNAAT – CPA	Visual readout in device-integrated lateral flow strip	Pressure-driven microfluidics	CT/NG; NG; TV; MG; HPV; HSV 1&2 (all CE-IVD)	NA
GeneXpert (Cepheid)	Multiplex; Level 2	Integrated in cartridge; 60–90 minutes depending on assay	Real-time PCR	Real-time fluorescence	Pressure-driven microfluidics (rotary valves)	CT/NG and TV (CE-IVD/FDA); HPV and HIV VL (CE-IVD)	NA
HG Swift (Hibergene Diagnostics)	Multiplex; Level 2	Not integrated; less than 60 minutes	Isothermal LAMP	Fluorometric	None	CT/NG; MG; HSV 1&2 (All CE-IVD)	NA
m-PIMA (Abbott)	Multiplex; Level 2	Integrated in cartridge; 60–70 minutes	Real-time PCR	Real-time fluorescence based on competitive reporter probe hybridization integrated micro array	Pressure-driven microfluidics	HIV VL (CE-IVD)	NA
SAMBA II (Diagnostics for the Real World)	Multiplex; Level 2	Integrated in assay module; ~2 hours	iNAAT – NASBA	Fluorescence	NA	HIV VL (CE-IVD)	CT/NG

Table 3.6 (continued): Commercially available point-of-care (POC) or near-POC platforms for STIs

Platform (manufacturer)	System type; setting	Sample preparation; TAT	Amplification technology	Detection technology	Fluidic handling	Available assays regulatory status	Pipeline assays
Solana (QuidelOrtho)	Multiplex; Level 2	Not integrated; 35–70 minutes depending on assay	iNAAT – HDA	Fluorescence; probe-based	None	TV and HSV 1&2 (CE-IVD/FDA)	NA
Truelab RT micro PCR (Molbio)	Multiplex; Level 2	Not integrated; 35–45 minutes, depending on assay	Real-time PCR	Real-time fluorescence	Pressure-driven macrofluidics	CT; NG; CT/NG; TV; HPV; HIV VL (all CE-IVD)	NA
io Diagnostic System (binx health, inc.)	Multiplex; Level 1, possible	Integrated in cartridge; ~30 minutes	Ultra-rapid PCR	Electrochemical	Pressure-driven macrofluidics	CT/NG (CE-IVD/FDA)	TV; MG
Sexual Health Click Test (Visby Medical)	Multiplex; Level 1	Integrated in device; ~30 minutes	Real-time PCR	Electrochemical	Pressure-driven microfluidics (rotary valves)	CT/NG/TV (FDA)	NA

CE-IVD: Conformité Européene (CE)-marked in vitro diagnostic medical device; CPA: crossprimingamplification; CT: *Chlamydia trachomatis*; FDA: U.S. Food and Drug Administration; HPV: human papillomavirus; HSV: herpes simplex virus; iNAAT: isothermal nucleic acid amplification test; LAMP: loop-mediated amplification; MG: *Mycoplasma genitalium*; NA: not available; NASBA: nucleic acid sequence-based amplification; NG: *Neisseria gonorrhoeae*; PCR: polymerase chain reaction; TAT: turnaround time; TV: *Trichomonas vaginalis*; VL: viral load.

Table 3.7: Pipeline for point-of-care (POC) or near-POC platforms for STIs

Platform (manufacturer)	System type; setting	Sample preparation; expected TAT	Amplification technology	Detection technology	Fluidic handling	Available assays	Pipeline assays
cobas Liat (Roche)	Multiplex; Level 2; Level 1 (possible)	Integrated in assay tube; ~20 minutes	Real-time PCR	Real-time fluorescence	Peristaltic actuation macrofluidics	NA	CT/NG; MG
DASH (Minute Molecular)	Multiplex; Level 1 possible	Integrated in cartridge; ~15 minutes	Real-time PCR	Fluorescence	Pressure-driven in mesofluidic chambers	NA	CT/NG
GeneXpert Xpress (Cepheid)	Multiplex; Level 1	Integrated in cartridge; ~20–40 minutes	Real-time PCR	Real-time fluorescence	Pressure-driven macrofluidics	NA	CT/NG: multiplex vaginal panel
ID NOW (Abbott)	Multiplex; Level 2, Level 1 (possible)	Semi-integrated; ~15 minutes	iNAAT – RPA or NEAR	Real-time fluorescence	Pressure-driven microfluidics	NA	CT/NG
NINAAT (Selfdiagnostics)	Multiplex; Home	Integrated; ~40 minutes	PCR-based iNAAT	Lateral flow	None	NA	CT/NG
Novel Dx (Novel Microdevices)	Multiplex; Level 1	Integrated in cartridge; ~30 minutes	Compatible with PCR and iNAAT		Microfluidics	NA	CT/NG
Q-POC (QuantuMDx Group)	Multiplex; Level 2	Integrated; ~30 minutes	Continuous flow endpoint real-time PCR	Label-free resistance changes in SiNW upon amplicon hybridization/ sequencing	Pressure-driven microfluidics	NA	CT/NG
Savanna (QuidelOrtho)	Multiplex; Level 2, Level 1 possible	Integrated in cartridge; ~30 minutes	Real-time PCR and iNAAT	NA	Pressure-driven microfluidics	NA	Vaginitis; Unspecified STIs
Talis One (Talis Biomedical)	Multiplex; Level 1	Integrated in cartridge; ~30 minutes	Real-time iNAAT	Fluorescence	Microfluidics	NA	CT/NG

CT: *Chlamydia trachomatis*; iNAAT: isothermal nucleic acid amplification test; MG: *Mycoplasma genitalium*; PCR: polymerase chain reaction; NA: not available; NEAR: nicking enzyme amplification reaction; NG: *Neisseria gonorrhoeae*; POC: point-of-care; RPA: recombinase polymerase amplification; SiNW: silicon nanowire; STI: sexually transmitted infection; TAT: turnaround time.

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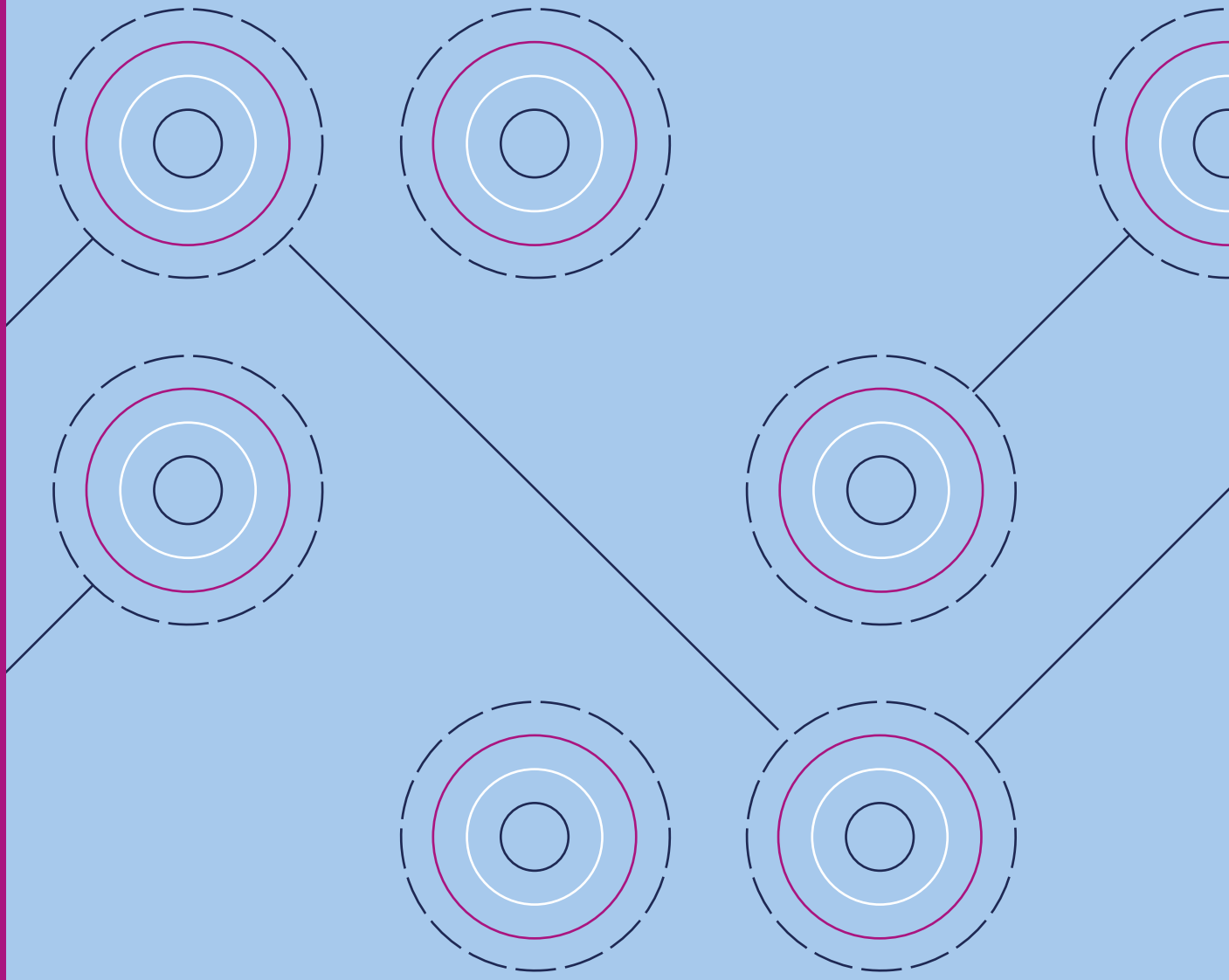
Chapter 4. Principles of molecular tests for the diagnosis of STIs



Chapter 4. Principles of molecular tests for the diagnosis of STIs

Susanne Jacobsson and David Whiley

4.1 Introduction

STIs can be caused by viruses, bacteria or parasites. Coinfection is common. Clinical specimens for STI testing may be taken from different anatomic sites, using various sampling devices and techniques. There is also wide variation between different geographical settings, in terms of the transport of specimens and storage capabilities, and resources for STI diagnostics that are available. There are, therefore, a large number of methods for effective diagnosis of STIs available worldwide. However, these differ in the quality of performance (i.e. sensitivity, specificity and reproducibility), when testing for a single infection, for a wide range of STIs, or across different anatomical sites. Historically, the laboratory methods used to detect etiological agents of STIs include bacteriological (and, to a lesser extent, tissue) culture, various serologic testing for the presence of specific antibodies, immunohistochemistry, antigen detection and microscopy. Similar to the diagnosis of many other infectious diseases, the direct detection of specific nucleic acids from STI pathogens using a range of nucleic acid amplification tests (NAATs) has become the new gold standard for the diagnosis of many STIs. This is due to the superior sensitivity, specificity and convenience of NAATs. Numerous NAATs are approved by the U.S. Food and Drug Administration (FDA) and/or other international, national or regional regulatory bodies, and are commercially available. Nevertheless, laboratory-developed tests (LDTs) using conventional polymerase chain reaction (PCR) or real-time PCR-based detection methods for STI diagnosis remain popular, especially for research and epidemiologic purposes. In this chapter, we explore the principles of molecular tests that are commonly used to diagnose STIs, focusing on explaining the technologies that are used in the tests. Note that the chapters discussing different STI pathogens will focus on application of the methods; molecular point-of-care (POC) tests are covered in Chapter 3.

A properly designed NAAT theoretically is capable of detecting as few as one target in a sample with very high specificity. However, achieving this level of limit of detection (LOD) requires a series of complex and sometimes time-consuming processes to successfully work in conjunction; hence the need for NAAT optimization. Of note, the analytical sensitivity of NAATs also can be influenced by a variety of factors. For example, inherent factors within the individual clinical specimen such as inhibitors, the specimen type, the copy number of target sequence, the nucleic acid extraction, amplification, and the detection technology used.

There are five critical stages in nucleic acid-based tests. These are:

- sample collection
- sample stabilization/storage/transport
- nucleic acid extraction/purification
- amplification
- detection.

All stages have a major bearing on the quality and reliability of laboratory testing results. For a NAAT to successfully diagnose STIs, a clinical specimen needs to be properly sampled from the appropriate anatomic site, and the nucleic acids of targets stabilized in the transport medium for storage and transfer. In the laboratory, efficient nucleic acid extraction/purification is crucial for sample preparation to produce a pure PCR template that is free of contaminating sequences, nucleases and inhibitors. Subsequently, well designed and optimized NAAT processing is essential for efficient amplification, to produce sufficient PCR amplicons. Last but not least, a proper detection strategy needs to be in place to produce clear and strong signals for positivity,

and also for negativity. The process of NAAT testing is considered to be of medium to high complexity, and generally requires well trained personnel and sophisticated laboratory settings.

Despite the complexities, molecular diagnostics of STIs using NAATs are ultimately very powerful. In many cases, NAATs are preferred over conventional diagnostic methods for their superior sensitivity and specificity, shorter turnaround time, high throughput, and opportunity for automation, multiplexing, and quantification. Due to their high sensitivity, NAATs are also effective in detecting organisms from asymptomatic infections, or those from an early phase of infection (before seroconversion). These can be applied to self-collected and noninvasive specimens, such as vaginal swabs from women and first-catch urine samples from men. The ability to multiplex and, thus, simultaneously test for various STIs from a self-collected sample in a single test reaction, has greatly enhanced screening practices and made it possible to use alternative locations for specimen collection, including at the patient's home. Self-collection is possible where a clinician-collected specimen is not required, providing a way to reach sexually active individuals who should be screened for STIs but lack access to health-care services or, for example, fear pelvic examinations. This is particularly important since most STIs are asymptomatic and detection depends on screening populations that ordinarily would not be tested.

The transition to NAATs is not, however, without limitations. For example, one of the strengths of NAATs is their ability to detect pathogens without the need for live microorganisms (making transport and storage of specimens less critical). This leads to one of the key weakness of NAATs, which is that isolates are not available for subsequent antimicrobial susceptibility testing of the target organism, or other phenotypic characterization. Antimicrobial susceptibility testing is particularly important for organisms displaying high levels of antimicrobial resistance (AMR) such as *Neisseria gonorrhoeae* and *Mycoplasma genitalium*. Indeed, *N. gonorrhoeae* is listed by WHO as a high-priority pathogen, for which new antibiotics are urgently needed *(1)*. Although there are some promising new commercial tests, as well as LDTs, for detecting *N. gonorrhoeae* and M. *genitalium* AMR markers, molecular detection of AMR for these organisms remains in its infancy. Further advances in the development of molecular resistance assays are urgently needed for testing for various STIs. An exception in this regard is molecular testing for HIV drug resistance, which is well established, and there are various commercial sequencing-based methods available. A further limitation of NAAT's ability to detect nucleic acid from non-viable organisms is that it can also pose problems for clinical interpretation of test-of-cure (TOC) results, unless sufficient time has been allowed for the residual nucleic acids to be cleared from the infected host before TOC sample collection. Hence, guidelines typically suggest waiting two or three weeks before doing a TOC using NAAT. Some promising research, however, suggests that RNA-based methods could be used more accurately for TOC, since mRNA allows for differentiation between viable infection and non-viable DNA *(2)*.

The high sensitivity of NAATs can also be problematic because, even a very low level of contamination in a testing environment can lead to false-positive results. The possibility of contamination, which is a risk that affects all molecular diagnostic laboratories, can come at almost any stage of nucleic acid-based processing. This includes sample-to-sample contamination of genomic DNA during sample handling and extraction (i.e. pre-PCR) or contamination of the laboratory environment with PCR amplicons (i.e. from post-PCR handling). There have also been recent reports of clean reagents being contaminated by synthetic nucleic acid control materials at oligonucleotide manufacturers *(3,4)*, although, to our knowledge the latter has not been reported for STIs. In some circumstances (i.e. where the PCR mix substitutes deoxyribonucleotide triphosphate [dNTP] for deoxythymidine triphosphate [dTTP] with deoxyuridine triphosphate [dUTP]), the addition of an enzyme such as uracil N-glycosylase to the PCR master mix preparation helps to reduce amplicon carry-over contamination by degrading the amplicons. However, it does not prevent false-positives caused by genomic DNA contamination directly from specimen to specimen. Thus, good molecular laboratory practices, unidirectional workflow, separate rooms for specimen nucleic acid extraction, PCR master mix preparation, and testing are prerequisites for a successful NAAT. In recent years, closed and fully automated robotic systems have increasingly become the norm for commercial platforms for STI testing. In doing so, they have provided additional means of reducing the risk of contamination (e.g. by minimizing human error). In doing so, these systems also eliminate many of the labour-intensive sample processing steps (aside from initial sample collection in the corresponding proprietary transport medium). However, they may not be affordable for resource-limited settings.

It should nevertheless be noted that, even where best practices have been followed, false-positives can still arise due to inherent issues with the target sequences, such as where the target organism and related species are close genetic relatives. For example, cross-reaction of certain NAATs for *N. gonorrhoeae* with commensal *Neisseria* spp. is well documented, particularly when analysing pharyngeal specimens where commensal *Neisseria* are ubiquitous *(5,6)*. Consequently, application of supplementary testing – i.e. presumptive positive specimens being confirmed by a second NAAT method before results are recorded as positive – has been implemented in many settings to minimize false-positive results. Issues with target sequences can also impact upon sensitivity. False-negative results caused by sequence variation or deletion of the specific target sequence in the organism have also been well documented, including some relating to STIs. For example, the Swedish new variant of *Chlamydia trachomatis* (SE-nvCT), the Finnish nvCT (FI-nvCT), and other similar diagnostic-escape nvCTs *(7–10)*.

These diagnostic-escape nvCTs have resulted in many commercial assays now incorporating two genetic targets, to minimize the potential for false-negative results caused by genetic variation *(8,11,12)*.

Currently, many commercially available and even LDT singleplex or multiplex NAATs are in use for STI diagnostics. Regardless of the method, appropriate safeguards need to be in place to ensure the quality and performance of the diagnostic NAAT. Many of these are mandated by national regulatory authorities such as the U.S. FDA and the European Union (EU). For diagnosis of STIs, it is recommended that only approved NAATs are used. If this is not possible, or if modifications to approved tests are warranted, then it is essential that the proposed NAAT is strictly validated before use, according to local requirements. Ideally, the test evaluation comparison should be made against at least one internationally approved NAAT. There are also various post-implementation measures to consider, which are very briefly summarized here. The assay should be used with appropriate positive, negative, and inhibition controls. In addition, participation in an appropriate external quality assessment (EQA) system is highly recommended. To avoid false-negative results, such as those caused by SE-nvCT, FI-nvCT or other diagnostic-escape mutants *(7–10)*, ongoing monitoring of sequence targets is recommended to ensure that the target sequences remain relevant. This may involve random retesting of samples with an additional target. Regarding the latter, however, it is important to note that there can be incompatibility between the different transport media used by the various platforms (e.g. different platforms use different transport media to stabilize samples during transportation, which may not be compatible for use on another platform). This may need to be considered before retesting of samples.

In high-income countries, future development of molecular diagnostics for STIs is likely to focus on expanding the portfolio of STI pathogen detection using POC applications (see Chapter 3), incorporation of quantitative detection, and the establishment of molecular typing or antimicrobial resistance markers, to go beyond mere diagnosis and to directly inform treatment guidelines and individual patient treatment *(13,14)*. Additional, routine use of genomics in clinical settings is becoming a reality, to answer detailed clinical questions. Although genome analysis of STIs is still in its infancy and lags far behind that of many other pathogens, genomics can reveal previously inaccessible aspects of pathogen biology. In recent years, DNA and RNA sequencing technologies have been used, not frequently in diagnostics, but to understand the biology, pathogenesis, virulence and epidemiology of STIs. Further, metagenomics allows identification of novel organisms by characterization of the collective genome of microbial communities in a sample. For low- and middle-income countries, having access to affordable, robust instrumentation with simple testing processes; less hands-on time; more capability of throughput; greater ability to implement closed systems to reduce risk of contamination; and availability of inexpensive test reagents remain the most important needs.

At the time of writing, two major categories of diagnostic molecular technologies exist for the identification of STIs using molecular methods. The first category, NAAT-based testing, which is the most widely used, is based on amplification of specific nucleic acid (DNA or RNA) from the target microorganism, to generate sufficient amplified products to be converted, through different technologies, into signals for detection. The second category, non-amplified nucleic acid-based technologies (i.e. nucleic acid hybridization [NAH] or nucleic acid probe [NAP]) uses non-amplified, nucleic acid probes (e.g. labelled with enzyme) that hybridize directly to the target template and subsequently through, for example, enzyme-substrate reactions, the signals are amplified and detected. The NAH assays have been continuously replaced by NAATs (or integrated as parts of NAATs). NAATs and NAHs are summarized as follows.

4.2 Amplified nucleic acid technologies

NAATs are the most sensitive, yet complex of molecular diagnostic tests. They generally perform better, in terms of sensitivity, than direct (non-amplified) probe-based tests as well as all other classes of diagnostic test (e.g. culture and microscopy-based methods). NAATs for STIs have advanced considerably over the least two decades. While some of the first-generation commercial NAATs (i.e. those that are partially automated) still exist, the second-generation fully automated commercial testing platforms now dominate the market. These are typically equipped with improved specimen-processing modules, to maximize nucleic acid yield, and are fully automated and closed, to achieve highly efficient and contamination-free molecular diagnostics and hence large-scale screening. Commercial amplification-based molecular diagnostic systems have focused mainly on assays for detection of *N. gonorrhoeae, C. trachomatis, M. genitalium*, herpes simplex virus (HSV), HIV, hepatitis B virus (HBV), hepatitis C virus (HCV) and human papillomavirus (HPV). In addition to qualitative detection of viruses, quantification of viral load in clinical specimens is valuable for diagnosis, prognosis and therapeutic monitoring for HIV, HBV and HCV. The key amplification technologies used by commercial systems are PCR, transcription mediated amplification (TMA), nucleic acid sequence based amplification (NASBA), and strand displacement amplification (SDA).

4.2.1 Polymerase chain reaction (PCR)

PCR was developed in 1983 *(15,16)* and is often heralded as one of the most important scientific advances in biology and medicine. It is now a fundamental, and often indispensable, technique that is widely used in molecular biology, microbiology, clinical diagnostics and many other applications. For clinical microbiology, PCR technology has revolutionized the detection and

characterization of infectious disease organisms, including the etiological agents of STIs.

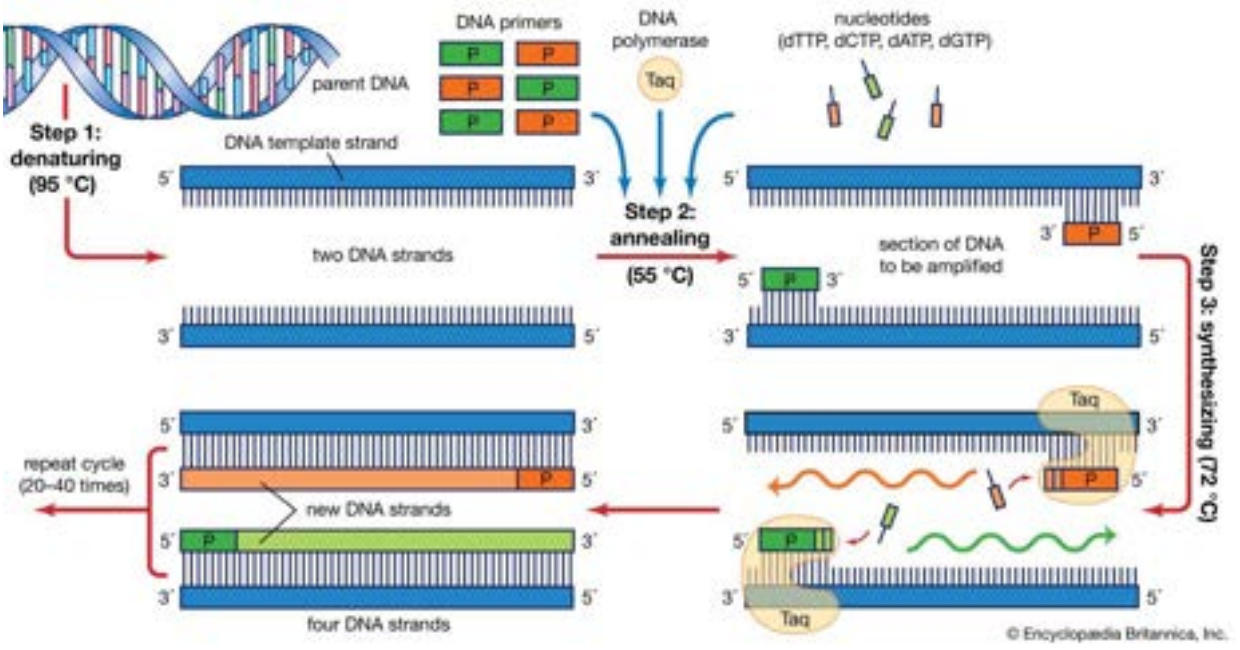
The name "PCR" comes from the DNA polymerase used to amplify a specific sequence of DNA by in vitro enzymatic replication. The initial heating step (usually at 94–95 °C) denatures the original target double-stranded DNA (dsDNA) into two complementary single strands of DNA, allowing forward and reverse primers to anneal at a lower temperature (dependent on primers) to their complementary sequences, flanking the specific sequence to be amplified. Next, the extension (usually at 72 °C) of the primers is catalysed by a heat-stable DNA polymerase (*Taq* DNA polymerase), which synthesizes two new strands of DNA, using dNTPs and the original strands as templates. This process (Fig. 4.1) results in the duplication of the original target DNA sequence, with each of the new molecules containing one old, and one new strand of DNA. Subsequently, each of these strands can be used to create two new copies, and so on. Numerous repetitive cycles of denaturing, annealing, and extension can result in exponential accumulation of a specific DNA fragment. The PCR cycles are repeated, typically up to a total of 35–45 cycles. This leads to billions of copies of the original DNA target sequence. The entire cycling process of PCR is completed in just a few hours. It is performed in a machine called a thermocycler, which is equipped with elements and/or fans for heating and cooling, to rapidly alter the temperature to allow DNA denaturation, primer annealing and extension. It is important to note that appropriate maintenance and verification of temperatures and ramp times of thermocyclers are crucial to ensure high-quality, reproducible results.

Traditionally, specific amplicons generated by PCR were detected by ethidium bromide-stained gel electrophoresis. Other amplicon detection methods include the use of capture and detection probes (labelled with enzymes or fluorescent molecules, among others) in a microwell format, and the sizing of PCR products using a genetic analyzer (Applied Biosystems) or bioanalyzer (Agilent Technologies). Many modifications and adaptations have been made to the original PCR technique to expand its usage and benefits, which include, but are not limited, to the following techniques: nested PCR, touchdown PCR, hot-start PCR, reverse transcriptase (RT) PCR (for RNA target), asymmetric PCR, digital PCR, multiplex PCR, semiquantitative PCR and quantitative real-time PCR (qPCR).

i. Real-time PCR

The principle of real-time PCR is identical to that of conventional PCR, except that the amplification and detection processes are combined to allow monitoring of PCR amplification as it occurs in real time. By contrast, conventional PCR is capable only of end-point measurement (i.e. to detect or visualize PCR products after amplification has been completed). The most significant advance in real-time PCR design is the incorporation of fluorescence-based detection technology, using either dsDNA-binding dyes, or specific fluorescent molecule-labelled probes (such as hydrolysis, hybridization, conformational probes or molecular beacons, for example) to allow not just the detection of the specific nucleic acid target, but also quantification of the target (qPCR).

Fig. 4.1: Polymerase chain reaction (PCR)



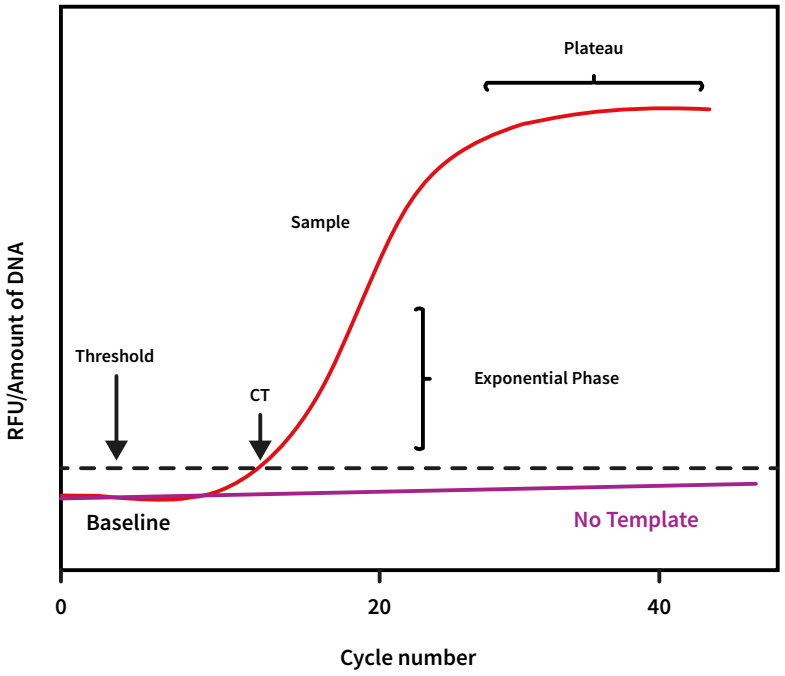
Source: Reproduced courtesy of Encyclopædia Britannica, Inc. *(17)*.

In addition, real-time PCR integrates and automates both amplification and detection in an instrument, which often requires less time and labour to obtain results, compared with performing conventional PCR. Other advantages of real-time PCR include: the use of shorter target sequences (< 150 bp); broader dynamic range of detection; higher analytical sensitivity and specificity; ability to multiplex and melt-curve analysis; and no requirement for post-PCR manipulation (minimizing the potential for post-PCR contamination).

To monitor amplification during real-time PCR, fluorescent reporter molecules are used to generate fluorescence signals. Most real-time PCR systems use a fixed light-emitting diode (LED) array for fluorescence excitation and a charge-coupled device camera or photomultiplier tube with proper filters for fluorescence detection. The increase in fluorescent signal is directly proportional to the number of PCR amplicons generated in the exponential phase of the reaction. The baseline of a real-time PCR refers to the level of signal (generally during the initial 5–15 cycles) in which there is little change in fluorescent signal, whereas the threshold is the level of signal that reflects a significant increase over the calculated baseline signal. The threshold can be set automatically by the instrument software or manually by the user. The cycle threshold (Ct) is the PCR cycle number at which the fluorescent signal of the reaction crosses the threshold. Accordingly, the presence of a Ct reflects the accumulation of a sufficient number of amplicons to be considered a positive reaction, and the Ct is inversely related to the amount of starting template. Real-time PCR data are usually plotted on a graph as PCR cycle number versus the intensity of fluorescence, which is related to the number of starting template. Fig. 4.2 shows a typical real-time PCR amplification curve.

Two common methods for the detection of amplification products in real-time PCR are: non-specific detection using fluorescent dyes that intercalate with any dsDNA, such as SYBR Green; and specific detection using target-specific fluorescent probes, such as the TaqMan probe (hydrolysis probe), molecular beacons, dual hybridization probes or tagging oligonucleotides. For SYBR Green, the unbound dye molecules exhibit very little fluorescence, but the fluorescence increases significantly when it is bound to dsDNA. As dsDNA accumulates, SYBR Green generates signals that are proportional to the concentration of the target DNA (Fig. 4.3A). This technology is, however, considered to lack very high specificity because the dye binds indiscriminately to all dsDNA formed during PCR and, consequently, any spurious amplification products may also contribute to the overall fluorescent signals. However, unlike certain probe types, including TaqMan probes, amplification that uses dsDNA-binding dyes can always be subjected to subsequent (i.e. post-PCR) melting curve analysis, for example, to differentiate between mutations or otherwise specific and non-specific PCR products based on melting peak temperatures and characteristics (Fig. 4.3B).

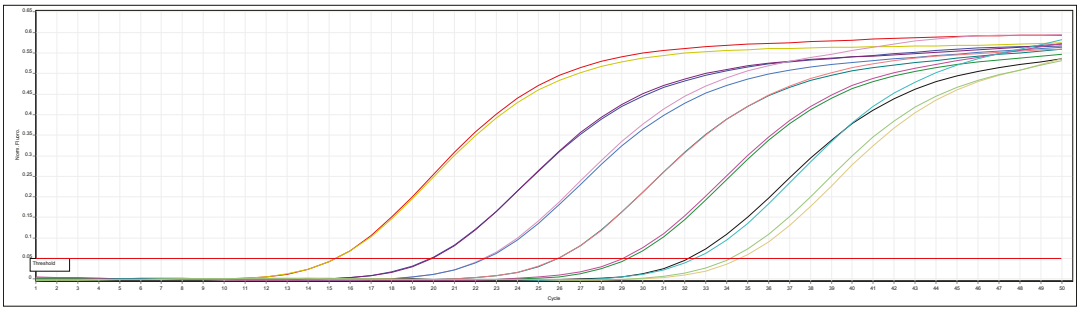
Fig. 4.2: Typical real-time polymerase chain reaction (PCR) amplification curve

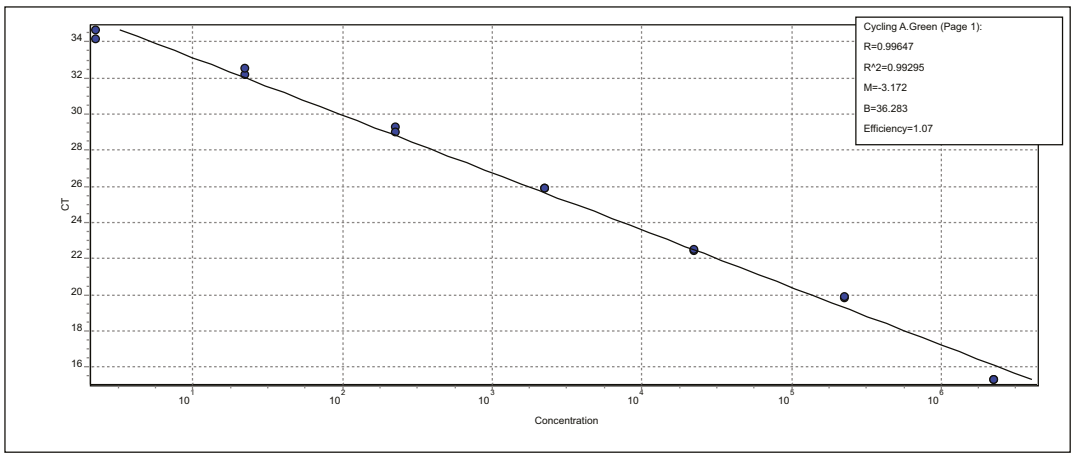


Source: Adapted from The Biotech Notes (https://thebiotechnotes.com/).

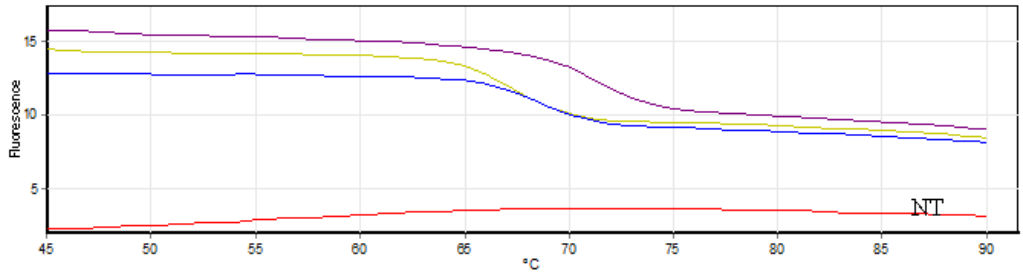
Fig. 4.3: Typical real-time PCR amplification curves

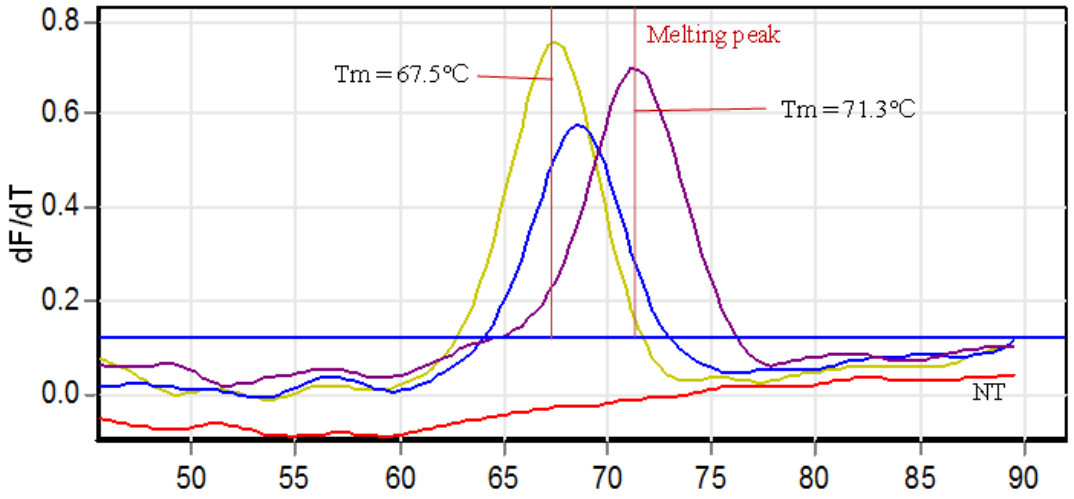
A. Specimens with a serial dilution of target DNA and the standard curve





B. Melting curve analysis using melting temperatures to enhance the specificity of SYBR Green-based real-time PCR



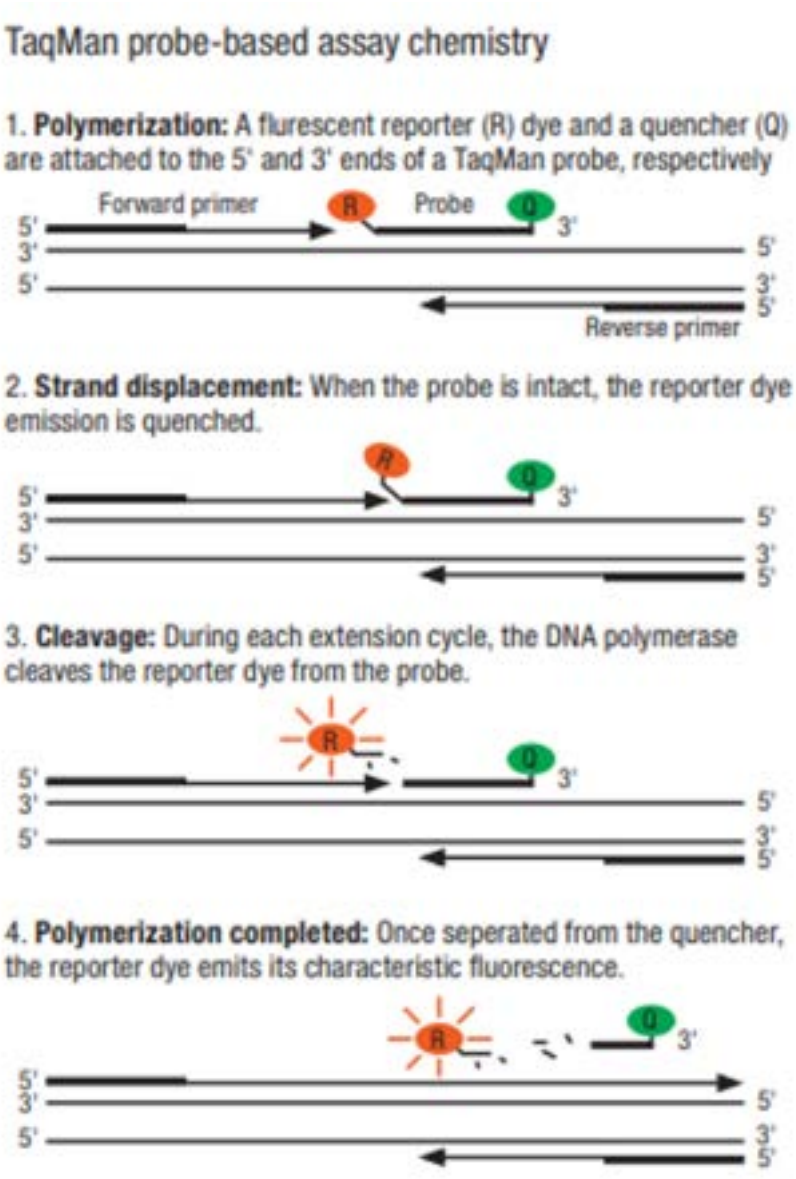


Source: Reproduced courtesy of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

a. Hydrolysis probes

The TaqMan probe (hydrolysis probe; Fig. 4.4) is the most commonly used target-specific probe in real-time PCR detection. The TaqMan probe-based PCR still utilizes a pair of specific PCR primers, but with the addition of an oligonucleotide TaqMan probe complementary to a specific DNA sequence of the template between the forward and reverse primers. The probe usually is designed with a high-energy dye, the reporter (fluorophore) at the 5' end, and a low-energy molecule, the quencher, at the 3' end (which quenches the fluorescence of the reporter). This dually labelled fluorescent probe emits little fluorescence when free in solution, due to the close proximity of the quencher to the reporter. During the real-time PCR amplification, the probe annealed to the template is cleaved by the 5' nuclease activity of the *Taq* DNA polymerase to release (separate from the quencher). Upon excitation by a light source, the reporter dye generates fluorescence that increases for each PCR cycle. TaqMan probe-based assays have been used widely in real-time PCR for gene expression, viral load determination, single nucleotide polymorphism (SNP) genotyping, bacterial identification, allelic discrimination, and verification of microarray results. They are widely used in clinical microbiology, including for STIs, owing to their simplicity and, importantly, the additional specificity they provide by being sequence-specific (as opposed to SYBR green, for example).

Fig. 4.4: TaqMan probe (hydrolysis probe)-based real-time PCR



Source: Thermo Fisher Scientific, 2023 *(18)*, reproduced courtesy of Thermo Fisher Scientific Inc., USA.

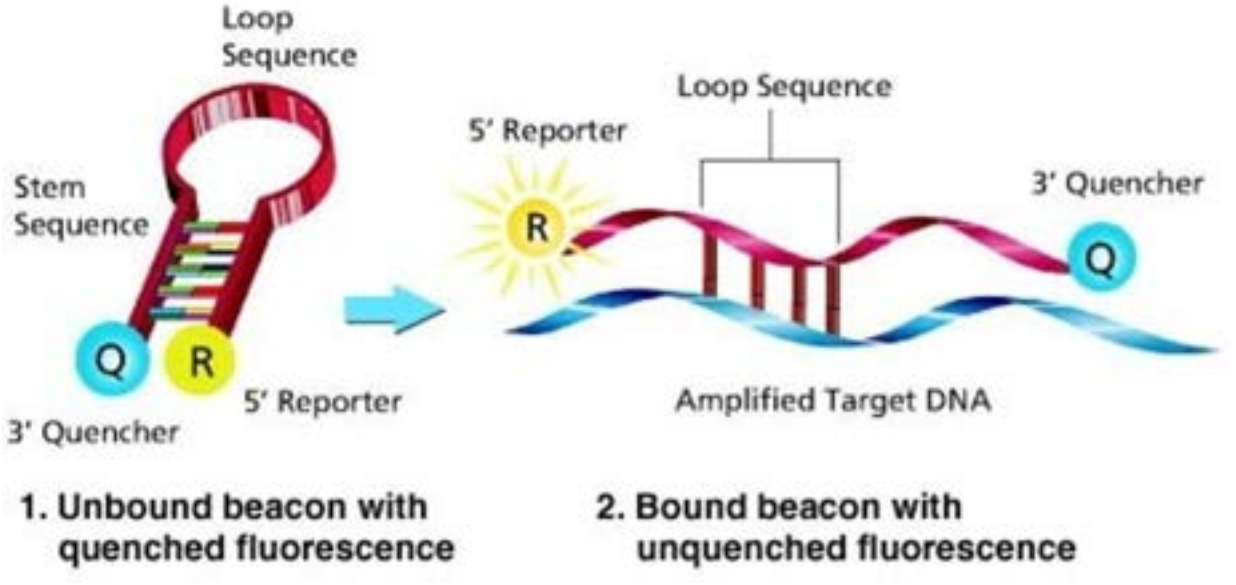
b. Hairpin probes

Molecular beacons (hairpin probes) are single-stranded oligonucleotide hybridization probes that form a stem-and-loop structure (Fig. 4.5). The loop contains a probe sequence that is complementary to a target sequence, and the stem is formed by the annealing of the complementary arm sequences that are located on both sides of the probe sequence. A fluorophore is covalently linked to the 5' end of one arm, and a quencher is covalently linked to the 3' end of the other arm. The stem keeps these two molecules in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer, thus molecular beacons do not fluoresce when they are free in solution. However, when they hybridize to a nucleic acid strand containing its complementary target sequence, they undergo a conformational change that enables them to fluoresce brightly. Accordingly, when the probe hybridizes to a target molecule, it forms a probe-target hybrid that is longer and more stable than the stem hybrid. The rigidity and length of the probe-target hybrid precludes the simultaneous existence of the stem hybrid. Consequently, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. Hence, molecular beacons essentially work in the opposite way to TaqMan probes, with a positive signal being generated via the coming together of fluorophores, whereas a positive signal for TaqMan probes occurs by the fluorophores separating. Molecular beacons are used in similar ways to TaqMan-based real-time PCR assays. However, an additional benefit of molecular beacons is that the probes can be used for post-PCR melting curve analysis for SNP and mutation detection.

c. Hybridization probes

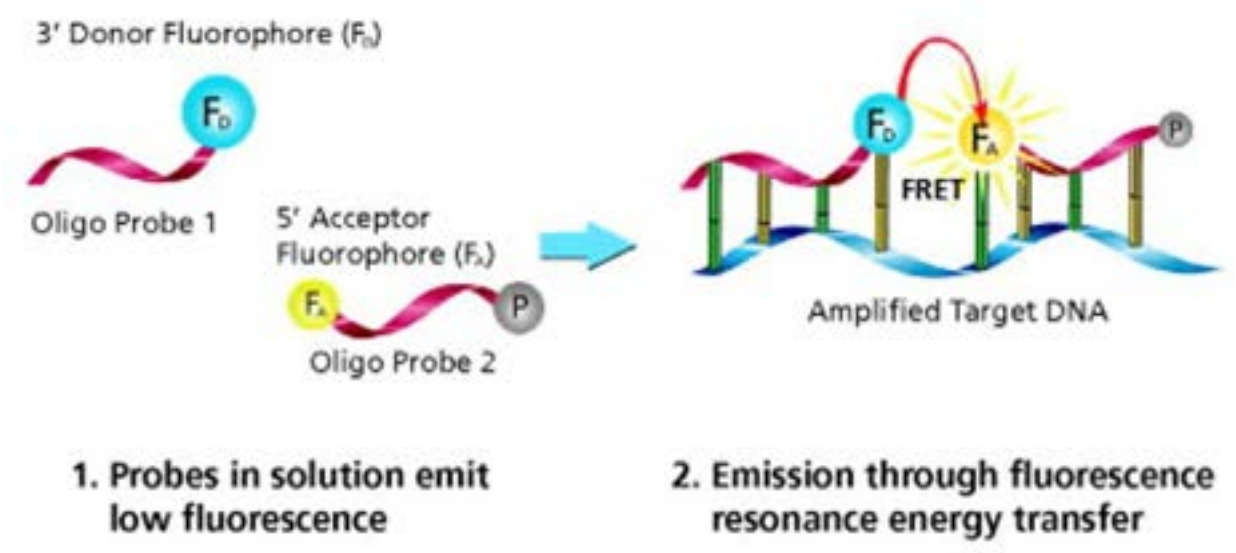
The dual hybridization or fluorescence resonance energy transfer (FRET) probe system (Fig. 4.6) consists of two partnering fluorescent probes that hybridize on the target sequence in close proximity (usually one to four nucleotides apart). The donor (anchor) probe is labelled with a fluorophore at the 3' end and the acceptor (reporter) probe with another fluorophore at the 5' end. The fluorophores are designed to allow the emission spectrum of one to overlap significantly with the excitation spectrum of the other. During PCR, the donor fluorophore is excited by an external light source, and the energy is transferred to the acceptor fluorophore, if positioned adjacent to the former. The excited acceptor fluorophore then emits light at a different wavelength, which then can be detected and measured. Thus, similar to molecular beacons, positive signal is generated by the fluorophores coming into close proximity, except that for the FRET probe system, there are two probes (rather than one) used to achieve this. While FRET probes are less commonly utilized for clinical (including STI) diagnostics compared to TaqMan probes, they remain useful because (similar to molecular beacons) they can also be subjected to melting curve analysis, and often are used in real-time PCR for allelic discrimination assays.

Fig. 4.5: Molecular beacon (hairpin probe)



Source: Merck KGaA, 2023 *(19)*, reproduced courtesy of Merck & Co Inc., Kenilworth, NJ, USA.

Fig. 4.6: Dual hybridization or fluorescence resonance energy transfer (FRET) probe system



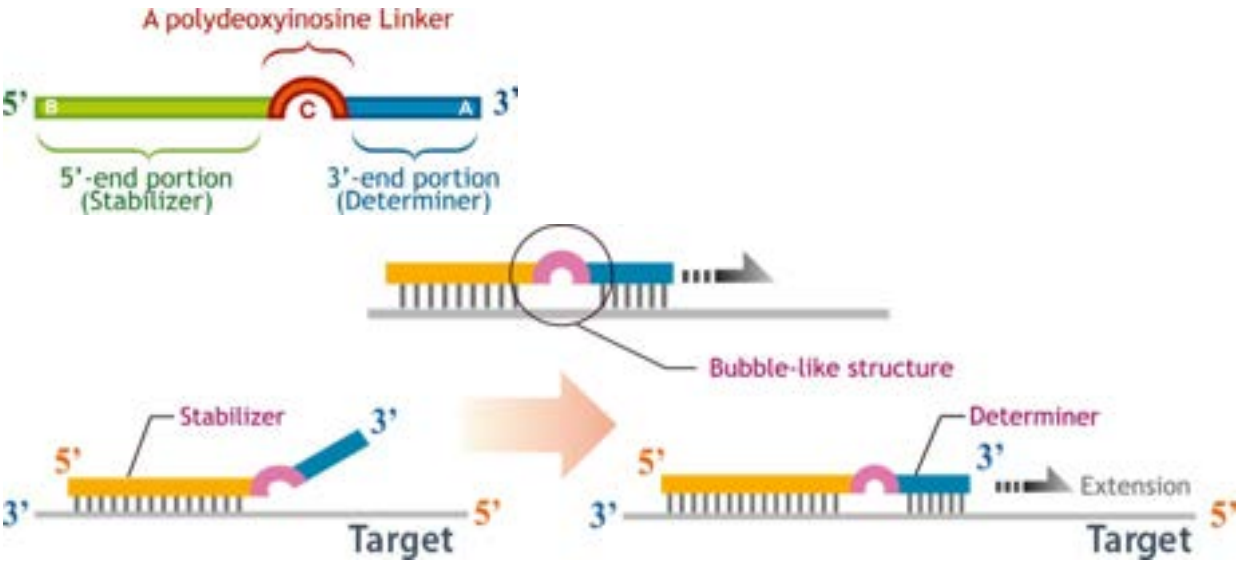
Source: Merck KGaA, 2023 *(20)*, reproduced courtesy of Merck & Co., Inc., Kenilworth, NJ, USA.

d. Tagging oligonucleotide

The key components for tagging oligonucleotide cleavage and extension (TOCE) technology are the dual priming oligonucleotides (DPOs). DPO is a structurally different primer composed of two functional priming portions connected by a polydeoxyinosine linker (poly-dI linker). The poly-dI linker forms a bubble-like structure at a certain annealing temperature and controls the two-step priming reactions required for the polymerase to extend a DPO primer. First, the longer 5' end portion binds to the template DNA and initiates annealing, and, thereafter, the shorter 3' end portion selectively binds to the target site. The DPO technology is then combined with the TOCE technology. This consists of a tagging oligonucleotide, called the pitcher, which hybridizes specifically to the target region and is cleaved away during extension. The released pitcher hybridizes to a dual-labelled artificial template, called the catcher. The pitcher-catcher formation induces extension on the catcher, resulting in the generation of a fluorescence signal (Fig. 4.7). This technology is used in the novel multiple detection temperatures (MUDT) technology and is well suited to highly multiplexed real-time PCR technology.

Fig. 4.7: Dual priming oligonucleotide (DPO) and tagging oligonucleotide cleavage and extension (TOCE) technology

A. The principle of DPO technology

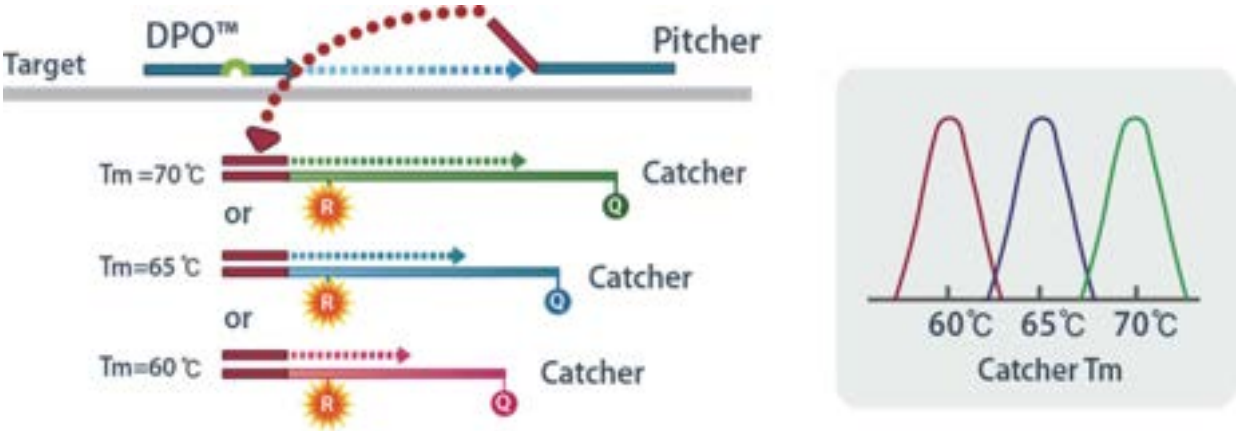


Step 1: First priming reaction

Step 2: Second priming reaction

Source: Seegene Inc. *(21)*, reproduced courtesy of Seegene Inc., Seoul, Republic of Korea.

B. Principles of TOCE technology



Source: Seegene Inc. *(22)*, reproduced courtesy of Seegene Inc., Seoul, Republic of Korea.

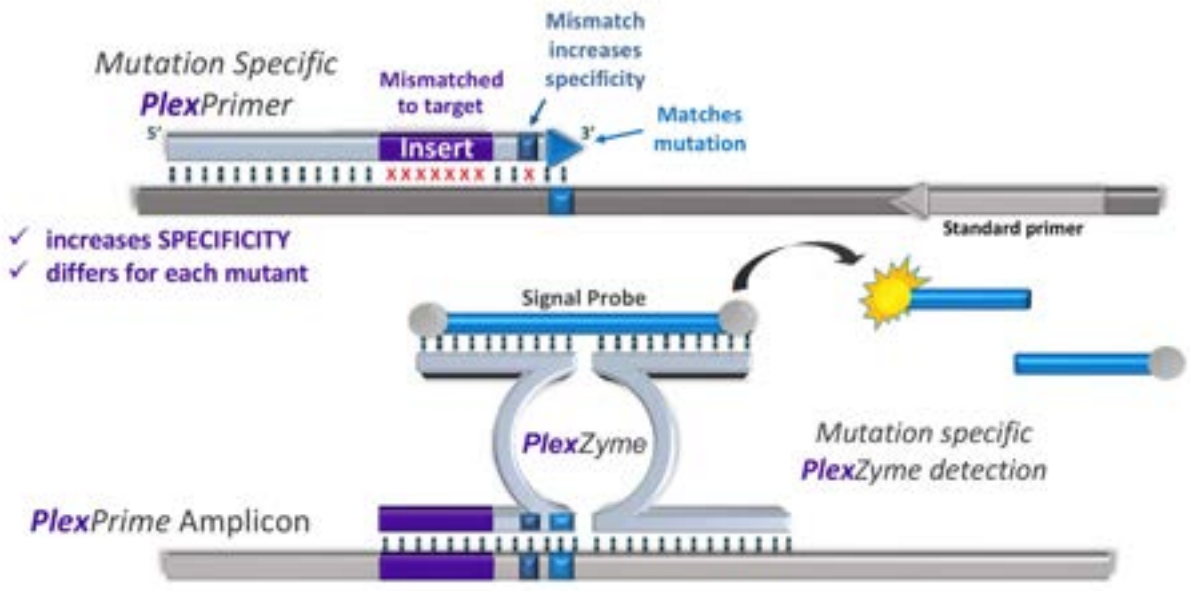
ii. PlexPCR

PlexPCR is another real-time PCR technology that is particularly suitable for detection and discrimination of mutations or SNPs in multiplex reactions. PlexPCR combines allele-specific primer amplification using PlexPrimers, which amplify the target nucleic acid sequence and produce amplicons that serve as a template for the following allele-specific detection by PlexZymes (also known as MNAzymes) *(23,24)*. The PlexPrimer contains three functional regions:

- a 5' target recognition region;
- a short 3' target-specific sequence; and
- a non-complemented intervening insert sequence.

The mismatched insert sequence is incorporated into the PlexPrime amplicons during amplification and is detected in real time using PlexZymes. PlexZymes are nucleic acid enzymes assembled from partzymes A and B, but only when the target amplicons are present. Each of the partzymes contain a probe binding arm, a partial catalytic core and a target binding arm. Partzyme A binds to the amplicon in the region that includes the complement of the insert sequence and the 3' target-specific sequence, and partzyme B binds adjacently downstream. Once the partzymes have assembled into PlexZyme enzymes, a universal probe can bind and an enzymatic cleavage of the probes between the fluorophore and quencher dye pairs generates fluorescence. The changes in fluorescence allow detection and/or quantification of the target nucleic acid in real time (Fig. 4.8). For multiplexed mutation detection, each PlexPrimer contains a different insert sequence designed to be allele-specific via the complementarity of the 3' terminus region with the target mutation.

Fig. 4.8: PlexPCR technology, PlexPrimer amplification coupled with PlexZyme detection



Source: SpeeDx Pty. Ltd *(25)*, reproduced courtesy of SpeeDx Pty. Ltd, Sydney, Australia.

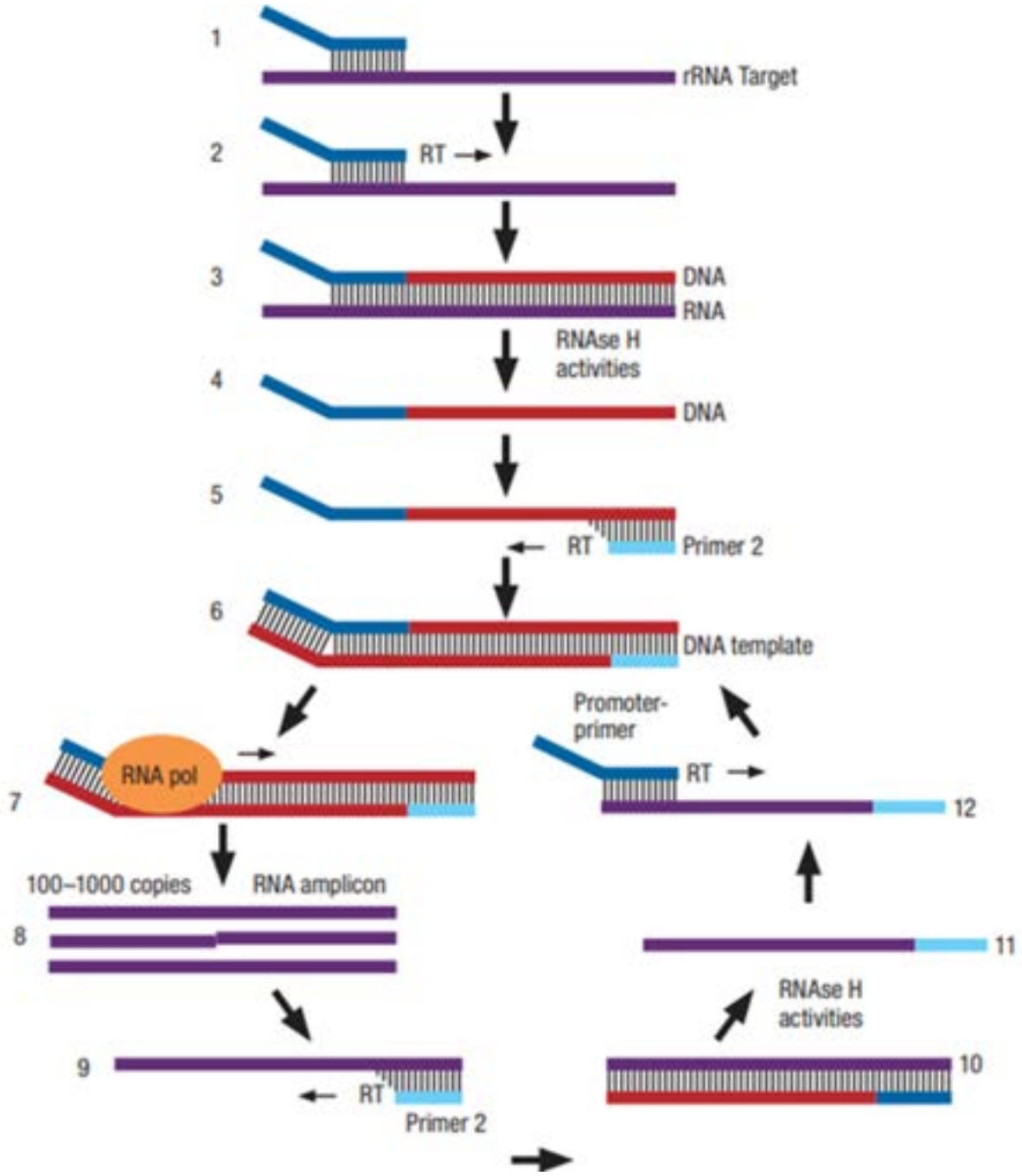
4.2.2 Transcription-mediated amplification (TMA)

The patented TMA technology *(26,27)* can be used to amplify either RNA or DNA, and produces RNA amplicon. This is in contrast to most other NAATs, which only produce DNA. An initial target capture (TC) process is performed before TMA to reduce inhibition of amplification and contamination. In this TC method, poly-T oligomers bound to magnetic particles are used to bind a capture probe containing poly-A tail and target-specific sequence, which then can specifically hybridize to the complementary sequence of the target RNA. The subsequent TMA process (Fig. 4.9), which amplifies the captured target sequence, uses two target-specific primers and two different enzymes (RT and RNA polymerase) for amplification. One of the target-specific primers contains a promoter sequence for the RNA polymerase, and when this primer hybridizes to the target RNA, a reverse transcription is initiated, creating a complementary DNA (cDNA) copy. The RNA in the resulting RNA/DNA heteroduplex is degraded by the RNase H activities of the RT. This enables the second primer to bind to the DNA copy and synthesize a new strand of DNA by the RT, creating a dsDNA molecule. Both strands of the created dsDNA molecule now contain promoter sequences for RNA polymerase and, therefore,

can be used as a template to initiate transcription. Subsequently, each of the newly synthesized RNA amplicons reenter the TMA process and serve as a template for a new round of replication, leading to an exponential expansion of the RNA target sequence. TMA can produce 100–1000 copies per amplification cycle, in contrast to PCR, which produces only two copies per cycle. TMA is isothermal; the entire reaction is performed at the same temperature in a water-bath or heat block, instead of a thermocycler.

The RNA amplicons produced in the TMA reaction can be combined with molecular torches for real-time detection or with a specific gene probe in hybridization protection assay (HPA) for chemiluminescence end-point detection. The HPA technique is described under non-amplified nucleic acid-based technologies, in section 4.3.

Fig. 4.9: Transcription-mediated amplification (TMA)

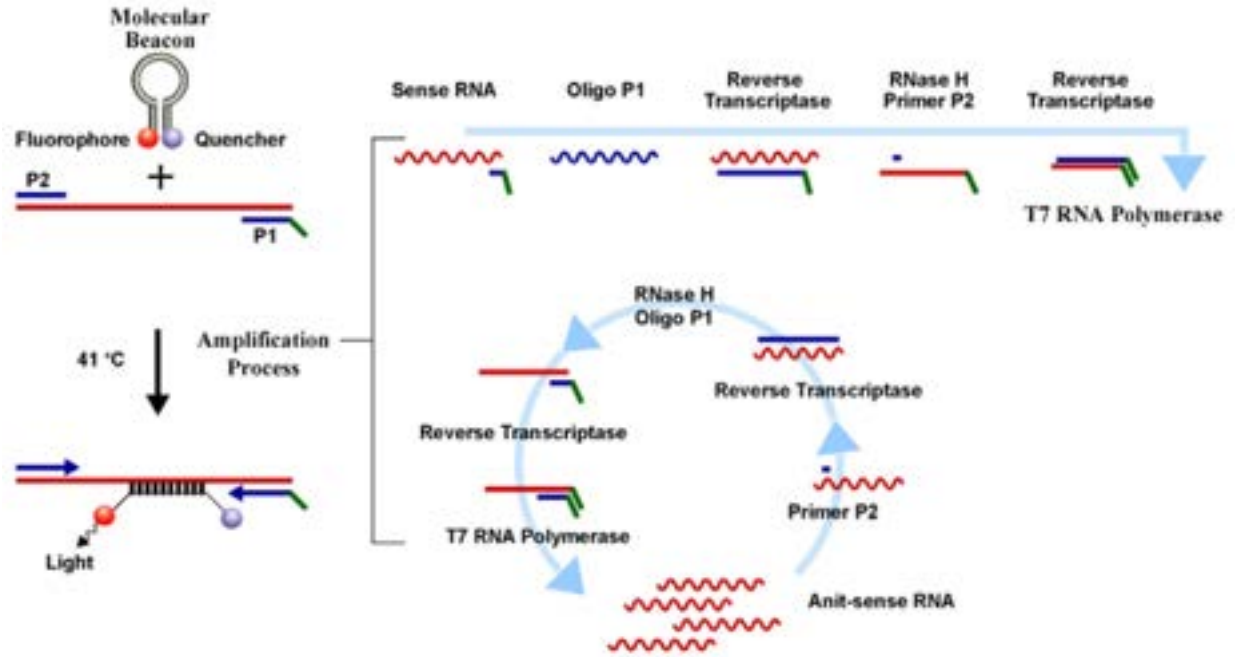


Source: Reproduced courtesy of Hologic Inc., San Diego, CA, USA.

4.2.3 Nucleic acid sequence-based amplification (NASBA)

NASBA *(27,28)* is another isothermal amplification technology for RNA or DNA target sequences. The technology is similar to TMA, except that NASBA uses three (instead of two) enzymes: avian myeloblastosis virus reverse transcriptase (AMV RT), RNase H, and T7 RNA polymerase. These enzymes, together with specific primers, enable the amplification of the target nucleic acid sequence (Fig. 4.10). Amplified RNA products can be detected by post-amplification hybridization using electrochemiluminescence labelled (ECL) probes, or by a real-time detection system using molecular beacons.

Fig. 4.10: Nucleic acid sequence-based amplification (NASBA)



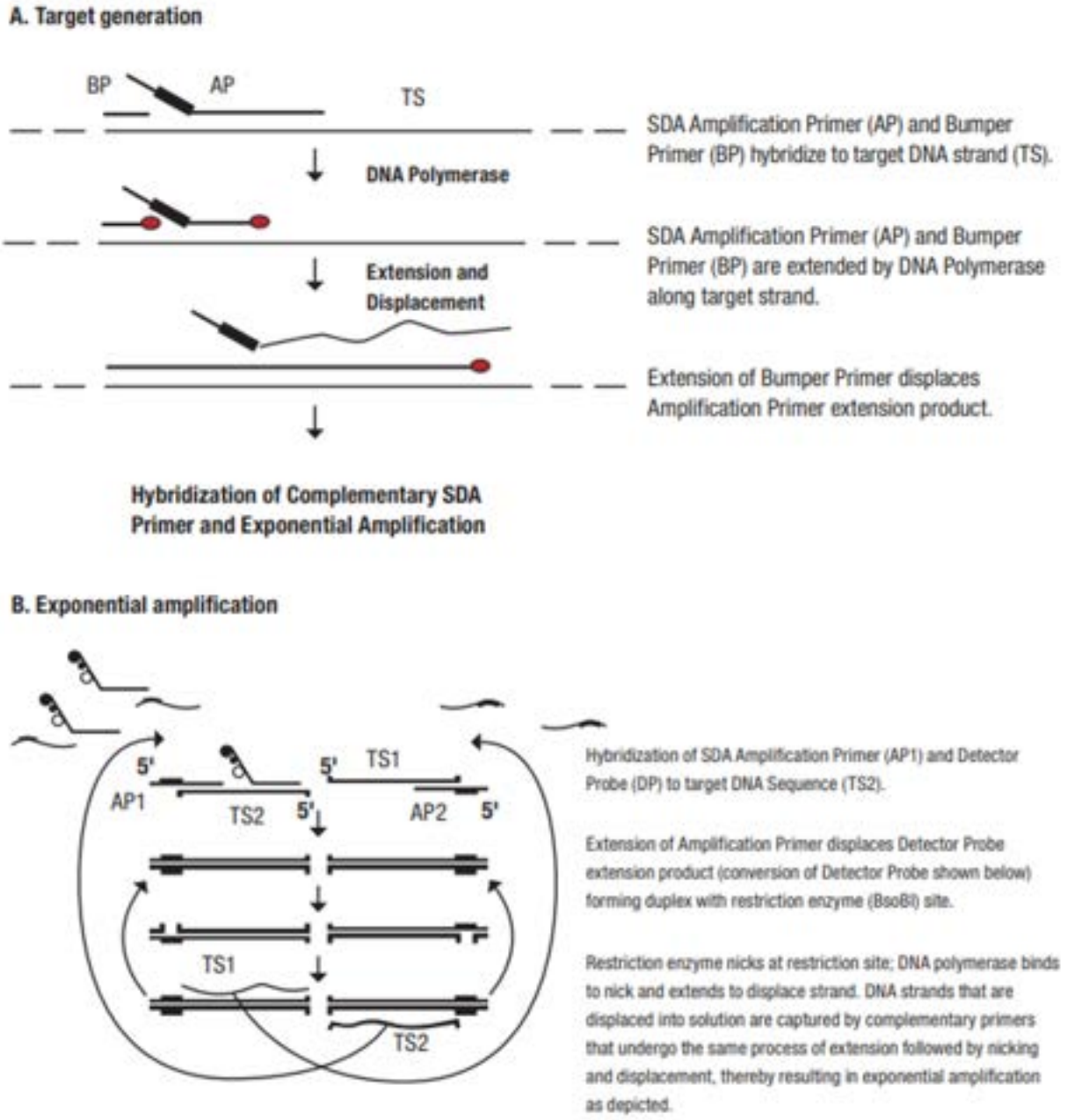
Source: PREMIER Biosoft, 2023 *(29)*, reproduced courtesy of PREMIER Biosoft, Palo Alto, CA, USA.

4.2.4 Strand displacement amplification (SDA)

SDA is an additional isothermal, in vitro enzymatic process that permits the amplification of target molecules from a single DNA or RNA template *(27,30)*. The patented technology is based on the combined action of a restriction enzyme, DNA polymerase, and two pairs of primers (SDA amplification primers and Bumper [Adapter] primers). The Bumper primers contain only target-specific DNA sequences, while the SDA amplification primer pairs contain a specific restriction endonuclease recognition sequence at its 5' end in addition to a sequence complementary to the target segment. Fig. 4.11 shows the SDA process, which consists of two phases. The first phase is the generation of dsDNA of the template of interest containing restriction endonuclease recognition site that feeds into the second phase of exponential amplification, where the restriction endonuclease nicks one of the two strands of the newly formed dsDNA, allowing the DNA polymerase to create a new dsDNA sequence from the displaced strand. This process, which resembles the rolling-circle replication of single-stranded phages and small plasmids, is repeated continuously until a sufficiently large amount of the DNA strand of interest is produced and can be identified by a detector probe.

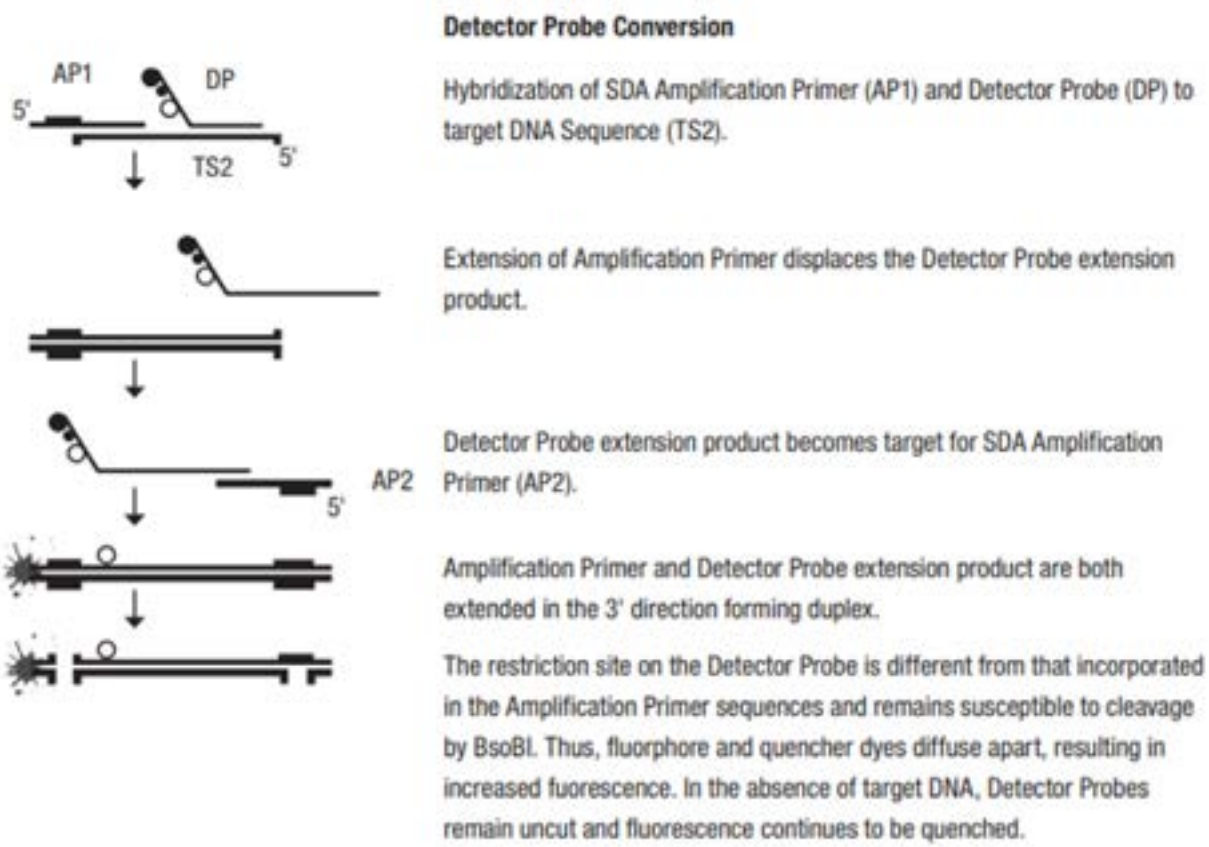
Currently, in a second-generation commercial platform, the real-time detection of SDA products occurs simultaneously with amplification, using fluorescent detector probe and fluorescence energy transfer (Fig. 4.12). The detector probe consists of a target-specific hybridization region at the 3' end and a hairpin structure at the 5' end. The loop of the hairpin contains the restriction endonuclease recognition sequence, and the 5' base is conjugated to the donor molecule, while the 3' base of the hairpin stem is conjugated to an acceptor molecule. In its native state, the hairpin maintains the donor and the acceptor molecules in close proximity and little fluorescence is observed. As the detector probe anneals to the target, the hairpin becomes linearized and extended by the polymerase. This extension creates a double-stranded detector probe with a cleavable restriction site, which the restriction enzyme promptly cleaves. The cleavage causes the physical separation of the donor from the quenching effects of the acceptor and allows real-time detection of fluorescence.

Fig. 4.11: Strand-displacement amplification (SDA)



Source: Reproduced courtesy of Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA.

Fig. 4.12: Second-generation strand-displacement amplification (SDA), in which amplified products are detected in real time using fluorescent detector probe and energy transfer



Source: Reproduced courtesy of Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA.

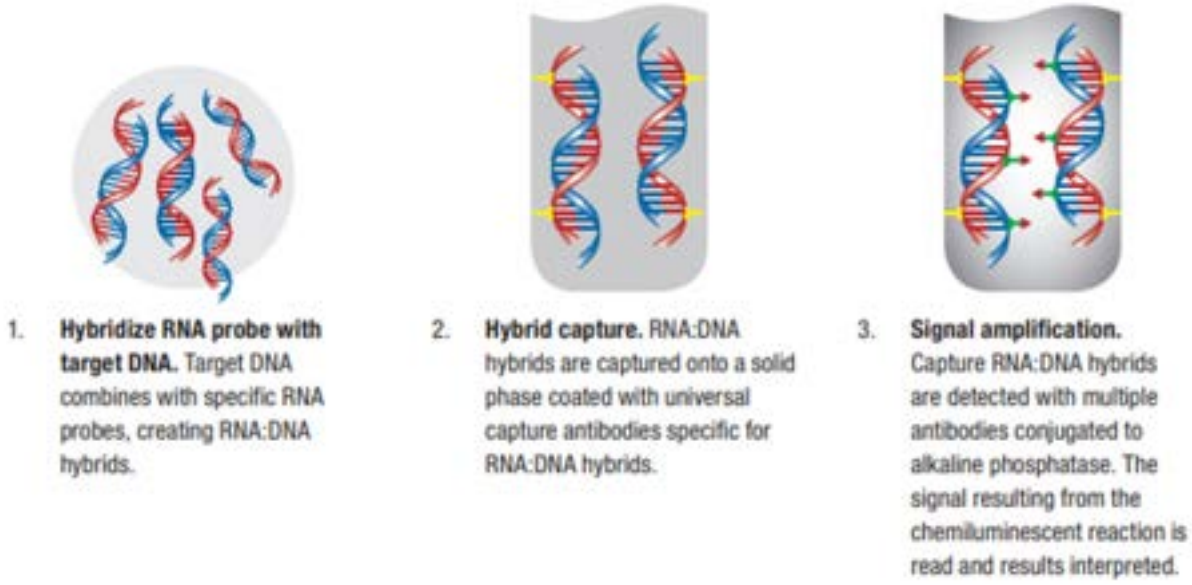
4.3 Non-amplified nucleic acid-based technologies

There are also non-amplified nucleic acid-based tests available for various STIs, including gonorrhoea, chlamydia, hepatitis and HIV. They are usually rapid, can be automated for large-scale screening, are relatively inexpensive, and only require moderate technical skill. The sensitivity of NAH assays in many cases exceeds that of conventional diagnostic methods such as culture or enzyme immunoassays (EIAs) but has, on the other hand, shown in some assays to be considerably less than that of NAAT-based methods. Most direct probe hybridization and detection assays are more likely to be used when large amounts of target DNA or RNA are expected to be present; for example, in a urethral swab or a bacterial culture. A reliable detection of the target without the use of signal amplification usually would require at least 10^4 copies of nucleic acid target per µl. However, amplification of the signal after probe hybridization can improve detection to as low as approximately 500 target molecules per µl, and for certain assays, provides quantitative capabilities.

4.3.1 Hybrid capture (HC)

HC technology is an in vitro NAH with signal-amplification using microplate chemiluminescence for the qualitative detection of nucleic acid targets *(31)*. Basic steps of the HC assay involve: the lysis of the virus or bacteria to release target DNA or RNA; hybridization of specific RNA or DNA probes to create RNA/DNA hybrids; capture of RNA/DNA hybrids onto a solid phase using universal antibodies specific for the hybrids; amplification of signal with antibodies conjugated to an enzyme (e.g. alkaline phosphatase); detection of signal when substrate (e.g. chemiluminescent dioxetane) is cleaved by the enzyme; and measurement of chemiluminescence produced in relative light units (RLUs) using a luminometer (Fig. 4.13).

Fig. 4.13: Hybrid capture technology

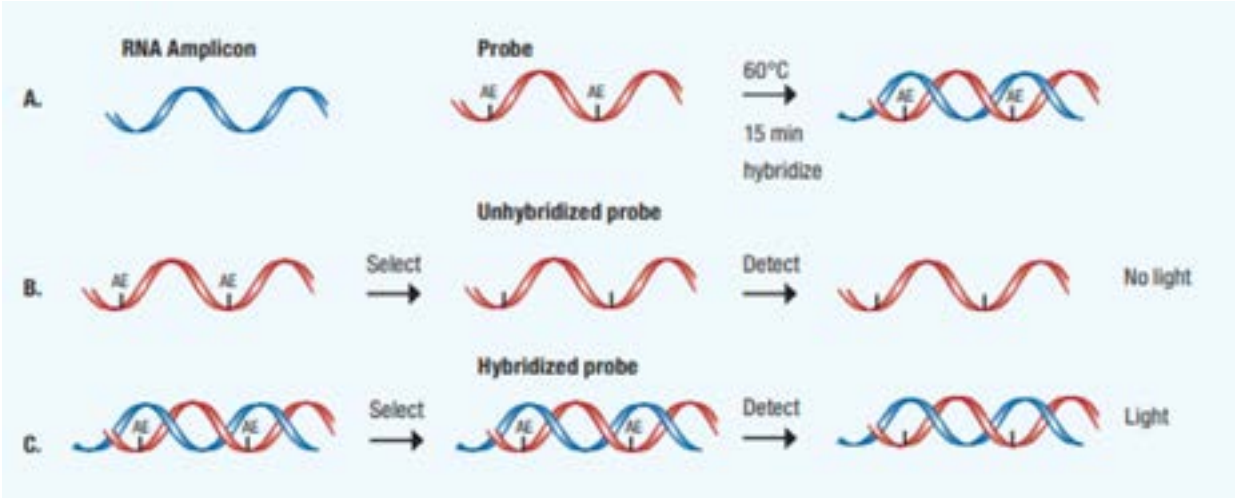


Source: Reproduced courtesy of Qiagen Inc., Hilden, Germany.

4.3.2 Hybridization protection assay (HPA)

HPA technology involves the detection of RNA or single-stranded DNA targets by means of a chemiluminescent DNA probe *(32,33)*. In the HPA process, sequence-specific DNA probes labelled with acridinium ester (AE) hybridize to the amplification products. Separation (selection) of hybridized from unhybridized probes is done by the addition of a selection reagent (alkali), which hydrolyses the AE label on unhybridized probes. When the probe binds to its specific target sequence, the AE label on the hybridized probe is protected within the double helix and is not hydrolysed. Upon the addition of detection reagent, only the AE label attached to the hybridized probe is left to produce a signal indicating that the target DNA or RNA is present. No chemiluminescence is emitted from the unhybridized probes (Fig. 4.14). HPA does not require the cumbersome wash steps needed with conventional probe tests and immunoassays. Furthermore, only one molecule of AE-labelled probe can bind to each RNA amplicon; thus, the chemiluminescent signal obtained is directly proportional to the number of target molecules in the initial sample. The dual kinetic assay (DKA) technology, which is a modification of the HPA technology, uses two types of probes labelled with two different AEs displaying different light-off kinetics in a single assay that enables the detection of two separate targets simultaneously. HPA or DKA technology is commonly used for the detection of RNA amplicons produced by TMA.

Fig. 4.14: Hybridization protection assay (HPA)



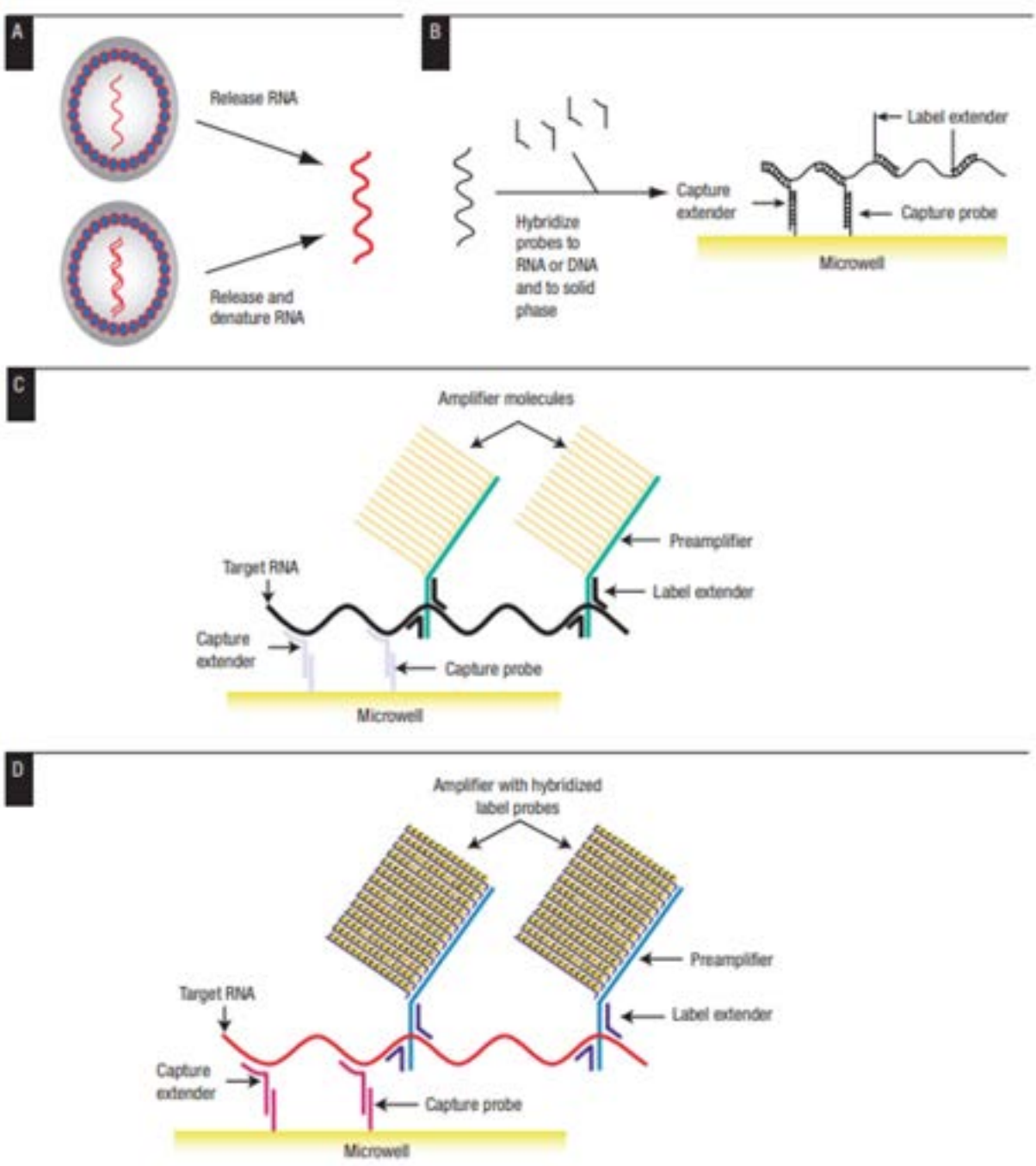
Source: Reproduced courtesy of Hologic Inc., San Diego, CA, USA.

4.3.3 Branched-chain DNA (bDNA) assay

Branched-chain DNA (bDNA) assays involve the use of a series of oligonucleotides in a sandwich nucleic acid hybridization method to detect and quantify a target by signal amplification *(34)*. The process of bDNA involves the lysis of target organisms, target capture, signal amplification and detection, as shown in Fig. 4.15. The initial step of a bDNA assay is to disrupt the target organisms using detergent and proteinase K to release nucleic acids (A). The first set of target-specific oligonucleotides (capture extender) then are hybridized with high stringency to both the target nucleic acids, as well as the capture probes that are attached to a microwell plate (B). The second set of oligonucleotides (label extenders) is designed to hybridize to contiguous regions on the target and to provide sequences for hybridization of a preamplifier oligonucleotide. The preamplifier forms a stable hybrid only if it hybridizes to two adjacent label extenders (C). Multiple bDNA amplifier molecules are then hybridized to the preamplifier to create a branched structure (D). Finally, alkaline phosphatase-labelled oligonucleotides that are complementary to bDNA amplifier sequences bind to the bDNA molecule by hybridization. The bDNA signal is the chemiluminescent product from the alkaline phosphatase and its specific substrate. In this way, the signal is amplified without copying the target nucleic acid sequence, and the amount of signal detected is directly proportional to the amount of bound nucleic acid.

bDNA technology has progressed from first-generation assays that were accurate and reproducible but relatively insensitive, to third-generation tests that are accurate, reproducible, highly sensitive, and amenable to full automation. In addition, bDNA assays do not require the amplification of a target sequence, thus cross-contamination between replicate samples or carryover is less likely in bDNA assays.

Fig. 4.15: Branched-chain DNA (bDNA) signal amplification assay



Source: Reproduced courtesy of the American Society for Clinical Pathology.

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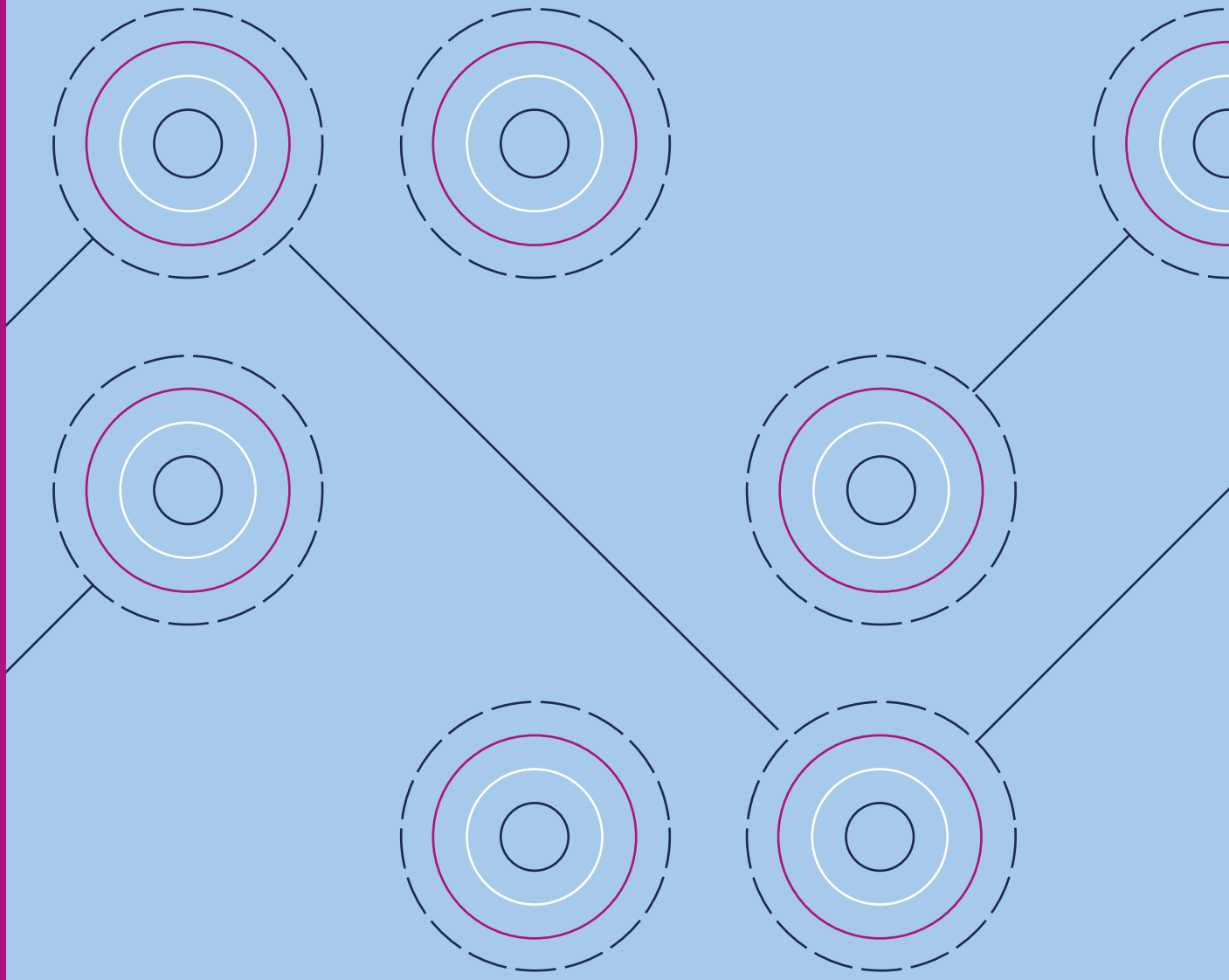
Chapter 5. Quality management of STI diagnostic testing



Chapter 5. Quality management of STI diagnostic testing

Michelle Cole and Rosanna Peeling

5.1 Introduction to quality management

The implementation of a quality management system (QMS) for STI diagnostic testing is paramount to ensuring that patient test results are reliable and accurate. This holds true in laboratory, as well as in non-laboratory settings that perform point-of-care (POC) testing. This chapter provides an overview of the key aspects of quality management in both of these settings. For an exhaustive list of all quality requirements the reader is advised to refer to the appropriate guidance for their setting.

The purpose of a quality system is to ensure that all patient results produced by the laboratory are of high quality, so that the right result goes to the right patient at the right time. To maintain a high quality of service, laboratories should strive to have a QMS and to become accredited *(1,2)* to a suitable national or international body, such as the International Organization for Standardization (ISO). The ISO 15189:2022 standard specifies the requirements for quality and competence in medical laboratories, is internationally recognized and can be used by laboratories to develop a QMS.[12] Different standards and different accreditation bodies are available, and some may be more applicable and/or achievable, depending upon the laboratory's function and setting. Accreditation involves regular external assessments by a representative of the accreditation body to provide assurance of ongoing compliance with the chosen standard. Having a QMS in place means that the laboratory is always striving for continual improvement. The main terms used in this chapter are defined in Box 5.1.

Accreditation by a recognized body is a time-consuming and expensive procedure. This can make it difficult to achieve for many laboratories. However, all laboratories and settings that issue patient results should work towards improving any procedure that affects the accuracy of information used to guide patient management. Schemes providing practical support and tools for developing countries in improving quality and moving towards accreditation have been developed and shown to work well *(3)*.

5.2 Quality management system (QMS)

5.2.1 The quality manual

It is good practice to describe the QMS of the laboratory in a quality manual. This informs the laboratory's own management and staff, provides information for users of the laboratory services and details how the laboratory fulfils the requirements of the accreditation body. The quality manual should be the key document to index all the quality, laboratory, clinical and management procedures.

[12] Available at: https://www.iso.org/standard/76677.html

Box 5.1: Key definitions

Accreditation	The assessment of the competence and impartiality of an organization and the compliance of their work to nationally and internationally recognized standards.
Audit	The systematic, independent and documented review and evaluation of a specific laboratory activity or examination procedure. The audit can also review and evaluate the overall system.
Corrective action	Action taken to eliminate the causes of non-conformities to prevent recurrence.
COSHH	Control of Substances Hazardous to Health.
Document control	Process to monitor and record the creation, review, approval, distribution and control of access of documents used in the laboratory.
External quality assessment (EQA)	Testing samples of known but undisclosed content from an independent source in a laboratory's routine testing procedure in order to provide assurance and comparison with others.
Internal quality assessment (IQA)	The periodical resubmitting and testing of randomly selected anonymized clinical samples within the same laboratory to ensure reproducibility of the results.
Internal quality control (IQC)	Used to detect problems or failure in one or more aspects of a test, such as reagents, equipment or operator error.
Point-of-care (POC) testing	A diagnostic test carried out at or near the person being tested. The results are returned to the person being tested during the same visit and the results can be used immediately for patient care and referral.
Preventative action	Action taken to prevent the occurrence of non-conformities.
Quality assurance (QA)	Planned and systematic activities implemented within the quality system that can be demonstrated to provide confidence that a laboratory service will fulfil requirements for quality.
Quality control (QC)	Includes IQC, evaluation, validation and verification of tests, and equipment, reagent and consumables validation and monitoring.
Quality management system (QMS)	A formal system that documents the structure, processes, roles, responsibilities and procedures required to achieve effective quality management.
Standard operating procedure (SOP)	A document with a detailed description of the individual procedure for each protocol or test used within the laboratory. The purpose of a SOP is to ensure the operations are performed correctly and always in the same manner. A SOP should be available at all times in the laboratory.
Risk assessment	A systematic process of evaluating the potential risks that may be involved in a laboratory/examination activity.
Validation of tests	The act of confirming a test meets the requirements for which it was intended.
Verification	Independent assessment of the performance criteria stated by a manufacturer, such as in the kit insert.

5.2.2 Organization and management

A staff organizational structure is necessary to describe management responsibilities and identify individuals and their roles and responsibilities. This will usually be a hierarchical structure, led by a director or head of department, who maintains oversight and overall responsibility for the laboratory services. The director (or equivalent) may delegate some duties and responsibilities to qualified personnel. The laboratory structure would usually consist of section or unit heads, laboratory and/or technical managers, health and safety and quality leads and a range of health-care professionals of different grades. The quality lead could

be either a dedicated role or an integral part of a wider job description. The quality manual should include an organogram of line management, as well as a brief description of each role. Each staff member should have a regularly reviewed, detailed job description that describes their role and responsibilities.

5.2.3 Staff training and registration

All staff within the laboratory should be trained in line with the requirements of their role and assessed to provide assurance of their competency to perform their duties. A programme of ongoing competency assessment is recommended, such as further training, cross-checking of worksheets and participation in IQA and EQA schemes. As well as training in daily duties, staff should receive appropriate QMS, health and safety, and information governance training. Where appropriate, to fulfil the duties of the role, staff should be registered to a national body and fulfil the continuing professional development (CPD) requirements for their professional title. Staff not registered with a professional body are also encouraged to maintain and develop their CPD. A training record (electronic or paper-based) should be kept for each staff member.

5.2.4 Communication

It is recommended that a series of meetings is organized, to enable the communication, implementation and review of the QMS, as well as supporting the function of the laboratories and laboratory personnel. Meetings should be minuted to capture key decisions, and actions managed through to completion and closure. Examples of key meetings may include the following.

- Management or senior staff meetings attended by the director or head of the laboratory, the laboratory manager, section heads, QA lead, laboratory safety lead and other appropriate senior staff. This group should ideally meet at least quarterly to discuss relevant matters, including finance, administration, staffing, equipment, IQA and EQA results, quality improvements, accreditation and other quality-related items, as well as raising matters affecting the delivery of the laboratory service.
- Section/unit level meetings at senior and all-staff levels are required to discuss laboratory, clinical and scientific issues along with finance, administration, staffing and operational issues. All relevant quality issues such as audits, IQA/IQC/EQA, stakeholder feedback, non-conformances and quality improvements should be included. Minutes and actions should be taken and reviewed.
- A management review meeting should be held at least annually to review the performance of the laboratory, encompassing key performance indicators and trending over time. Quality objectives for the upcoming year can be agreed.

Other specialized meetings, such as technical, clinical governance, safety and training should be held when appropriate.

5.2.5 Standard operating procedures (SOPs)

All laboratory procedures should be documented as SOPs that are easy to follow, based on a standard template. This template should include principal details of the reagents and methodology, including internal controls and interpretative criteria. SOPs should be written by individuals performing the method/test/procedure and authorized by another member of staff who is also familiar with the content. SOPs should cross-reference to risk assessments and safety information, as well as other relevant SOPs and documentation required to fulfil and appropriately monitor the undertaking of the procedure. The SOPs should be readily accessible in the laboratory for daily use, reviewed regularly and document controlled. In some settings, local SOPs can be informed by national SOPs.

5.2.6 Document control

It is advisable that all documentation used in any examination process is document controlled, to ensure the correct version is being used. Document control is a process to monitor and record the creation, review, approval, distribution of and access to documents used in the laboratory, such as SOPs, worksheets, manuals, policies and risk assessments. Cross-referencing to relevant documentation is important. Documents must be reviewed regularly to ensure they remain fit for purpose, and updated as required.

5.3 Quality assurance (QA)

QA involves planned and systematic activities to provide confidence that a laboratory service is meeting its defined quality requirements, and that results are both accurate and reproducible. Accuracy is defined as close agreement between the mean value obtained from a large series of test results and an accepted reference value. Reproducibility means the ability to produce the same diagnostic result, irrespective of variations in operator, reagent batches, laboratory or validated ancillary equipment.

There are two main QA elements: quality assessment, including IQA, EQA and audit; and quality control (QC), which encompasses evaluation and validation of tests, IQC, and equipment evaluation and monitoring (Fig. 5.1).

Fig. 5.1: Different elements contributing to quality assurance and quality control

Quality assessment		Quality control		
Internal and external assessment	Audits	Evaluation, verification and validation of tests	Internal quality control	Equipment, reagent and consumables validation and monitoring

Source: Michelle Cole and Rosanna Peeling.

5.3.1 Quality assessment

i. Internal quality assessment (IQA)

IQA involves the periodic resubmission and testing of randomly selected, anonymized clinical samples within the same laboratory. The IQA sample result is matched to the original after testing to show reproducibility. All IQA specimens should be tested blindly, undergo routine analysis and receive no special treatment. For the scheme to provide both useful and relevant information, IQA should be performed regularly for all sample types, but the number of IQA samples and frequency of testing may vary depending on the assay. It is recommended that at least 1% of samples are subject to IQA on a monthly basis. IQA samples must not be tested by the same person who selected the samples and the results should be analysed by an independent person, with any discrepancies repeated and investigated.

ii. External quality assessment (EQA)

EQA is the independent evaluation and performance assessment of laboratory examination procedures, where the QMS is effectively challenged. Any issues identified can be investigated and corrective and preventative actions implemented to improve the quality of the service. EQA provides a number of benefits for the laboratory:

- gives staff an insight into their laboratory's performance, with comparison (benchmarking) against other laboratories, nationally and/or internationally;
- highlights any deficiencies in the examined tests;
- identifies possible problem areas; and
- demonstrates to clients, colleagues and accreditation bodies that there is a commitment to quality.

EQA schemes, which are sometimes also referred to as proficiency testing schemes, are available from many providers.[13] Where no EQA scheme exists, blinded sample exchanges between laboratories can be implemented to assess a broad range of techniques and procedures performed in the clinical laboratory.

EQA samples should, as far as possible, be treated the same as clinical samples. As EQA samples are usually clearly identifiable, there is the potential for them to be handled in ways that go beyond normal laboratory procedures (e.g. handled by senior staff, subjected to repeat testing), so measures to avoid this should be put in place. Where possible, residual samples should be stored until the EQA scheme results are known, to enable tests to be repeated if a discrepancy has occurred. Any discrepancies must be investigated and any corrective and/or preventative actions implemented.

iii. Audits

A programme of internal and external audits of laboratory procedures should be part of a good QMS and will be required for accreditation. Audits are an opportunity to assess the performance of the laboratory and provide assurance of compliance, for example with locally defined processes, Standards or regulatory requirements. They also provide an opportunity to identify improvements. Internal audits are normally performed by someone within the same organization but should be independent of the area of work audited. External audits are performed by those outside of the organization, including accreditation bodies

5.3.2 Quality control (QC)

i. Evaluation, verification and validation

Evaluation, verification and validation of tests is essential in any laboratory to provide an evidence-based assessment of the performance capabilities of a test before it is incorporated into the service offered.

- Evaluation is a systematic process that compares different systems designed to perform the same, or similar, functions. Examples of evaluations within microbiology include comparison of different nucleic acid amplification tests (NAATs) to detect the same target, compare different culture media to

[13] For example, those listed here: https://www.eptis.org/index.htm

isolate the same organism, or perform antimicrobial susceptibility testing; or comparison of different equipment with the same function, such as enzyme-linked immunosorbent assay (ELISA) plate readers. Evaluation findings should be fed back to interested parties, such as via publication of results. Where performance is equivalent, then ease of use, cost, time or ease of procurement are additional factors to be considered.

- Verification of commercial assays or equipment is performed to ensure the performance criteria stated by the manufacturer, such as in the instructions for use, is independently verified locally before being used routinely. The verification document should be reviewed and approved by appropriate staff.
- Validation of examination procedures – including in-house assays and any commercial assays or equipment that are used in a way that is not described in the instructions for use – are required to ensure the system is fit for purpose and any results issued are acceptable. A validation file for each method or system should be produced, and should include a range of information with different emphasis, depending on whether the laboratory is using a commercial system or has developed a system in-house. Typically, the file would include sections such as sensitivity, specificity and negative/positive predictive values results from different sample panels (i.e. positives, negatives, challenging, cross-reactive), reproducibility, precision and accuracy data, uncertainty of measurement, relevant publications, quality control data, including EQA, IQA, and IQC, SOPs, worksheets, error logs, and any customer complaints.

ii. Internal quality control (IQC)

IQC is used to detect and monitor any issues or failure in one or more components of a procedure that is used to generate patient results. The IQC should include positive and negative controls as a minimum, should appropriately challenge the examination procedure (i.e. be close to clinical decision limits) and should verify that the results are of an acceptable quality. For instance, for culture systems, one or more known control strains can be used to ensure the medium supports the growth of the desired organism and, if it is a selective medium, that it also inhibits the organisms not required.

For molecular testing, IQC should include a nucleic acid extraction control, an amplification (positive) control and inhibition control. This will prevent false-negative results by detecting failed extraction or amplification, failure of one or more reagents, or thermal cycling, and inhibition of amplification. A no template control can detect contamination, which may lead to a false-positive result.

For commercial assays that come with kit controls, such as serological testing, then both the QC samples supplied with the kit and external QCs independent of any controls provided with commercial assays should be included in every examination procedure and tested in the same manner as the patient's specimens.

The acceptable IQC range should be established using a number of measurements (minimum of 20), taken over a period of time. The mean values, standard deviations (SD), and coefficient of variation (CV) can then be calculated. Statistical process control charts (such as Levey–Jennings [LJ] or Shewhart charts) can be used to indicate any possible issues with the test performed and to detect any trends over time that might indicate areas of concern.

iii. Equipment validation and monitoring

Equipment should be validated to ensure it is fit for purpose and meets the expected requirements. This should be done before use and following any changes. All procedures should be risk assessed, and SOPs established, to ensure safe and correct usage. Regular equipment monitoring is essential in all laboratories *(4,5)*. This can be achieved by maintaining an equipment inventory, in which all appropriate equipment activities including calibration and maintenance are recorded, along with equipment worksheets that can record times of use, settings used, time, temperatures and name of operator. Microbiological safety cabinets should be monitored and recorded regularly for airflow; the temperature of all incubators, water baths, refrigerators and freezers should be recorded daily. Any failures should be investigated, and corrective and preventative actions put in place.

iv. Reagents and consumables

All reagents and consumables should be stored and used within the time frame as recommended by the manufacturer. New batches should be recorded when used, and their performance verified (i.e. by acceptable IQC). All chemicals should be risk assessed before use (i.e. Control of Substances Hazardous to Health [COSHH]) and if appropriate their usage detailed in a SOP.

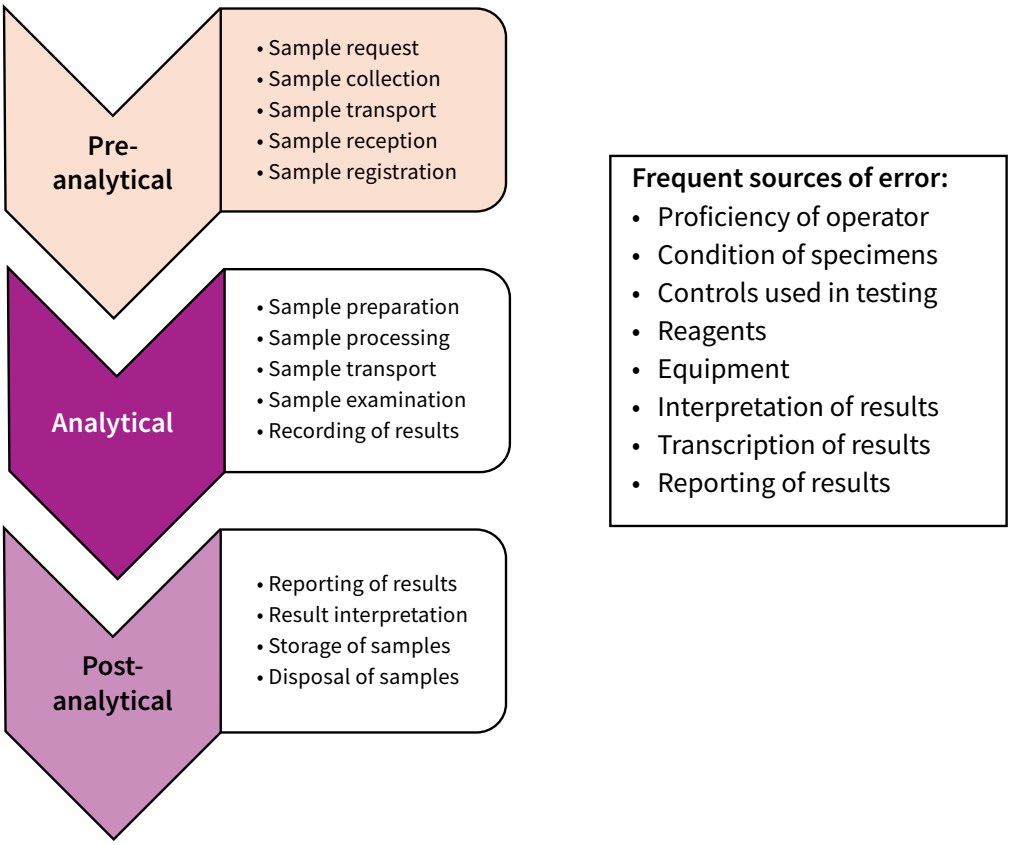
v. Nonconformity

A nonconformity, can be defined as a failure to meet a required specification. This may relate to a product or service, or an error that is identified at any stage during a process. This can take many forms; examples include IQC, IQA and EQA failures, errors in following processes, temperatures outside of acceptable range or an incorrect test result report issued. It is essential to record instances of nonconformity, to investigate and implement appropriate root-cause corrective actions and review them at regular intervals, to ensure lessons are learned and the risk of recurrence is reduced.

In practical terms, when a specimen is being processed by a laboratory or at a POC test site, potential sources of error can be divided into pre-analytical, analytical and post-analytical errors. As part of its quality management, a laboratory or a POC programme supervisor should pay close attention to errors that are made in each phase and

strive to investigate the root causes of error to ensure corrective action is taken. Fig. 5.2 shows the processing steps in the three phases and frequent sources of error in the testing process.

Fig. 5.2: Phases of testing and frequent sources of error in the testing process



Source: Michelle Cole and Rosanna Peeling.

5.4 Quality indicators

Laboratories should consider setting quality indicators to monitor and review the laboratory processes that contribute to test results *(6)*. For example, targets for turnaround times can be used to attempt to minimize the interval between the sample being taken and the patient receiving the results. IQA and EQA results can be reviewed to ensure intra-laboratory and inter-laboratory comparability of results, respectively.

- A QMS is crucial to maintain and improve the quality of the results produced by a laboratory.
- All laboratories should strive to improve quality and work towards accreditation.
- The QMS should be described within a quality manual
- An organizational structure and regular meetings of different levels of staff are necessary to maintain and review the QMS of the laboratory.
- Staff should be suitably trained, deemed to be competent and, if relevant, registered with a professional body.
- Laboratory procedures should be documented as SOPs and all documentation used in any examination process document controlled.
- QA encompasses quality assessment and quality control.
- Quality assessment includes internal and external quality assessments and audits.
- QC includes IQC, evaluation, validation and verification of tests, and equipment, reagent and consumables validation and monitoring.
- NCs should be identified, investigated to identify the root cause, and corrective and preventative actions implemented.
- Laboratories should use indicators to monitor the quality of their laboratory tests.

5.5 Special considerations for quality assurance (QA) of STI point-of-care (POC) testing

Rapid POC tests are sold in kits, as health-care products that are regulated. Where the quality of a laboratory is governed by international standards as described, the quality of tests and testing outside the laboratory system require management through a quality continuum (Fig. 5.3). QA starts with regulatory review of the accuracy of a test as claimed and of the quality of manufacturing through on-site audits of the manufacturing process (International Standards Organization ISO 15189:2022).[14] Once a test is selected, procured and shipped, its arrival at its destination should trigger a check for quality in case there are circumstances during transport and storage that may compromise the quality of the tests. A mechanism for batch or lot testing with positive and negative controls on tests should be performed on arrival. The quality of tests needs to be maintained while they are distributed to POC sites, no matter how far away, or the mode of transport used. The quality of the test used and the quality of testing can be assured by the performance of proficiency panels (or EQA panels) at regular intervals, using unknown positive and negative control samples. When errors occur, they should be investigated, and corrective action taken, with the aim of improving performance and not laying blame. Test results need to be reported to those who will act on the results to fully realize the impact of testing.

Unlike laboratory-based tests, those that can be used at the POC are intended to be used by health providers with no laboratory training or expertise. Since 2016, WHO has introduced a special initiative to increase access to STI testing and screening by defining the required target product profiles of POC tests for STIs. This also includes evaluating the performance and operational characteristics of commercially available tests, in collaboration with partners such as the Centers for Disease Control and Prevention (CDC) in the USA, which has developed guidance for QA of POC HIV tests *(7–9)*. POC tests are used to detect nucleic acids (DNA or RNA), antigen or antibodies in a few simple steps. They are commercially available as instrument-based assays or single use, disposable lateral flow or flow-through tests. To minimize operator error and reduce the need for extensive training, the instrument-based assays usually use a sample-in-answer-out format, in which the steps after loading the specimen are automated. The test results are displayed on the instrument screen or monitor with data transmission capability, which means that EQA results can also be transmitted in real time to a central QA database. This allows errors to be investigated and corrective action taken without delay *(10)*. The disposable single-use rapid tests are simple to use, but are more prone to interpretation error compared to instrument-based assays. This is because the results are often read by eye, and thus are highly dependent on the visual acuity of the operator and their competency to perform the test.

Fig. 5.3: Quality assurance for point-of-care diagnostic tests and testing

PQ: pre-qualification.

Source: Developed by X-S Chen and RW Peeling, reproduced from WHO, 2021 *(11)*.

[14] Available at: https://www.iso.org/standard/76677.html

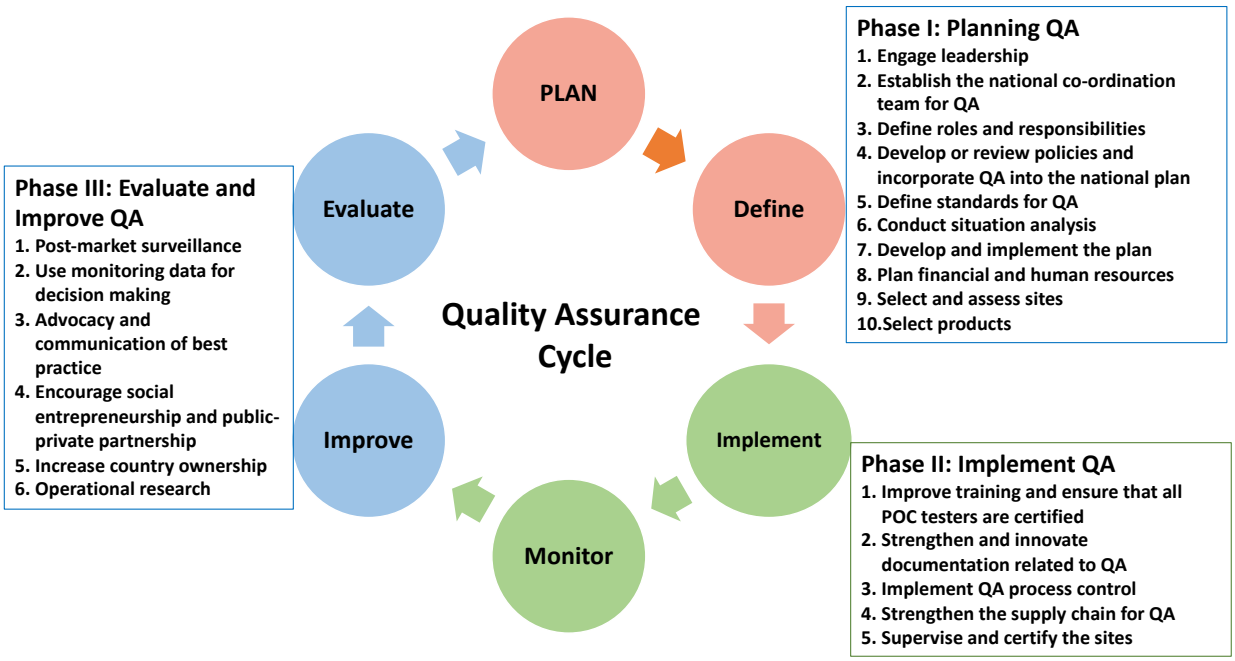
5.5.1 Organization of QA programmes for POC testing

WHO, in collaboration with the CDC and other partners, developed a handbook on how POC QA programmes should be planned, defined, implemented, monitored, improved and evaluated in a continuous cycle of quality improvement *(12)*. Although the handbook was developed for HIV tests, it can be applied to all POC tests. How countries develop their POC testing QA programme is dependent upon the organization of their laboratory system, human and financial resources, and their experience with organizing QA programmes for HIV POC tests *(9,13–15)*.

The most important starting point for a POC testing programme is that it should be integrated within the national laboratory network *(13,14)*. The following are steps to establish a POC testing programme.

i. Identify the need for POC testing in country.
ii. Develop the POC QA model to ensure quality testing at every testing site, regardless of how remote they are.
iii. Identify sources of proficiency panels for implementing an EQA programme.
iv. Appoint appropriate QA officers, who can monitor performance and recommend to management means of identifying and reducing errors.
v. Implement the QA programme in a coordinated manner with partners.
vi. Monitor costing and cost-effectiveness of an EQA programme.
vii. Ensure the sustainability of the national QA programme.

Fig. 5.4: The quality assurance (QA) cycle for point-of-care (POC) testing



Sources: Fonjungo et al; 2016 *(9)*, WHO, 2015 *(12)*.

5.5.2 Preparation of POC EQA panels

Unlike EQA panels for laboratories, EQA panels for all POC testing sites can amount to hundreds, if not thousands of panels a year. There are few publicly funded EQA programmes that can meet the enormous demand for POC testing programmes in each country. It is therefore recommended that the national or lead laboratory of the QA programme subscribe to an international proficiency programme to assure their quality practice. The EQA panels can then be sourced or made by the national laboratory using a variety of methods.

Sources of materials:

- Blood banks: blood that is past its expiry date for use provides large volumes, but it is essential to ensure that blood is safe by testing for HIV, hepatitis, syphilis and other blood-borne pathogens circulating in the region, such as Chagas.
- Biobanks: samples tend to be in small volumes and there may be safety concerns if not tested for transmissible blood-borne pathogens. They should be collected with informed consent.
- Antibodies from animals with humanized immune systems such as mice or bovines: these need to be specially developed. Even though they are safe and can come in large volumes, they may be costly and need to be carefully characterized for appropriateness of substitution for human antibodies *(16)*.

The preparation of the EQA is usually performed by the national laboratory, which performs further characterization and also aliquots, stores and distributes the panels. The national laboratory would then collate results, as well as investigate discrepancies and implement any necessary corrective action.

An example of proficiency panels:

For HIV and syphilis tests, panels can be made using the "Dried Tube Specimen" method developed by the CDC *(17)*:

- 45 µl of positive and negative sera are air dried in a small tube with a small volume of trypan blue dye in a biological safety cabinet overnight.
- The tubes are capped, and are stable for one year at room temperature.

5.5.3 Frequency of EQA

The frequency of EQA, also known as proficiency testing, for POC testing is largely dependent on the number of POC testing sites, how many panels can be distributed and the cost of the programme. It is important to ensure that all POC testing sites have proficiency testing at least once a year before increasing the frequency of testing.

At a POC testing site, EQA panels need to be performed by everyone who is performing POC testing and results should be reported to the QA officer in charge. POC testing sites are often based at busy clinics that, unlike laboratories, can be distracting to those conducting diagnostic testing. Often, there can also be a high turnover of staff performing testing. The QA officer needs to work with the testing site staff to identify sources of errors and their causes, develop a proactive plan for correction to prevent/reduce further errors in the future, and monitor whether the errors have been corrected. Sources of error at a POC testing site are shown in Table 5.1.

Table 5.1: Sources of error at a point-of-care (POC) testing site

Systematic errors	Personal errors
• Standard operating procedures (SOPs) not explicit enough	• Distractions at work
• Inadequate training	• Recording errors
• Lack of supervision	• Mixing up specimens
• Supervisors signing off on results without checking	• Not following SOPs
• Temperature of incubators, fridges and freezers not monitored	• Transcription errors
• Too much workload	• Not reporting results to the person who should be taking action on the test results

QA is an essential component of diagnostic testing, regardless of whether it is performed in the laboratory or at a POC testing site. Poor diagnostic quality leads to poor patient outcomes, increasing morbidity and mortality, and ineffective public health interventions. QA is often viewed as a luxury or as an added cost for a laboratory or POC testing system but, in reality, diagnostic QA systems are cost-effective and investment in QMSs should be valued as a means of improving patient outcomes and reducing health-care costs by preventing misdiagnosis, repetition of testing, incorrect treatment and inappropriate public health actions.

5.6 References

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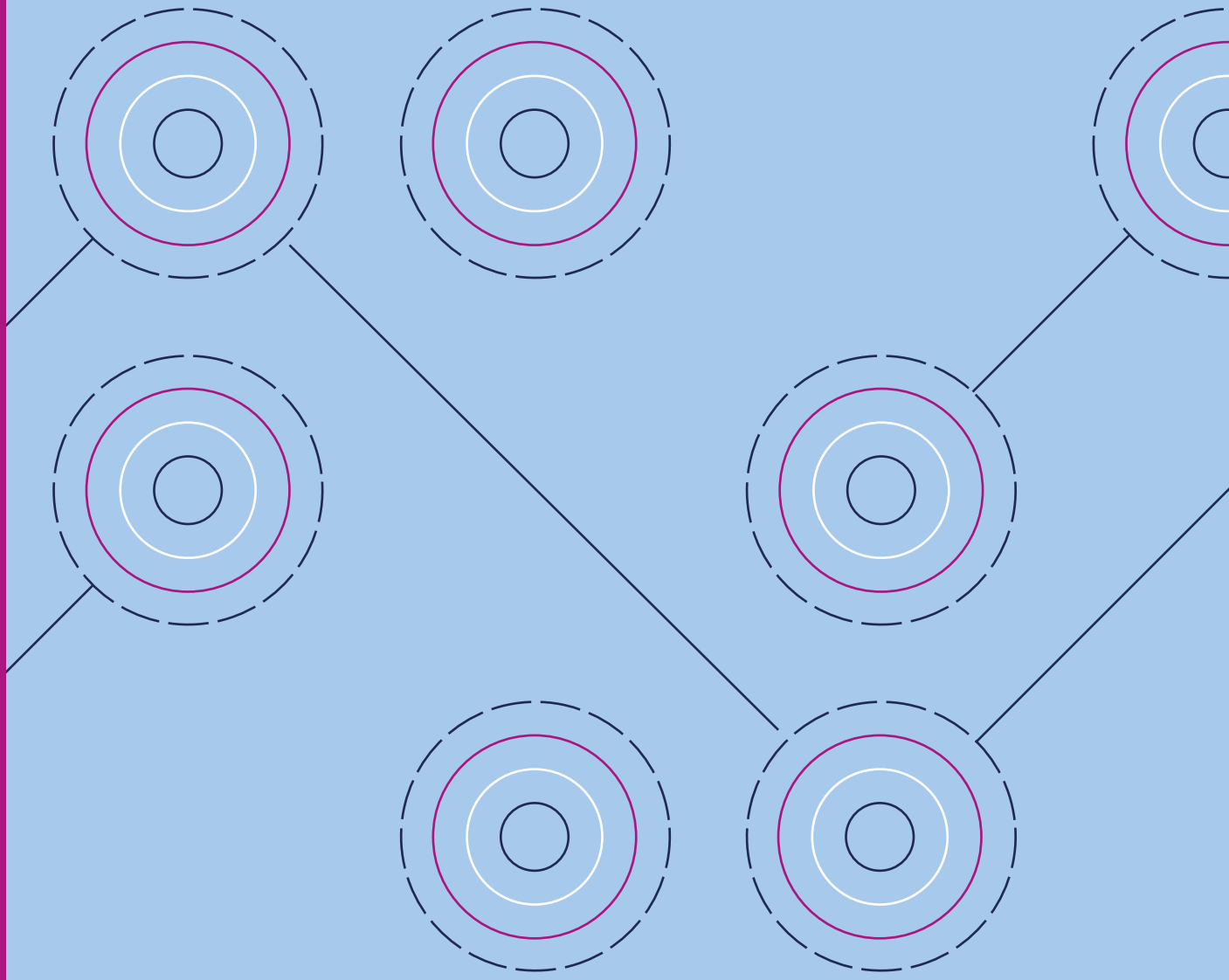
Chapter 6. Urogenital mycoplasmas



Chapter 6. Urogenital mycoplasmas

Jørgen Skov Jensen, Catriona Bradshaw and Magnus Unemo

6.1 Introduction

Mycoplasmas are members of the class Mollicutes. They are very small free-living bacteria usually ranging from 0.3–0.5 µm in size. Mycoplasmas lack the rigid cell wall of other bacteria, making them resistant to penicillins and other antimicrobials that act on the cell wall. *Mycoplasma* genitalium and *Mycoplasma* hominis and the two ureaplasma species Ureaplasma urealyticum (previously known as U. urealyticum biovar 2) and *Ureaplasma parvum* (previously known as U. urealyticum biovar 1) are commonly found in the human urogenital tract. It is important to stress that before U. urealyticum and U. parvum were recognized as separate species, both were designated U. urealyticum, making interpretation of the results of previous studies difficult. Table 6.1 presents disease associations.

M. genitalium is found in 1–3% of sexually active men and women in population-based studies *(1,2)*. Ureaplasmas can be found in the cervix or vagina of 40–80% of sexually active, asymptomatic women, and *M. hominis* in 20–50% *(3)*. Accordingly, ureaplasmas and *M. hominis* should be considered primarily as commensals when detected in the lower genital tract. Particularly, *M. hominis* is strongly associated with more severe forms of bacterial vaginosis *(4)* and seems to disappear when the dysbiosis is corrected *(5)*. *M. hominis* and the ureaplasmas are recognized as a cause of extragenital disease in patients with B-cell deficiencies (hypo- and agammaglobulinaemia, including iatrogenic B-cell deficiency) and in preterm infants *(6)*. Ureaplasmas may also cause life-threatening hyperammonaemia syndrome in transplant recipients *(7)*.

Table 6.1: Disease associations of urogenital mycoplasmas

Species	Disease associations						
	Urethritis	Cervicitis	Bacterial vaginosis	Endometritis and/or PID	Preterm birth	Infertility (women)	HIV transmission
M. genitalium	++++	+++	+/-	+++	+/-	+	+
M. hominis	-	-	++++	+/-	+/-	-	ND
Ureaplasmas (undifferentiated)	+/-	-	+	ND	+/-	+/-	ND
U. urealyticum	+/-	-	-	-	+/-	+/-	ND
U. parvum	-	-	-	-	+/-	+/-	ND

ND: not determined; PID: pelvic inflammatory disease.
Key: ++++ strong association, +++ association in most studies, + association only from a few studies, +/- conflicting results, - no association.

M. genitalium has been strongly and uniformly associated with non-gonococcal urethritis (NGU) in more than 30 studies, and has been detected in the urethra of 15–25% of men with symptomatic NGU compared to about 5–10% of those without this disease *(8)*. In studies that evaluated the association with non-chlamydial NGU (NCNGU), the association has generally been stronger, showing that *M. genitalium* and *C. trachomatis* act as separate causes of NGU. In several studies, *M. genitalium* has been found in more than one third of men with NCNGU *(8)*. Among sexual health clinic populations, approximately 90% of *M. genitalium*-infected men have microscopic signs of urethritis and almost three quarters report symptoms *(9,10)*.

Several clinical studies have shown a strong correlation between *M. genitalium* and persistent or recurrent NGU, particularly in the context of using doxycycline for syndromic treatment for NGU. *M. genitalium* has generally been eradicated from no more than one third of the infected patients after treatment with standard doses of doxycycline *(11)*. *M. genitalium* has also been found in as many as 41% of men with persistent or recurrent urethritis after treatment with doxycycline *(12,13)*.

In contrast to the consistency of studies associating *M. genitalium* with NGU, the role of the ureaplasmas in this disease has been more controversial and there is no evidence supporting a role for *M. hominis* as a cause of urethritis *(6)*. Importantly, detecting ureaplasmas in a man with NGU does not necessarily indicate that this organism is the cause of the disease given the high colonization rate. This is the case even if quantitative culture or nucleic acid amplification test (NAAT) is applied. The division of the human ureaplasmas into two species, *U. urealyticum* and *U. parvum*, led to studies suggesting that *U. urealyticum* may be associated with NGU in younger men with fewer partners *(14)* or when present in high titres *(15)*. However, these findings have not been confirmed in other studies. In general, routine STI screening of asymptomatic men and women or routine STI testing of symptomatic individuals for *M. hominis*, *U. urealyticum* and *U. parvum* is not recommended *(3,16)*.

M. genitalium has consistently been associated with cervicitis, but the association is somewhat weaker than that between *M. genitalium* and male urethritis *(17)*, possibly due to the difficulty and different criteria used in diagnosing cervicitis in women *(8)*. However, in a number of studies, the association has been as strong as that for *C. trachomatis (10)*. In studies where signs of urethritis have been reported, urethritis in women has been significantly associated with *M. genitalium* infection *(8)*.

M. genitalium has been detected in the endometrium of 60% of women with *M. genitalium* detected at the cervix, and its presence in endometrial biopsies has been associated with histological endometritis and with recurrent pelvic inflammatory disease *(18,19)*. A significantly higher proportion of women with tubal factor infertility have antibodies against *M. genitalium* compared to women with infertility from other causes *(20)*, and non-human primate studies have shown *M. genitalium* causes fallopian tube damage *(21)*. *M. genitalium* has been detected in the synovial fluid of a patient with sexually acquired reactive arthritis (SARA) *(22)* and clinical experience has shown that SARA is not uncommon after *M. genitalium*-positive NGU. However, systematic studies have not been undertaken. It has been shown that HIV-infected women with higher loads of *M. genitalium* were more likely to shed HIV *(23)* and it has also been reported that *M. genitalium* infection enhances susceptibility to HIV acquisition *(24)*. There is no indication that *M. hominis* or ureaplasmas may play a similar role.

- *M. genitalium* is a common cause of urethritis in men and women, and causes cervicitis and upper genital tract infection in women.
- *M. hominis* and ureaplasmas are commonly detected in healthy individuals. Their association with urogenital infection in either men or women remains to be proven.

6.2 Overview of available diagnostic methods for *M. genitalium*

Appropriate diagnosis of *M. genitalium* is limited to NAATs, as culture is extremely slow (several months), challenging and insensitive *(25)*. To date, no serological assays, antigen detection assays or rapid point-of-care (POC) tests have proven effective for diagnosis of urogenital *M. genitalium* infections.

- NAAT is the only practical method for diagnosis of *M. genitalium*, and should ideally include macrolide resistance testing (see sections 6.4 and 6.5).

6.3 Collection, transport and storage conditions of specimens

Sample collection should be performed as described for *C. trachomatis* (Chapter 8). For commercially available NAATs, the sampling (swabs and/or collection devices), transport and storage conditions of specimens are frequently assay-dependent. Accordingly, specimens should be collected, placed in the manufacturer's collection device, stored and transported in accordance with the instructions for use for the specific NAAT. In general, swabs and transport medium should not contain inhibitory substances for NAATs. It is also appropriate to use a transport system that is compatible with the NAAT for *C. trachomatis*, as testing for this organism should have the highest priority. However, it should be noted that the organism load of *M. genitalium* is 100-fold lower than that of *C. trachomatis (15)*, so transport systems that dilute the specimen unnecessarily should be avoided. Unfortunately, no clear guidance can be given regarding

the optimal specimen for detection of *M. genitalium*, as collection devices, sample preparation methods and detection systems have varied between studies. If only one specimen is tested from each patient, it appears that first-void urine from men and vaginal swabs from women contain the highest load of bacteria. It is also important to consider extracting nucleic acid using a method that involves a concentration step.

6.4 Detection of *M. genitalium* by nucleic acid amplification test (NAAT)

The only appropriate method for diagnosis of *M. genitalium* is by NAAT. A range of assays has become commercially available and some have received U.S. Food and Drug Administration (FDA) clearance.[15] Several other assays have received the far less stringent European "Conformité Européenne" (CE) mark for in vitro diagnostic (IVD) medical devices (CE-IVD).

At the time of writing, three assays have received FDA clearance.

The first was the CE-IVD-marked Aptima *Mycoplasma genitalium* (AMG) assay (Hologic Inc.). The AMG assay uses target capture (TC), transcription-mediated amplification (TMA) and a hybridization protection assay (HPA) to detect 16S rRNA of *M. genitalium* in self- and clinician-collected urogenital specimens from symptomatic and asymptomatic subjects. It is performed on the automated Panther system (Hologic Inc.) *(26)*. *M. genitalium* can also be detected in the same Aptima samples as those used for the *C. trachomatis* and *N. gonorrhoeae* Aptima assays. The multi-copy 16S rRNA target together with the efficient TC, TMA and HPA technologies provide a very high sensitivity that is difficult to achieve by polymerase chain reaction (PCR)-based methods. Thus, the FDA approval was based on a reference standard of three other research-use only TMA assays also developed by Hologic Inc. *(27)*. The clinical specificity was over 98% for most sample types, although female rectal and male meatal swabs were slightly below 98%. One concern has been the cross-reactivity with three other *Mycoplasma* species (*M. amphoriforme, M. pirum* and *M. alvi*), which were reproducibly detected at less than 50 genome equivalents per reaction *(28)*. The first two species have been detected in human specimens in respiratory and rectal samples, respectively, but as judged from the high clinical specificity, this may not be clinically important. At the time of writing, no macrolide resistance assay is available on the platform, and due to the high sensitivity of the TMA assay, PCR-based typing methods may be unable to provide a resistance type in 25–45% of the TMA-positive specimens *(29)*. The Panther Fusion platform supports laboratory-developed tests (LDTs), but at present, no assays with appropriate sensitivity have been presented. Recently, Hologic has made analyte-specific reagents available for detection of macrolide resistance mutations. However, no validation of the reagents has yet been presented.

The Roche cobas TV/MG test for the detection of *Trichomonas vaginalis* and *M. genitalium* was the second to receive FDA approval. It runs on the automated cobas 6800/8800 platforms and on the same samples as those for the *C. trachomatis* and *N. gonorrhoeae* cobas assay. The *M. genitalium* component of the assay has dual targets for *M. genitalium*: the *mgpB* "A" region, which is a single copy conserved target, and the *mgpB* "EF" region, which has approximately nine copies per cell but is generally believed to be more variable between *M. genitalium* strains. The multi-copy nature, however, increases the analytical sensitivity and likely also clinical sensitivity. Importantly, test results can be masked to report only the *M. genitalium* or the *T. vaginalis* component, which prevents reporting *M. genitalium* results for asymptomatic screening samples, thus improving antimicrobial stewardship. At present, no macrolide resistance detection assay is available. However, the cobas 6800/8800 platforms support LDTs, so addition of a mutation detection assay should be possible *(30)*. As part of the FDA approval, the cobas MG assay was compared with the Aptima MG assay (Hologic) and had a sensitivity and specificity of 77% and 100%, respectively, for vaginal swabs and 96% and 99.5% for male urine samples. The cobas assay was more sensitive than the two LDTs used for reference *(30)*.

The most recently FDA-approved assay is the Abbott Alinity m STI Assay. The Alinity m STI assay is a reverse transcription-PCR (RT-PCR) assay for use on the automated Alinity m System for the direct, qualitative detection and differentiation of rRNA from *C. trachomatis*, DNA from *N. gonorrhoeae*, rRNA from *T. vaginalis* and rRNA from *M. genitalium*. The reverse transcription step aims to increase the sensitivity of detection by using the multi-copy nature of the rRNA target for three of the organisms. Importantly, the assay read-out can be defined at assay set-up to report only the pathogens requested. However, the data is available in the system for epidemiological purposes. The *M. genitalium* component is approved for male urine and for vaginal swabs (clinician-collected and self-collected in a clinical setting) and endocervical swabs, with vaginal swabs being the recommended sample type for women. Comparative studies with other assays have not yet been published. However, according to the data presented for the FDA approval[16] where the Aptima MG test was used for reference, the sensitivity and specificity of the Alinity *M. genitalium* component was 98.1% and 99.2%, respectively, for clinician-collected vaginal swabs; and 95.4% and 98.6%, respectively, for self-collected vaginal swabs compared to 82.8% and 99.2%, respectively, for endocervical swabs. For male urine, comparable

[15] The U.S. FDA's list of microbial tests is available at: https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial

[16] Letter to Stacy Ferguson, Associate Director Regulatory Affairs, Abbott Molecular, Inc., from U.S. FDA, RE: K202977, Trade/Device Name: Alinity m STI Assay, 29 April 2022 (https://www.accessdata.fda.gov/cdrh_docs/pdf20/K202977.pdf).

figures were 98.1% and 97.5%, respectively. At the time of writing, no macrolide resistance assay is available on the platform. However, the RT-PCR approach appears to increase the sensitivity for the diagnostic PCR, so a 23S rRNA-based macrolide-resistance mutations (MRM) detection assay should be able to achieve comparable sensitivity with the diagnostic assay.

A range of CE/IVD-marked NAATs are commercially available, but none have the same level of validation as the FDA-approved assays. Several of the assays have combined detection of *M. genitalium* and macrolide resistance mutations. Examples are the ResistancePlus MG (SpeeDx) *(31)*, the S-DiaMGRes assay (Diagenode), the RealAccurate TVMGres assay (PathoFinder) *(29)* and the Allplex MG & AziR assay (Seegene) *(32)*. Unfortunately, none of the assays have been validated head-to-head with other assays on a relevant collection of unselected clinical specimens. Use of macrolide-resistance testing for *M. genitalium* has been shown to improve first-line cure and antibiotic stewardship *(33)*, and is recommended in many national or regional guidelines, including the Asian, Australian, European, the United Kingdom of Great Britain and Northern Ireland, and USA guidelines *(34–38)*. No true rapid POC tests are currently available, but the SpeeDx ResistancePlus MG FleXible assay detects *M. genitalium* and MRM using the GeneXpert platform (Cepheid) and is a near-patient assay providing results in less than 3 hours from sample set-up to report *(39)*. Recently, commercially available assays that detect a range of *parC* mutations have become available. Examples are the Allplex MG & MoxiR Assay (Seegene), LightMix Modular *parC* kit (TIBMOLBIOL) and MGMO qPCR (NYtor) *(40)*. These assays detect mutations in the quinolone resistance-determining region and although the mutations (with the exception of the ParC S83N) are associated with elevated minimum inhibitory concentrations (MICs) against moxifloxacin in vitro *(41)* their presence does not always correlate with treatment failure *(42)*. Recent studies from Australia suggest that the use of assays that detect the most common ParC S83I mutation, or even a wildtype ParC S83 target, are likely to provide guidance to clinicians about appropriate selection of antimicrobials, and specifically the likelihood that moxifloxacin will fail or be effective *(43)*. However, further studies in additional geographic settings are imperative and primary detection of *parC* mutations in clinical samples is currently not recommended in any of the treatment guidelines. However, detection of quinolone resistance-associated mutations (QRAMs) in moxifloxacin treatment failures is helpful to exclude reinfections or poor compliance, thus determining the relevance of third-line therapy.

Some assays detect *M. genitalium* together with several other genital organisms such as ureaplasmas and *M. hominis*. Most of these highly multiplexed assays suffer from suboptimal sensitivity in diagnosing *M. genitalium*, as this organism is often present at very low levels, even in symptomatic patients *(15,44)*. Consequently, the use of these assays is not recommended. For assays without a recommended sample transport medium and nucleic acid extraction procedure, efforts should be made to optimize the sample preparation step, primarily by considering sample input volume in relation to elution volume.

It is of utmost importance that laboratories performing *M. genitalium* diagnostics carefully validate their LDTs and participate in external quality assurance programmes; see Chapters 1, 4 and 5 regarding selection of diagnostic tests, NAATs and their quality assurance and validation of non-approved NAATs. New assays may also become available rapidly *(45)*. It is important to assess the relevant literature continually for high-quality evaluations of new assays to determine the best fit for each laboratory.

- The only appropriate method for diagnosis of *M. genitalium* is by NAAT. Some diagnostic NAATs have received FDA clearance[17] and several others have the far less stringent European CE/IVD mark. It is of utmost importance that laboratories performing *M. genitalium* diagnostics carefully validate and quality assure any LDTs.
- The *M. genitalium* diagnosis should ideally always include macrolide resistance testing, to guide first-line treatment (see also section 6.5).
- Sample preparation and assay sensitivity should be optimal for *M. genitalium* testing, as this pathogen is present in 100-fold lower concentrations than *C. trachomatis*. Most highly multiplexed assays have a lower sensitivity than assays with only one or a few targets.

[17] The U.S. FDA's list of microbial tests is available at: https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial

6.5 Antimicrobial resistance and susceptibility testing of *M. genitalium*

M. genitalium antimicrobial resistance has become a major problem that is seriously impacting the management and control of this infection. Classic antimicrobial susceptibility testing of *M. genitalium* is complicated and is only feasible in specialized reference laboratories. Broth dilution MIC determination with a standardized inoculum is used as the reference method, but MIC determination of *M. genitalium* growing only in cell culture has also been successfully applied *(46)*. However, as *M. genitalium* is cultivable only in a few laboratories in the world, and since growth is too slow to allow meaningful results for the individual patient, molecular testing for the resistance-mediating mutations is the mainstay for clinical management.

Several clinical studies have shown that tetracyclines such as doxycycline are inferior to azithromycin in eradicating *M. genitalium (11)*. Although in vitro MICs suggest that most isolates of this species should be tetracycline susceptible *(47,48)*, clinical experience contradicts this, with less than 40% cured. No molecular mechanisms of resistance in the 16S rRNA gene have been found to explain the low efficacy *(49)*, and no correlation between MIC and cure has been demonstrated *(48)*. However, a recent study has suggested that tetracycline resistance in *M. genitalium* can be selected by long-term doxycycline treatment. The resistance is caused by alterations in intrinsic efflux pumps resulting in increased MICs of doxycycline, tetracycline and minocycline *(50)*. Macrolide resistance has been documented by isolation of *M. genitalium* strains from patients failing treatment with azithromycin *(51)*, and the main resistance-mediating mutations have been shown to be in region V of the 23S rRNA gene, primarily A2058G and A2059G (*E. coli* numbering), but other mutations in the same positions also increase the azithromycin MIC dramatically *(52)*. These mutations can be detected directly from clinical specimens by NAATs or sequencing of PCR amplicons (see section 6.4), enabling clinically useful information in the absence of culture. Treatment with azithromycin 1 g single dose leads to development of resistance or selection of pre-existing resistant variants in at least 10% of cases *(51,53,54)*, and data suggest that the community level of macrolide resistance in *M. genitalium* is highly dependent on the use of azithromycin 1 g single dose for treatment of *C. trachomatis* infection and presumptive treatment of STI syndromes. This is illustrated by a low prevalence of resistance in countries using doxycycline as the primary drug for NGU (e.g. Sweden) and resistance as high as 100% in Greenland, where azithromycin is used for NGU treatment and where the prevalence of *C. trachomatis* infections is extremely high *(28,55,56)*.

Moxifloxacin has proven to be an effective second-line treatment *(57)*, but strains with combined high-level quinolone and macrolide resistance have been isolated *(41,47)* and are increasingly being reported *(55,58,59)*. Although several QRAMs, particularly in ParC positions S83 and D87, have been found to increase the in vitro MIC *(41,47)*, there have been few studies that have sufficient power to determine their impact on treatment outcomes *(60)*, and some QRAMs such as the ParC S83N do not significantly increase MIC *(41)*. Data from Australia suggest the most common QRAM ParC S83I to be most consistently associated with failure of moxifloxacin, which results in failure rates of around 60% *(61)*. Data suggests this mutation is becoming more common, while other mutations affecting ParC S83 and D87 are less common, and their contribution to failure of moxifloxacin less defined. Mutations in *gyrA* appear to contribute to moxifloxacin failure as they have been suggested to have an additive effect on moxifloxacin and sitafloxacin MICs *(41,61)*. GyrA mutations are less common than ParC mutations and generally co-occur with ParC S83I. The role of the analysis for QRAM is currently under evaluation and expert advice should be sought for selection of third-line therapy.

- Macrolide resistance in *M. genitalium* is very common in patients failing treatment with azithromycin. Moxifloxacin is the most frequently used second-line treatment.
- Macrolide resistance in *M. genitalium*, mediated by specific mutations in the 23S rRNA gene, can be detected directly from clinical specimens.
- Mutations in the *M. genitalium* quinolone resistance-determining regions, particularly in *parC*, have been found in patients failing quinolone treatment and their prevalence appears to be increasing. While the clinical correlates between these *parC* mutations and treatment outcome are suboptimal, the ParC S83I is the most common QRAM and there is increasing evidence that it significantly impacts the efficacy of moxifloxacin, i.e. reducing cure.

6.6 References

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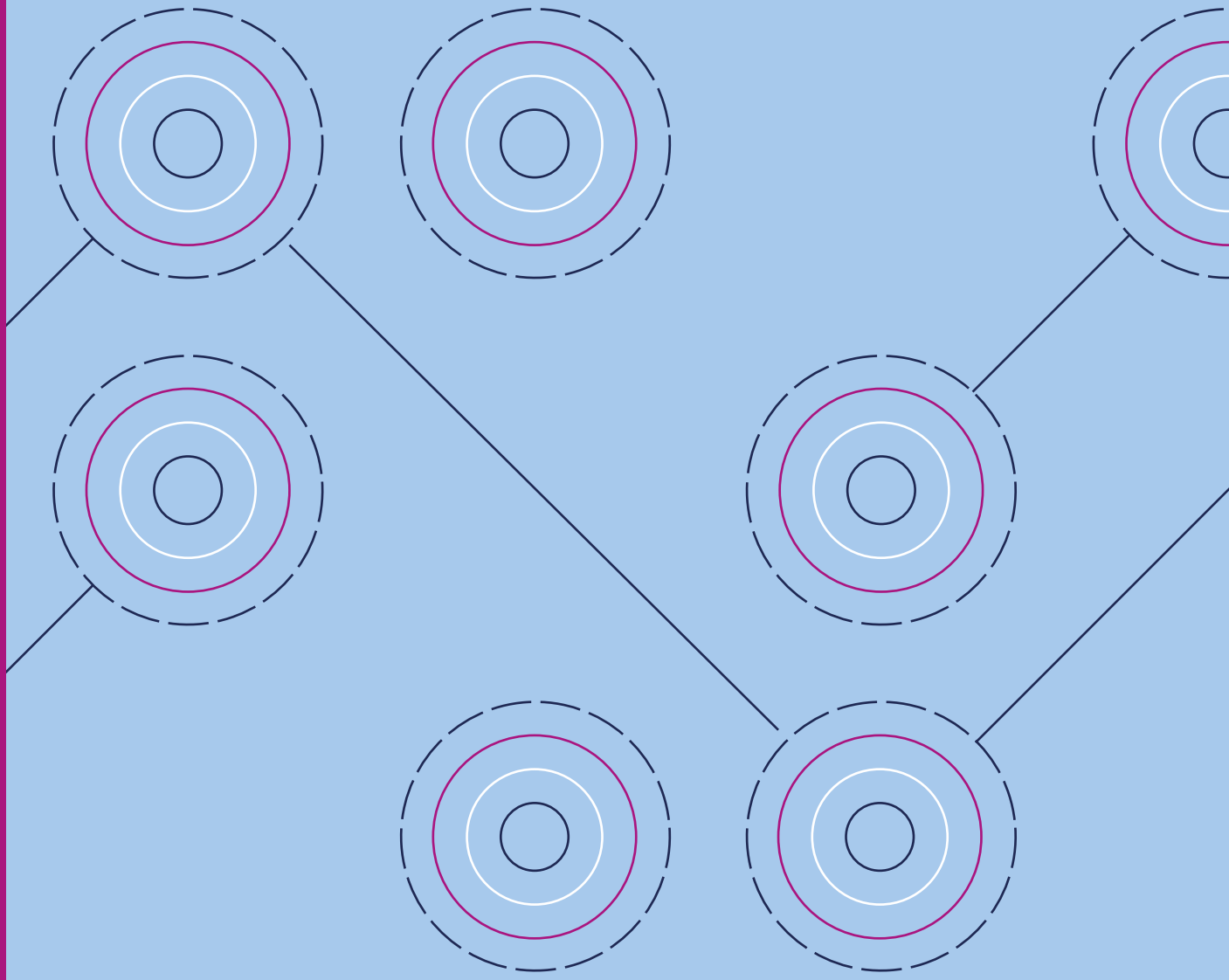


Chapter 7. Gonorrhoea

Magnus Unemo, Daniel Golparian and Michelle Cole

7.1 Introduction

Gonorrhoea, caused by *Neisseria gonorrhoeae* (gonococcus), is an ancient STI. In 2020, WHO estimated 82 million new cases among adults. This places gonorrhoea as the second most prevalent of the estimated curable bacterial STIs, that is, after *Chlamydia trachomatis (1)*. Gonorrhoea, including its severe complications and sequelae, causes substantial morbidity and economic costs, and remains a major public health concern globally. It is also of grave concern that the bacterium has developed resistance to all antimicrobials introduced as first-line treatment of gonorrhoea, and it is feared that gonorrhoea may become untreatable in certain circumstances *(2–7)*.

The genus *Neisseria* contains two species primarily pathogenic to humans, *N. gonorrhoeae* and *Neisseria meningitidis*, and many usually nonpathogenic *Neisseria* species, for example, *Neisseria lactamica, Neisseria sicca, Neisseria cinerea, Neisseria flavescens, Neisseria subflava* and *Neisseria mucosa*. These species predominantly inhabit the upper respiratory tract as commensals, but may be found infrequently in the lower urogenital tract. Gonococci are Gram-negative, aerobic, capnophilic (prefer enhanced concentration [3–7%] of carbon dioxide [CO_2]), non-flagellated, non-sporulating, and oxidase- and catalase-producing cocci, which typically are arranged in pairs (diplococci) with adjacent sides concave, that is, in microscopy they have a characteristic kidney or coffee bean morphology. *N. gonorrhoeae* is fastidious and requires complex nutritionally enriched culture medium for in vitro growth *(4)*.

N. gonorrhoeae infects humans only, colonizes mucosal surfaces and is the etiological agent of lower urogenital tract infections, namely urethritis in men and cervicitis in women. Asymptomatic urogenital infection occurs in a minority of men (≤ 5–10%) but is more common (≥ 50%) in women. Infection of the rectum (proctitis) and the oropharynx, mostly asymptomatic, can occur in both sexes depending on sexual behaviour, but is predominantly found in men who have sex with men (MSM). If undetected and not treated or inappropriately treated, infection can ascend to the upper genital tract and cause complicated gonococcal infection (e.g. pelvic inflammatory disease (PID) and related sequelae, such as ectopic pregnancy and infertility) in women, and penile oedema and epididymitis in men. Conjunctivitis can occur in adults but, most commonly, infection of the eye presents as ophthalmia neonatorum in the newborn. Disseminated gonococcal infection (DGI), which is a distinct entity, can occur in both sexes but is infrequently encountered *(4)*. Table 7.1 summarizes the clinical manifestations of gonococcal infections.

- *N. gonorrhoeae* causes the second most common bacterial STI globally, which includes a spectrum of diseases in a variety of sites, including urogenital, oropharyngeal, rectal and conjunctival.
- Complications and sequelae associated with untreated *N. gonorrhoeae* infections include PID, ectopic pregnancy, infertility, penile oedema, epididymitis and DGI.

7.2 Overview of available diagnostic methods

Gonorrhoea is frequently asymptomatic, especially in women, and in the oropharynx and rectum in both sexes. Symptoms, if present, can be nonspecific (see Table 7.1). Accordingly, laboratory procedures are needed for diagnosis, case-finding and test of cure. The diagnosis of gonorrhoea is established by the identification of *N. gonorrhoeae* in genital or extra-genital secretions. Table 7.2 summarizes the recommended methods for the diagnosis of gonorrhoea and the performance and other characteristics of these methods.

A correctly prepared, Gram-stained and **microscopy**-examined smear to identify Gram-negative intracellular diplococci in polymorphonuclear leukocytes (PMNLs) is sensitive (95%) and specific (97%) for the diagnosis of gonorrhoea in symptomatic men with urethral discharge. In women, however, smears of cervical secretions detect only 40–60% of culture-positive specimens, which may reflect the lower number of gonococci in women. False-positive results can occur, and specificity (80–95%) is dependent on the experience of the microscopist. Direct microscopic examination is not recommended for the diagnosis of rectal and oropharyngeal infections because of the large number of other bacterial species present and low sensitivity. Screening of asymptomatic individuals by microscopy is not recommended *(4)*.

Table 7.1: Clinical manifestations of gonococcal infections[a]

Uncomplicated gonorrhoea	
Urethra	Copious purulent discharge Scant clear discharge Dysuria
Cervix	Copious purulent discharge Burning or stinging pain Tenesmus Blood in stools
Rectum	Copious purulent discharge Burning or stinging pain Tenesmus Blood in stools
Oropharynx	Mild pharyngitis Mild sore throat Erythema
Conjunctiva	Copious purulent discharge Keratitis and corneal ulceration; perforation, extrusion of lens; scarring, opacification of lens Blindness
Complicated gonorrhoea[b]	
Male complications	Penile oedema Tyson's gland abscess Cowper's gland abscess Seminal vesiculitis Epididymitis Infertility (rare)
Female complications	Endometritis Salpingitis Bartholin abscess Lymphangitis Tubo-ovarian abscess Ectopic pregnancy Infertility
Disseminated gonococcal infection (DGI)	
—	Bacteraemia Fever Dermatitis (skin lesions: macular, erythematous, pustular, necrotic, haemorrhagic) Tenosynovitis Joints; septic arthritis Endocarditis Meningitis

[a] Gonococcal infection may be asymptomatic, particularly in women, and in the oropharynx and rectum in both sexes.

[b] Gonorrhoea also significantly facilitates HIV acquisition and transmission.

Historically, **culture** of *N. gonorrhoeae* was the gold standard for the diagnosis of both genital and extra-genital gonorrhoea. Culture is, under optimized circumstances, sensitive and highly specific for urethral samples in men and cervical samples in women, is relatively inexpensive, and importantly allows antimicrobial susceptibility testing. Antimicrobial resistance (AMR) in gonococci is a severe problem worldwide; as culture is the only method allowing complete antimicrobial susceptibility testing, it is crucial to maintain and, where necessary, strengthen the culture capacity nationally and internationally *(4)*.

Throughout the last three decades, **nucleic acid amplification tests (NAATs)** have been developed and introduced for the detection of specific *N. gonorrhoeae* DNA/RNA. These are generally more sensitive than culture for the diagnosis of gonorrhoea, especially for oropharyngeal and rectal samples *(8–17)*. Several commercially available NAATs have received U.S.

Food and Drug Administration (FDA) clearance[18] for these sample types based on strong performance characteristics *(11–13)*. However, the specificity of some gonococcal NAATs has been suboptimal in the diagnosis of gonorrhoea and, in particular, for oropharyngeal samples, which causes low positive predictive value (PPV), particularly in low-prevalence populations *(4,17)*. Notably, the first NAATs that can be performed in 30 minutes or under by non-laboratory personnel, that is, point-of-care (POC) NAATs, have now received FDA clearance *(18,19)*.

To date, there are **no direct immunofluorescence assays, enzyme immunoassays or rapid antigen-based POC tests** for antigen detection with appropriate performance characteristics (sensitivity and specificity) commercially available for the diagnosis of uncomplicated or complicated gonorrhoea. Some promising assays may be in the pipeline *(20)*, but further evaluations are still pending. Furthermore, there is no commercial and internationally approved method for the **detection of gonococcal antibodies in serum**, and these methods cannot differentiate current from past infection. These methods should not be used because of their suboptimal sensitivity and specificity for the diagnosis of gonorrhoea in individual patients.

Table 7.2: Common diagnostic tests (as of August 2022) for the detection of *Neisseria gonorrhoeae*

	Microscopy[a]	Culture	NAAT (laboratory-based)[b]
Specimen types			
Endocervical swab	Yes[a]	Yes	Yes
Vaginal swab	No	Yes[c]	Yes
Urine			
Female	No	No	Yes[d]
Male	No	No	Yes
Urethral swab	Yes[a]	Yes	Yes
Rectal swab	No	Yes	Yes[e]
Oropharyngeal swab	No	Yes	Yes[e]
Conjunctival swab	Yes	Yes	No[e]
Description			
Performance			
Sensitivity[f]	Low to high[a]	Moderate to high	Very high
Specificity[f]	Moderate to high[a]	Very high[g]	Moderate to very high
Cost	Low	Moderate	Very high
Instrumentation	Microscope	Routine microbiology	Large footprint
Throughput/automation	Moderate/no	Moderate/no	High/yes
Technical complexity	Low	Moderate	High
Level of laboratory infrastructure	Peripheral	Peripheral to intermediate	Intermediate to central
Multiple STI pathogens from one sample	No	No	*Chlamydia trachomatis, Mycoplasma genitalium, Trichomonas vaginalis* and HPV on several platforms
Other comments			
—	—	Strict sample collection, transport and storage are crucial to maintain viability. Only method that allows complete antimicrobial susceptibility testing	NAATs have generally a superior sensitivity compared to culture, especially for oropharyngeal and rectal samples. However, specificity can be suboptimal, and confirmation using supplementary NAAT may be required.

NAAT: nucleic acid amplification test; HPV: human papillomavirus.

[a] Microscopy has high sensitivity and specificity in symptomatic men (with urethritis), low sensitivity in asymptomatic men, and endocervical infections, and is not recommended for vaginal, urine, rectal and oropharyngeal specimens *(4,17)*.

[b] The first rapid, sensitive and specific NAATs that can be performed in 30 minutes or less by non-laboratory personnel, that is, POC NAATs, have now received U.S. FDA clearance *(18,19)*.

[c] Not an ideal specimen; it is mainly applied for prepubertal girls or women who have had a hysterectomy.

[d] Because of suboptimal sensitivity, urine is not the ideal sample to detect *N. gonorrhoeae* in women *(17,21)*.

[e] For rectal and oropharyngeal specimens, several commercially available laboratory-based NAATs have received U.S. FDA clearance *(11–13)*.[19] Data indicate that other appropriate NAATs and POC NAATs also perform well for these sample types, as well as conjunctival samples.

[f] Sensitivity and specificity estimates vary widely depending on the different sensitivity and specificity of assays of the same methodology, as well as assays used for comparison (i.e. the gold standard).

[g] High specificity requires use of appropriate species-identifying assays.

[18] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

[19] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

For appropriate performance characteristics of all diagnostic methods, it is crucial to follow precisely the standard operating procedures for performance and recommendations from the manufacturer with regard to collection, transport and storage of samples, as well as performance, including quality control (QC) of the specific assay.

- Microscopy is sensitive and specific in symptomatic men with urethral discharge.
- Culture, in optimized circumstances, is sensitive and highly specific for urethral samples in men and cervical samples in women, is inexpensive and allows complete antimicrobial susceptibility testing.
- NAATs generally have superior sensitivity compared to culture, especially for oropharyngeal and rectal samples. However, the specificity of some of the gonococcal NAATs has been suboptimal in the diagnosis of gonorrhoea and, in particular, for oropharyngeal samples, resulting in low PPVs in low-prevalence populations.

7.3 Collection, transport and storage of specimens

Appropriate anatomical sites for specimen collection depend on the sex, age and sexual behaviour of the individual; clinical manifestations of the infection; and the diagnostic testing method, including its performance characteristics (sensitivity and specificity). The primary collection site in women using culture and microscopy is the endocervical canal; for NAATs, it is the vagina and alternatively the endocervical canal. Secondary sites include the urethra, rectum and oropharynx. In heterosexual men, specimens for culture and microscopy should be collected from the urethra, and for NAATs, a first void (catch) urine specimen. In MSM and men and women with indicative clinical signs and/or sexual practice (oral and/or anal sex), the rectum and oropharynx should also be sampled. NAATs have higher sensitivity for these infections than culture and can increase case-finding *(8–17,21)*. For culture, which requires live microorganisms, the sample must be collected from sites with columnar or cuboidal epithelial cells. Charcoal-coated (if charcoal is not included in the non-nutritive transport medium) sterile Dacron or Rayon swabs are ideally used. For diagnostics using NAATs, recommendations from the manufacturer regarding collection, transport and storage of samples must be followed in detail. The use of antiseptics, analgesics and lubricants when collecting specimens should be avoided because these may inhibit gonococci. All specimen collection should be performed before initiating antimicrobial treatment.

Endocervix: Insert a swab 2–3 cm into the cervical os and rotate gently for 5–10 seconds. Endocervical samples should not be taken in prepubertal girls or women who have had a hysterectomy; instead, specimens should be sampled from the vestibule of the vagina and a urine specimen (for NAAT diagnostics) should also be sampled. For NAATs, strictly follow the manufacturer's instructions.

Urethra: Take urethral specimens at least 1 hour after the patient has urinated. Collect discharge directly on a swab. If no discharge is evident, in men the urethra is stripped towards the orifice to evacuate exudate. If no exudate is obtained, insert a thin swab 2–3 cm into the urethra and gently rotate for 5–10 seconds. In women, massage the urethra against the pubic symphysis and use the same technique as for men. Culturing both the endocervix and urethra when testing women can increase case-finding. For NAATs, strictly follow the manufacturer's instructions.

Vagina (NAATs only): Strictly follow the manufacturer's instructions. If no instructions are provided, the swab should be rotated against the posterior vaginal walls for 5 seconds. Vaginal swabs may be patient- or clinician-obtained (before inserting the speculum) *(17,21–23)*. Menstruation does not create poor sample quality, but during menstruation urine collection may be preferred.

First void (catch) urine (NAATs only): Strictly follow the manufacturer's instructions. If no instructions are provided, do not have the patient clean the genital area and catch 10–20 ml (≤ 25 ml) of first void urine in a sterile collection container at least 1 hour after the patient has urinated. Urine specimens from females are substantially less sensitive compared to vaginal swabs *(17, 21)*.

Penile meatal swabs (NAATs only): Penile meatal swabs work in some NAAT assays *(24–26)*. However, performance is variable and no assays have claims for this sample type. When a penile meatal swab is required (e.g. when liquids cannot be transported or if patient cannot provide a urine specimen), it should be collected by passing a swab along the meatus to capture material from the urethral opening.

Rectum: Insert a swab 2–3 cm into the rectum and rotate it against all the rectal walls for 10 seconds. If faecal contamination occurs, discard the swab and use another to obtain the specimen. In symptomatic patients, anorectal specimens should be obtained ideally under direct vision after insertion of a proctoscope. However, self-collection is preferred by most patients, works well and may increase uptake of screening and reduce clinical time *(27,28)*. For NAATs, strictly follow the manufacturer's instructions.

Oropharynx: Swab the region of the posterior pharynx above the inferior edge of the soft palate and both tonsils, including the tonsillar crypts. Specimen collection is best performed by a clinician due to the induction of a gag reflex. For NAATs, strictly follow the manufacturer's instructions.

Conjunctiva: Retract the inferior eyelid and move a thin swab across the surface of the inferior palpebral conjunctiva towards the median corner of the eye.

Table 7.3 provides a summary of the procedures for appropriate collection, transport and storage of specimens.

Table 7.3: Sample collection, transport and storage

Anatomical site	Collection device	Sampling procedure[a]	Microscopy	Culture	NAAT
Endocervix	Swab/plastic[b] (OR endocervical brush OR assay-specific collection kit for NAATs)	Use a vaginal speculum and clean the ectocervix. Insert swab 2–3 cm and rotate for 5–10 seconds.	Roll onto slide (thin layer) and air-dry (see Chapter 2). *The sensitivity for endocervical samples is suboptimal.*	Bedside inoculation should be performed on selective gonococcal medium and incubated immediately. If bedside collection is not performed, gonococcal non-nutritive or nutritive transport medium should be used. Specimens in non-nutritive transport medium should be inoculated at the laboratory as soon as possible and at the latest within 48 hours (see also section 7.5).	Place sample into the manufacturer's collection device, and transport and store according to the manufacturer's instructions. *If transport medium is not available from the manufacturer, use appropriate transport medium stabilizing the nucleic acid, e.g. Genelock tubes.*
Urethra (collected ≥ 1 hour after last void)	Swab/aluminium[c] (OR assay-specific collection kit for NAATs)	Collect discharge directly on a swab. Insert swab 2–3 cm into the urethra and gently rotate for 5–10 seconds.	Roll onto slide (thin layer) and air-dry (see Chapter 2).	*For transport and storage, see the endocervical sample for culture.*	*For transport and storage, see the endocervical sample for NAAT.*
Vagina	Swab/plastic[b] (OR assay-specific collection kit for NAATs)	Rotate swab against all posterior vaginal walls for 5 seconds.	NA	Specimen type for prepubertal girls or women who have had a hysterectomy. For transport and storage, see endocervical sample for culture.	Transport and storage, see endocervical sample for NAAT.
Urine (collected ≥ 1 hour after last void)	Sterile urine cup	Patient should not clean the genital area. Catch first void urine (≤ 25 ml).	NA	NA	*For transport and storage, see the endocervical sample for NAAT.*
Rectum	Swab/plastic[b] (OR assay-specific collection kit for NAATs)	Insert swab 2–3 cm into the rectum and rotate it against all the rectal walls for 10 seconds.	NA	*For transport and storage, see the endocervical sample for culture.*	*For transport and storage, see the endocervical sample for NAAT.*[e]
Oropharynx	Swab/plastic[b] (OR assay-specific collection kit for NAATs)	Swab the posterior pharynx and both tonsils, including the tonsillar crypts.	NA	*For transport and storage, see the endocervical sample for culture.*	*For transport and storage, see the endocervical sample for NAAT.*[e]
Conjunctiva	Swab/aluminium[d] (OR assay-specific collection kit for NAATs)	Purulent discharge should be removed with a swab. Retract the inferior eyelid. Swab the surface of the inferior palpebral conjunctiva.	Roll onto slide (thin layer) and air-dry (see Chapter 2). Mainly for neonates	*For transport and storage, see the endocervical sample for culture.*	*For transport and storage, see the endocervical sample for NAAT.*[e]

NA: not applicable; NAAT: nucleic acid amplification test.

[a] For NAATs, sample in strict accordance with the manufacturer's instructions.

[b] Dacron or Rayon swabs on a plastic shaft.

[c] An appropriate *N. gonorrhoeae* non-nutritive transport medium such as Amies, Stuart, Copan's ESwab or VCM swabs (stored at +4°C before transport), or a nutritive (growth) transport medium such as Jembec, Transgrow, Gono-Pak or InTray GC system (stored at +36 ± 1°C before transport) should be used.

[d] Dacron or Rayon swabs on an aluminium shaft.

[e] For rectal and oropharyngeal specimens, several commercially available laboratory-based NAATs have received U.S. FDA clearance *(11–13)*[20]. Data indicate that other appropriate NAATs and POC NAATs also perform well for these sample types and conjunctival samples.

[20] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

- Specimen collection, transport and storage conditions vary according to detection assay and may have a significant influence on testing sensitivity.
- Selection of appropriate specimens and detection assays is crucial for effective diagnosis.

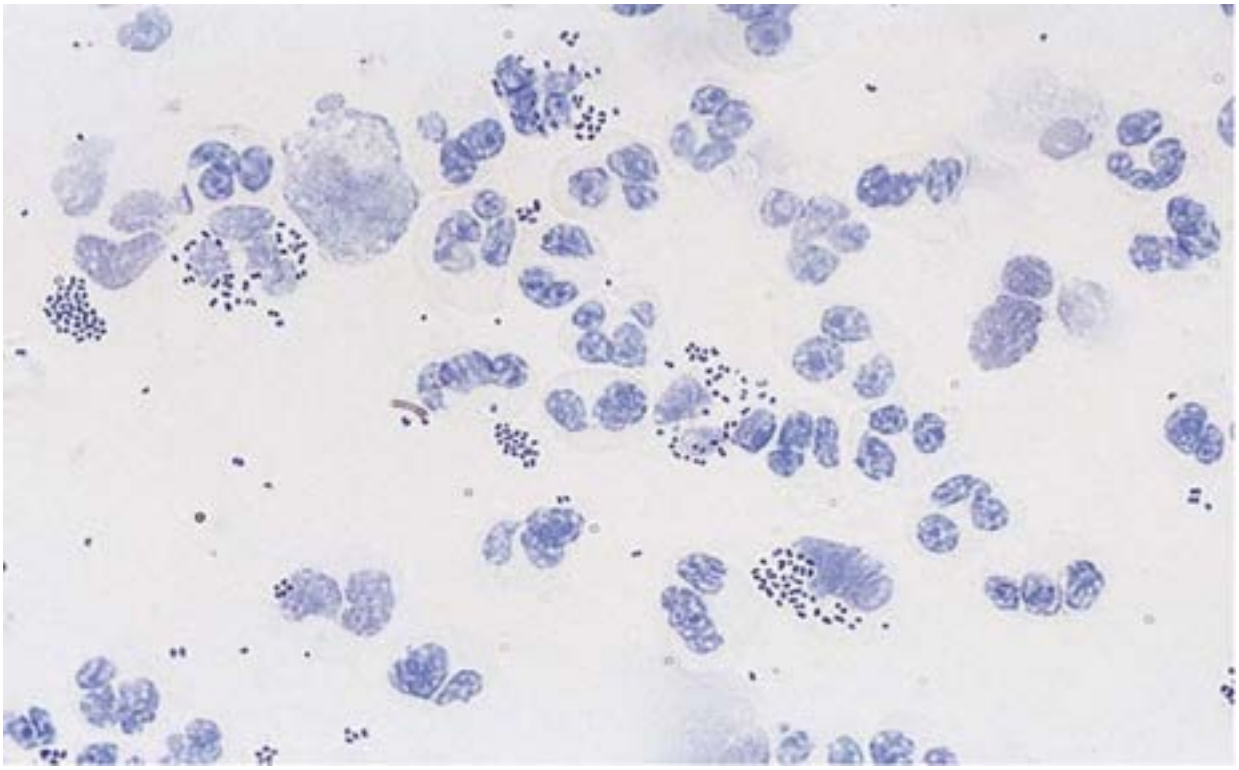
7.4 Presumptive diagnosis: microscopy

7.4.1 Preparation of slides for staining

Prepare the smear as described in Chapter 2 (Microscopy). Fix the dried smear by heating either on a hotplate or by passing the slide rapidly through a flame three times, while keeping the film side uppermost. Avoid overheating because this distorts the cells. The slide should feel merely warm when touched with the back of the wrist.

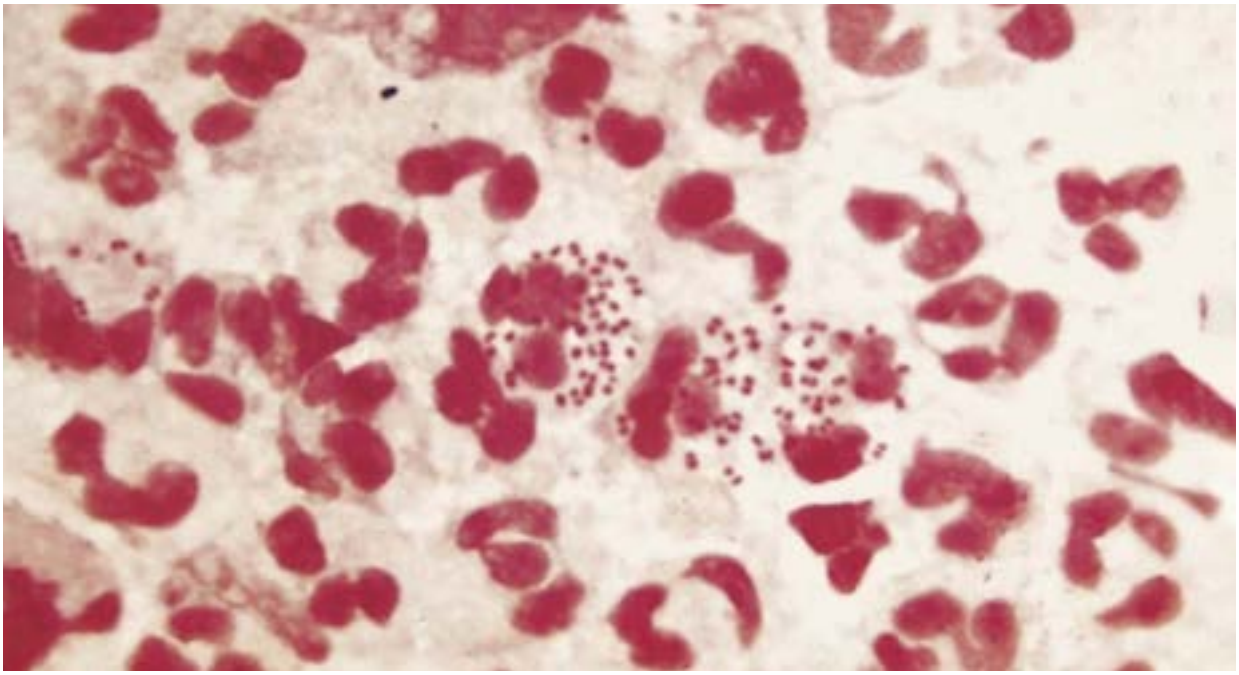
Apart from being simple and quick, microscopy after staining with methylene blue (Fig. 7.1) is a reliable method for the diagnosis of gonorrhoea in men with purulent urethritis *(29)*, but does not allow differentiation of Gram-negative cocci and therefore lacks ideal specificity. The Gram stain to identify intracellular Gram-negative diplococci within PMNLs is the method of choice for the presumptive diagnosis of *N. gonorrhoeae* (Fig. 7.2). Extracellular microorganisms may be observed; however, they alone are insufficient for diagnosis but are sometimes used in combination with clinical symptoms.

Fig. 7.1: Microscopy of methylene blue stain of a male urethral exudate showing intracellular diplococci within PMNLs (1000×)



Source: Reproduced with permission from Morse et al., 2010 *(30)*.

Fig. 7.2: Microscopy of Gram stain of a male urethral exudate showing Gram-negative intracellular diplococci within PMNLs (1000×)



Source: Reproduced with permission from Morse et al., 2010 *(30)*.

7.4.2 Gram stain procedure

1. Cover the fixed smear with crystal violet for 30 seconds. Gently rinse with cold tap water.
2. Flood the slide with iodine solution for 30 seconds. Gently rinse with cold tap water.
3. Decolourize with acetone, acetone-ethanolor ethanol alone until the purple colour stops flooding out of the smear. It is best to hold the slide in a gloved hand near running water. The time of decolourization will depend on which chemical agent is used and the thickness of the smear – it will be shortest (typically a few seconds) for acetone and require longer (up to a minute) for ethanol. Excessive decolouration must be avoided, otherwise Gram-positive bacteria will appear as Gram-negative. Disregard the thick portions of an uneven smear, which may stain blue.
4. Rinse quickly under running water to stop decolouration and drain off excess water.
5. Counterstain with safranin or fuchsin for 1 minute.
6. Rinse with running water and gently blot the slide with absorbent paper.

7.4.3 Methylene blue stain procedure

1. Cover the smear with methylene blue stain for 30–60 seconds.
2. Rinse with running water and gently blot the slide with absorbent paper.

7.4.4 Smear reading and interpretation

Use a bright-light microscope and immersion oil of good quality, and examine the slide with a 100× objective (10× ocular). Gonococci appear as Gram-negative diplococci within PMNLs. Always describe exactly what is seen on the smear: epithelial cells, PMNLs, morphologies of bacteria and intracellular or extracellular location. A slide should be examined for at least 2 minutes before concluding that it does not contain any Gram-negative intracellular diplococci.

7.4.5 QC of microscopy using Gram-stained smears

QC should be performed at regular intervals using a range of bacteria giving different Gram reactions and/or control specimens. This should always be performed when using a new batch of reagents.

- Microscopy is sensitive and specific in symptomatic men with urethral discharge.
- However, microscopy has lower sensitivity in asymptomatic men and endocervical infections, and does not provide a definitive diagnosis for these infections.
- Microscopy is not recommended for the diagnosis of rectal and oropharyngeal infections.

7.5 Culture and presumptive and definitive identification of *N. gonorrhoeae*

7.5.1 Transport and culture

Culture is essential for antimicrobial susceptibility testing (see section 7.8). Gonococci are highly susceptible to environmental conditions (temperature, desiccation, oxidation and toxic substances) and transport of specimens from the clinic to the laboratory (Table 7.3) will reduce the viability of the microorganisms. Urogenital specimens inoculated directly onto a nutritious selective culture medium (see the following sections) in the consultation room is the optimal method; if this is not feasible, the swabs should be inserted into a non-nutritive transport medium such as Stuart, Amies, Copan's ESwab or VCM swabs (see Annex 1) or inoculated on a nutritive (growth) transport system, such as Transgrow *(31,32)*, Jembec *(32,33)*, Gono-Pak or InTray GC. Using a non-nutritive transport medium, the isolation rate after transport of specimens at room temperature (20–25 °C) is approximately 100% within 6 hours and relatively high also after 24 hours. After 48 hours, however, the number of gonococci decreases and recovery may no longer be possible, especially in specimens from asymptomatic patients that contain small numbers of microorganisms. When a transit time of more than 48 hours is expected, nutritive (growth) transport systems that incorporate a culture medium and provide an atmosphere with enhanced concentration of CO_2 should ideally be used. Maximum survival and recovery of gonococci from non-nutritive transport medium is obtained when the inoculated transport medium tubes are stored in the refrigerator at +4 °C before transport to the laboratory. These media delay the growth of gonococci, preventing loss of viability. In contrast, maximum survival from nutritive (growth) transport media is obtained when specimens are preincubated in the transport medium at 36 ± 1 °C overnight before transport to the laboratory; best results are obtained if transport time does not exceed 2 days *(32)*.

Regarding culture on selective culture agar media, the prevalence of gonococcal strains susceptible to the concentration of antimicrobials generally used in media is negligible in most countries. It is recommended that nutritious selective culture agar media, such as the Thayer–Martin *(34)*, modified Thayer–Martin medium (MTM; see Annex 1) or New York City agar *(35)* are used for routine diagnosis of gonorrhoea. If resources allow, ideally also a non-selective culture plate is used for each urogenital sample. Some gonococcal strains may be susceptible to a 3–4 mg/L vancomycin concentration in the selective culture media *(36)*; therefore, selective supplements with a reduced concentration of vancomycin of 2 mg/L or with lincomycin (1 mg/L), which is less inhibitory to contaminating bacteria than vancomycin, can also be used. If rectal and oropharyngeal samples are routinely tested, a high concentration of vancomycin is advisable.

7.5.2 QC of culture media

Each batch of medium should be controlled for sterility, and its ability to sustain the growth of gonococci and inhibit other contaminating bacterial species. To evaluate the growth ability and QC of all diagnostic methods, the 2016 WHO *N. gonorrhoeae* reference strains *(37)* are available from WHO sources (see section 7.8.3[i]). To control the inhibition of non-gonococcal microorganisms reference strains of, for example, *Escherichia coli* (strain 25922, ATCC), *Staphylococcus epidermidis* (strain 12228, ATCC), *N. sicca* (strain 9913, ATCC) and *Candida albicans* (strain 14053, ATCC) can be used.

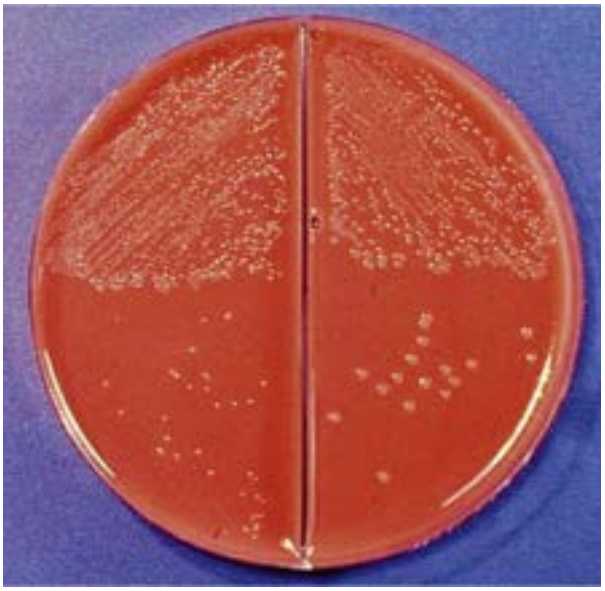
7.5.3 Culture inoculation and incubation

Roll the swab containing the specimen over approximately one quarter of the surface of the plate, ideally Petri dishes with a diameter of 90 mm. Using a sterile bacteriological loop, spread the inoculum over the remaining part of the medium to ensure the growth of isolated colonies. Alternatively, the specimen can be inoculated over the entire surface of the plate in a Z pattern and then streaked out; this can yield more isolated colonies. Incubate the inoculated plates immediately at 36 ± 1 °C in a humid atmosphere (approximately 70–80% humidity) enriched with 5 ± 1% CO_2 (candle extinction jar with moistened cotton wool ball or towels [Fig. 7.3], jar with CO_2-generating envelopes or CO_2 incubator with water bowl or other equipment for enhanced humidity). Examine the plates after 18–24 hours and, if negative, again after 48 hours. After 24 hours of incubation, typical colonies may vary from a diameter of 0.5 to 1 mm in size, being grey to beige-white, transparent to opaque and convex to flat (appearance varies depending on the gonococcal strain and culture medium [Fig. 7.4]). After further incubation, they may reach 3 mm in diameter and become less smooth. Frequently, a mixture of different colony types appears on a plate.

Fig. 7.3: Candle extinction jar for incubation of *Neisseria gonorrhoeae* culture plates

Photography credit: Magnus Unemo.

Fig. 7.4: Typical colonies of *Neisseria gonorrhoeae* on gonococcal-selective (left: MTM) and non-selective culture agar media (right: MTM without antimicrobials added), showing slight growth inhibition by selective antimicrobials



MTM: modified Thayer–Martin medium.

Source: Reproduced with permission from Morse et al., 2010 *(30)*.

7.5.4 Presumptive identification of *N. gonorrhoeae* after culture

A presumptive identification of colonies with a gonococcus-like appearance on selective media can be made using a Gram stain (see section 7.4.2) and an oxidase test. An oxidase test detects the presence of cytochrome c oxidase; the test is best performed by rubbing a few colonies directly onto a filter paper strip moistened with the reagent (1% aqueous solution of N,N,N,N tetramethyl-para-phenylenediamine dihydrochloride) prepared either in the laboratory (in-house) or commercially (e.g. BactiDrop oxidase). A positive test changes in colour from colourless to purple in a few (maximum of 30) seconds (Fig. 7.5). Alternatively, the oxidase test can be performed by placing a drop of oxidase reagent on a few representative colonies on a pure growth culture. Care should be taken because the reagent is toxic to *N. gonorrhoeae*; if only a few colonies are present, they should be subcultured before testing (Fig. 7.5). The observation of oxidase-positive, Gram-negative diplococci with typical colonial morphology on selective media from genital specimens offers a sufficient and reliable identification of *N. gonorrhoeae* for presumptive diagnosis and is highly predictive of *N. gonorrhoeae* if grown on gonococcal-selective agar from a high-risk patient. In situations where resources are limited, this is sufficient to initiate treatment. However, to provide a definitive diagnosis of gonorrhoea, it is necessary to confirm the identification of *N. gonorrhoeae* by eliminating closely related species, such as *N. meningitidis, N. lactamica* and *N. cinerea,* which might also grow on selective gonococcal agar media. It is advisable to always confirm the identification of Gram-negative, oxidase-positive colonies from all extra-genital sites because the likelihood of isolating *Neisseria* species other than *N. gonorrhoeae* at these sites is higher, particularly in the oropharynx. Any gonococcal isolates that are to be characterized further, for example, using antimicrobial susceptibility testing (see section 7.8) or by phenotypic or genetic typing should also have their identification confirmed.

7.5.5 QC of reagents for oxidase test

QC should be performed at regular intervals and always when using a new batch of reagents. Oxidase-positive strains, such as a *N. gonorrhoeae* (2016 WHO reference strain *[37]*), and oxidative-negative reference strains, such as *S. epidermidis* (strain 12228, ATCC) or *E. coli* (strain 25922, ATCC), can be used.

Fig. 7.5: Oxidase-positive purple colonies of *Neisseria gonorrhoeae* on a culture plate (left) and a filter paper (right, which also shows a negative reaction that remained yellow), after reaction with 1% aqueous solution of N,N,N,N tetramethyl-para-phenylenediamine dihydrochloride

Source: Reproduced with permission from Morse et al., 2010 *(30)*.

7.5.6 Confirmation of identification of *N. gonorrhoeae* after culture

Several approaches can be used for confirmation: biochemical tests; matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometry (MS); use of immunological reagents; or use of *N. gonorrhoeae*-specific molecular detection. The choice among these approaches depends on the number of isolates to be tested, the species-confirmatory assays available, expertise and cost. No test is 100% sensitive and specific. Especially in reference laboratories, clinical isolates of *N. gonorrhoeae* are frequently identified using several different species-confirmatory tests.

i. Tests that differentiate between *Neisseria* species

Traditionally, the ability of *N. gonorrhoeae* to produce acid from its use of glucose (and no other tested carbohydrates), detected by colour change of a pH indicator due to lowered pH, in comparison to, for example, *N. meningitidis*, which additionally uses maltose, has been a common method of identification (Table 7.4). This unique pattern of carbohydrate use is detected by the inoculation of pure cultures into cysteine trypticase agar (CTA) containing glucose, maltose and sucrose, respectively, at a final concentration of 1–2% after 24-hour incubation (Fig. 7.6) *(38)*. It is essential to perform this test using a pure culture; this may necessitate at least one subculture resulting in a longer turnaround time to confirm the identification than more rapid tests described below.

The rapid carbohydrate utilization test (RCUT) *(39,40)*, which relies on preformed enzymes and not on growth, using liquid media inoculated with a heavy pure growth, produces results in 4 hours and is also effective.

Fig. 7.6: Acid production from carbohydrate utilization in cysteine trypticase agar (CTA) medium

Key:

Tubes from left to right are: CTA base medium containing no carbohydrate; CTA medium containing 1% glucose; and CTA medium containing 1% maltose.

The inoculated bacterial isolate is *N. gonorrhoeae* because only glucose is used, which results in the yellow colour.

Source: Reproduced with permission from Morse et al., 2010 *(30)*.

Table 7.4: Carbohydrate (sugar) use and enzyme activity of different *Neisseria* species and other oxidase-positive species such as *Moraxella catarrhalis* and *Kingella denitrificans*

Species	Reactivity							
	Glucose	Maltose	Lactose	Saccharose (sucrose)	Fructose (levulose)	ONPG	GGT	GGT
N. gonorrhoeae	+	–	–	–	–	–	–	+ (–)
N. meningitidis	+	+	–	–	–	–	+ (–)	– (+)
N. lactamica[a]	+	+	+	–	–	+	–	+
N. polysaccharea[a]	+	+	–	+/-	–	–	–	+
N. cinerea[a]	– (+)	–	–	–	–	–	–	+
Species	**Reactivity**							
N. subflava[a,b]	+	+	–	+/–	+/–	–	–	+
N. sicca	+	+	–	+	+	–	–	+
N. mucosa	+	+	–	+	+	–	–	+
N. flavescens	–	–	–	–	–	–	–	+
M. catarrhalis	–	–	–	–	–	–	–	– (+)
K. denitrificans	+	–	–	–	–	–	–	+

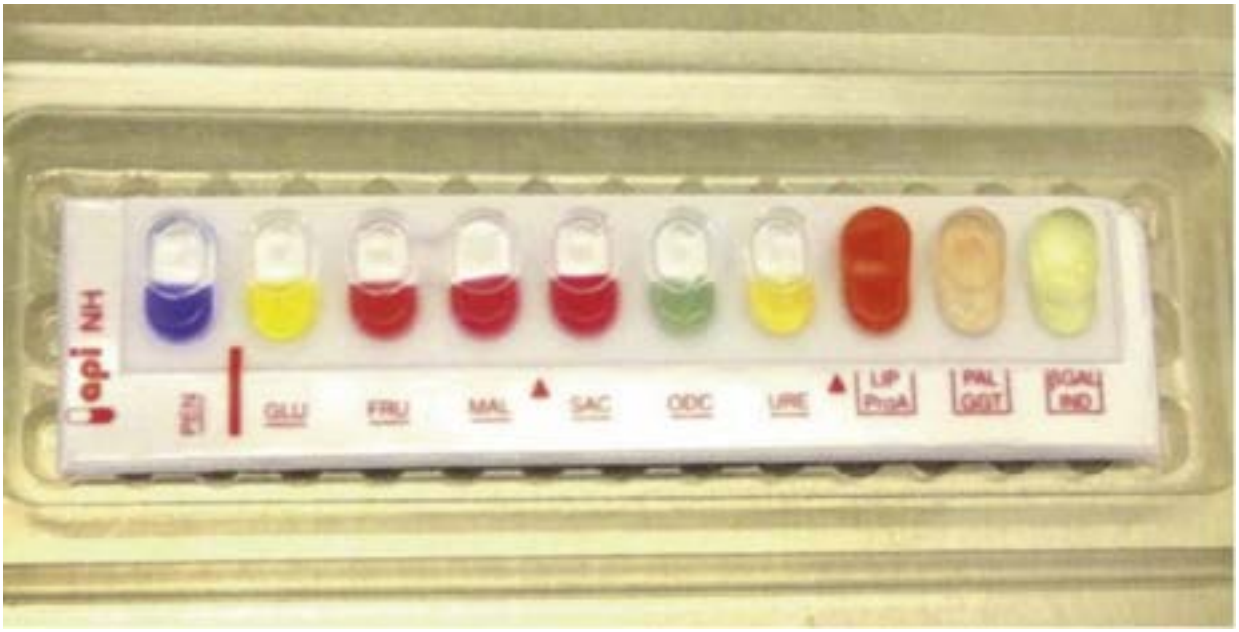
ONPG: ortho-nitrophenyl-β-galactosidase; GGT: ɣ-glutamyl aminopeptidase (transferase); PIP: prolyliminopeptidase (hydroxyproline aminopeptidase; proline arylamidase).
[a] *N. lactamica* usually grows on gonococcal-selective medium, but also other non-pathogenic saprophytic *Neisseria* species may grow.
[b] Includes the biovars subflava, flava and perflava, which differ in their activity against saccharose and fructose.
+/–: not consistent for the species; + (–): mostly positive but negative strains exist; – (+): mostly negative but positive strains exist.

The rapid detection of preformed enzymes also requires a pure culture (which should always be taken from a non-selective medium), but does not require overnight incubation for the test and provides faster results. For all these tests, it is important to precisely follow the instructions from the manufacturer. Primarily, these tests detect different enzymes in the aminopeptidase pathway and include, for example, Gonochek-II *(38,40)*. The Gonochek-II test is used to differentiate the *Neisseria* species via the detection of three preformed enzymes (prolyliminopeptidase [PIP], ɣ-glutamyl aminopeptidase and β-galactosidase). A change to a blue colour indicates that there is hydrolysis of 5-bromo-4-chloro-3-indolyl β-galactoside by β-galactosidase, which is indicative of *N. lactamica*. A yellow colour indicates hydrolysis of ɣ-glutamyl-p-nitroanilide by ɣ-glutamyl aminopeptidase, which is a characteristic of *N. meningitidis*. In the absence of a colour change, the primary lid is removed and replaced with the secondary lid, which has a diazo dye (colour developer) incorporated, and the tube is inverted. If a red colour is observed, this indicates hydrolysis of L-proline 4-methoxynaphtylamide and the presence of PIP, giving a presumptive identification of *N. gonorrhoeae*.

Some saprophytic *Neisseria* species, such as *N. cinerea*, *N. polysaccharea* and *N. subflava*, may appear on specialized gonococcal media after 24–48 hours of incubation; these species possess PIP and, therefore, may produce misleading false-positive results with the Gonochek-II kit. False negatives can also occur with isolates of *N. gonorrhoeae* that lack expression of a functional prolyliminopeptidase enzyme; worldwide dissemination of a PIP-negative gonococcal clone has been described *(41)*. Occasional isolates of *N. meningitidis* from urogenital sites lack ɣ-glutamyl aminopeptidase.

Species verification using preformed enzyme tests alone is not recommended. However, a combination of carbohydrate use and detection of preformed enzymes, which is commercially available in rapid kits, gives a more reliable identification. It is essential to strictly follow the instructions from the manufacturer for these tests. API NH and RapID NH *(37)* are two examples of such kits, which unfortunately may be too expensive for lower-resource settings. They both contain dehydrated substrates in a series of cupules or wells, which are filled with a suspension of the colonies to be identified; after 2–4 hours of incubation at 37 °C, colour reactions are recorded. A profile number is produced and identification is obtained by comparison with a database (on paper or online). The API NH has 10 wells allowing 13 identification tests, which include a β-lactamase test, 4 carbohydrate use tests and 8 biochemical tests for different enzyme–substrate reactions (Fig. 7.7). The RapID NH consists of 2 carbohydrate cupules and 11 biochemical wells for different enzyme–substrate reactions (see the main reactions for distinguishing *Neisseria* species in Table 7.4).

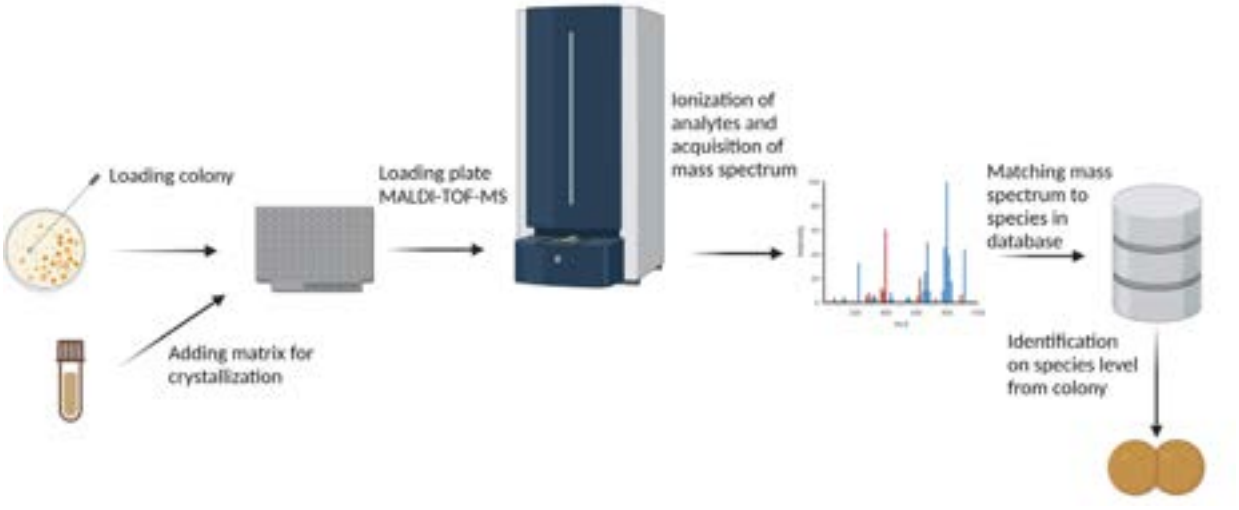
Fig. 7.7: API NH identification kit demonstrating the profile of *Neisseria gonorrhoeae*



Source: Reproduced with permission from Morse et al., 2010 *(30)*.

In better-resourced laboratories, MALDI–TOF MS is most frequently used for species verification of most bacterial species including *N. gonorrhoeae (42)*. The MALDI–TOF MS equipment has a high throughput and is expensive; however, the cost per species-verified isolate is low and the method is very rapid and easy to perform. When using MALDI–TOF MS for species verification of *N. gonorrhoeae*, it is important to precisely follow the instructions from the manufacturer. A schematic of using MALDI–TOF MS for species verification is shown in Fig. 7.8. Briefly, for the direct method, pure colonies of the cultured isolate are mixed with a matrix solution for lysis of the cells and crystallization of the bacterial proteins on a MALDI–TOF plate. The plate is loaded onto the MALDI–TOF MS and within a few minutes specific mass spectra are generated. These mass spectra are then matched with other mass spectra in a large database, and the species or genus of the cultured isolate is determined along with an associated score or numerical value. An extraction step, as described by the manufacturer, can be performed before adding to the MALDI–TOF plate. This extraction step can contribute to a more reliable species identification; if appropriately validated locally, it can be sufficient for *N. gonorrhoeae* identification. An additional species identification test must be used if a species identification score is not obtained.

Fig. 7.8: Schematic of matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometry (MS) for species verification



Source: Created with BioRender.com.

ii. QC of biochemical and other phenotypic species-verifying assays

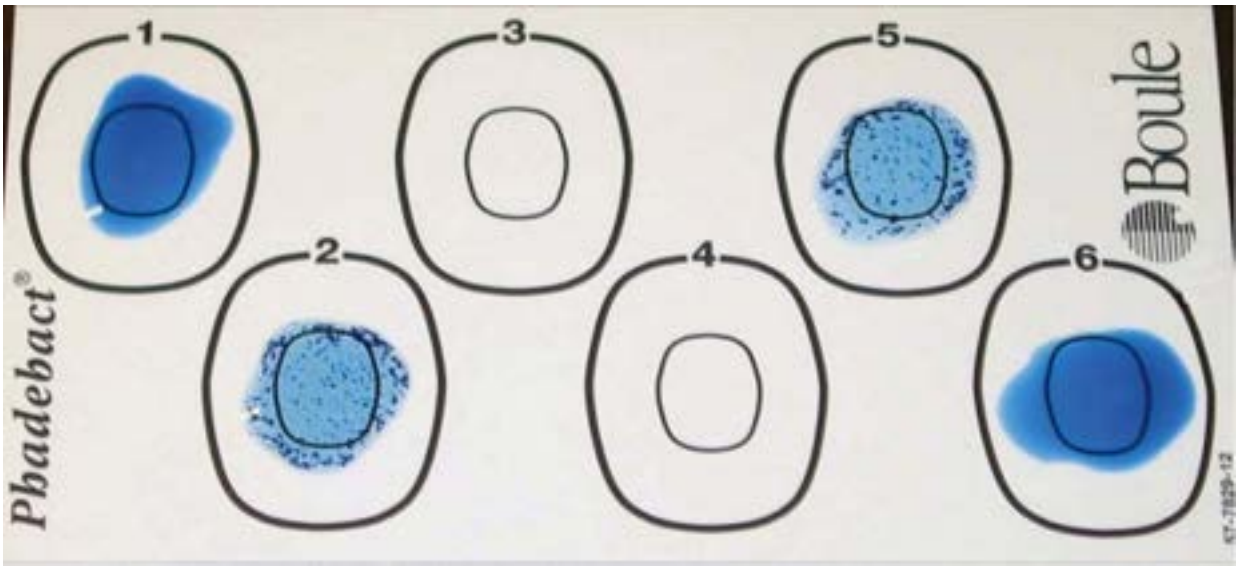
Each new batch of commercially available assays or in-house reagents should be quality-controlled using reference strains of appropriate bacterial species, such as *N. gonorrhoeae* (2016 WHO reference strain *[37]*), *N. meningitidis* (strain BAA-335, ATCC), *N. lactamica* (strain 23970, ATCC), *N. sicca* (strain 9913, ATCC) and *N. cinerea* (strain 14685, ATCC). Furthermore, the same reference strains should be tested as controls on a regular basis and results should be monitored over time.

iii. Tests specific for *N. gonorrhoeae*

Confirmation by tests that are specific for *N. gonorrhoeae* can also be immunologically or molecularly based. These relatively expensive assays may be performed directly with colonies on the selective isolation plate and do not require the use of a pure subculture. This means that an isolate can be identified ≥ 24 hours earlier than it is possible with the rapid carbohydrate or enzyme–substrate assays.

The most popular of the commercially available kits has been the Phadebact Monoclonal GC test *(38,40)*, which contains a mixture of monoclonal antibodies directed at the major outer membrane porin (PorB). These antibodies are absorbed, via their Fc segment, onto protein A from *Staphylococcus aureus*; when mixed with gonococcal antigen, agglutination occurs. The test requires a light suspension of the test microorganism (approximately a density of 0.5 according to the McFarland nephelometric standards; see Annex 1) made in the manufacturer's buffer or 0.9% sterile phosphate-buffered solution (PBS), which is then boiled for 10 minutes and allowed to cool before use. The suspension should be boiled immediately after it has been prepared because any delay will result in lysis of the bacteria and release of bacterial DNA, which can cause auto-agglutination and difficulty in reading the test. One drop of the suspension is mixed for 2 minutes with each of two reagents, which allows serogrouping into WI (IA; PorB1a) and WII/III (IB; PorB1b) in addition to identification (Fig. 7.9). The reagent is coloured blue to aid reading the agglutination reaction. This test has a high sensitivity and specificity but it is a mixture of specific antibodies rather than a single antibody to a conserved antigen. False negatives are unusual but occur. Unfortunately, this test is expensive and it may be discontinued soon.

Fig. 7.9: Reaction of two *Neisseria gonorrhoeae* strains (A, wells 1 and 2; B, wells 5 and 6) with Phadebact co-agglutination reagents (WI, wells 1 and 5; WII/III, wells 2 and 6)



Source: Reproduced with permission from Morse et al., 2010 *(30)*.

GonoGen II *(38,40)* also uses a similar panel of antibodies, but in this test the monoclonal antibodies are adsorbed onto suspended metalsol particles. When the culture is emulsified in the solubilizing buffer, the outer membrane of the microorganism is stripped off, releasing the PorB-containing complexes into solution. These released PorB complexes then are captured by the antibody and metalsol particles. The sample and reagent mixture then is filtered through the special matrix device; the PorB antibody and metalsol complexes are held back by the matrix, resulting in a red spot. Antibody and metalsol particles that have not bound PorB will pass through the matrix giving a negative result (a white to pale pink ring).

iv. QC of immunological identification tests

Each new batch of commercially available assays or in-house reagents should be quality-controlled using *N. gonorrhoeae* reference strains (positive controls for both serogroup WI [IA, PorB1a; e.g. 2016 WHO reference strain F, G or N *(37)*] and WII/III [IB, PorB1b; e.g. 2016 WHO reference strain K *(37)*]), as well as a negative reference strain of other closely related species, for example, *N. lactamica* (strain 23970, ATCC). Furthermore, the same reference strains should be tested as controls on a regular basis and results should be monitored over time.

Molecular confirmation of gonococcal identity can be performed using the NAATs described in section 7.6 or other validated and quality-assured NAATs.

- Culture, under optimized conditions, is sensitive and highly specific for urethral samples in men and cervical samples in women. It is also relatively inexpensive and provides viable gonococcal isolates for antimicrobial susceptibility testing.
- Because of the high level of AMR in gonococci worldwide, it is essential to keep and, in several settings, strengthen the culture capacity to allow surveillance of AMR.
- For sensitive and specific culture, sample collection, transport, storage and culture methodology must be optimized and quality assured.

7.6 Molecular detection

7.6.1 Introduction and molecular assays

Molecular detection of specific nucleic acid (DNA/RNA) sequences of *N. gonorrhoeae* is most commonly performed using commercially available kits that detect both *N. gonorrhoeae* and *C. trachomatis* in the same kit simultaneously, often at little or no extra direct cost. The first developed molecular tests were the non-amplified nucleic acid hybridization (NAH) assays or nucleic acid probe (NAP) assays such as PACE 2 (Gen-Probe) and Hybrid Capture 2 (HC2) CT/NG (Digene Corporation). NAH/NAP assays rely on the binding of specific complementary nucleic acid probes and subsequent signal amplification to detect binding. However, NAH assays are substantially less sensitive than NAATs and should not be used diagnostically when NAATs are available and affordable.

Presently, NAATs are used in many countries for screening of *N. gonorrhoeae* (Table 7.5). Gonococcal NAATs detect different regions of DNA or ribosomal RNA specific to *N. gonorrhoeae*. The target sequence is amplified using a variety of methods to produce multiple copies that can be easily detected. For basic information regarding NAH/NAP assays and different NAAT technologies, see Chapters 4 and 8 *(C. trachomatis)*. Gonococcal NAATs are highly sensitive and specific, with some variation between NAATs, and can be used with specimens taken less invasively and self-collected specimens (e.g. urine and penile meatal swabs in men and vaginal swabs in women). This allows both a greater number of patients to be seen in a clinic or primary care setting as well as providing the prerequisites for effective screening. NAATs can usually be performed within a working day, giving a faster turnaround time than culture (minimum of 2–3 days); they are often used in combination with robotics, thereby enabling a high throughput. The sensitivity of NAATs is higher than culture sensitivity, especially for oropharyngeal and rectal samples, and this reflects their greater analytical sensitivity but also higher tolerance to inadequacies in the collection, transport and storage process. Disadvantages of using NAATs for detection of *N. gonorrhoeae* include the cost of equipment and reagents, the fact that not all NAATs are licensed for extra-genital specimens, the inability to perform antimicrobial susceptibility testing and the suboptimal specificity of some NAAT assays (see sections 7.6.2–7.6.4). Despite these disadvantages, the use of appropriate NAATs in addition to culture (for antimicrobial susceptibility testing) should be encouraged even in resource-constrained settings.

This can be facilitated by the creation and support of regional reference laboratories that can provide diagnostic services using these methods. Regional reference laboratories offer many advantages because of larger testing volumes, rigorous adherence to good laboratory practices and improved technical expertise. Use of regional laboratories may reduce costs while using assays with the highest sensitivity, and thus result in equivalent or reduced turnaround times. Notably, the first NAATs that can be performed in ≤ 30 minutes by non-laboratory personnel, that is, POC NAATs, have now received FDA clearance *(18,19,43).*

- NAATs generally offer the highest sensitivity, especially for oropharyngeal and rectal samples, are usually highly specific and can be used with samples taken less invasively and self-collected samples.
- Molecular assays are more tolerant to inadequacies in collection, transport and storage conditions, and are objective.
- The specificity of some of the gonococcal NAATs is suboptimal (see sections 7.6.2–7.6.4), which results in low PPVs in low-prevalence populations; supplementary NAATs that target another sequence may be required for confirmation.
- New NAATs and POC NAATs are rapidly becoming available (see Chapter 3 and WHO, 2023 *[44]*).
- If internationally approved NAATs cannot be used, we strongly recommend that, before use, the effectiveness of the proposed NAAT for the local settings is strictly validated and quality-assured against at least one internationally approved NAAT.

New NAAT assays are rapidly becoming available and cannot be anticipated in this chapter. It is important to continually assess the relevant literature for high-quality evaluation of new assays to determine the best fit for each laboratory.

7.6.2 Specimen types for *N. gonorrhoeae* NAATs

A range of different specimen types can be used with NAATs, including invasively taken specimens, such as liquid Pap specimens, cervical swabs and urethral swabs (men); and specimens taken less invasively, such as vaginal swabs, penile meatal swabs and urine (men and women). However, different manufacturers produce NAATs that are licensed for different specimen types (Table 7.5), so it is essential to check the manufacturer's instructions or conduct extensive in-house validation. Urine is not the optimal sample, because of reduced sensitivity, to detect *N. gonorrhoeae* in women *(17,21)*. It is essential to precisely follow the instructions from the manufacturer regarding approved samples; collection, transport and storage of samples; and performance of NAATs.

NAATs are generally more sensitive than culture for the diagnosis of gonorrhoea, especially for oropharyngeal and rectal samples *(8–17)*. Several commercially available NAATs have recently received FDA clearance[21] for these sample types based on strong performance characteristics *(11–13)*. However, in many countries it is recommended that a positive test at either of these sites should be ideally confirmed using a supplementary test (NAAT with another target sequence) to avoid false-positive results *(17,45)*.

[21] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

Table 7.5: U.S. Food and Drug Administration-approved NAATs for detection of *Neisseria gonorrhoeae* (August 2022)

Assay (manufacturer)	Sample type	Testing location	Footprint	Technical skill required	Time to clinical results
RealTime CT/NG (Abbott)	Endocervical samples, vaginal and urethral swabs, urine. Conjunctival samples[a]	Laboratory	Large, floor	High	1–2 days
Alinity m STI (Abbott)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Large, floor	High	1–2 days
CTQx/GCQx (Becton Dickinson)	Endocervical samples, vaginal swabs, urine	Laboratory	Large, floor	High	1–2 days
CTGCTV2 for MAX system (Becton Dickinson)	Endocervical samples, vaginal swabs, urine	(On-site) laboratory	Medium, benchtop	High	4 hours if on-site lab
Xpert CT/NG (Cepheid)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine. Conjunctival samples[a]	On-site laboratory	Small, benchtop-large, floor	Low	90 min
Aptima Combo 2 (Hologic)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Laboratory	High	1–2 days
Aptima *N. gonorrhoeae* (Hologic)	Endocervical samples, vaginal	Laboratory	Laboratory	High	1–2 days
cobas CT/NG 4800 (Roche)	Endocervical samples, vaginal and urethral swabs, urine	Laboratory	Laboratory	High	1–2 days
cobas CT/NG 6800/8800 (Roche)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Laboratory	Moderate	1–2 days
cobas CT/NG 5800 (Roche)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Large, floor	Moderate	1–2 days
health io CT/NG (binx)	Vaginal swabs, penile urine	Clinic	Small, benchtop	Low	30 min
Visby Medical Sexual Health CT/NG/TV (Visby Medical)	Vaginal swabs	Clinic	Very small, handheld/benchtop	Low	30 min

CT: *Chlamydia trachomatis*; NAAT: nucleic acid amplification test; NG: *Neisseria gonorrhoeae*; TV: *Trichomonas vaginalis.*

[a] These assays have been well validated for use with conjunctival samples. There is reason to assume that other NAATs would also perform adequately. However, no NAAT has a U.S. FDA claim for this specimen type.

7.6.3 Specificity of *N. gonorrhoeae* NAATs

Historically, there has been concern over the specificity of the NAAT target chosen because the species in the *Neisseria* genus are genetically closely related and many of the commensal *Neisseria* species are found especially in the oropharynx but also sometimes in the rectum and lower genital tract. Identification of a target sequence specific to *N. gonorrhoeae* has been a challenge; cross-reactivity has been reported with many non-gonococcal *Neisseria* species for some NAATs, including *N. meningitidis, N. cinerea, N. flavescens, N. lactamica, N. sicca* and *N. subflava (17,46–53)*. The most recent generation of commercially available NAATs has improved markedly in this regard, but it is still a factor to be considered when selecting the appropriate kit for the specimen type to be tested because not all NAATs for gonorrhoea are equal *(17,21)*.

7.6.4 Sensitivity, specificity and prevalence: the effect on PPV

The prevalence of gonorrhoea in the population being tested must be considered together with the sensitivity and specificity of the NAAT being used because this will affect the PPV of the test and hence the number of false positives obtained. This is demonstrated in Table 7.6. A PPV of more than 90% (using a single NAAT or screening NAAT plus supplementary NAAT with different target) has been suggested as a minimum when using NAATs to detect *N. gonorrhoeae*.

Even when the sensitivity and specificity of a NAAT is greater than 95%, the PPV in a population of both 1% and 5% is for most NAATs still less than 90% whereas, at a prevalence of 10%, the PPV of most (but not all) NAATs is greater than 90%.

7.6.5 NAATs not approved by the U.S. FDA

Laboratory-developed (or commercial non-FDA-approved) NAATs that target, for example, the *cppB* gene, cytosine DNA methyltransferase (*CMT*) gene, *opa* genes and *porA* pseudogene have been described *(48, 54–57)* and are used extensively in some countries. Several of these target the *porA* pseudogene and *opa* genes either separately or in combination. The *porA* pseudogene is absent in commensal *Neisseria* species (and the *porA* gene in *N. meningitidis* is sufficiently different); consequently this target is highly specific for *N. gonorrhoeae*. Nevertheless, several countries have reported rare *N. gonorrhoeae* strains containing a meningococcal *porA* instead of a gonococcal *porA* pseudogene, resulting in false-negative NAAT results *(58–62)*. NAATs that target the *cppB* gene can vary in sensitivity and specificity; some isolates of *N. gonorrhoeae* do not carry this gene and conversely some strains of *N. meningitidis* do have this gene and cross-react. Furthermore, the NAATs targeting the *CMT* gene can cross-react with commensal *Neisseria* species. Hence, the NAATs targeting the *cppB* and *CMT* genes are not recommended. Laboratory-developed NAATs may require more technical expertise; however, the sensitivity and specificity of these tests can be adequate and they can be a less expensive and effective option, particularly for small numbers of samples. Multiplex commercial polymerase chain reaction assays detecting, for example, *N. gonorrhoeae, C. trachomatis, M. genitalium* and *Trichomonas vaginalis* also have been developed *(63,64)*.

Accordingly, globally there are many commercially available or even laboratory-developed *N. gonorrhoeae* NAATs in use. If any NAATs that have not been approved by the FDA are used, regional, such as European Union, and/or other national regulatory processes should provide safeguards on the quality and performance of diagnostic NAATs. It is strongly recommended that only internationally approved NAATs are used. If this is not possible, it is essential that the proposed NAAT, before use, is strictly validated for local requirements against at least one internationally approved NAAT and subsequently used with appropriate positive, negative and inhibition controls, as well as participation in an appropriate external quality assessment (EQA) system.

Table 7.6: Effect of prevalence on positive predictive value (PPV) for single tests

Test	A	B	C
Sensitivity	97.8%	96.4%	98.0%
Specificity	99.2%	97.9%	99.7%
PPV			
10% prevalence	93%	84%	97%
5% prevalence	87%	73%	95%
1% prevalence	55%	35%	77%

7.6.6 QC and quality assurance of NAATs

Internal quality controls should be included in each test run. Internal quality assessment (IQA) should be performed regularly by retesting samples to which the original result has been blinded. The number and frequency of these IQAs will be dependent on the total number of tests performed, ideally 1–5% of the total tested each month. EQA can be achieved by using panels of specimens from appropriate EQA providers, such as United Kingdom National External Quality Assessment Services[22] or the Quality Control of Molecular Diagnostics,[23] which deliver to many countries, or by a more informal exchange of samples between laboratories.

7.7 Point-of-care (POC) tests (rapid diagnostic tests)

No POC test (rapid diagnostic test) for antigen detection with appropriate performance characteristics, especially sensitivity, is yet commercially available; therefore, none can be recommended for the diagnosis of uncomplicated or complicated gonorrhoea. However, some promising antigen-based POC assays may be in the pipeline *(20)*; but, further evaluations are pending. In resource-constrained, high-prevalence populations, decreases in sensitivity may be acceptable in exchange for the ability to test and treat while the patient is on site *(65–67)*. In these settings, antigen-based POC tests could be used to increase the specificity of the syndromic management algorithms, which will reduce overtreatment and also find many asymptomatic infections, especially in women *(67)*. Furthermore, the first sensitive and specific NAATs that can be performed in ≤ 30 minutes by non-laboratory personnel, that is, POC NAATs, have now received FDA clearance. These are the health io CT/NG test (binx) and Visby Medical Sexual Health (CT/NG/TV) test *(18,19,43)*. If affordable and accessible, these POC NAATs can be exceedingly valuable for rapid diagnosis at POC, for example, at the clinic or in the field.

- No rapid antigen-based POC test with appropriate sensitivity is commercially available; therefore, none can be currently recommended for the diagnosis of gonorrhoea.
- However, the first rapid POC NAATs, with appropriate sensitivity and specificity, have now received U.S. FDA clearance *(18,19,42)*. These and similar POC NAATs can be exceedingly valuable for rapid diagnosis and immediate treatment of the patient.
- New NAATs and POC NAATs are becoming available rapidly (see Chapter 3 and WHO, 2023 *[44]*).

7.8 Antimicrobial susceptibility testing

7.8.1 Introduction

N. gonorrhoeae has developed resistance to all previous first-line antimicrobial treatments for gonorrhoea, for example, sulphonamides, penicillin, tetracycline, spectinomycin, fluoroquinolones, early-generation cephalosporins and macrolides *(2–7)*. In most global settings, the third-generation, extended-spectrum cephalosporin ceftriaxone, given as high-dose monotherapy or together with azithromycin, is the only remaining option for first-line empirical antimicrobial therapy of gonorrhoea *(7)*. Nevertheless, in the past two decades, gonococcal strains with in vitro and clinical resistance to ceftriaxone and azithromycin have emerged globally *(2–7,17,68–84)*. Some extensively drug-resistant (XDR) gonococcal strains with ceftriaxone resistance in combination with high-level resistance to azithromycin have also been detected *(69–72)*. The emergence and international spread of multidrug-resistant and sporadic XDR gonococcal strains and treatment failures with ceftriaxone mean that gonorrhoea has become a major public health concern, alerting about the future prospect of untreatable gonorrhoea in certain circumstances and settings *(3–7)*. Thus, it is essential to monitor the antimicrobial susceptibility of *N. gonorrhoeae* locally, regionally and globally. WHO has revitalized the WHO Global Gonococcal Antimicrobial Surveillance Programme (GASP) *(5–7)* and is currently also substantially expanding its Enhanced GASP (EGASP), which includes standardized and quality-assured protocols, epidemiological data, whole-genome sequencing (WGS) and, where feasible, test of cure *(85)*. Notably, WGS in conjunction with AMR and epidemiological data has been used for several years in the European GASP (Euro-GASP) *(83,84)*. WHO has additionally published the WHO Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae (3)*.

The agar dilution method is the recommended gold standard method for antimicrobial susceptibility testing or determination of the minimum inhibitory concentration (MIC) (in µg/ml or mg/L) of gonococcal isolates to antimicrobial drugs. However, this method can be laborious and less suited for routine antimicrobial susceptibility testing, especially if testing a low number of isolates. Therefore, the standardized and quality-assured ETEST method (an MIC gradient strip test), which adequately correlates with the agar dilution method, is commonly used. There are also other MIC gradient strip tests commercially available; however, the performance characteristics of these can vary in the antimicrobial susceptibility testing of *N. gonorrhoeae (86)*.

[22] The UK NEQAS website can be accessed at https://ukneqas.org.uk

[23] The QCMD website can be found at https://www.qcmd.org/

A qualitative determination of antimicrobial susceptibility can be obtained using disc diffusion assays. Several disc diffusion methods are in use; however, these require pronounced standardization and appropriate QCs to attain a high level of reproducibility and correct interpretation to adequately reflect the MIC values of the different antimicrobials. Disc diffusion methods are inexpensive but only recommended for use when MIC determination cannot be performed because of limited resources or similar reasons. If using a disc diffusion method, it is recommended that the finding of any new, emerging or rare AMR is confirmed using MIC determination. β-lactamase production is often determined by a chromogenic cephalosporin test using nitrocefin discs or nitrocefin solution.

All methods for antimicrobial susceptibility testing should be performed from pure, fresh (18–24 hours) *N. gonorrhoeae* cultures taken from non-selective culture media. Isolates should have been appropriately species-verified and subcultured at least once. In antimicrobial susceptibility testing, it is important to precisely follow all the steps of the nominated method, including selection and use of agar medium, reagents (antimicrobial powder, ETEST strips, discs and buffers), inoculation, incubation and interpretation.

- Because of the high level of AMR in gonococci worldwide, it is essential to monitor the antimicrobial susceptibility of *N. gonorrhoeae* nationally and internationally.
- MIC determination is performed using the agar dilution method or ETEST.
- Qualitative disc diffusion methods require standardization and appropriate QCs; importantly, they do not measure and only reflect the MIC. These methods should only be used when MIC determination cannot be performed, for example, due to resource constraints.
- In all antimicrobial susceptibility testing, it is essential to follow the nominated method precisely, which should be appropriately standardized, validated and quality-assured.

7.8.2 Choice of antimicrobials included in antimicrobial susceptibility testing

The list of antimicrobials to be tested should include drugs nationally or regionally recommended and used for the treatment of gonococcal infections, as well as drugs recommended by the local antimicrobial susceptibility surveillance programme. However, especially at reference laboratories, additional antimicrobials can be tested, such as antimicrobials recommended for treatment in other settings, antimicrobials of value for local longitudinal studies and antimicrobials that may be candidates for future treatment of gonorrhoea. Notably, in WHO EGASP, the mandatorily tested antimicrobials are ceftriaxone, cefixime and azithromycin; ciprofloxacin and gentamicin are optional.

7.8.3 Determination of MIC (agar dilution and ETEST)

i. Recommended agar medium

The medium recommended for the determination of MIC of different antimicrobials, using agar dilution or ETEST, for *N. gonorrhoeae* isolates is an appropriate gonococcal agar base, such as Difco GC Medium Base, supplemented with 1% defined growth supplement[24] or 1% IsoVitaleX/Vitox. As an example, see the GCVIT medium described in Annex 1.

ii. Interpretative criteria

The most frequently applied interpretative criteria for susceptibility and resistance using MIC determination are described in Table 7.7. These criteria are from the Clinical and Laboratory Standards Institute (CLSI) *(87)* and from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) *(88)*.

[24] In accordance with the CLSI *(84)*: 1.1 g L-cysteine, 0.03 g guanine HCl, 3 mg thiamine HCl, 13 mg para-aminobenzoic acid, 0.01 g B12, 0.1 g cocarboxylase, 0.25 g nicotinamide adenine dinucleotide, 1 g adenine, 10 g L-glutamine, 100 g glucose and 0.02 g ferric nitrate (in 1 l H2O). Cysteine-free growth supplement is required for agar dilution tests with carbapenems and clavulanate.

Table 7.7: Minimum inhibitory concentration (MIC) interpretative criteria for categorizing *Neisseria gonorrhoeae* into susceptibility categories according to the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)

Antimicrobial	MIC (mg/L)	
	Susceptible (S) CLSI/EUCAST	Resistant (R) CLSI/EUCAST
Ceftriaxone	≤ 0.25/≤ 0.125	NA/> 0.125
Cefotaxime	≤ 0.5/≤ 0.125	NA/> 0.125
Cefixime	≤ 0.25/≤ 0.125	NA/> 0.125
Benzylpenicillin	≤ 0.06/≤ 0.06	> 1/> 1
Ciprofloxacin	≤ 0.06/≤ 0.03	> 0.5/> 0.06
Ofloxacin	≤ 0.25/≤ 0.125	> 1/> 0.25
Spectinomycin	≤ 32/≤ 64	> 64/> 64
Azithromycin[a]	NA/≤ 1	NA/> 1
Tetracycline	≤ 0.25/≤ 0.5	> 1/> 0.5

NA: not available.

[a] EUCAST recommends no clinical breakpoints for azithromycin. However, the epidemiological cut-off value (MIC > 1 mg/L) that indicates the presence of acquired macrolide resistance determinants is shown.

Sources: CLSI, 2023 *(84)*, EUCAST, 2023*(88)*.

iii. QC of MIC determination (agar dilution and ETEST)

An appropriate selection of the 2016 WHO *N. gonorrhoeae* reference strains *(37)* should be included, ideally with each batch of antimicrobial susceptibility testing and always when a new batch of antimicrobial powder, agar medium or ETEST strips are used. The MIC of each antimicrobial and reference strain should be documented on a QC chart and monitored over time. For acceptable MIC values of different antimicrobials in the QC, see Table 7.8. The 2016 WHO *N. gonorrhoeae* reference strains *(37)* can also be used for QCs in other phenotypic and genetic laboratory tests, including genomics, for *N. gonorrhoeae* and in EQA systems.

It is not necessary to use all 14 2016 WHO *N. gonorrhoeae* reference strains *(37)* for QC in the antimicrobial susceptibility testing performed in routine practice. Notably, the WHO EGASP *(85)* uses only the WHO L, M, U and ATCC 49226 reference strains, while Euro-GASP *(83, 84)* uses the WHO G, K, M, P, Q and, when spectinomycin is tested, O reference strains *(37)* for QC. The 2016 WHO *N. gonorrhoeae* reference strains *(37)* and other WHO strains are available from the WHO Collaborating Centre for Gonorrhoea and Other STIs, in Örebro, Sweden; the WHO Collaborating Centre for STI and AMR, in Sydney, Australia; and at the National Collection of Type Cultures (NCTC), in the United Kingdom, named as NCTC 13477–13484 and 13817–13822.[25]

[25] Further information from the NCTC is available at: https://www.culturecollections.org.uk/collections/nctc.jsp

Table 7.8: Acceptable antimicrobial susceptibility categorization, according to EUCAST, and minimum inhibitory concentration (MIC) ranges (mg/L) for the 2016 WHO *Neisseria gonorrhoeae* reference strains (*37*) and the ATCC *Neisseria gonorrhoeae* strain 49226 recommended by CLSI *(84)*

	WHO *N. gonorrhoeae* reference strain														ATCC *N. gonorrhoeae* strain[a]
	F	G	K	L	M	N	O	P	U	V	W	X	Y	Z	
Antimicrobial															
Ceftriaxone	S (< 0.002)	S (0.004–0.016)	S (0.032–0.125)	R (0.064–0.25)	S (0.008–0.032)	S (0.002–0.008)	S (0.016–0.064)	S (0.002–0.008)	S (0.001–0.004)	S (0.032–0.125)	S (0.032–0.125)	R (1–4)	R (0.5–2)	R (0.25–1)	S (0.004–0.016)
Cefixime	S (< 0.016)	S (< 0.016)	R (0.25–1)	S (0.125–0.5)	S (< 0.016)	S (< 0.016)	S (< 0.016–0.032)	S (< 0.016)	S (< 0.016)	S (< 0.016)	R (0.125–0.5)	R (2–8)	R (1–4)	R (1–4)	S (0.004–0.032)
Azithromycin	S (0.064–0.25)	S (0.125–0.5)	S (0.125–0.5)	I (0.25–1)	S (0.125–0.5)	S (0.064–0.25)	S (0.125–0.5)	R (1–4)	R (2–8)	R (> 256)	I (0.25–1)	I (0.25–1)	R (0.5–2)	R (0.5–2)	S (0.5–1)
Ciprofloxacin	S (0.002–0.008)	R (0.064–0.25)	R (> 32)	R (> 32)	R (1–4)	R (2–8)	S (0.004–0.016)	S (0.002–0.008)	S (0.002–0.008)	R (> 32)	R (> 32)	R (> 32)	R (> 32)	R (> 32)	S (0.001–0.008)
Spectinomycin	S (16–64)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	R (> 1024)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	S (8–32)	S (8–32)

SIR categorization: S, susceptible; I, susceptible, increased exposure; R, resistant.

Note that the exact MICs shown should be used and interpreted with caution because they were derived using one specific ETEST method only and, accordingly, may differ using other methods. However, the identified resistance phenotypes (SIR categorization) should be consistent between different methods.

[a] Acceptable ranges of MICs (CLSI) *(87)*.

7.8.4 Agar dilution method for MIC determination

i. Introduction

Agar dilution is the recommended gold standard method for quantitative antimicrobial susceptibility testing or to determine the MIC of gonococcal isolates to antimicrobials. Antimicrobials are incorporated into a gonococcal agar base supplemented with 1% defined growth supplement or 1% IsoVitaleX/Vitox (see section 7.8.3[i]) in serial twofold dilutions. Other media and supplements used for agar dilution, such as diagnostic sensitivity test agar with 5% lysed horse blood and 1% IsoVitaleX/Vitox as used in some countries,[26] should be fully validated and quality-assured before use. *N. gonorrhoeae* isolates to be tested are grown overnight on non-selective gonococcal agar medium and then suspended in Mueller–Hinton (MH) broth, sterile saline solution or equivalent solution. Then, approximately 10^4 colony-forming units (CFU) are inoculated onto the surface of the antimicrobial-containing media and two plates of antimicrobial-free control medium with a Steer's replicator, multipoint inoculator or a calibrated loop. Plates are finally incubated overnight and subsequently examined for growth. The MIC of the antimicrobial for an isolate is the lowest concentration that inhibits its growth. A modification of the full MIC agar dilution method is the agar dilution breakpoint technique, which is similar but with the agar medium containing only one or two concentrations of antimicrobials, which can be used to categorize isolates as being resistant (using an agar plate with a concentration at the breakpoint for resistance) or being susceptible or non-susceptible (using an agar plate with a concentration at breakpoint for susceptibility). The breakpoint technique is useful for screening many isolates.

ii. Preparation of antimicrobial solutions

Appropriate antimicrobial powders or tablets to dissolve should be obtained directly from pharmaceutical companies or other validated providers. Because most antimicrobials are not 100% pure, the concentration incorporated in the agar plates should be based on the activity or potency (active drug per mg) of the antimicrobial, as specified by the manufacturer. The instructions from the manufacturer regarding dissolving the antimicrobial powder, expiry date and storage instructions must be followed in detail. Antimicrobials should be incorporated into the agar medium in twofold dilutions, for example, using a scheme in which one part of antimicrobial solution is added to nine parts of agar (see section 7.8.4[iii]).

For example, prepare stock solutions by dissolving 128 mg or a weight equivalent to 128 mg of active ingredient in a minimal amount of appropriate solvent (usually 5–10 ml, follow the instructions from the manufacturer precisely) and dilute further with distilled water (or other recommended solvent) to an exact volume of 25 ml. These stock solutions contain antimicrobial product at a concentration of 5120 mg/L, which is practical for preparing the working solutions. If not otherwise recommended by the manufacturer, the solvents given in Table 7.9 can be used to prepare stock solutions. Stock solutions can be sterilized using membrane filtration (0.22-µm filter) and stored in aliquots in tightly sealed vials at −20 °C or, preferably, at −70 °C for up to 6 months. When thawed, solutions have to be used immediately and they should not be refrozen for later use.

Table 7.9: Solvents for the preparation of antimicrobial solutions for minimum inhibitory concentration (MIC) determination using the agar dilution method[a]

Antimicrobial	Solvent
Ceftriaxone	Distilled water
Cefixime	0.1 mol/L phosphate buffer, pH 7.0
Benzylpenicillin	Distilled water
Ciprofloxacin	Water or 0.1 mol/L HCl
Azithromycin	95% ethanol or anhydrous (glacial) acetic acid[b]
Erythromycin	95% ethanol or anhydrous (glacial) acetic acid[b]
Spectinomycin	Distilled water
Gentamicin	Distilled water
Kanamycin	Distilled water
Tetracycline	Distilled water
Chloramphenicol	95% ethanol

[a] If the solvent and the procedure for dissolving the antimicrobial powder is given by the manufacturer, follow the instructions from the manufacturer precisely.

[b] For anhydrous (glacial) acetic acid, use one half volume of distilled water, then add anhydrous (glacial) acetic acid drop wise until dissolved, not exceeding 2.5 µl/ml.

[26] The GRASP (gonococcal resistance to antimicrobials surveillance programme) protocol can be found at: https://www.gov.uk/government/publications/gonococcal-resistance-to-antimicrobials-surveillance-programme-grasp-protocol/gonococcal-resistance-to-antimicrobials-surveillance-programme-grasp-protocol

For further use, prepare a series of twofold working dilutions for each antimicrobial concentration 10 times higher than the final concentrations to be obtained in the agar. An example of a standardized scheme for preparing the working dilutions is shown in Table 7.10.

The range of concentrations used in the antimicrobial susceptibility testing should be adapted to each antimicrobial and to local resistance levels. For many antimicrobials, wide variations exist in the susceptibility patterns of gonococcal isolates from different countries. To reduce the number of concentrations that have to be tested, an approximation of the local variation in susceptibility for the examined antimicrobials is required. If unknown, it may be obtained by determining the lower and upper limits of the susceptibility ranges for these antimicrobials on a small number of isolates. In general, it is more relevant and important to know the upper rather than the lower limits of the MIC values.

Table 7.10: Preparing dilutions of antimicrobial agents for minimum inhibitory concentration (MIC) determination using the agar dilution method

Antimicrobial solution							
Step	Concentration (mg/L)	Source	Volume (ml)	+	Volume of distilled water (ml)	=	Concentration of working dilution (mg/L)	Final concentration at 1 : 10 dilution in agar (mg/L)
1	5120	Stock	1		NA		NA	NA
2	5120	Stock	1		1		2560	256
3	5120	Stock	1		3		1280	128
4	5120	Stock	1		7		640	64
5	640	Step 4	1		1		320	32
6	640	Step 4	1		3		160	16
7	640	Step 4	1		7		80	8
8	80	Step 7	1		1		40	4
9	80	Step 7	1		3		20	2
10	80	Step 7	1		7		10	1
11	10	Step 10	1		1		5	0.5
12	10	Step 10	1		3		2.5	0.25
13	10	Step 10	1		7		1.25	0.125
14	1.25	Step 13	1		1		0.625	0.06
15	1.25	Step 13	1		3		0.3125	0.03
16	1.25	Step 13	1		7		0.156	0.016
17	0.156	Step 16	1		1		0.08	0.008
18	0.156	Step 16	1		3		0.04	0.004
19	0.156	Step 16	1		7		0.02	0.002

NA: not applicable (the main stock, undiluted)

iii. Preparation of plates for the agar dilution method

For each antimicrobial concentration to be tested, prepare a volume of, for example, 89 ml of appropriate gonococcal agar base, such as Difco GC Medium Base (3.6 g gonococcal agar base and 89 ml distilled water) in a glass bottle, which is enough to prepare four 90-mm plates. Autoclave the agar medium in the bottle, then allow it to cool down to a temperature of 50 °C in a waterbath before aseptically adding sterile supplements (1 ml enrichment/IsoVitaleX/Vitox) and 10 ml of antimicrobial working solution, that is, in the concentration to be tested (see Table 7.10). Immediately mix gently by inverting the bottle three times, remove the bottle top, flame the mouth of the bottle and pour approximately 20–25 ml of medium into plates (90-mm diameter) to give a layer of approximately 3.5–4.5 mm. Make sure to eliminate bubbles by gently swirling the plates or quickly flaming the agar surface, for example, with a Bunsen flame. Once the agar has solidified at room temperature, store the plates inverted in sealed plastic bags at 4 °C until use. Under these conditions, there is no significant loss of antimicrobial activity for up to two weeks. However, for penicillin, which is less stable, it is recommended that plates are used within one week. Agar plates without antimicrobials incorporated to be used as a negative control should also be prepared.

iv. MIC determination using agar dilution

Preparation of bacterial inoculum: Use a sterile loop or swab to collect *N. gonorrhoeae* from an 18–24-hour pure culture on non-selective gonococcal agar medium and prepare a homogenized suspension of cells (equivalent to a 0.5 McFarland nephelometric standard, approximately 10^8 CFU per ml) in 1 ml of MH broth or sterile saline solution (suspension must be used within 15 minutes). Dilute the suspension 1 : 10 in MH broth or sterile saline solution to obtain 10^7 CFU/ml. Carefully transfer 0.5 ml of each suspension into the corresponding replicator or multipoint inoculator well.

Inoculation of plates: The agar plates must be dried before inoculation, that is, by placing them in an incubator in an inverted position with the lids ajar. The replicator or multipoint inoculator should transfer approximately 1–2 µl of each suspension onto the agar surface in circular areas with diameters of 5–7 mm, giving a final bacterial inoculum of approximately 10^4 CFU per spot. If the replicator or multipoint inoculator transfers a smaller or larger volume of suspension, the dilution of the inoculum has to be adjusted, that is, to give the final inoculum of approximately 10^4 CFU per spot. When testing small numbers of isolates, a sterile 1-µl plastic loop may be used. Inoculate a negative control plate (containing no antimicrobial) first, followed by the series of plates containing the different concentrations of antimicrobial, starting with the lowest concentration for each antibiotic. Finally, inoculate a second negative control plate to ensure that there has been no contamination during the inoculation. The 2016 WHO *N. gonorrhoeae* reference strains *(37)* are recommended for QC (see Table 7.8). Allow the inocula to dry and incubate the plates inverted for 20–24 hours at 36 ± 1 °C in a 5 ± 1% CO_2-enriched atmosphere with high humidity (70–80%). Sterilize the pin heads and wells by wrapping in foil and autoclaving, by placing them in a hot oven at 160 °C for 2 hours or by dipping them in 70% ethanol followed by flaming.

Reading of results: The results of the 2016 WHO *N. gonorrhoeae* reference strains (see Table 7.8 for the acceptable MICs of different antimicrobials *[37]*) and the negative control plates (should have pure and confluent gonococcal growth on both plates) must be reviewed and approved before reading other results. If not approved, perform troubleshooting and repeat the testing. For tested isolates, record the MIC as the lowest concentration of antimicrobial agent that completely inhibited growth (Fig. 7.10).

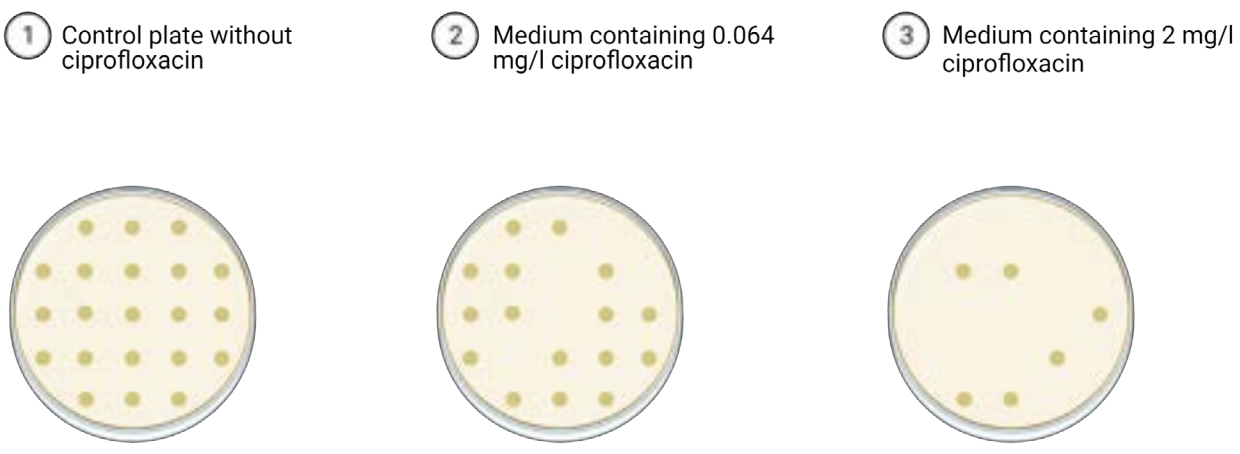
Interpretation of results: Interpret the results for the tested isolates into categories of susceptibility (see Table 7.7).

7.8.5 ETEST method to determine MIC

i. Introduction

ETEST is a quantitative technique (MIC gradient strip test) to determine the MIC of antimicrobials against microorganisms. ETEST uses plastic strips calibrated with an MIC scale in µg/ml (mg/L) and a code to identify the antimicrobial. A predefined concentration gradient of antimicrobial is immobilized on the other surface of the strip. Once applied to the surface of an agar plate, the antimicrobial diffuses into the medium. *N. gonorrhoeae* inoculated onto the medium (before adding the strip) will show an elliptical zone of inhibition of growth after overnight incubation, if any susceptibility to the test antimicrobial is present. The MIC should be read as the intersection of the ellipse and the gradient scale marked on the strip. Regarding the storage of ETEST strips, performance of ETEST and reading of the ETEST results, follow the instructions from the manufacturer precisely. Notably, other MIC gradient strip tests are commercially available; however, the performance characteristics of these can vary highly in their antimicrobial susceptibility testing of *N. gonorrhoeae (86)*. All MIC gradient strips used should be fully validated and quality-assured before being implemented into routine use.

Fig. 7.10: Results of ciprofloxacin minimum inhibitory concentration (MIC) determination for *Neisseria gonorrhoeae* using the agar dilution method



Source: Created with BioRender.com

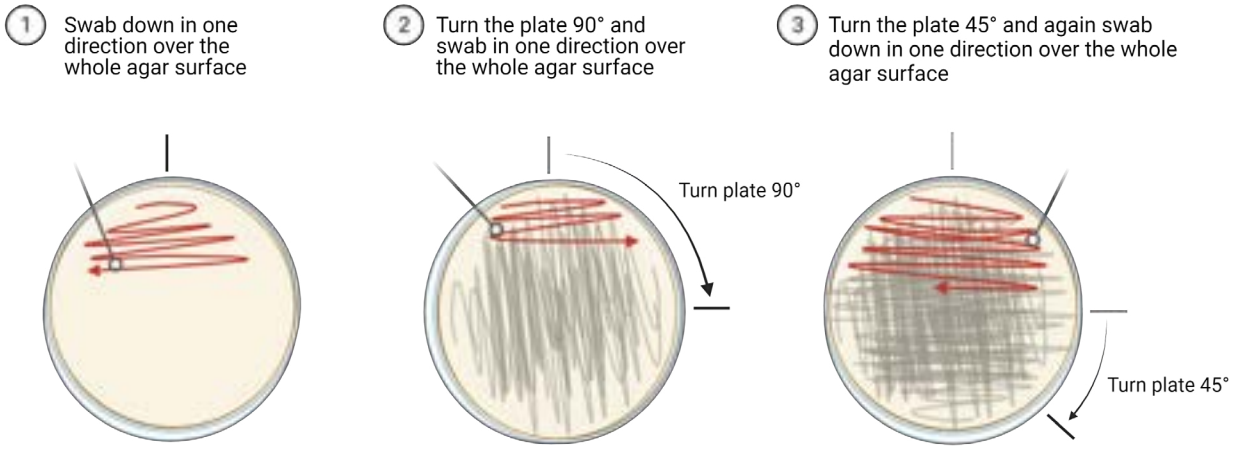
ii. Procedure to determine MIC using ETEST

1. Dry (should be free of visible moisture but do not over-dry them) the number of GCVIT agar plates (see Annex 1) needed.
2. Allow the ETEST strips to reach room temperature for approximately 30 minutes. An opened ETEST package should be stored in an airtight container with desiccant.
3. Use a sterile loop or swab to collect *N. gonorrhoeae* from an 18–24-hour pure culture on non-selective gonococcal agar medium and prepare a homogenized suspension of cells (0.5 McFarland nephelometric standard, approximately 10^8 CFU per ml) in 1 ml of sterile saline solution or PBS (suspension must be used within 15 minutes). Do not use a nutrient broth to prepare the suspension.
4. Dip a fresh sterile swab into the suspension and remove excess fluid by pressing and rotating the swab against the tube wall.
5. Swab the entire agar surface of the GCVIT plate evenly in three directions (Fig. 7.11) to produce a confluent lawn.
6. Replace the lid of the plate and allow the agar surface to dry for approximately 10 minutes.
7. Press the ETEST applicator onto an ETEST strip to pick it up (or use sterile forceps), place it on the agar surface and push the piston down to release the strip.
8. Confirm that the strip is in complete contact with the agar and remove possible air pockets carefully by streaking with a loop from lower to higher concentration of the antimicrobial.
9. Place a maximum of four ETEST strips per 140–150-mm plate and one strip per 90-mm plate.
10. Once applied to the agar, the ETEST strip should not be removed (the antimicrobial is immediately released).
11. Immediately incubate the inverted plates for 20–24 hours at 36 ± 1 °C in 5 ± 1% CO_2-enriched humid (70–80%) atmosphere (CO_2 incubator or, if not available, candle extinction jar with some additional humidity).

iii. Reading and interpretation of ETEST results after incubation

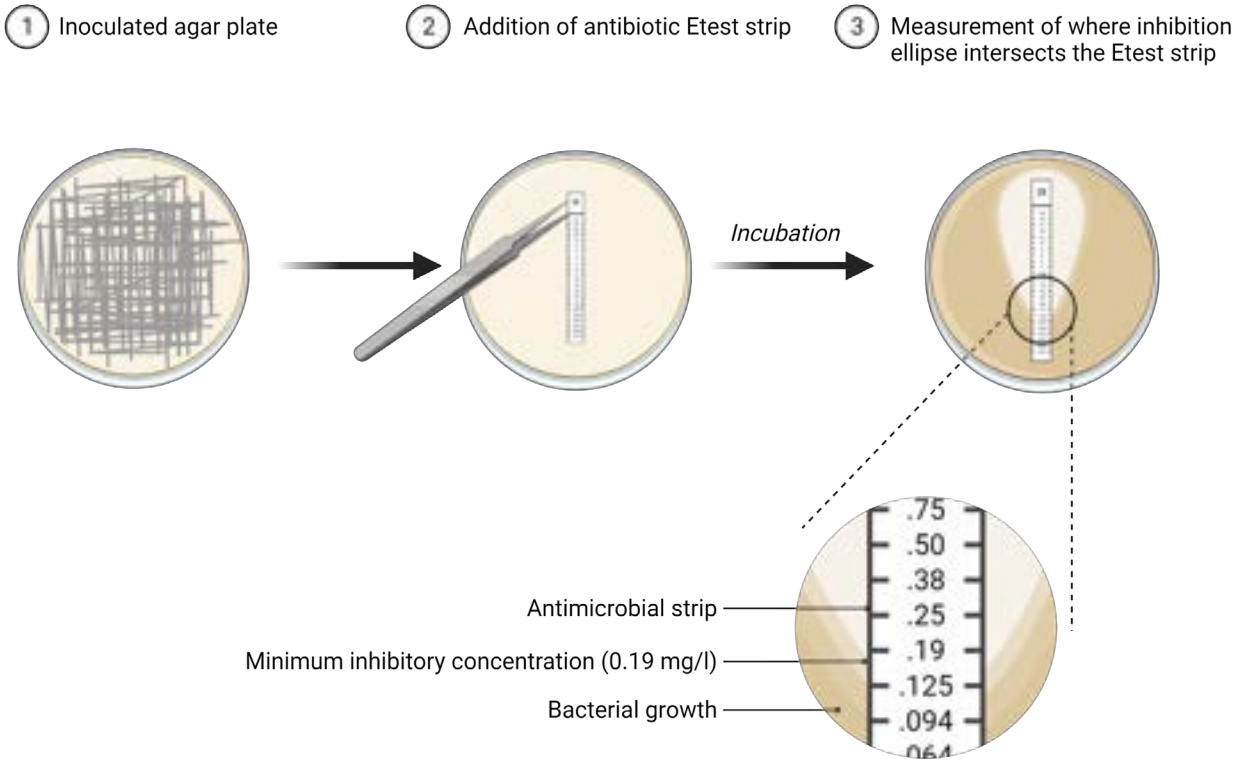
1. The results of the 2016 WHO reference strains (see Table 7.8 for the acceptable MICs of different antimicrobials *[37]*), when included as QC, must be reviewed and approved before reading other results. If not approved, perform troubleshooting and repeat the testing. For tested isolates, read the plates only when sufficient growth is seen and the inhibition ellipse is clearly visible. Otherwise, the testing has to be repeated with more growth.
2. Read the exact MIC where the ellipse (zone) intersects the MIC scale on the ETEST strip (Fig. 7.12). If the ellipse of inhibition intersects the ETEST strip in-between two MIC values, always read the highest value. Always read the end-point at complete inhibition of all growth, including hazes, microcolonies and isolated macrocolonies.
3. Growth along the entire strip implies no inhibition (i.e. the MIC is higher than the highest concentration on the strip). If the inhibition ellipse is below the strip and does not intersect the strip, the MIC is lower than the lowest concentration on the strip.
4. Interpret the results for the tested isolates into categories of susceptibility (see Table 7.7).

Fig. 7.11: Swabbing of culture plates for ETEST



Source: Created with BioRender.com

Fig. 7.12: Performance and results of minimum inhibitory concentration (MIC) determination using the ETEST for *Neisseria gonorrhoeae*, showing that the MIC of cefixime (IX) is determined at the point the ellipse crosses the strip (0.19 mg/L)



Source: Created with BioRender.com.

7.8.6 Disc diffusion method

Disc diffusion assays are qualitative techniques for categorizing isolates as susceptible, intermediate (or decreased) susceptible or resistant to different antimicrobials. Thus, these methods do not determine the exact MIC of antimicrobials against microorganisms; however, they should reflect the MICs. Disc diffusion assays are in general not recommended by WHO for *N. gonorrhoeae* and these assays should only be used when MIC determination cannot be performed, for example, because of resource constraints. Disc diffusion methods use commercially available discs that are impregnated with a known concentration of antimicrobial. The antimicrobial in the disc diffuses into the surface of the agar inoculated with the bacterial isolate and produces a concentration gradient that is highest close to the disc and proportionally decreases with the distance from the disc. After incubation, a zone of inhibition is visible that is measured and subsequently interpreted into a susceptibility category. Several disc diffusion methods are in use internationally; however, all require pronounced standardization and appropriate QC to attain a high level of reproducibility and sufficient reflection of the MIC of the examined antimicrobials. The main differences between these are the potency of discs (content of antimicrobial) and agar used, which result in different breakpoints for susceptibility categorization. The CLSI disc diffusion method *(87)* has been used in several regions. Furthermore, the calibrated dichotomous sensitivity (CDS) disc diffusion test *(89,90)* has been used for gonococcal antimicrobial susceptibility testing and surveillance in, for example, WHO Western Pacific and South-East Asia Regions. For details regarding the CDS method for *N. gonorrhoeae*, see the 2013 edition of WHO's *Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus (91)*.

7.8.7 Detection of plasmid-mediated resistance to penicillin

N. gonorrhoeae may carry plasmids that produce an enzyme (β-lactamase [penicillinase]) that inactivates penicillins such as benzylpenicillin, penicillin, ampicillin and amoxicillin. Several qualitative methods have been used to detect β-lactamase production by microorganisms.

The chromogenic cephalosporin method is simple, sensitive, specific and widely used to detect β-lactamase in gonococci. When the β-lactam ring of the chromogenic cephalosporin, nitrocefin, is hydrolysed by β-lactamase, a colour change from yellow to red occurs. The test is available commercially in a variety of formats; lyophilized nitrocefin can also be purchased for

laboratory-developed tests *(92)*. Other less standardized and quality-assured methods to detect β-lactamase, such as the acidometric method and the iodometric test, are also available.

i. Nitrocefin disc method

1. Hydrate a nitrocefin disc on a glass slide or in a clean empty plate with sterile distilled water.
2. Sample several colonies from overnight pure gonococcal culture with a sterile loop and streak onto the surface of the disc.
3. A positive reaction usually produces a red colour within 1 minute. However, weak positive reactions may take slightly longer to develop, but this is rare. A negative result will show no colour change (remains yellow).

ii. Nitrocefin solution method

The nitrocefin solution method is performed either by dropping the reagent directly onto colonies growing on selective or non-selective media or by inoculating the solution on a glass slide or filter paper with colonies. Commercially available nitrocefin solution or a solution prepared in-house using nitrocefin powder can be used.

Direct plate test

1. Add one drop of nitrocefin solution directly onto pure isolated gonococcal colonies on the agar medium. Agar dilution or ETEST agar plates may be used after reading the MICs of the examined antimicrobials.
2. A colour change of the nitrocefin solution from yellow to red within 1 minute indicates a positive result. However, rare weak positive reactions may take slightly longer to develop. A negative result will show no colour change (remains yellow) (Fig. 7.13).

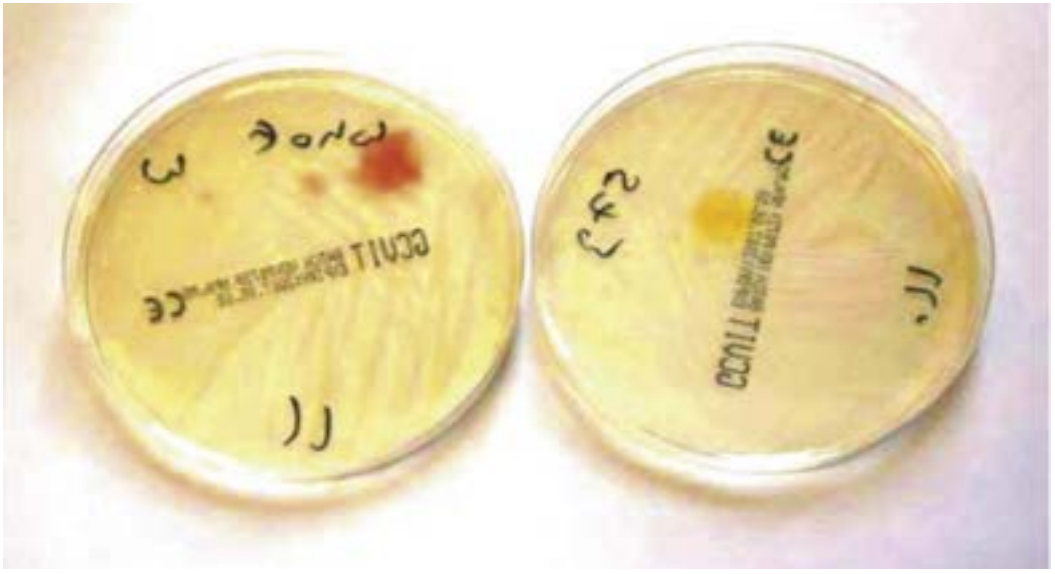
Slide/filter paper test

1. Add one drop of nitrocefin solution onto a clean glass slide or filter paper.
2. Sample several colonies from overnight pure gonococcal culture with a sterile loop and emulsify into the nitrocefin drop.
3. A colour change from yellow to red within 1 minute indicates a positive result. Rare weak positive reactions may take slightly longer to develop. A negative result will show no colour change (remains yellow).

iii. Acidometric method

Place a strip of filter paper in an empty, clean Petri dish. Saturate the paper with penicillin solution (0.05 mol/L phosphate buffer, pH 8.0, 0.2 g/L bromocresol purple and 50 g/L buffer-free benzylpenicillin [stored frozen]). With a bacteriological loop, spread 10–20 colonies over an area of approximately 5 mm of the filter paper. Incubate the inoculated filter paper at room temperature for 30 minutes with the Petri dish lid on. β-lactamase activity will result in a colour change from blue to yellow, usually visible in less than 10 minutes.

Fig. 7.13: β-lactamase activity in *Neisseria gonorrhoeae* determined on culture plate with chromogenic cephalosporin (nitrocefin test): the medium on the left demonstrates a positive reaction (red) and the medium on the right shows a negative reaction (remains yellow)



Source: Reproduced with permission from Morse et al., 2010 *(30)*.

iv. Iodometric test

A penicillin-iodine mixture is freshly prepared by adding 1.1 ml of an iodine solution (1.5 mg potassium iodide and 0.3 g iodine in 100 ml of 0.1 mol/L phosphate buffer, pH 6.4, stored in a brown bottle at 4 °C) to a vial containing 0.15 ml benzylpenicillin solution (1 million IU per ml, stored at −20 °C). The reagent mixture should be used within 1 hour. A loopful of the test microorganism is removed from the colonial growth on an agar plate and emulsified in one drop of the penicillin-iodine mixture on a glass plate. One drop of a starch solution (4 g/L in distilled water, autoclaved and stored at 4 °C) is added, giving a deep purple colour to the mixture. A negative result is indicated when this colour remains for 5 minutes. A colour change to colourless within 5 minutes (normally within 1 minute) indicates a positive test.

7.9 Preservation of *N. gonorrhoeae* isolates

To maintain the viability of *N. gonorrhoeae* strains on gonococcal agar media, it is necessary to subculture at least every 48 hours and to avoid subculturing continually for more than seven days. Thus, effective methods for long-term preservation of gonococcal strains are crucial.

7.9.1 Conservation on chocolate agar slopes

Storage up to 9 months (strains are viable during transport for up to five days):

- A pure overnight culture on gonococcal agar media is heavily inoculated onto a 3-ml volume chocolate agar slope in a polycarbonate bijou bottle with screw cap (5-ml volume; a plastic bottle must be used) and immediately incubated with the screwcap loosened for a minimum of 24 hours at 36 ± 1 °C, 5 ± 1% CO_2-enriched atmosphere or until visible growth is present on the agar surface. Sterile liquid paraffin is then used to completely fill the agar slope, the screwcap is fully tightened and the bijou bottle slope is then stored at 37 °C. When the gonococci are required for testing, a sterile bacteriological loop is inserted through the paraffin overlay to remove some *N. gonorrhoeae* growth that is inoculated on gonococcal-selective culture medium. After incubation, for 48 hours at 36 ± 1 °C, 5 ± 1% CO_2-enriched humid atmosphere, gonococcal colonies are readily discernable and can be subcultured for appropriate examination (globules of paraffin will also be present but can be easily distinguished from the gonococcal colonies). The original paraffin overlaid slope can be returned to storage for further use *(93,94)*.

7.9.2 Conservation by freezing or lyophilization

Storage up to 1–3 months:

- All growth on a plate full of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing 0.5 ml of a sterile nutritive broth (e.g. nutrient broth, tryptic soy broth, brainheart infusion) with 15–20% glycerol, suspended with a sterile pipette and immediately frozen at –20 to –25 °C. Extended storage at this temperature is not desirable because gonococci will quickly lose their viability.

Long-term storage:

- All growth on a plate full of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing approximately 0.5–1.0 ml of a sterile cryoprotective nutritive broth with 15–20% glycerol, suspended with a sterile pipette and immediately frozen at ≤ −70 °C.
- All growth on a plate full of pure overnight culture on gonococcal agar media can be inoculated in a small cryovial containing sterile cryoprotective medium (e.g. nutritive broth with 15–20% glycerol), suspended with a sterile pipette and immediately frozen in liquid nitrogen.
- All growth on a plate full of pure overnight culture on gonococcal agar media can be inoculated in a small vial containing approximately 0.5–1.0 ml of a sterile microbank fluid with cryobeads (commercially available), suspended by inverting five times; subsequently, as much liquid as possible is removed and the vial is immediately frozen at ≤ −70 °C.
- All growth on a plate full of pure overnight culture on gonococcal agar media can be inoculated into sterile nutritive broth (e.g. nutrient broth, tryptic soy broth, brainheart infusion) with 15–20% glycerol (alternatively, if no nutritive broth is available, sterile skimmed milk can be used) and preserved by lyophilization.

To note, conservation of viable gonococci is enhanced when the culture is fresh in the morning or at lunchtime after the overnight subculture; later in the day the colonies may become sticky and difficult to collect.

7.10 Retrieval of frozen *N. gonorrhoeae* isolates

Remove the cryotube containing the isolate from the freezer or liquid nitrogen and do not allow it to completely thaw. Using the tip of a sterile Pasteur pipette, gently remove a small sample of the frozen bacterial suspension (or one cryobead) and transfer it to a gonococcal agar culture medium. Use a loop to streak the inoculum for single isolated colonies and immediately incubate the culture plate for 24 hours at 36 ± 1 °C, 5 ± 1% CO_2-enriched humid atmosphere. Return the cryotube immediately to the freezer.

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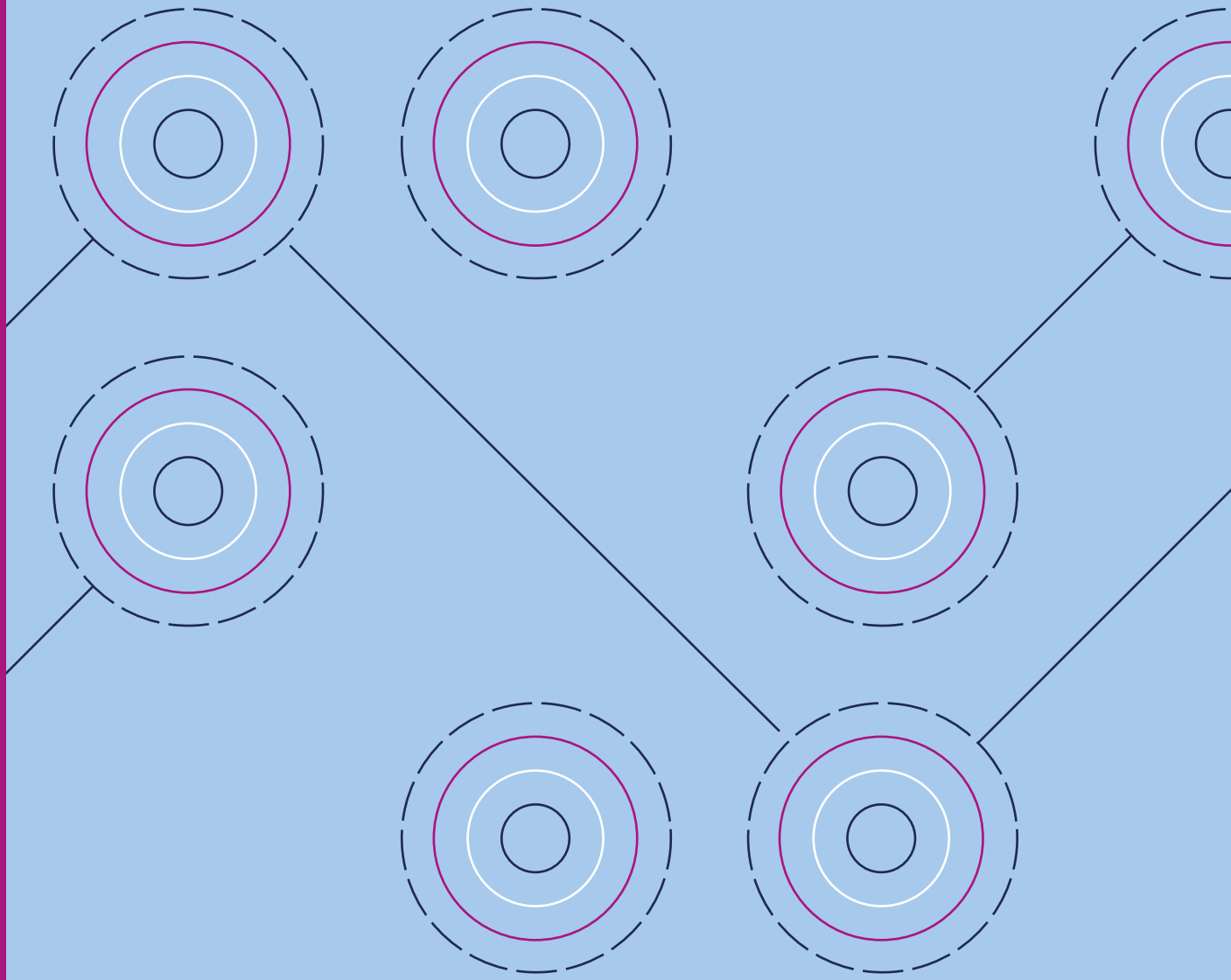


Chapter 8. Chlamydial infections

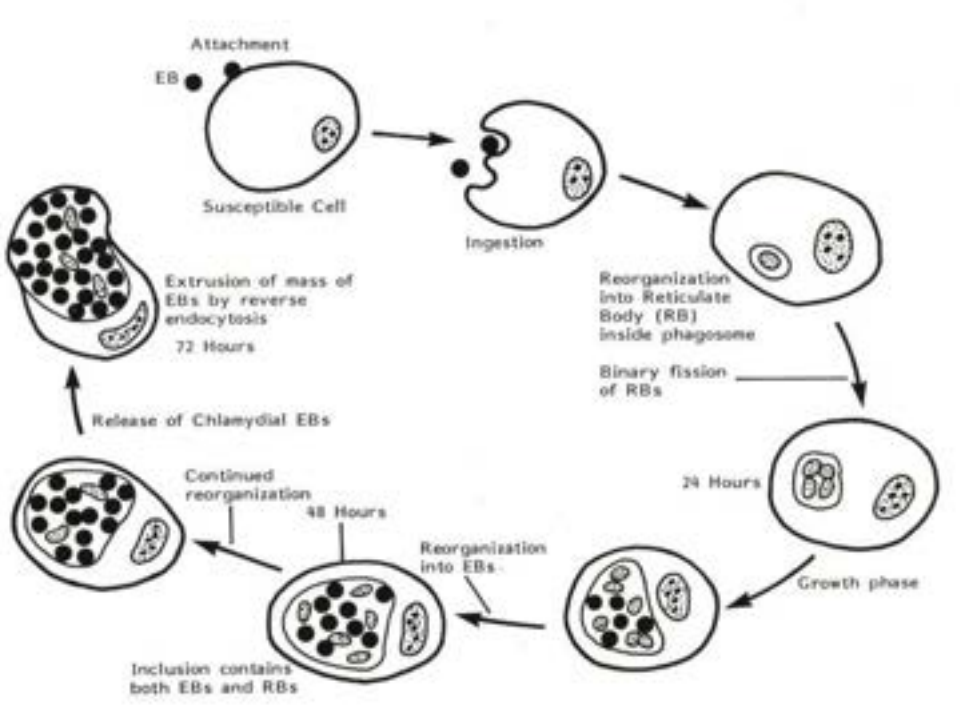
Barbara Van Der Pol and Magnus Unemo

8.1 Introduction

Chlamydia trachomatis, the etiological agent of chlamydia, causes substantial morbidity and economic costs worldwide. In 2020, WHO estimated 129 million new cases of urogenital chlamydia among adults globally *(1)*. This places chlamydia as the most prevalent bacterial STI. The burden of this infection primarily falls on women, who have higher rates of disease than men, by causing pelvic inflammatory disease (PID), tubal factor infertility (TIF) *(2)* and poor birth outcomes *(3)*.

C. trachomatis has a unique intracellular life cycle (Fig. 8.1). The bacterial species has been classified into three biovars, each containing several serovars or genotypes (depending on the method used for classification). The biovars were defined based on the type of infection, common localization of infection (tissue tropism) and relative virulence of the disease (Table 8.1): those that cause trachoma, the leading cause of preventable blindness worldwide and endemic in many developing countries; those that cause genital infection, a leading bacterial STI globally; and those that cause lymphogranuloma venereum (LGV), a genital ulcer disease that affects lymphoid tissue (see Chapter 14). The trachoma (ocular) biovar consists of serovars A–C, which are found predominantly in conjunctival infections. Tissue tropism is not absolute because these organisms, especially serovar B, may also be isolated from genital infections, but the frequency of these occurrences is rare.

Fig. 8.1: The chlamydial life cycle



Source: Courtesy of Barbara Van Der Pol.

The predominant biovar consists of serovars D–K, which are transmitted sexually and infect the genital epithelium, causing urethritis in men and cervicitis (and urethritis) in women (Table 8.2). In persons who engage in receptive anal or oral intercourse, these serovars are responsible for infections in those body sites *(4)*. Chlamydial infections in the genital compartment are asymptomatic in up to 50% of men and up to 90% of women; the proportion that are subclinical may be even higher for anorectal and oropharyngeal infections. If undetected and not treated, urogenital infection can ascend to the upper genital tract and cause epididymitis in men and PID and related sequelae (ectopic pregnancy and TIF) in women. These serovars may also be isolated from ocular infections in neonates, acquired during passage through an infected birth canal, but are largely not responsible for trachoma. Neonates also may develop chlamydial pneumonia because of exposure to these serovars during vaginal delivery (not to be confused with infection by *Chlamydia pneumoniae*).

Finally, the LGV biovar, consisting of serovars L1–L3 with subvariants such as L2a and L2b, is also a sexually transmitted biovar but with tissue preference for lymphoid cells and more aggressive disease progression. LGV infections are accordingly more invasive and likely to cause systemic infection than the other biovars. LGV is endemic in many developing settings worldwide. Since 2003, outbreaks of LGV proctitis and proctocolitis have been documented among men who have sex with men (MSM) especially in Europe, North America and other well resourced settings, which previously only observed sporadic cases (see Chapter 14). Most anorectal infections are caused by serovars D–K.

- *C. trachomatis* causes the most common bacterial STI globally, which includes a spectrum of infections in a variety of sites (i.e. genital, anorectal, oropharyngeal, ocular, lymph nodes, and bronchial).
- Negative outcomes associated with untreated *C. trachomatis* infections include PID, ectopic pregnancy, TIF, epididymitis, prostatitis and others.

8.2 Overview of available diagnostic methods

Although chlamydial infections are highly prevalent, the knowledge regarding this STI was very limited in public health settings before the 1980s due to limitations with diagnostic methods. Early diagnostic techniques were developed around trachoma control efforts rather than the STI. **Serology** has been used to distinguish between acute and chronic infection and to obtain population-based estimates of lifetime exposure, but lacks appropriate sensitivity and specificity for diagnosis of acute infection.

Table 8.1: Characteristics and infections associated with different serovars of *Chlamydia trachomatis*

Serovar	Characteristics	Tissue tropism/biovar	Infection
A-C (including Ba)	Non-invasive	Epithelial cells/trachoma	Endemic blinding trachoma
D–K	Non-invasive	Epithelial cells/genital	Urogenital, anorectal, oropharyngeal, conjunctivitis, neonatal pneumonia
L1, L2, L2a, L2b, L3	Invasive	Lymphatic cells/LGV	Lymphogranuloma venereum

Table 8.2: Clinical manifestations of infection with *Chlamydia trachomatis*

Genital infection	Primary	Sequelae
Women	Cervicitis, copious purulent discharge, friable cervix, dysuria, pelvic pain, cervical motion tenderness	Pelvic inflammatory disease, ectopic pregnancy, salpingitis, tubal factor infertility
Men	Urethral discharge, dysuria, testicular pain	Epididymitis, prostatitis
Non-genital infections		
Rectal	Discharge, rectal pain, blood in stool	Proctitis
Oropharyngeal	Pharyngitis, mild sore throat	
Lymph nodes	Lymphatic inflammation	
Ocular	Conjunctivitis	Scarring, blinding trachoma
Neonatal pneumonia	Pneumonia	

This assay cannot distinguish current from past infection. Culture was standardized in the 1970s, making isolation of the microorganism a useful diagnostic tool. **Culture** offers proof of infection; however, the need to maintain microorganism viability requires stringent transport and storage conditions and this service is now offered only in specialized reference laboratories. **Antigen detection assays, direct immunofluorescence assays (DFAs) and solid phase enzyme-linked immunosorbent assays (ELISAs)** were developed in the early 1980s, making diagnosis of chlamydial infections more available given the lack of a viability requirement. As additional ELISAs were developed, several rapid, **antigen-based point-of-care (aPOC)** assays became available. DFA, lab-based ELISA and aPOC suffered from low sensitivity relative to culture and had suboptimal specificity. While antigen detection offers rapid proof of active infection, decreased sensitivity (as low as 50–60% compared to molecular diagnostic methods) limits the utility of these tests in most settings. High-prevalence populations with very limited probability of return for treatment could benefit from these tests if no other options are available. Their use should be restricted to diagnostic use among patients with symptoms and they should never be used for population screening among asymptomatic individuals.

The next major advance in chlamydia diagnostics was the use of nucleic acid sequences rather than antigens as detection targets. The sensitivities of the initially developed **non-amplified nucleic acid probe (NAP) assays** were similar to that of culture, but again there were questions regarding specificity. The addition of *Neisseria gonorrhoeae* to the test menu made this assay extremely attractive. **Nucleic acid amplification tests (NAATs)** were the next diagnostic development. NAATs use enzymatic methods to amplify target DNA or RNA exponentially into billions of copies. Amplification technology can differ and amplified products are detected using a variety of means, giving each assay unique performance characteristics. Like the NAP test, NAATs combined chlamydia and gonorrhoea testing from a single sample. Several NAATs can now be performed in as little as 30 minutes and performed by non-laboratory personnel, thus offering a new POC option: **POC NAAT**. Because of the superior performance characteristics, for example, sensitivity, specificity, range of specimen types, automation and independence from maintaining microorganism viability, NAATs are strongly recommended for the diagnosis and screening of chlamydial infections. While the performance estimates of NAATs may vary due to differences in nucleic acid extraction efficiency, and amplification and detection chemistries, as a class, this type of test is consistently the highest quality diagnostic tool available for the detection of *C. trachomatis*.

Issues associated with assay evaluation and performance variability *(5,6)* underscore the need for verification of any assay adopted by the laboratory and strict quality assurance (QA) within each laboratory, not only before adoption of a method, but also on a recurring basis. For further information on this topic, see Chapters 1 and 5. For adequate performance characteristics of all diagnostic methods, it is crucial to precisely follow the recommendations from the manufacturer concerning collection, transport and storage of samples, as well as performance of the specific assay, including use of quality controls. When commercially available assays with evaluation by a regulatory body (e.g. the U.S. Food and Drug Administration (FDA)[27] or the European Union CE In Vitro Diagnostics Certification processes), these are preferred over laboratory-developed tests (LDTs) that may not have been subjected to the same level of scrutiny as approved assays. The characteristics of diagnostic tests with FDA clearance (July 2022) for detection of *C. trachomatis* are summarized in Table 8.3.

Table 8.3: Diagnostic tests with U.S. FDA clearance (as of July 2022) for the detection of *Chlamydia trachomatis*

	NAAT (laboratory-based)	Culture	aPOC	POC NAAT
Specimen types				
Endocervical swab	Yes	Yes	Yes	No
Liquid cytology medium	Yes	No	No	No
Vaginal swabs				
Self-obtained	Yes	No	Yes (poorly performing)	Yes
Clinician-collected	Yes	No	Yes (poorly performing)	Yes
Specimen types				

[27] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

Table 8.3 (continued): Diagnostic tests with U.S. FDA clearance (as of July 2022) for the detection of *Chlamydia trachomatis*

	NAAT (laboratory-based)	Culture	aPOC	POC NAAT
Urine				
Female	Yes	No	No	No
Male	Yes	No	No	Yes
Male urethral swab	Yes	Yes	Yes	No
Rectal swab	Yes (most modern assays)	Yes	No	No[a]
Oropharyngeal swab	Yes (most modern assays)	Yes	No	No[a]
Conjunctival swab	No[a]	Yes	No	No[a]
Performance				
Sensitivity	Very high	Moderate to high	Low to moderate	Very high
Specificity	Very high	Very high	High	Very high
Cost	Very high	High	Low	High
Transport and storage	2–30 °C (see IFU)	4 °C up to 24 hours, –70 °C after 24 hours	NA	2–8 °C, up to 4–24 hours (see IFU)
Instrumentation	Large footprint	Routine microbiology/ virology	Small to none	Small
Throughput/automation	High/yes	Low/no	Low/no	Low/no
Technical complexity	High	High	Low	Low
Level of laboratory infrastructure	Reference	Reference	Site	Site
Multiple pathogens from one sample (discharge-causing STIs, vaginal infections and human papillomavirus, depending on the assay)	Yes	No	No	Yes
Other comments	• Due to the superior performance characteristics, NAATs are strongly recommended for diagnosis and screening. • Potential for laboratory contamination requires strict adherence to protocols. Some require large batch size, which may delay turnaround time.	• Strict collection and transport is crucial to maintain viability; this is the major barrier to high sensitivity with this assay. Potential to obtain viable isolates is useful for additional testing, such as genotyping and antibiotic susceptibility testing.	• These tests perform poorly compared to other options, but may be better than no testing in high-prevalence populations when return for treatment is unlikely as these tests support test and treat at the same visit.	• These tests have performance that is near that of laboratory-based NAATs. Given the low technical requirements and rapid results, these support test and treat in settings where financial resources are available.

aPOC: antigen point-of-care; IFU: instructions for use; NA: not applicable; NAAT, nucleic acid amplification test.

[a]Data indicate that appropriate NAATs and POC NAATs perform well for these sample types, but not all manufacturers have a claim for these specimens.

Source: Adapted from Unemo and Papp, 2010 *(7)*, pp. 40–63.

- The field of chlamydia diagnostics is rapidly evolving and the reader is encouraged to search Google Scholar for *chlamydia trachomatis* diagnostics for up-to-date publications.
- Because of superior performance characteristics, NAATs, including POC NAATs are strongly recommended for the diagnosis and screening of chlamydial infections. However, the choice of tests is dependent on the resources available, the population served and the available level of laboratory support.

8.3 Nucleic acid amplification tests (NAATs)

Molecular detection of specific nucleic acid sequences and subsequent validation and commercialization of such assays have vastly improved laboratory detection of *C. trachomatis*. NAATs have superior performance characteristics compared to any other test types for the detection of chlamydial infections *(8–11)*; thus, they are the assay type recommended for both the diagnosis of genital and extragenital sites, and screening for chlamydial infections by the Centers for Disease Control and Prevention (CDC) in the USA *(12)*, the European Centre for Disease Prevention and Control (ECDC), the International Union against STIs (IUSTI) *(13)* and other regulatory and advising bodies. Confirmatory testing of *C. trachomatis*-positive NAAT samples is no longer recommended. Validated and effective NAATs have several advantages that are independent of the manufacturer. They are clearly the most sensitive assays available, which creates opportunities of sample pooling in resource-limited settings; they are independent from microorganism viability; most commercially available assays can simultaneously test for both chlamydia and gonorrhoea; and these assays have a broader range of useful sample types that includes less invasively collected penile urine samples, penile meatal swabs and vaginal swabs in addition to the previously required endocervical and urethral swabs. Additionally, these assays are well suited to automation, which results in increased standardization and QA of extraction, amplification and detection, as well as significantly increased throughput. However, NAAT assays also share some common difficulties. Laboratory-based NAATs require substantial instrumentation and are not appropriate for many laboratory settings in resource-constrained countries. These assays also are susceptible to environmental contamination due to the exponential amplification of target sequences. Any action that involves an open amplified sample is a potential source for aerosol formation and environmental contamination. Once laboratory facilities and equipment (e.g. pipettors) become contaminated, recovering from carry-over contamination may be extremely difficult. Most commercially available assays have built-in safeguards (chemical or mechanical) to reduce the potential for such contamination events, which is another reason to use these assays rather than LDTs.

Another issue involves the potential for false-negative results. Nucleic acid extraction efficiency is critical to the detection process, but it is difficult to guarantee for each sample unless an assay includes a process control within each amplification reaction. Amplification requires precise salt, nucleotide and enzyme concentrations to proceed efficiently, which requires high precision in pipetting. The enzyme that promotes amplification may also be sensitive to the components of blood, mucus and urine. Therefore, a negative result may actually reflect a lack of target nucleic acid due to inadequate sample collection or extraction, or a lack of amplification rather than a lack of target sequence. The proportion of samples that are inhibitory can vary according to test method and, using some methods, may be as high as 7.5% in certain populations *(14)*. Finally, diagnostic escape *C. trachomatis* mutants can evolve, by mutations involving the NAAT target(s), and result in false-negative results *(15–19)*. However, the performance of NAATs is still strongly preferable to that of culture, which can provide false-negative results due to loss of microorganism viability, and other non-NAAT methods, which have sensitivity lower than (or at best equal to) culture while also having suboptimal specificity. Therefore, despite the need for appropriate laboratory caution, NAAT advantages substantially outweigh the disadvantages. Use of NAATs should be encouraged even in resource-constrained settings by creating regional reference laboratories that can provide diagnostic services using these methods. This option is enabled by the high stability of the samples once placed into an assay transport buffer. Regional reference laboratories provide many advantages that result from larger testing volumes, rigorous adherence to good laboratory practices and improved technical expertise. Use of regional laboratories may reduce costs while using assays with the highest sensitivity. Despite the need to send samples out to a regional facility, the high volume and access to high-throughput automation may result in equivalent or reduced turnaround times. For these reasons, laboratories that cannot afford to perform the most sensitive NAAT assays should consider using the services of a regional reference laboratory rather than using a less sensitive diagnostic tool. As POC NAATs become more widely available, even smaller, more remote laboratories and clinics will have the capacity to test patients on site using highly sensitive molecular assays. This is the ultimate goal for the near future. Given the rigorous evaluation required before approval of a diagnostic test by the FDA, which includes multisite clinical trials with comparisons against appropriate standards, the performance of FDA-approved assays is well documented. Therefore, we use this level of evaluation as the standard to determine high-quality assays. Table 8.4 summarizes the features of FDA-cleared molecular diagnostic tests for the detection of *C. trachomatis*.

Table 8.4: U.S. FDA-approved *Chlamydia trachomatis* NAATs (July 2022)

Assay (manufacturer)	Sample types	Testing location	Footprint	Technical skill required	Time to clinical results
RealTime CT/NG (Abbott)	Endocervical samples, vaginal and urethral swabs, urine. Conjunctival samples[a]	Laboratory	Large, floor	High	1–2 days
Alinity m STI (Abbott)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Large, floor	High	1–2 days
CTQx/GCQx (Becton Dickinson)	Endocervical samples, vaginal swabs, urine	Laboratory	Large, floor	High	1–2 days
CTGCTV2 for BD MAX system (Becton Dickinson)	Endocervical samples, vaginal swabs, urine	(On-site) laboratory	Medium, benchtop	High	4 hours if on-site laboratory
Xpert CT/NG (Cepheid)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine. Conjunctival samples[a]	On-site laboratory	Small, benchtop to large, floor	Low	90 minutes
Aptima Combo 2 (Hologic)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Large, floor	High	1–2 days
Aptima CT (Hologic)	Endocervical samples, vaginal swabs, urine	Laboratory	Large, floor	High	1–2 days
cobas CT/NG 4800 (Roche)	Endocervical samples, vaginal and urethral swabs, urine	Laboratory	Large, floor	High	1–2 days
cobas CT/NG 68/8800 (Roche)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Very large, floor	Moderate	1–2 days
cobas CT/NG 5800 (Roche)	Endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine	Laboratory	Large, floor	Moderate	1–2 days
binx health io CT/NG (binx health)	Vaginal swabs, penile urine	Clinic	Small, benchtop	Low	30 minutes
Visby Medical Sexual Health CT/NG/TV (Visby Medical)	Vaginal swabs	Clinic	Very small, handheld/ benchtop	Low	30 minutes

CT: *Chlamydia trachomatis*; NAAT: nucleic acid amplification test; NG: *Neisseria gonorrhoeae*; TV: *Trichomonas vaginalis.*

[a]These assays have been well validated for use with conjunctival samples. There is reason to assume other assays would perform as well. No assay has a U.S. FDA claim for this specimen type.

8.3.1 Collection, transport and storage conditions of specimens for NAATs

NAATs have the advantage of reliance on nucleic acid materials that do not require viable or intact microorganisms. NAATs are highly sensitive when using self-obtained samples; vaginal swabs or penile urine are the optimum genital sampling types. Because of the invasive nature of sample collection with no improvement in case-finding, endocervical (see the culture sample collection method if there is a need for this sample type to be used) and urethral swabs should not be the first choice of specimen for NAAT. The transport and storage conditions of specimens (Table 8.5) are assay-dependent in many cases. This section presents some general guidelines, but details related to any specific diagnostic assay should be taken from the appropriate assay instructions for use (IFU) documents.

Table 8.5: Detailed sample collection instructions for nucleic acid amplification tests (NAATs)

Site	Collection device[a]	Sampling procedure[b]	IFU[c] for storage conditions and stability
Vagina	Swab/plastic	Clinicians or patients can obtain samples. Rotate the swab to come into contact with the vaginal walls on all sides.	Place into the manufacturer's collection device. Many assays can support dry swabs received in no medium.
Urine	Sterile urine cup	Do not have patient clean the genital area. Obtain first portion of the void (< 25 ml preferably). ≥ 1 hour after urination.	Mix well and aliquot into the manufacturer's collection device using the provided transept. Fill the tube to the window.
Endocervix	Swab/plastic, liquid cytology brush or broom	Insert the collection device 2–3 cm and rotate the swab 360° in the endocervical os.	Place into the manufacturer's collection device or use liquid cytology medium if the sample is intended for Pap testing.
Rectum	Swab/plastic	Insert swab into the anorectum until the material is fully inserted and rotate 360°.	Most manufacturers have a claim for this sample type. Verify in the assay IFU.
Oropharynx	Swab/plastic	Swab the posterior pharynx and the tonsillar pillars.	Most manufacturers have a claim for this sample type. Verify in the assay IFU.
Conjunctiva	Swab/aluminium	Swab the surface of the inferior palpebral conjunctiva.	No manufacturers currently have a claim for this sample type. However, this sample type works well in several NAATs.

IFU: instructions for use.

[a]Avoid wooden-shaft swabs: use plastic-shaft swabs for larger surfaces (e.g. vaginal sampling) or aluminium-shaft swabs for smaller surfaces (e.g. conjunctiva).

[b]See assay IFU for additional collection instructions specific to the assay in use.

[c]Storage and stability conditions vary according to assay, so refer to the IFU of the assay in use.

i. Persons assigned female sex at birth

Vaginal swabs (optimal) may be patient- or clinician-obtained with equal utility *(20,21)*. Insert the assay-provided swab into the vagina 1–2 cm and rotate to sample the vaginal walls completely. If collected by a clinician, these samples should be collected before the insertion of a speculum and the sample should not be collected from the vaginal fornix because the material pooled in this area is often too concentrated and results in invalid test results. The patient should be asked about recent use of vaginal lubricants, drying agents or cleansing products because these may have an inhibitory effect on NAATs. Ideally, no products should have been used in the previous 24 hours. Menstruation does not create poor quality samples; however, in these cases urine collection may be preferred. Vaginal swab collection during pregnancy does not pose a health hazard. Several POC NAAT tests have claims only for vaginal swabs, making this the only possible sample type.

Urine from biological females is significantly less sensitive than other sample types (i.e. vaginal swabs *(22)* or endocervical specimens) missing 10–20% of infections *(23)*. This is because few infections are in the urethra and thus urine is only capturing material from the cervix that drips into the urine specimen. In addition to the low quantity of microorganisms, the dilution factor of urine decreases sensitivity. However, in cases where vaginal sampling is a contraindication, urine NAAT is a better choice than performing other tests than NAAT. The urine should be the **first-catch** portion, ideally limited to ≤ 25 ml, and the patient should not have urinated within the previous hour.

Endocervical samples are difficult to collect and highly invasive. However, NAATs are useful for testing residual liquid-based cytology (LBC) medium, allowing screening to be performed for women undergoing Pap testing. This should only be used if vaginal swab sampling (in addition to Pap sample collection) is not possible because the sensitivity of this sample type is lower due to the dilution in 10–20 ml of cytology preservative medium.

ii. Persons assigned male sex at birth

Penile urine (optimal) specimens are the optimal sample type because this sampling method washes the microorganisms from the urethra where infection occurs. The urine should be the **first-catch** portion, ideally limited to ≤ 25 ml, and the patient should not have urinated during the previous hour.

Penile meatal swabs have been shown to work in several NAAT assays *(24–26)*. However, performance is variable and no assays have claims for this sample type. When a meatal swab may be useful (e.g. if trying to avoid transporting liquids or for patients who have trouble providing a urine specimen), it should be collected by passing a swab along the meatus to capture material from the urethral opening.

If a **urethral swab** is collected for a microscopy smear, the swab may be placed into a NAAT transport tube with buffer and sent to the lab for testing.

iii. Non-genital samples

Non-genital sampling is required in some cases, that is, depending on clinical signs and sexual practice. Several commercially available NAATs have U.S. FDA clearance[28] for these sample types based on strong performance characteristics *(27,28)*.

Anorectal swabs are used to detect rectal *C. trachomatis* strains (LGV or genital strains) in anyone reporting receptive anal intercourse; the presence of symptoms is not required. Self-collection of samples for use with NAATs is preferred by most patients, works well and may increase uptake of screening and reduce the burden of clinical time *(29,30)*. Use of NAATs with extra-genital samples in MSM increases case-finding substantially compared to genital-only testing *(31–34)* and all NAATs perform similarly for detection using this sample type *(35)*. As NAATs are capable of detecting shed DNA/RNA, testing for rectal infections can be performed using anorectal swabs that do not involve insertion past a sphincter and are thus more comfortable for the patient. Full insertion of the swab material and a complete rotation of the device is sufficient for this sample type. For suspected LGV, see Chapter 14.

Oropharyngeal samples should be taken from the posterior pharynx; both tonsillar pillars should be used to detect throat infections potentially transmitted during oral sex, preferably using a NAAT test *(35)*. Collection is best performed by a clinician because of the induction of a gag reflex. However, if circumstances dictate patient collection, strongly council the patient to swab all appropriate surfaces to avoid a false-negative result.

Conjunctival samples should be taken by retracting the inferior eyelid and using a swab moving across the surface of the inferior palpebral conjunctiva towards the median corner of the eye. This sample type works well in several assays and presumably can be performed on any of the commercially available NAATs.

- Specimen collection, transport and storage conditions vary according to detection assay and may have a significant influence on the sensitivity of testing.
- Selection of the appropriate specimen and detection assay are crucial for effective diagnosis.

8.3.2 Laboratory-based NAATs

Five companies currently have U.S. FDA-approved commercially available laboratory-based NAATs for the detection of *C. trachomatis*. The assays marketed by these manufacturers are described in the next sections. All commercially available and approved NAATs detect LGV as *C. trachomatis*-positive, but without distinguishing the results as L1–L3-positive. For this purpose, genotyping is necessary (see Chapter 14). Globally, there are many additional commercially available or even laboratory-developed *C. trachomatis* NAATs in use *(36,37)*. If non-FDA-approved NAATs are used, regional (such as the European Union [EU]) or other national regulatory processes should provide safeguards on the quality and performance of the diagnostic NAAT. Use of internationally approved NAATs is strongly recommended. If this is not possible, it is strongly recommended that the effectiveness of the proposed NAAT for the local setting is strictly validated and quality-assured before use against at least one internationally approved NAAT and subsequently used with appropriate positive, negative and inhibition controls; participation in an appropriate external quality assessment system is also strongly recommended (see Chapters 1 and 5). For basic information regarding different NAAT technologies, see Chapter 4. A brief description of FDA-approved NAATs is provided below, with assays in alphabetical order of manufacturer.

- Molecular amplification technologies (NAATs) offer the best sensitivity with excellent specificity.
- Molecular assays reduce the need for strict transport and storage conditions and remove subjective analysis (e.g. microscopy).
- If internationally approved NAATs cannot be used, it is strongly recommended that the effectiveness of the proposed NAAT for the local settings is strictly validated and quality-assured against at least one internationally approved NAAT before use.
- New assays are becoming available rapidly and cannot be anticipated in this document (see WHO, 2023 *[38]*). It is important to assess the relevant literature continually for high-quality evaluations of new assays to determine the best fit for each laboratory (i.e. search Google Scholar for *Chlamydia trachomatis* diagnostics and check the U.S. FDA's list of microbial tests).[29]

[28] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

[29] The U.S. FDA's list of microbial tests is available at https://www.fda.gov/medical-devices/in-vitro-diagnostics/nucleic-acid-based-tests#microbial.

i. Abbott NAATs

- The **Abbott RealTime CT/NG** and CT-only assays are run on the automated **m2000 system** (Abbott Laboratories). This assay has replaced the FDA-approved LCx assay, which was one of the first commercially available NAATs. The RealTime polymerase chain reaction (PCR) assay uses the m2000sp, an automated sample preparation instrument that extracts DNA using a magnetic particle-based capture system. After extraction, the m2000sp instrument loads the master mix into the PCR tray, adds the purified nucleic acids, which are ready for real-time amplification and detection in the m2000rt *(39)*. This real-time thermal cycler detects amplification by fluorescence emitted when probes bind specifically to amplified target sequences during each amplification cycle. The assay now includes dual targets, that is, two sequences on the cryptic plasmid (7–10 copies per microorganism), for *C. trachomatis* to ensure detection of the Swedish new variant of *C. trachomatis* (nvCT), which caused thousands of false-negative reports in Sweden and other Nordic countries using NAATs at that time available from Abbott and Roche *(15–17)*. The system can detect multiple signals that allow the assay to detect *C. trachomatis*, *N. gonorrhoeae*, and a non-competitive internal control to measure potential inhibition in each sample. The internal control sequence is based on plant DNA that is added during the DNA extraction step to provide a measure of extraction efficiency and potential PCR inhibition. The system is capable of processing 96 samples per run, including three controls, allowing 186 samples and six controls to be tested in approximately 8 hours.

  Approved samples include urine, vaginal, endocervical and urethral swabs. Conjunctival samples have been tested as part of trachoma elimination projects and the assay works well for this purpose *(40,41)*. Swab and urine samples are collected using the multi-Collect Specimen Collection Kit provided by the manufacturer and are stable at 2–30 °C for up to 14 days before testing. The kit is a single collection device for all sample types that can run simultaneously on the m2000 system.

  The RealTime CT/NG assay has excellent analytical sensitivity and compares well even with the Aptima Combo 2 (AC2) assay (Hologic) *(42)*. The DNA purification process is designed to remove potential inhibitors, while the internal control provides a warning if inhibition exists. The process is designed to remove naturally occurring fluorophores that might interfere with test performance. The use of homogeneous fluorescence detection technology with specific PCR primers combines amplification and detection into a one-step closed system. The closed nature of the system reduces the potential for carry-over contamination; use of a negative control provides the user with a method for rapidly identifying environmental contamination.

- The **Alinity m STI** assay is a next-generation NAAT from Abbott that can process up to 300 samples per 8-hour shift. This assay is run on the **Alinity m** platform and can simultaneously detect *C. trachomatis, N. gonorrhoeae, Trichomonas vaginalis* and *Mycoplasma genitalium* making it the first FDA-cleared quadriplex assay available for discharge-causing STIs. The assay detects ribosomal RNA (rRNA) for *C. trachomatis*, *T. vaginalis* and *M. genitalium* and detects a chromosomal DNA sequence for *N. gonorrhoeae*. The assay uses magnetic microparticle nucleic acid extraction followed by a PCR assay for amplification and real-time detection of fluorescent outputs. There are no hands-on steps after starting the assay. The assay includes a human cellular control and an internal control to assess specimen adequacy and serve as extraction and amplification controls. No open samples are handled after amplification to minimize the potential for carry-over contamination. The assay detects the Swedish nvCT; the Finnish and other nvCTs were not assessed *(15–19)*.

  Approved sample types for chlamydia include vaginal swabs (patient- or clinician-collected), endocervical, oropharyngeal and anorectal swabs, and penile urine. All sample types are collected using the Alinity multi-Collect Specimen Collection Kit and sample tubes are then placed directly into the Alinity m system for testing. Specimens in the transport buffer are stable for up to 60 days if stored at 2–8 °C. When compared to the m2000 and Hologic Aptima Combo 2 assays, sensitivity and specificity estimates were 98.5% and 99.3%, 94.5% and 99.4%, and 97.2% and 99.5% for self-obtained vaginal swabs, endocervical swabs and penile urine, respectively (data from FDA submission documents[30]). Sensitivity and specificity estimates for oropharyngeal and anorectal samples were 93.3% and 99.9%, and 94.5% and 99.6%, respectively. This assay was only recently approved by the FDA, but has been available in Europe where comparative studies have supported the use of this assay *(43,44)* in laboratories performing NAAT testing. Eventually, Abbott will phase out the m2000 to be replaced by the Alinity m platform.

ii. Becton Dickinson NAATs

- Becton Dickinson's second-generation assay is the U.S. FDA-approved **ProbeTec ET CTQx/GCQx on the Viper System with XTR Technology.** The Q^x test uses isothermal strand displacement amplification to amplify and detect target sequences simultaneously at 52.5 °C. Amplification of a sequence of the chlamydial cryptic plasmid occurs in a sealed plate with a fluorescent energy transfer read-out. This assay is semi-automated and can generate 278 results during a single 8-hour work shift. The manual steps involve preheating the samples to improve the lysis process. After preheating, samples are transferred to the Viper instrument and only a single intervention

[30] The U.S. FDA submission documents can be accessed at https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm?ID=K202977.

(plate sealing) is performed after that point. This assay uses duplex chemistry that includes amplification detection paired with each chlamydial or gonococcal detection reaction to verify extraction success and the absence of inhibition. The system is highly robust with excellent time motion characteristics *(45)*.

Approved samples for the ProbeTec tests include endocervical, patient-collected vaginal and urethral swabs, as well as urine. Vaginal swabs may be collected and sent to the laboratory without transport buffer if they are stored at 2–30 °C, transported to the laboratory and placed into buffer within 14 days. Swabs in buffer are stable under these conditions for up to 30 days. Urine, stored at 2–30 °C must be transferred (2–3 ml) to the buffer within a day of collection and is then stable for another 30 days under the same storage conditions. Endocervical samples in LBC may be used in the assay by transferring 0.5 ml LBC to a Q^x transport tube. These samples can then be stored for up to 30 days like the other sample types. When compared to ProbeTec (no longer available) and Aptima Combo 2, sensitivity has been estimated at 96.5%, 91.3%, 93.0%, 92.1% and 98.0% for vaginal swabs, endocervical swabs, female urine, urethral swabs and male urine, respectively. All specificity estimates were ≥ 98.3% *(46)*.

- Becton Dickinson's newest assay is the **CTGCTV2** assay for the **BD MAX system**. This assay is a true triplex real-time PCR assay that is performed on a benchtop instrument *(47)*. The assay is "unitized" in that specimen processing and DNA extraction are performed in a single strip per sample that contains all of the necessary reagents and consumables. The instrument transfers extracted samples in master mix to a microfluidic amplification card (with space for 24 reactions) from which the amplified product is detected in real-time using fluorophore emissions. The samples for this assay do not require pre-warming; the collection tubes are placed directly into racks and strips and amplification cards are added as needed. The instrument can test 1–24 strips during a run, thus providing a solution with a highly sensitive NAAT that can be run in response to a limited number of tests. This is ideal for settings that have a hospital laboratory running small volumes of samples as the need for batching at approximately 96 samples to be cost-efficient is removed. This may be a tool that is appropriate for laboratories that want to test locally (to avoid shipping costs and delays) and have staff that can perform the automated testing in small batches. Testing is complete in approximately 3.5 hours, but samples cannot be added once the instrument starts a run. Thus, it is unlikely that this solution would support test and treat on the same day as the clinic visit.

  Approved sample types include endocervical specimens (swab or LBC), vaginal swabs and urine from men or women *(46)*. Because the assay is a triplex that includes testing for *T. vaginalis*, the preferred sample type for women is the vaginal swab (either self- or clinician-collected). Sensitivity estimates from clinical studies performed in the United States for this assay were 98.4%, 94.5%, 92.7% and 96.7% for vaginal swabs, endocervical swabs, LBC and male urine, respectively. Female urine performed similarly to female urine in other tests, but was not evaluated against full patient infection status that included other genital sites of infection (see the CTGCTV2 for BD MAX package insert for details).

iii. Cepheid NAATs

- The Cepheid **Xpert CT/NG** assay performed on the GeneXpert instrument is a widely available NAAT that can be performed at near-POC and takes approximately 90 minutes to complete. The assay is a cartridge-based test that uses 5′ exonuclease real-time PCR. Individual cartridges are self-contained to minimize the potential for carry-over contamination *(49)*. The Xpert CT/NG assay contains a cellular control *(50)* to provide indirect evidence of specimen adequacy and a sample-processing control to verify that negative results are not due to inhibition. The assay requires a single transfer step followed by insertion of the cartridge into an instrument bay, making it simple to use. The GeneXpert instrument comes in 2-, 4-, 8-, 16- and 48-bay sizes allowing the user to support the throughput necessary for the clinic's needs. An automated 80-bay system (GeneXpert Infinity) is available for use in very high-throughput clinics or in reference laboratories. Many laboratories around the world have access to GeneXpert and are familiar with its use because of the tuberculosis assay on the same system.

  Approved sample types include endocervical, vaginal, anorectal and oropharyngeal swabs, as well as urine from both men and women *(51,52)*. In a large US clinical trial enrolling 1722 women and 1387 men, sensitivity was estimated at 98.7%, 97.4%, 97.6% and 97.5% for vaginal and endocervical swabs, and female and male urine, respectively *(53)*. The Xpert package insert estimates the oropharyngeal and rectal sample sensitivities to be 95.9% and 86.0%, respectively. The assay has also been validated for use with ocular specimens *(41)*.
- An updated version of the Xpert CT/NG will have a shorter run time and will be appropriate for use by non-laboratory trained personnel.

iv. Hologic NAATs

- The U.S. FDA-approved **Aptima Combo 2** assay is based on the principle of rRNA target capture (intended to reduce or eliminate the inhibition of amplification), that is, isolation of target rRNA sequences using capture oligonucleotides and DNA magnetic beads, followed by amplification using transcription-mediated amplification technology of a sequence of the 23S rRNA of *C. trachomatis*. Amplification is detected using the kinetics of light emission from labelled DNA probes complementary to the target region. Data confirming the lack of inhibition using this assay have been obtained primarily through the use of negative patient samples spiked with laboratory strains of *C. trachomatis (54)*. The updated Aptima Combo 2 assay

(55) targets two different *C. trachomatis* 23S rRNA sequences, that is, to ensure detection of the Finnish and other similar diagnostic escape nvCTs *(18)*.

Approved sample types include urine, endocervical samples (swabs or LBC), and vaginal, urethral, rectal and oropharyngeal swab specimens *(9,35,56)*. Swabs are collected using the manufacturer's transport medium and are then stable for up to 60 days at room temperature, making them ideal for transport to distant laboratories. First-catch urine is stable for up to 24 hours after collection; once placed in the manufacturer's medium, it is stable at room temperature for up to 30 days. The Aptima Combo 2 test also detects *N. gonorrhoeae* (16S rRNA); during one working day, approximately 250 samples are processed using the automated Panther system. The popularity of this assay is rapidly increasing in many countries because of the extremely high sensitivity, specificity, extended sample stability and process automation.

As samples are sealed after amplification, the potential for carry-over environmental contamination is expected to be very low. However, environmental monitoring is strongly recommended in the absence of an enzymatic or other control measure for the degradation of the amplified product. The manufacturer strongly recommends stringent adherence to cleaning and decontamination procedures. Reproducibility should be monitored.

- Confirmatory assays detecting *C. trachomatis*-specific 16S rRNA (**Aptima CT**) and *N. gonorrhoeae*-specific 16S rRNA (**Aptima GC**; another sequence of 16S rRNA compared to the one used in Aptima Combo 2) are available; these assays are, when required, ideal for confirmation of positive results and for repeat testing *(56)*. These assays are not available in the USA at this time, but are widely available in other countries.

v. Roche NAATs

- The U.S. FDA-approved **cobas CT/NG v.2** assay uses PCR technology to amplify target DNA sequences using microorganism-specific biotinylated primer pairs *(57)*. The chlamydial target is located on the cryptic plasmid. After a three-temperature amplification process, products are hybridized to magnetic beads coated with species-specific probe sequences located internal to the primer sequences. The primer targets used in all currently available cobas assays have been redesigned to include dual targets for chlamydia (one plasmid target and one chromosomal target) to ensure detection of the Swedish nvCT *(15–17)*. The detection process is based on biotin-avidin interactions. The **cobas 4800** instrument can test approximately 192 samples, including specimens and controls, during an 8-hour period. Swabs are collected in a cobas transport medium. Urine aliquoted into transport medium and swabs are then stable at 2–30 °C for up to one year. This assay includes a measure of inhibition based on an irrelevant DNA sequence preloaded into every reaction tube. This is intended to give the diagnostician evidence that a negative result is truly negative and not merely affected by the contents of the sample. The assay also uses an enzyme, uracil-N-glycosylase in the amplification mix that degrades previously amplified sequences based on the presence of deoxyuridine triphosphate rather than deoxythymidine triphosphate in the amplified product. This provides the user with a safety net to protect against minor splashes and aerosol formation that routinely occur when handling many samples and enhances the reproducibility of results. The only hands-on step after the run begins is to seal the amplification plate and move it to a thermal cycler.

  Approved specimen types include endocervical samples (including swabs and LBC), first-catch urine and vaginal swabs (patient- or clinician-obtained). Sensitivity is highest for vaginal swabs (93.5%) with estimates from a large USA-based clinical trial of 4279 women *(58)*. Penile urine is the only approved sample type for men with this assay, with an estimated sensitivity of 97.6% *(59)*.

  This assay has the advantage of allowing the user to recover and store extracted DNA after the extraction step, which can be useful for running LDTs or sequencing. In addition, as the light cycler is a separate instrument, using the open channel option, LDTs can easily be added to the laboratory's menu. However, this assay and the 4800 instrument will be phased out in the next few years.
- In 2021, the **CT/NG** assay for use on the **cobas 6800 and 8800** systems was approved by the U.S. FDA. The assay continues to use both a plasmid and a chromosomal target to detect *C. trachomatis*, which reduces the likelihood of diagnostic evasion through mutation in target regions. Other aspects of the extraction, amplification and detection steps are similar to the assay run on the 4800. The instruments are very large and have substantial power requirements. However, reagents can be stored on board for up to 90 days using the internal refrigeration units so that the footprint contains the equivalent of a large refrigerator, as well as a molecular analyser *(60)*. For very large reference laboratories, the 6800 (which can generate 384 results per day) and the 8800 (which can generate 1056 results in the same time period) can also be connected to the Roche haematology, chemistry and immunology instruments for extremely high efficiency. However, for smaller throughput laboratories, Roche developed the 5800 analyser, which is substantially smaller and can run the same tests (i.e. use the same reagent kits) generating 96 results per day.

  Approved specimen types to detect *C. trachomatis* include endocervical specimens (swabs and LBC), vaginal, anorectal and oropharyngeal swabs, and urine from men and women *(27,61).* From a study of over 1000 men and over 3800 women, the estimated sensitivities of the newest-generation assay are 99.2%, 95.6%, 93.3%, 92.5% and 100% for vaginal

swabs (self-obtained), female urine, endocervical swabs, endocervical samples in LBC and male urine, respectively. The optimal sample type for women is clearly the vaginal swab, although other sample types are acceptable. Rectal and oropharyngeal sensitivity estimates are 95.1% and 100%, respectively.

vi. Other laboratory-based NAATs

The assays described previously are based on the information available at the time of preparation of this manual. Several new NAATs are currently under development and evaluation; given the rapidly changing nature of this field, we can expect a proliferation of new tests (see WHO, 2023 *[38]*). Laboratories must review the literature as descriptions of these new assays become available. Additionally, in many settings, U.S. FDA-approved assays are not readily available or cannot be performed due to the requirement for specialized instrumentation or high cost. Thus, commercially available assays that have not been reviewed by the FDA and LDTs are used. If non-FDA-approved NAATs are used, regional (such as the EU) or other national regulatory processes should provide safeguards on the quality and performance of diagnostic NAATs *(36,37)*. It is also critical that laboratories engage in rigorous validation of the performance of these NAAT assays before using them to generate patient results (see also Chapters 1 and 5 regarding NAATs and their validation and QA).

8.3.3 POC NAATs

POC NAATs must not be confused with currently available aPOC assays that perform poorly. POC NAATs perform at the same level of sensitivity and specificity as laboratory-based NAATs but with the advantage of being done at site (POC) in support of test-and-treat schemes. The Cepheid Xpert assay is in fact a near-patient NAAT in settings where it is used on site, but it is often housed in a laboratory and was thus described in section 8.3.2. Two FDA-approved POC NAATs are described in this section, but several new POC NAATs are in development; if this is an affordable option, continue to check the literature and development pipeline for improved options (see WHO, 2023 *[38]*). For all of these NAAT POC tests, the major hurdle for adoption is cost and access to electricity.

- The **binx health io CT/NG** assay on the **binx health io** system is a POC NAAT that uses real-time PCR with an electrochemical output *(62)*. Samples are collected into transport buffer and placed into a cartridge specific for the CT/NG assay using a fixed-volume pipette to ensure accurate delivery of sample volume. The cartridge is immediately placed into the binx health io instrument where microfluidic processors control the extraction and amplification and detection steps. There is no additional user interface required. Results are generated in 30 minutes and the data can be transmitted to a laboratory information system or electronic medical record to facilitate patient management. The footprint of the instrument is smaller than 900 cm^2 and the instrument can be powered by 110 V or 220 V power sources *(63)*. Each instrument can test only one sample at a time.

  Based on data that suggest that the optimal sample types for detection of *C. trachomatis* are vaginal swabs and penile urine, these are the sample types for which the assay has claims. Sensitivity estimates from a USA-based clinical study were 96.1% (99.1% specificity) and 92.5% (99.3% specificity) for vaginal swabs and penile urine, respectively *(64)*. This assay does not have claims for anorectal or oropharyngeal samples.
- The **Visby Medical Sexual Health CT/NG/TV** test is a 30-minute POC NAAT assay used to detect *C. trachomatis, N. gonorrhoeae* and *T. vaginalis*; it is performed in a completely disposable cartridge with no external instrumentation. This PCR system uses a chromatographic lateral flow system for microorganism-specific detection of amplified target sequences. The cartridge does require power via an AC adaptor and cannot be moved while the assay is running. If running several samples simultaneously, the testing staff will need to ensure a bench space where the assay can be immobile. Because of the lateral flow output (similar to a rapid HIV test), there is no electronic capture of data. The assay has been cleared by the FDA for use with vaginal swabs (97.6% sensitivity, 98.3% specificity) *(65)*.

8.4 Antigen detection assays

Antigen detection tests work by using capture antibodies that bind to microorganism-specific antigens. For *C. trachomatis*, the antigens are associated with the major outer membrane protein, other membrane-exposed proteins or lipopolysaccharides (LPS). The antibodies used to capture the chlamydial antigens may be monoclonal, with excellent specificity for the antigen target, or polyclonal, which are less expensive to produce. The antigen–antibody complex is either observed directly (usually due to a fluorescent tag) or indirectly by using a second antibody tagged with a chemical moiety that will produce a colour change on binding to the antibody–antigen complex. These assays take many forms and these are described in the next sections. The advantage of antigen detection tests is the lack of need to maintain chlamydia viability and, in some cases, the ability to test at the POC, which enables test-and-treat service provision during a single clinic visit. The disadvantage is that most of these assays have somewhat lower sensitivity than culture and substantially lower sensitivity than NAATs. Thus, false-negative results may lead to untreated infections that are responsible for downstream sequelae.

Table 8.6: Detailed sample collection instructions for antigen detection assays

Site	Collection device[a]	Sampling procedure[b]	IFU[c] for storage conditions and stability
Endocervix	Swab/plastic	Insert the collection device into the endocervical os and rotate swab 360°. Collection of endocervical cells is critical to the DFA procedure.	For DFA: roll onto slide (thin layer) and air-dry. Fix and stain according to the assay IFU. For ELISA: follow assay IFU.
Vagina	Swab/plastic	Rotate the swab to come into contact with the vaginal walls on all sides. Not appropriate for DFA.	Verify that the antigen assay in use supports use of this sample type. Follow assay IFU.
Urethra	Swab/aluminium	Insert swab 2–3 cm into the urethra and rotate 360°. Collection of cuboidal epithelial cells is critical to the DFA procedure.	For DFA: roll onto slide (thin layer) and air-dry. Fix and stain according to assay IFU. For ELISA: follow assay IFU.
Penile Urine	Sterile urine cup	Do not have patient clean the genital area. Obtain first portion of the void (< 25 ml preferably). Not appropriate for DFA. ≥ 1 h after urination.	Follow assay IFU.
Conjunctiva	Swab/aluminium	Swab the surface of the inferior palpebral conjunctiva. Not appropriate for ELISA.	Roll onto slide (thin layer) and air-dry. Fix and stain according to assay IFU.
Nasopharynx (for suspected cases of neonatal pneumonia)	Swab/aluminium	Swab the nasopharynx or take the tracheobronchial aspirate. Not appropriate for ELISA.	Roll onto slide (thin layer) and air-dry. Fix and stain according to assay IFU.

DFA: direct immunofluorescence assay; ELISA: enzyme-linked immunoassay.

[a]Avoid wooden-shaft swabs: use plastic-shaft swabs for larger surfaces (e.g. cervical or rectal sampling) or aluminum-shaft swabs for smaller surfaces (e.g. conjunctiva).

[b]See assay instructions for use (IFU) for additional collection and storage instructions specific to the assay in use.

[c]Storage and stability conditions vary according to assay, so refer to the IFU of the assay in use.

8.4.1 Collection, transport and storage conditions of specimens for antigen detection assays

Table 8.6 outlines the sample collection, transport and storage of specimens for antigen detection.

8.4.2 DFA

DFA uses a fluorescein-tagged anti-chlamydia antibody to allow direct microscopic visualization of *C. trachomatis* elementary bodies in cellular smears collected from the conjunctiva, urethra or the endocervix or nasopharynx.

A single DFA stain produced by IMAGEN (Thermo Fisher Scientific) is commercially available in the USA and can be used to stain smears collected from the urethra, endocervix, conjunctiva and neonatal nasopharynx for the visualization of chlamydial elementary bodies. This assay uses a fluorescein-tagged polyclonal antibody specific for LPS that is broadly reactive and will stain *Chlamydia pneumoniae* as well as *C. trachomatis*. The most common use for DFA is staining of conjunctival smears, predominantly in neonates in developed countries. The DFA offers the advantages of rapid turnaround time, high specificity and detection of non-viable microorganisms. In addition, no other commercial assay has a regulatory claim for conjunctival samples or the capacity to assess specimen quality directly. However, DFA suffers from substantially lower sensitivity than that of NAATs (or culture), is laborious and unsuitable for high-throughput diagnostics, and requires skilled microscopists. Negative results should be interpreted with caution due to the poor performance of these tests, which may result in missed infections.

8.4.3 Laboratory-based ELISA

ELISAs are a laboratory-based class of tests that use anti-chlamydia antibodies to capture surface antigen. ELISAs usually involve a sandwich assay design that involves a colorimetric output often using avidin–biotin and horseradish peroxidase reactions. These assays are suitable for batching of 96 tests per run and the testing is only moderately complex. Samples are pipetted into wells and incubated, which is followed by washing and adding chemical reactants. Results are usually interpreted based on optical densities from an

automated reader. The assays are manual in nature and require laboratory equipment. Furthermore, ELISAs suffer from low sensitivity and suboptimal specificity (especially ELISAs detecting LPS) compared to NAATs, and may require confirmative testing of positive results. Negative results should be interpreted with caution due to the poor performance of these tests, which may result in missed infections. Due to the limitations of existing ELISAs, these tests should not be used when any other testing options are available.

8.4.4 Antigen-based POC assays

Unlike laboratory-based ELISAs, aPOC tests that use similar antibody–antigen capture technology have advantages that make their use reasonable in specific settings. Several aPOC tests, which are commonly based on lateral flow through a fixed matrix and antigen membrane capture on immunochromatographic strips, have been developed to diagnose *C. trachomatis* infections. These tests are performed very much like HIV rapid antibody tests, making them suitable for POC testing in support of test-and-treat strategies. Many of these tests have been developed and commercialized, but most have not undergone appropriate and comprehensive evaluation. Currently two such assays are commonly available in the USA, the Clearview (Alere) and the QuickVue (Quidel) assays. These aPOC assays obtained FDA approval before the introduction of NAATs and would not obtain approval if compared to today's performance requirements. Accordingly, when compared to NAATs, these rapid tests display clearly insufficient sensitivity and they should only be used when adequate laboratory facilities are lacking. Despite the low sensitivity of the traditional aPOC tests, in resource-constrained, high-prevalence populations, decreases in sensitivity may be acceptable in exchange for the ability to test and treat while the patient is on site *(65)*. In a decision analysis study by Gift et al., when the patient return-for-treatment rate was 65% or lower, rapid POC diagnostics provided an increase in the number of patients treated even though fewer infections were identified *(66)*. In these settings, the aPOC tests could also be used to increase the specificity of the syndromic management algorithms, which will reduce overtreatment and screen for asymptomatic infections.

Over the last several years, substantial advancements in microfluidics, nanoparticles, aptamers and biosensing technologies have shown promise for the development of drastically improved lateral flow antigen detection tests. To date, none of these technologies has been developed to detect chlamydial antigens, but this is an area that should be closely monitored over the immediate future. An unexpected benefit of the global severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic has been the rapid development of new, rapid and inexpensive diagnostic assays for that infection. Many manufacturers are already beginning to assess the potential for applying their technology to the field of STI diagnostics and detection of *C. trachomatis* is likely to be an early outcome of this developmental work.

- For laboratories with fluorescence microscopy capacity, the DFA may offer an affordable and rapid diagnostic test for trachoma or neonatal infections, but sensitivity is limited and the technical skill level required is high.
- There is rarely an advantage to laboratory-based ELISAs due to poor sensitivity, limited sample types, transport and result management time.
- Rapid, inexpensive aPOC tests offer expanded opportunities for reaching non-clinic-based populations and providing immediate treatment. In some situations, the impact of lower cost and increased treatment may offset the lower sensitivity of aPOC tests compared to NAATs.

8.5 Methodologies for use in reference laboratories only

8.5.1 Culture

Until the early 1980s, the main and gold standard method to diagnose *C. trachomatis* infection was the centrifuge-assisted inoculation of clinical specimens onto susceptible viable cells in tissue culture, followed by the demonstration of characteristic chlamydial inclusions after incubation. Briefly, the specimen is collected using Dacron swabs or cytobrushes and placed into a transport medium, for example, sucrose-phosphate-glutamate (SPG) buffer (see Annex 1) containing fetal bovine serum and antimicrobials, such as vancomycin, gentamicin and nystatin, to inhibit growth of other bacteria and fungi. While this method should now be reserved for use in reference laboratories, it is important to maintain the ability to obtain patient-derived isolates on occasions, and this requires the use of tissue culture.

i. Collection, transport and storage conditions of specimens for culture testing

The anatomical sampling sites will vary depending on the clinical presentation and history of the patient and the overall sensitivity and specificity of the assay. For culture, which requires live microorganisms, the sample must be collected from sites with columnar or cuboidal epithelial cells, which are most likely to be actively infected. Thus, the endocervical os should be sampled in women and the urethral epithelium should be sampled in men. Rectal samples should be collected for MSM. Obtain **endocervical samples** by inserting

the collection device 2–3 cm into the os and rotating a full 360°. Endocervical samples are not taken in girls of prepubertal age; instead, specimens should be sampled from the vestibule of the vagina. Collect **urethral samples** by inserting the swab 2–3 cm into the urethra, followed by full rotation to obtain cellular material. **Rectal samples** should be collected by inserting the collection device past the second sphincter and rotating the swab a full 360°. The details are summarized in Table 8.7. Sample inadequacy has been described as a common problem that will negatively affect culture sensitivity. Samples should be placed immediately in transport buffer (e.g. SPG; see Annex 1) that contains antibiotics to control overgrowth of yeast and bacteria while not effective against *C. trachomatis.* All samples must be kept at or below 4 °C for no more than 24 hours before inoculation into tissue culture monolayers. If samples cannot be tested within 24 hours, they must be stored at −70 to −80 °C until inoculation. Samples will not retain viability if stored under other conditions. Storage at −20 °C will not maintain viability.

ii. Culture of *C. trachomatis* in McCoy cell line

Splitting flasks

1. Check media for sterility before use.
2. Check monolayers visually for confluence and lack of microbial contamination.
3. Proceed using sterile technique working in a biohazard containment hood. Aspirate medium from confluent flask using a sterile pipette and a vacuum flask.
4. Rinse the monolayer with 10 ml glucose-potassium-sodium-phosphate (GKNP) solution (see Annex 1) and aspirate.
5. Add 4 ml trypsin and incubate cells at room temperature until the monolayer is loosened from the flask (approximately 3–7 minutes). Lightly tap the sides of the flask to remove the cells.
6. Add 4 ml Iscove's modified Dulbecco's medium (IMDM) with vancomycin and gentamicin (IMDM-VG) (see Annex 1) to inactivate the trypsin and mix well. Use the liquid to rinse off any remaining cells on the back of the flask.
7. Add 1 ml of the cell suspension to each new 175-cm^2 flask and bring the total volume up to 75 ml with IMDM-VG. When seeding a 75-cm^2 flask, add 0.5 ml and bring the total volume up to 35 ml.
8. Incubate flasks at 37 °C for 48–96 hours with the lids tightly capped.

Seeding microtitre plates and vials

1. Follow steps 1–6 above.
2. Each plate to be seeded requires 15 ml of a diluted cell suspension. Calculate the total volume needed based on the number of plates desired. Example: 10 plates require 150 ml of IMDM-VG. Each vial requires 1 ml of Dulbecco's Modified Eagle's Medium (see Annex 1); calculate the total volume needed based on the number of vials desired.
3. For each 50 ml of IMDM-VG for the microtitre plates, add 1 ml of the cell suspension from the trypsinized flask. Example: 150 ml of IMDM-VG needs 3 ml of cells. For each 100 ml of IMDM-VG per vial, add 1 ml of the cells.
4. Load a haemocytometer with the diluted cell suspension. Count 5 squares (the 4 corners and the middle); the mean number of cells per square is multiplied by 10^5 to give the number of cells per ml. Microtitre plates require 1–1.4 × 10^6 cells per ml (10–14 cells per square); vials require 7–10 × 10^5 cells per ml to be confluent in 48 hours. If the number of cells counted is out of range, adjust the concentration by adding additional IMDM-VG or by adding more cells from the flask as appropriate. Re-count and adjust until the correct concentration is achieved.
5. Add 200 µl to each well. Seal the plates with sealing film. Add 1 ml to each vial and cap tightly. Incubate at 37 °C until use (48–96 hours).

Table 8.7: Detailed sample collection instructions for culture

Site	Collection device[a]	Sampling procedure
Endocervix	Swab/plastic-shaft, liquid cytology brush or broom	Use a cleaning swab to remove excess mucus before sample collection. Insert collection device into the endocervical os. Rotate swab 360°.
Urethra	Swab/aluminium	Insert swab 2–3 cm into the urethra and rotate 360°. Collection of cuboidal epithelial cells is critical.
Rectum	Swab/plastic	Insert swab into the rectum past the second sphincter and rotate 360°.
Oropharynx	Swab/plastic	Swab the posterior pharynx and the tonsillar pillars.
Nasopharynx (for suspected cases of neonatal pneumonia)	Swab/aluminium	Swab the nasopharynx or take tracheobronchial aspirate.
Conjunctiva	Swab/aluminium	Swab the surface of the inferior palpebral conjunctiva.

[a]Avoid wooden-shaft swabs: use plastic-shaft swabs for larger surfaces (e.g. cervical or rectal sampling) or aluminium-shaft swabs for smaller surfaces (e.g. urethra or conjunctiva)

Inoculating microtitre plates

1. Check the McCoy cell monolayer for confluence. Cells should be touching and slightly crowded. Media should be clear.
2. The following specimens should be tested in the microtitre format: cervical, urethral, rectal, oropharyngeal and conjunctival.
3. Vortex specimens for 5 seconds then sonicate, if possible, for 20 seconds. During sonication, hearing and eye protection must be worn.
4. Specimens that exhibit gross bacterial contamination (e.g. cloudy transport medium or pH change in the medium) may cause toxicity in the tissue culture. These specimens should be tested undiluted and diluted at 1 : 2 and 1 : 10 (in SPG).
5. Working in a biohazard containment hood, place one half of each specimen into the cryovial with the same specimen number.
6. Aspirate plates containing confluent monolayers.
7. Add 100 µl from each specimen and layer it onto the cell monolayer. Each specimen is inoculated onto three consecutive monolayers. After completing the inoculation of each plate, add 200 µl IMDM with vancomycin, gentamicin and amphotherin B (IMDM-VGA) into each well.
8. Seal plates with sealing film and centrifuge at 1400*g* for 1 hour at 30 °C. Incubate at 37 °C for 48 hours.
9. Store specimens at –70 °C until the results are available. All positive specimens are stored for more than 2 weeks.
10. Remove media from plates. Fix monolayers by covering with methanol for 10 minutes. Remove the methanol.

Inoculating vials

1. Only exceptional specimens are cultured in vials. Some examples include endometrial and tubal biopsies and lymph node tissue.
2. Check McCoy cell vials for confluence; cells should be touching and slightly elongated. Medium should be clear.
3. Vortex specimens for 5 seconds then sonicate for 20 seconds. During sonication, hearing and eye protection must be worn.
4. Specimens that exhibit gross bacterial contamination (e.g. cloudy transport medium or pH change in the medium) may cause toxicity in the tissue culture. These specimens should be tested undiluted and diluted at 1 : 2 and 1 : 10 (in SPG).
5. Working in the hood, label and aspirate three vials per specimen. Add 0.2 ml of specimen to each vial. Overlay with 1 ml IMDM-VGA.
6. Centrifuge at 2500*g* for 1 hour at 30 °C. Incubate at 37 °C for 72 hours.
7. Store all specimens at –70 °C until the results are available. All positive specimens are stored indefinitely.
8. Aspirate medium from 1 vial per specimen and fix by covering the monolayer with methanol for 10 minutes.

Staining with fluorescent antibodies

1. Follow the package insert instructions for the specific anti-chlamydial antibody used.
2. Layer the antiserum onto the fixed monolayer (after removal of methanol). Microtitre wells receive 40 µl each using a multichannel pipettor.
3. Incubate at room temperature for 30 minutes.
4. Rinse gently three times with phosphate-buffered saline (see Annex 1).
5. Plates are read as is; 13-mm round coverslips are removed from the vials and, using mounting fluid, placed on glass slides with 22 × 50 mm coverslips. Keep stained cultures in the dark until they are read. Cultures should be read the same day they are stained.

In most laboratories, culture is no longer an appropriate diagnostic test because it is significantly less sensitive than NAATs, has a significantly longer turnaround time, demands invasive samples, requires more restrictive handling conditions to preserve the viability of *C. trachomatis*, is technically complex and lacks internationally standardized and quality-assured methods. Culture is now requested predominantly for use in medico-legal cases because of the assumption of 100% specificity (but cross-contamination between samples occurs) and for test-of-cure (TOC) testing requested fewer than 14–28 days after treatment. In fact, the specificity of NAATs is such that these assays should be acceptable for medico-legal cases; reliance on culture reflects the slow pace of change in legal standards. TOC is generally not recommended and rarely performed given the efficacy of antimicrobial treatments now available; however, some recent studies showed suboptimal eradication efficacy of azithromycin 1 g, especially of rectal chlamydial infections. If TOC is performed, this should be performed more than 14–28 days post-treatment at which point, in most cases, NAATs are appropriate because DNA shedding from the initial infection should be complete by this time. However, the ideal time point for TOC may be shorter for RNA-based NAATs. Therefore, unless isolates of microorganisms are desirable for research purposes, including antimicrobial susceptibility testing, there is no longer a valid justification for culture as a routine diagnostic method. This is consistent with the recommendations in the current CDC sexually transmitted diseases laboratory guidelines *(67)*, as well as the European guidelines on the management of *C. trachomatis* infections *(13)*.

Isolates that are recovered in those settings in which culture is maintained should be preserved in a specimen repository for epidemiological studies. These studies may include investigation of antimicrobial susceptibility

(see section 8.5.2) and genotyping, including whole-genome sequencing. Genotyping can be performed using molecular techniques and does not require viable organisms *(68–72)*. Therefore, repositories of NAAT-positive samples should also be maintained in regions throughout the world.

- Culture has suboptimal sensitivity compared to commercially available and internationally approved NAATs and cannot be recommended for diagnostics if an appropriate NAAT is available and affordable.
- Culture capability should be maintained in some reference laboratories and repositories of isolates should be stored for potential future phenotypic and genetic studies.

8.5.2 Antimicrobial susceptibility testing

There is no unambiguous evidence of the emergence of acquired homotypic (phenotypically and genetically) and stable resistance to recommended antimicrobials in clinical *C. trachomatis* isolates, although case reports suggested bacterial resistance as a cause of treatment failure. Furthermore, in vitro antimicrobial susceptibility testing of *C. trachomatis* has never been performed routinely; a universally accepted, standardized, reproducible and quality-assured method, as well as reliable and evidence-based correlates between in vitro activity and in vivo efficacy (clinical treatment outcome), are lacking *(73)*.

The antimicrobial susceptibility testing of *C. trachomatis* is labour-intensive, requires tissue culture expertise and is only feasible in reference laboratories. However, the emergence and spread of clinically relevant antimicrobial resistance in *C. trachomatis* in the future should not be excluded. This highlights the need for appropriate evaluation of current antimicrobial susceptibility testing methods and the development of an effective, standardized, objective and quality-assured method, as well as appropriate correlates between in vitro activity and treatment outcome. This method may be useful in the future for monitoring possible antimicrobial resistance, clinical treatment studies and assessment of the in vitro activity of new antimicrobials. In the event of spread of clinically relevant antimicrobial resistance in *C. trachomatis*, repositories of isolates will be useful for retrospective evaluations in research settings.

8.5.3 Serology

Serological methods of diagnosing chlamydia infections were among the earliest techniques available. These methods identify and in some cases titrate the level of antibody response to chlamydial antigens. While the first assays, complement fixation and microimmunofluorescence, relied on whole microorganisms, subsequent assays were developed that are specific for responses to individual proteins or antibody classes. Serology may aid in the diagnosis and screening for complicated *C. trachomatis* infections (reactive arthritis, PID, ectopic pregnancy, TIF), be diagnostic in neonatal pneumonia and LGV infections (see Chapter 14) and be valuable in research and epidemiological studies, for example, for the cumulative history of exposure of a sample population to chlamydial infection *(74)*. Nevertheless, always interpret serological results with caution and not out of context.

In an acute, primary chlamydial infection, specific immunoglobulin M, but also immunoglobulins G and A, may be detected. However, systemic antibody response may be delayed or not measurable after uncomplicated urogenital infection. In contrast, high levels of antibodies to *C. trachomatis* can persist long after infection has been cleared. Accordingly, due to the low sensitivity and specificity, measurement of chlamydial antibodies has limited value to diagnose acute *C. trachomatis* infection and should not be used for routine diagnosis of uncomplicated *C. trachomatis* infections *(74)*.

- Do not use serology to diagnose uncomplicated urogenital *C. trachomatis* infection.
- Only use serology as a possible aid in the diagnosis of and screening for complicated *C. trachomatis* infections, neonatal pneumonia and LGV infections, and in epidemiological studies.

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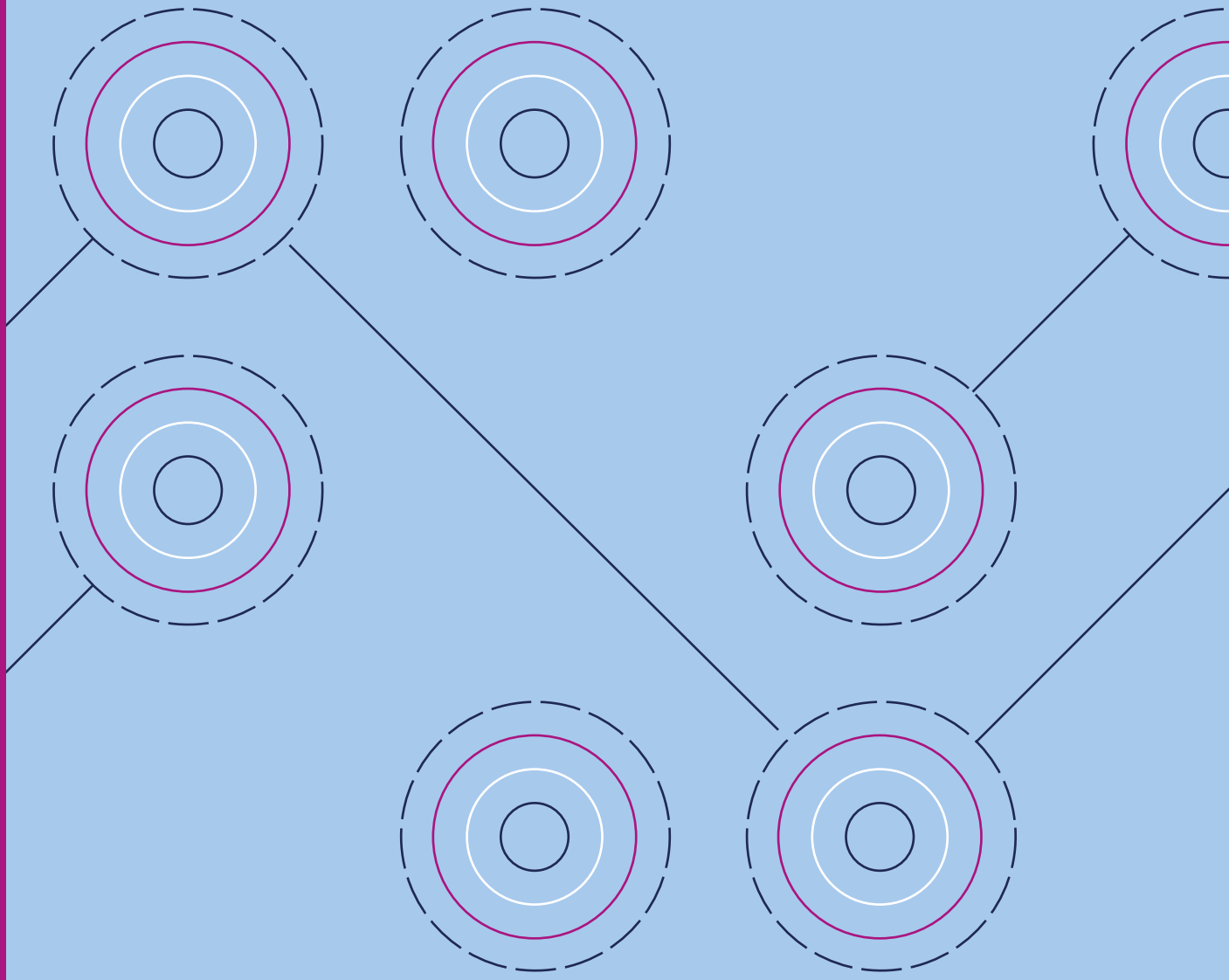
Chapter 9. Trichomoniasis



Chapter 9. Trichomoniasis

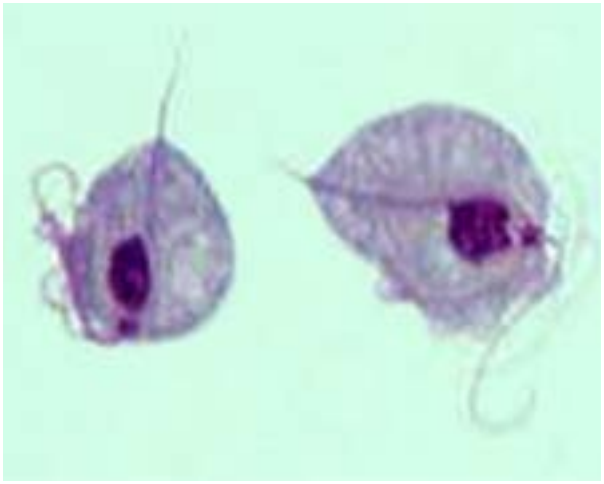
Barbara Van Der Pol, Yaw Adu-Sarkodie and Patricia Kissinger

9.1 Introduction

Trichomonas vaginalis is the etiological agent of the most prevalent non-viral STI worldwide. In 2020, there were an estimated 156 million new cases of *T. vaginalis* globally among adults aged 15–49 years *(1)*. Previous estimates have indicated that the global prevalence rate among men is far lower than women, and rates also vary by continent, with the highest rates in Africa (11.7% and 1.2% for women and men, respectively) and the lowest in Europe (1.6% and 0.2%) *(2)*. Despite this high prevalence, STI control efforts have historically underemphasized this pathogen and it is not a reportable infection. Although *T. vaginalis* may cause an abnormal vaginal discharge (trichomoniasis) in women and may be responsible for as much as 10–12% of non-gonococcal urethritis cases in men *(3)*, the infection may be asymptomatic in at least 50% of women and 70–80% of men *(4,5)*. Laboratory diagnosis is therefore essential to supplement syndromic management strategies for the treatment of infection.

T. vaginalis is a motile, ovoid, pear-shaped, flagellated protozoan (10–20 µm long) (Fig. 9.1). The organism has four free, anterior flagellae and a fifth flagellum embedded in an undulating membrane that extends around the anterior two thirds of the cell. The flagellae move the protozoan with a jerky movement that is critical to microscopic detection of the organism. *T. vaginalis* adheres to the mucous membranes associated with squamous epithelium and does not invade the mucosa. In men and women, the organism can elicit a robust inflammatory response that results in the fulminate discharge of overt disease. See Table 9.1 for a description of the clinical manifestations of *T. vaginalis* infection.

Fig. 9.1: *Trichomonas vaginalis* stained with Giemsa stain in high magnification



Source: Reproduced courtesy of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.[31]

Table 9.1: Clinical manifestations of *Trichomonas vaginalis* infection

Genital infection	Primary	Sequelae
Women	Fulminate, purulent or frothy white to yellow discharge, dysuria, pelvic pain, itching, strawberry cervix (more often seen on colposcopy)	Adverse pregnancy outcomes *(6)*, increased risk of HIV acquistion *(7)* and possible transmission *(8)*.
Men	Urethral discharge, dysuria, testicular pain	Possible epidydimitis and prostatitis

[31] Available at: https://www.cdc.gov/dpdx/trichomoniasis

T. vaginalis has been associated with poor birth outcomes such as low birth weight, preterm delivery, pelvic inflammatory disease among HIV-infected women, and premature rupture of membranes *(6,9)*. One small study also showed an association between maternal *T. vaginalis* infection and intellectual disability in children born to infected mothers *(10)*. Although rare, *T. vaginalis* infection can be transmitted perinatally *(11)* and cause vaginal and respiratory infections in neonates *(12,13)*. Among men, *T. vaginalis* can cause urethritis, prostatitis and reduced fertility *(14)*.

T. vaginalis has also been associated with HIV transmission. A meta-analysis of 19 studies found that persons with *T. vaginalis* living in sub-Saharan African were 1.5 times more likely to acquire HIV than those without the infection *(15)*. Another review of the literature found that most studies demonstrated excess risk for HIV acquisition in the presence of *T. vaginalis* but less consistency in the transmission of HIV *(16)*. In men, treatment of *T. vaginalis* urethritis results in a 0.5–2 log decrease in the seminal fluid HIV viral load *(17,18)*. Similar results have been observed in women treated for vaginal discharge *(19,20)*. Given that genital compartment viral load is one of the most significant risk factors for transmission to an uninfected partner, appropriate diagnosis and treatment of *T. vaginalis* infections should be a public health priority as part of an HIV infection elimination strategy. Similarly, data have been available for many years indicating that the presence of discharge-causing STI increases the risk of HIV acquisition. Most of these studies, however, were done prior to the introduction of pre-exposure prophylaxis and highly active anti-retroviral treatment and it is unknown if *T. vaginalis* infections have the same impact on HIV transmission in the presence of these two strong HIV prevention strategies.

The epidemiology of *T. vaginalis* infection differs from other infections that cause genital discharge in two important aspects. First, the age distribution of infection is distinct compared to chlamydia and gonorrhoea, which tend to peak in women aged 15–25 years, whereas *T. vaginalis* peak infection prevalence extends to older women. However, improved data as a result of more screening suggests that this may not be as stark a difference as earlier described *(21)*. Despite the high prevalence of *T. vaginalis* among older women, more than 50% of the cases in the USA are among women of reproductive age *(22)*. This is important because of the potential for poor *T. vaginalis*-related birth outcomes in this age group. The age-specific distribution of infection in men has not been studied adequately. The second difference is that, despite the sexual transmission of this pathogen, the gender distribution of laboratory-diagnosed *T. vaginalis* infections is highly skewed, with a female-to-male ratio as high as 4 : 1 *(23–25)*. This distribution is demonstrated in the infection rates of male partners to infected women, which ranges from 22% to 72% *(4,26)*. This is likely due to a more transient infection in men resulting from the constant washing action of the urethra by urine and the trichomonicidal property of zinc found in prostatic secretions *(27)*. The environment of the male urethra, unlike the vaginal milieu, makes it less permissive for the organism to remain in that location. Thus, there is a brief window of opportunity for detection of the organism in men. As a result, there are limited data regarding the true epidemiology of *T. vaginalis* infection in men *(25,28)*.

The inflammatory response in trichomoniasis in both women and men is significant and substantially increases the risk of HIV transmission and acquisition, as well as the probability of perinatal morbidity *(29)*.

- *T. vaginalis* is the most common global non-viral STI.
- Untreated infections enhance the risk of HIV acquisition and increase the probability of adverse pregnancy outcomes such as pre-term birth, premature rupture of membranes and small for gestational age.

9.2 Overview of available diagnostic methods

Clinical diagnosis of trichomoniasis is based on the odour, quality and quantity of vaginal discharge, the vaginal pH, and possible presence of cervical friability. Vaginal pH, usually > 6.0, fulminate or frothy white discharge, and punctate cervical friability ("strawberry cervix") are suggestive of infection with *T. vaginalis.* However, the absence of these clinical signs is not sufficient evidence to rule out infection, especially when the infection is asymptomatic in nearly 50% of women and 70–80% of men *(23,30)*. Accordingly, to enhance the sensitivity and specificity of diagnosis, wet preparation microscopy (at a minimum) or laboratory methods (preferably) are required.

There are four main classes of diagnostic assays: wet preparation microscopy, antigen detection, culture, and nucleic acid amplification tests (NAATs). **Wet preparation microscopy** may be performed in clinical settings, and in combination with testing for bacterial vaginosis, where a good quality microscope and trained microscopists are available. This is an ideal first-line diagnostic method, if adequately performed and interpreted, as it provides a definitive diagnosis with high specificity if positive. However, there should be caution in ruling out infection based solely on negative microscopy for three reasons. First, trichomonads are highly temperature-sensitive and lose their motility in as few as 10 minutes following sample collection *(31)*. Since motility is a hallmark feature, this loss may result in false-negative results. Second, the size of trichomonads is similar to that of white blood cells (lymphocytes or small neutrophil granulocytes), which are often present as a result of the inflammatory process. Thus, the trichomonads may be obscured by or mistaken for these cells. Additionally, *T. vaginalis* morphology closely resembles other trichomonads found in humans (e.g. *Pentatrichomonas*

hominis and *Trichomonas tenax*) *(32,33)*. Finally, in many women and in most men, the organism load may be below the limit of detection for microscopy. In addition to vaginal swab samples, microscopy may be performed on urethral exudates or urine sediment from men, but this technique suffers from low sensitivity, probably also due to low organism load *(30)*.

Antigen point-of-care (aPOC) tests are now available in many settings. These tests are approved only for vaginal swab samples. The latest generation of these tests, the OSOM Trichomonas Rapid Test (Genzyme Diagnostics), has superior sensitivity compared to microscopy *(34)* and can provide results in approximately 30 minutes, i.e. while the patient waits. As with other point-of-care (POC) assays, the opportunity to treat infections immediately is an advantage of this test over tests that need referral to a central laboratory. Performance of any commercially available test should be in compliance with the manufacturer's instructions for use (IFU).

Laboratory-based culture has been available for many years and in the last 1–2 decades, commercially available culture kits, such as InPouch TV culture system (BioMed Diagnostics), have become available. Vaginal swabs, urethral swabs and urine sediment from men are specimens cleared for use in these commercial systems (see Table 9.2). This method has a turnaround time of up to 7 days and requires an incubator, a quality microscope and well trained microscopists. However, culture substantially increases the sensitivity beyond that of wet preparation microscopy *(30,35)*. Details of culture are provided below and, if using a commercial product, the IFU should be followed.

Finally, **NAATs** are available for detection of specific *T. vaginalis* DNA or RNA. For programmes that employ NAATs for chlamydia and gonorrhoea, inclusion of *T. vaginalis* testing may be a reasonable strategy. At the time of this writing, most of the U.S. Food and Drug Administration (FDA)-cleared laboratory-based chlamydia/gonorrhoea NAATs also have claims for detection of *T. vaginalis (36–41)*. The sensitivity and specificity of the FDA-approved NAATs are very high *(42–45)*. Data demonstrate substantially improved case finding with use of NAAT tests for detection of *T. vaginalis* compared to culture or microscopy *(46,47)*. NAATs that can be used at the point-of-care (POC NAATs) are also becoming more widely available. Several such assays have been cleared by the FDA *(40,48,49)* and are described in section 9.4.3 below.

A number of methods have been described for the detection of antibodies to *T. vaginalis*. However, antibody tests have a low sensitivity and suboptimal specificity for the detection of current *T. vaginalis* infection and should not be used for routine diagnosis of trichomoniasis.

Table 9.2 summarizes the performance characteristics of available diagnostic tests for detection of *T. vaginalis*. For appropriate performance of all diagnostic methods, it is crucial to follow precisely the assay IFU and recommendations from the manufacturer concerning collection, transport and storage of samples, as well as performance of the specific assay, including quality controls.

- Culture and some aPOC tests have higher sensitivity than microscopy.
- Commercially available, U.S. FDA-cleared NAATs have significantly superior sensitivity relative to other diagnostic methods.
- Antibody testing is not an appropriate clinical diagnostic tool.

Table 9.2: Features of diagnostic tests for detection of *Trichomonas vaginalis*

	Microscopy	aPOC test	Culture	NAAT
Specimen types				
Endocervical swab	No	No	No	***Yes***
Liquid cytology medium	No	No	No	***Yes***
Vaginal swabs				
Self-obtained	Yes	Yes	Yes	Yes
Clinician-collected	Yes	Yes	Yes	Yes
Urine				
Female	No	No	No	Yes
Male	Yes	No	Yes	Yes
Male urethral swab	Yes	No	Yes	Yes

Table 9.2 (continued): Features of diagnostic tests for detection of *Trichomonas vaginalis*

	Microscopy	aPOC test	Culture	NAAT
Performance				
Sensitivity	Low	High[a]	Moderate–high	Very high
Specificity	Very high	Very high	Very high	Very high
Cost	Low	Moderate	Moderate	High
Transport and storage	NA	NA	Ambient	Ambient
Instrumentation	Microscope	None	Incubator, Microscope	Large footprint
Throughput/ automation	Low/No	Low/No	Low/No	High/Possible
Technical complexity	Moderate (microscopy skills)	Low	Moderate (microscopy skills)	High
Level of laboratory infrastructure	Peripheral	Peripheral	Intermediate-central	Central
Other comments	• False-negative results are more likely than real negative results so clinical context is critical.	• Infections identified may be treated before the patient leaves the clinic.	• Strict attention to accurate microscopy is required. • Potential to obtain viable isolates is useful for additional testing such as genotyping and antimicrobial susceptibility testing.	• Potential for laboratory contamination requires strict adherence to protocols. • Some require large batch size, which may delay turn-around time.

aPOC: antigen point-of-care; NAAT: nucleic acid amplification test; NA: not applicable.

[a]Refers to OSOM Trichomonas Rapid Test (Genzyme Diagnostics).

9.3 Collection, transport and storage conditions of specimens

Because collection of samples for microscopy or culture requires capture of viable organisms, the sampling methods are somewhat more invasive than those needed for NAAT testing (see Tables 9.3 and 9.4).

Women

Vaginal swab samples are optimal for detection of *T. vaginalis*. For culture or microscopy, sampling of the posterior fornix should be done using a Dacron or rayon swab on a plastic shaft. Cotton swabs on wooden shafts are acceptable for microscopy or culture inoculation, but are not recommended for aPOC test or NAAT. Therefore, to avoid confusion, it is practical to avoid swabs with wooden shafts. Clinicians should collect samples prior to insertion of a speculum during pelvic examinations, or patients may self-obtain samples. Clinicians should provide instructions to ensure that patients understand how to collect their own samples. Provision of appropriate instructions has been found to be the determining factor *(50)* in patient acceptability of self-sampling. Residual samples – vaginal, cervical or urine – collected for chlamydia/gonorrhoea testing (see Chapter 8) may be used for *T. vaginalis* NAAT.

Table 9.3: Sample collection for detection of *Trichomonas vaginalis*

Sample type	Collection device	Sampling procedure
Vaginal, clinician-collected	Swab/plastic[a]	Sample the posterior fornix prior to insertion of speculum.
Vaginal, patient-collected	Swab/plastic[a]	Rotate the swab 360°, touching all of the vaginal walls
Urethra (only for symptomatic men)	Swab/aluminium[a]	Collect urethral exudates > 1 hour after previous void.
Urine	Sterile urine cup	Do not have patient clean the genital area. Obtain first portion of the void (in general less than 25 ml), > 1 hour after previous void.

[a]Dacron or Rayon swabs. Do not use wooden-shaft cotton swabs

Table 9.4: Specimen handling for *Trichomonas vaginalis* tests

Sample type	Microscopy	aPOC test	Culture	NAAT
Swabs	Elute in ≤ 0.5 ml saline, place one drop of saline on slide with coverslip.	Place into kit extraction buffer and follow IFU. Only use for cleared sample types	Place directly into culture medium.[a] Incubate at 37 °C.	Place into manufacturer's collection device. Can be sent dry to laboratory. Store and transport according to IFU.
Urine	Centrifuge at 500g for 5 min. Place sediment into ≤ 0.5 ml saline. Place one drop of saline on slide with coverslip.	NA	Centrifuge at 500g for 5 min. Place sediment into culture medium. Incubate at 37 °C.	Place into manufacturer's collection device. Store and transport according to IFU.

aPOC: antigen point-of-care; NAAT: nucleic acid amplification test (includes laboratory-based NAATs and POC NAATs); NA: not applicable; IFU: manufacturer's instructions for use.

[a]Culture medium may be Diamond's medium or InPouch Culture system. For sites that do not have access to culture medium, swabs can be placed in tubes containing Amies medium and stored at 4°C for transport to arrive in the central laboratory within 24 hours.

Men

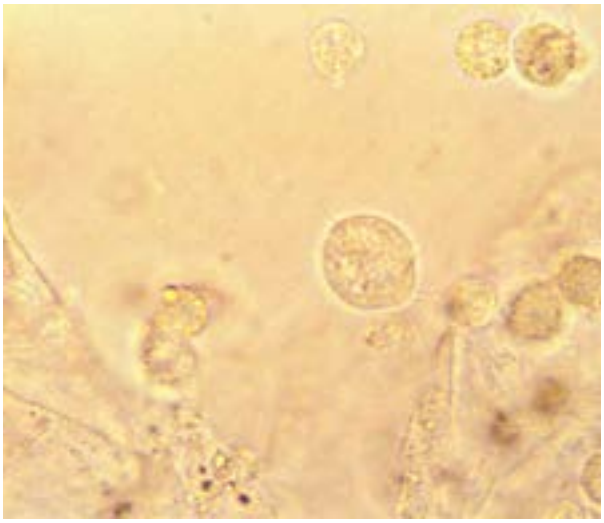
Since men with trichomoniasis are often asymptomatic and have a lower parasite burden than infected women, diagnosis can be challenging *(51)*. Urethral exudates may be collected using a Dacron or rayon swab on an aluminium shaft. These samples may be used for wet preparation microscopy, culture or NAAT. First-void urine may be centrifuged to obtain sediment that is appropriate for culture. Non-centrifuged urine is adequate for NAAT testing. While most tests are FDA approved for urine only, one study found that self-collected meatal swabs for men were four times more sensitive than urine *(52)*.

9.4 Diagnostic methods

9.4.1 Microscopy

Immediately following collection, swabs should be eluted into ≤ 0.5 ml of sterile saline at room temperature and a slide should be prepared with a drop of the saline sample and a coverslip. The slide should be read at 100× magnification within 10 minutes of collection to look for motile trichomonads. Confirmation of pear-shaped morphology, including visualization of flagella, should be performed using 400× magnifications (Fig. 9.2). Non-motile cells cannot be diagnosed as trichomonads and, accordingly, immediate microscopy is crucial because the trichomonads quickly lose their motility *(53)*. The sensitivity of microscopy is limited (as low as 40–65% for women in some settings and even lower for samples from men) *(23,30)* and negative results should be interpreted with caution. In some settings, microscopy is the first-line diagnosis and screening, and negative samples are referred to a central laboratory for further evaluation, particularly in symptomatic individuals. However, when the strictly required motility and the morphology are identified, the specificity of microscopy is excellent and all patients with positive microscopy results should be considered infected.

Fig. 9.2: *Trichomonas vaginalis* in wet preparation microscopy



Source: WHO, 2013 *(54)*, reproduced by permission of the Division of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium.

9.4.2 Antigen point-of-care (aPOC) detection tests

Several antigen detection assays have been developed for detection of *T. vaginalis (34,35,55)*. Requirements for equipment and costs of reagents vary, as does assay performance. Several of these assays are intended for use only in symptomatic women, making them less useful than other options. The latest generation of these tests, e.g. OSOM Trichomonas Rapid Test (Genzyme Diagnostics), has superior sensitivity compared to microscopy *(34,56)*. Procedures will vary by manufacturer and the manufacturer's IFU should be followed precisely for each specific assay.

Table 9.5: Point-of-care diagnostic tests for *Trichomonas vaginalis*

Test	Sample type	Sensitivity Specificity	Complexity Time Instrumentation required
Wet mount microscopy	Women only Vaginal swab	Sensitivity: 44–68% Specificity: 100%	Low Results in 5 minutes Microscope
OSOM aPOC *(44)*	Symptomatic women only Vaginal swab	Sensitivity: 83–92% Specificity: 99–100%	Low Results in 10 minutes No instrumentation needed
Amplivue® POC NAAT *(57)*	Women only Vaginal swab	Sensitivity: 97–100% Specificity: 97–99%	Moderate[a] Results in ~45 minutes Small instrument
Cepheid Xpert® POC NAAT *(40)*	Women & Men Endocervical or vaginal swabs and urine	Sensitivity: 95–100% Specificity: 98–99%	Low-Moderate Results in ~40 minutes Small-medium instrument
Solana® POC NAAT *(48)*	Women only Vaginal swab or urine	Sensitivity: 90–99% Specificity: 97–99%	Moderate[a] Results in < 1 hour Small instrument
Visby POC NAAT *(58)*	Women only Vaginal swabs		Low Results in 30 minutes No instrument, power required

aPOC: antigen point-of-care; POC NAAT: point-of-care nucleic acid amplification tests.

[a]Although described as POC tests, these tests require trained laboratorians and are unlikely to be used in clinical settings, so they are described with the lab-based NAATs below.

- Microscopy must be performed and interpreted within 10 minutes for optimal results, and has the highest sensitivity in symptomatic women.
- Well validated aPOC tests have substantially higher sensitivity than microscopy and provide rapid results with minimal technical expertise.
- NAATs and POC NAATs have the best performance but may be too expensive unless tests for *C. trachomatis/N. gonorrhoeae* are already being used and *T. vaginalis* is included in those assays.

9.4.3 POC NAATs

POC NAATs must not be confused with currently available aPOC assays. POC NAATs perform at a similar level of sensitivity and specificity as laboratory-based NAATs but with the advantage of being carried out in near-patient settings in support of test-and-treat schemes. Several of these assays can be used at the POC if there is a qualified on-site laboratory. These assays are described in the section on laboratory-based NAATs (section 9.4.4) because they require trained laboratory technicians and are considered to be moderately complex to perform. Two FDA-approved assays that can be run by non-laboratorians are described below. Several new POC NAATs are in development so if these tests are an affordable option, continue to check the literature for improved options. For all of these POC NAATs, the major hurdle for adoption will be cost and access to electricity.

The Cepheid Xpert TV assay performed on the GeneXpert instrument is a widely available molecular assay that can be performed at the POC and takes less than 1 hour to complete (this time to result is expected to be reduced in the near future). The assay is a cartridge-based test utilizing 5' exonuclease real-time PCR. Individual cartridges are self-contained in order to minimize potential for carry-over contamination *(59)*. The Xpert TV assay contains a cellular control *(60)* to provide indirect evidence of specimen adequacy and a sample processing control to verify that negative results are not due to inhibition. The assay requires a single transfer step followed by insertion of the cartridge into an instrument bay, making it simple to use. The GeneXpert instrument comes in 2-, 4-, 8-, 16- and 48-bay sizes, allowing the user to support the throughput necessary for the clinic's needs. An automated 80-bay system is available for use in very high throughput clinics or in reference laboratories. Many laboratories around the world already have access to and are familiar with the GeneXpert because of the tuberculosis assay that uses the same system.

Approved sample types include endocervical and vaginal swabs as well as urine from both men and women. In a large USA clinical trial enrolling 1867 women and 4791 men, sensitivity was estimated at 96.4, 98.9, 98.4 and 97.2% for vaginal and endocervical swabs and female and male urine, respectively *(61)*.

The ***Visby Medical Sexual Health test*** is a 30-minute polymerase chain reaction (PCR) assay for detection of *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* that is performed in a completely disposable cartridge with no external instrumentation. This PCR system uses a chromatographic lateral flow system for organism-specific detection of amplified target sequences. The cartridge requires power via an AC adapter and cannot be moved while the assay is running. If running several samples simultaneously, the testing staff will need to ensure a bench space where the assay can be immobile. As a result of the lateral flow output (similar to a rapid HIV test), there is no electronic capture of data. The assay has been cleared by the FDA for use with vaginal swabs (97.6% sensitivity) *(58,62)*.

9.4.4 Laboratory-based NAATs

NAATs offer the greatest flexibility in sample collection methods as well as the highest sensitivity of all available diagnostic methods. Residual genital samples used for diagnosis of chlamydia and gonorrhoea using NAATs are appropriate for detection of *T. vaginalis* nucleic acids. Laboratories that routinely run chlamydia and gonorrhoea NAATs should consider testing for *T. vaginalis*. While the sensitivity and specificity of NAATs is excellent, these assays may be affected by environmental contamination and thus warrant strict adherence to good laboratory practice. It is also strongly recommended that the performance of any laboratory-developed test for *T. vaginalis* NAAT is strictly validated, before use in diagnostics, against at least one internationally validated NAAT, ideally an FDA-cleared assay, and subsequently used with an appropriate quality assurance system (see Chapter 5). The current FDA-cleared assays for detection of *T. vaginalis* molecular targets are described in Table 9.6. It is important to follow specific details in the IFU for each of these assays.

Table 9.6: Laboratory-based nucleic acid amplification tests (NAATs) for *Trichomonas vaginalis*

Assay[a]	Platform features	Complexity Time to results
Alinity m STI Panel Laboratory -based	Very large instrument. True quadriplex CT/NG/TV/MG	High complexity; fully automated
AmpliVue For use on-site or in central laboratory	Detects only *T. vaginalis*	Moderate complexity; pipetting required. Results are available in < 1 hour.
BD CTGCTV2 on MAX Laboratory-based	Medium instrument, bench-top. Can run 48 samples per 8 hours.	High complexity; partially automated. Time to results ~3.5 hours.
BD ProbeTec ET on Viper Laboratory-based	Large instrument. Can test 92–276 samples per 8 hours. Throughput is lower if also testing for CT/NG. Being phased out.	High complexity; partially automated. Time to results ~6 hours.
Hologic Aptima TV Laboratory-based	Large instrument. Approximately 250 samples per 8 hours. Detects rRNA so extremely sensitive.	High complexity; fully automated.
Roche cobas 6800/8800 TV/MG Laboratory-based	Gigantic footprint. Combo with assay. 384/1056 samples per 8-hours for the 6800/8800. Reagents stored on-board up to 90 days	Moderate complexity; fully automated. Run requires ~5 hours.
Solana For use on-site or in central laboratory	Detects only *T. vaginalis*	Moderate complexity. Results available in < 1 hour.

CT: *Chlamydia trachomatis*; MG: *Mycoplasma genitalium*; NG: *Neisseria gonorrhoeae*; TV: *Trichomonas vaginalis*.

[a]All samples can also be used for chlamydia/gonorrhoea testing unless noted otherwise. See manufacturer's instructions for use (IFU) for performing the assay.

- U.S. FDA-cleared NAATs have very high sensitivity and are especially useful in settings where chlamydia/gonorrhoea testing is being performed using NAATs.
- NAAT diagnostics are expensive and require specialized equipment, reagents and technical expertise.

i. Abbott Alinity m *T. vaginalis* assay

The Alinity m STI Assay is the next generation of NAAT from Abbott that can process up to 300 samples per 8-hour shift. This assay is run on the Alinity m platform and can simultaneously detect *C. trachomatis, N. gonorrhoeae, T. vaginalis* and *Mycoplasma genitalium,* making it the first FDA-cleared quadriplex assay available for discharge-causing STI. The assay detects ribosomal RNA (rRNA) from *T. vaginalis*. The assay utilizes magnetic micro-particle nucleic acid extraction followed by a PCR assay for amplification and real-time detection of fluorescent outputs. There are no hands-on steps after starting the assay. The assay includes a human cellular control and an internal control to assess specimen adequacy and to serve as an extraction and amplification control. No open samples are handled following amplification to minimize potential for carry-over contamination.

Approved sample types for *T. vaginalis* detection include vaginal swabs (patient- or clinician-collected) and endocervical swabs as well as urine. All sample types are collected using the Alinity multi-collect specimen collection kit. Sample tubes are then placed directly into the Alinity m system for testing. Specimens in the transport buffer are stable for up to 60 days if stored at 2–8 °C. When compared to the m2000 and Hologic AC2 assays, sensitivity/specificity estimates were 99.7/97.2% for self-obtained vaginal swabs, 97.7/96.9% for endocervical swabs, 97.7/99.1% for female urine, and 98.7/99.2% for male urine, respectively.[32] This assay was only recently approved by the U.S. FDA, but has been available in Europe where comparative studies support the use of this assay in laboratories performing NAAT testing *(63,64)*.

ii. AmpliVue *T. vaginalis* assay

The Amplivue Trichomonas Assay (Quidel) is another rapid test providing qualitative detection of T. vaginalis that has been FDA approved for vaginal specimens from symptomatic and asymptomatic women. The test has a sensitivity of 90.7% and specificity of 98.9% compared to the Aptima assay *(49,65)*. This test utilizes an isothermic process with helicase-dependent amplification. While it requires less than 1 hour to run and the system is small, it is more complex than many clinical settings have the capacity to perform. Given the cost and complexity, this adds little improvement over the antigen-based Osom assay.

iii. Becton Dickinson *T. vaginalis* assays

Becton Dickinson's second-generation assay is the FDA-approved ProbeTec ET CTQx/GCQx on the Viper System with XTR (Viper). The Qx test uses isothermal strand displacement amplification to amplify and detect target sequences simultaneously at 52.5 °C. Amplification of a sequence of the *Trichomonas* adhesion protein (ap65-I) occurs in a sealed plate with a fluorescent energy transfer read-out *(45)*. This assay is semi-automated and can generate 278 results during a single 8-hour work shift. The manual steps involve preheating of samples to improve the lysis process. Following preheating, the samples are transferred to the Viper instrument and only a single intervention (plate sealing) is performed after that point. This assay uses a duplex chemistry that includes an amplification detection paired with each chlamydial or gonococcal detection reaction to verify extraction success and the absence of inhibition. The system is highly robust with excellent time-motion characteristics.

Approved samples for the ProbeTec tests include endocervical and patient-collected vaginal swabs. Vaginal swabs may be collected and sent to the laboratory without transport buffer if they will be stored at 2–30 °C, transported to the laboratory and placed into buffer within 14 days. Swabs in buffer are stable in these conditions for up to 30 days. Urine, stored at 2–30 °C, must be transferred (2–3 ml) to buffer within a day of collection and is then stable for another 30 days under the same storage conditions. When compared to Hologic Aptima TV, sensitivity has been estimated at 98.3% for vaginal swabs with a specificity of 99.0% *(45)*.

Becton Dickinson's newest assay is the CTGCTV2 assay on the BD MAX system. This assay is a true triplex real-time PCR assay that is performed on a benchtop-size instrument *(66)*. The assay is "unitized" in that specimen processing and DNA extraction are performed in a single strip per sample that contains all of the necessary reagents and consumables. The instrument transfers extracted samples in master mix to a microfluidic amplification card (with space for 24 reactions) from which amplified product is detected in real-time using fluorophore emissions. The samples for this assay do not require a pre-warm so the collection tubes are placed directly into racks and strips and amplifications cards are added as needed. The instrument can test 1–24 strips during a run, thus providing a solution with a highly sensitive NAAT that can be run in response to a limited number of tests. This is ideal for settings that have a hospital laboratory running small volumes of samples because the need for batching at approximately 96 samples for the process to be cost efficient is removed. This assay may be appropriate for laboratories that want to test locally (to avoid shipping costs and delays) and have staff that can perform the automated testing in small batches. Testing is complete in approximately 3.5 hours, but samples cannot be added once the

[32] Data from the U.S. FDA submission documents can be found at: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm?ID=K202977

instrument starts a run. It is therefore unlikely that this solution would support test and treat on the same day as the clinic visit.

Approved sample types include endocervical specimens, vaginal swabs and urine from men or women. Since the assay is a triplex that includes testing for *T. vaginalis*, the preferred sample type for women is the vaginal swab (either self- or clinician-collected). Sensitivity estimates from the clinical studies performed in the USA for this assay were 98.4%, 94.5% and 97.9% for vaginal swabs, endocervical swabs and male urine, respectively. Female urine performed similarly to female urine in other tests, but was not evaluated against full patient-infection status that included other genital sites of infection (see BD CTGCTV2 on MAX package insert for details) *(36)*.

iv. Hologic Aptima *T. vaginalis* assay

The FDA-approved APTIMA TV (ATV) assay (Hologic) is based on the principle of rRNA target capture (intended to reduce or eliminate inhibition of amplification), i.e. isolation of target rRNA sequences using capture oligonucleotides and DNA magnetic beads, followed by amplification using transcription-mediated amplification technology targeting a sequence of the small ribosomal subunit of *T. vaginalis*. Amplification is detected using the kinetics of light emission from labelled DNA probes complementary to the target region. The assay can be performed in parallel with the Aptima Combo 2 (chlamydia/gonorrhoea) assay from a single specimen.

Approved sample types include endocervical and vaginal swab specimens. Swabs are collected using the manufacturer's transport medium and are then stable for up to 60 days at room temperature, making them ideal for transport to distant laboratories. During one working day (8 hours), approximately 250 samples can be tested using the automated Panther system. Estimates of sensitivity/specificity from the USA clinical studies for FDA submission were 100%/98.2% and 100%/98.1% for vaginal and endocervical swabs, respectively.[33]

Since samples remain sealed following amplification, the potential for carry-over environmental contamination is expected to be very low. However, environmental monitoring is strongly recommended in the absence of an enzymatic or other control measure for degradation of amplified product. The manufacturer strongly recommends stringent adherence to cleaning and decontamination procedures. Reproducibility should be monitored.

v. Roche *T. vaginalis* assay

In 2021, the Roche TV/MG assay for use on the cobas 6800/8800 systems was approved by the FDA. The assay uses a target on 5.8S rRNA, of which about 200 copies per organism are present, for detection of *T. vaginalis*. Other aspects of the extraction, amplification and detection methods are similar to other cobas assays *(67)*. Specimens used for *C. trachomatis* and/or *N. gonorrhoeae* testing on this platform can be tested in parallel for *T. vaginalis*. The instruments are very large and have substantial power requirements. However, reagents can be stored on board for up to 90 days utilizing the internal refrigeration units so the footprint contains the equivalent of a large refrigerator as well as a molecular analyser. For very large reference laboratories, the cobas 6800 (which can generate 384 results per day) and the 8800 (which can generate 1056 results in the same time period) can also be connected to Roche haematology, chemistry and immunology instruments for extremely high efficiency. However, for smaller throughput laboratories, Roche has developed the cobas 5800 System, which is substantially smaller and can run the same tests and use the same reagent packs to generate 96 results per day.

Approved specimen types for detection of T. vaginalis include urine and endocervical and vaginal swabs from women and urine and meatal swabs from men. From a study of approximately 1000 women and 1000 men, the estimated sensitivities of this assay were 99.4% for vaginal swabs, 97.7% for female urine, 97.6% for endocervical swabs, 94.7% for endocervical samples in LBC, 100% for meatal swabs and 100% for male urine *(37)*.

vi. Solana *T. vaginalis* assay

The Solana Trichomonas Assay is a rapid in vitro diagnostic test for the qualitative detection of nucleic acids isolated from clinician-collected vaginal swab specimens or urine specimens obtained from asymptomatic or symptomatic women. The assay utilizes helicase-dependent amplification and fluorescent probe-based detection in the Solana instrument to determine assay results. Sensitivities and specificities are > 98% for both symptomatic and asymptomatic persons for vaginal swabs and > 92% for urines *(44,57)*. This assay is similar to the Amplivue assay in that it is too complex for most site labs and offers little advantage over the Osom test.

Fig. 9.3: InPouch TV Culture System

Photography credit: Barbara Van Der Pol.

[33] For data from the assay package insert, see https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfPMN/pmn.cfm?ID=K122062

9.4.5 Culture

Culture has been the cornerstone for *T. vaginalis* diagnosis for many years. *T. vaginalis* is an anaerobic organism that grows more slowly under aerobic conditions. Nowadays, culture is usually performed using modified Diamond's medium or the commercial InPouch TV culture system (BioMed Diagnostics) (Fig. 9.3). Cultures should be incubated for up to 5–7 days. It is important to note that *T. vaginalis* should be grown at the bottom of the culture tube or pouch and, accordingly, the tube or tubes should be incubated in a vertical position. Furthermore, culture medium should be pre-reduced and culture tubes slightly opened before placement in an anaerobic jar for incubation at 37 °C.

Diamond's original medium T (see Annex 1) has subsequently been modified according to Fouts and Kraus, i.e. with streptomycin replaced by netilmicin *(68)*. The modified medium improves the sensitivity to approximately 75% relative to NAATs. However, culture of T. vaginalis has some disadvantages; the sensitivity of culture is low compared to results obtained with NAAT (especially for men), it is a time-consuming procedure, and it requires that patients return for results. Other culture media have also been described *(69–71)* (see Annex 1). Swabs should be eluted or urine sediment inoculated directly into culture medium at the clinical site. For sites that do not have access to culture medium due to short shelf-life or other reasons, swabs can be placed in tubes containing Amies medium and stored at 4 °C for transport to arrive in the central laboratory within 24 hours. Cultures should be gently mixed and a drop taken from the bottom of the tube (highest concentration of trichomonads) for wet preparation microscopy each day for up to 7 days. If it is not possible to perform examination daily, examination after 3–4 days and again at 7 days will detect almost all positive specimens.

In the mid-1990s, a culture system became commercially available that increased the use of culture for diagnosis *(72,73)*. This system, the InPouch TV culture system, utilizes a self-contained two-chamber pouch (Fig. 9.3). Swab samples are eluted or urine sediment is inoculated into the medium in the first section, the medium is forced into the second section, and the pouch is sealed. Pouches are transferred to the laboratory for incubation at 37°C for up to 5 days. The pouch system can be concluded earlier than standard culture since the entire volume is assessed each time. Sealed pouches are read by placing the entire pouch on the microscope stage, using 100× magnifications. Pouch holders that fit into the slide clips are available and should be used to assist with movement of the pouch during microscopy. The entire culture is read by carefully scanning from end to end. It is important to note that the thickness of the pouch requires that all planes of focus be evaluated; this requires movement top to bottom as well as side to side. Slides should be read daily until day 5. If no trichomonads are identified by day 5, the culture is reported as negative.

- Culture has sensitivity similar to appropriate aPOC tests, lower than NAATs and POC NAATs, and may be used also for testing men.
- Culture is necessary for obtaining isolates for susceptibility testing.

9.5 Antimicrobial resistance

Isolates of *T. vaginalis* with decreased susceptibility and resistance to metronidazole (in general first-line treatment) and tinidazole (usually second-line therapy) have been described, but occur rarely *(74,75)*. Nevertheless, in certain settings low-level metronidazole resistance has been identified in 0–9.6% of specimens tested *(76)*. The origin of the antimicrobial resistance is unclear, but many of the resistant *T. vaginalis* strains appear to be aero-tolerant. Organisms that are facultative are generally resistant to metronidazole. However, the resistance data are scant and additional studies in this area are needed to determine the mechanisms, prevalence and epidemiology of this resistance. However, as most organisms are susceptible, antimicrobial susceptibility testing *(27,77)* is not routinely performed. Nevertheless, some reference laboratories need to maintain capacity to perform antimicrobial susceptibility testing of *T. vaginalis* isolates, particularly when patients appear to fail metronidazole therapy. Patients returning with continued symptoms should be evaluated in the context of possible behavioural exposures and adherence to treatment regimens. Examination of the influence of the *T. vaginalis* viruses that often co-exist with *T. vaginalis* infection failed to find an association *(78)*. A review of eight studies of clinically resistant *T. vaginalis* found that high-level in vitro metronidazole resistance was rare; low-moderate level was described in most of the cases. However, clinical resistance of trichomoniasis to metronidazole has been widely reported *(76)*. Others studies have published similar findings *(79,80)*.

IMPORTANT: Clinicians should be aware that antimicrobial resistance has been described and this issue should be considered for people with continuing symptoms.

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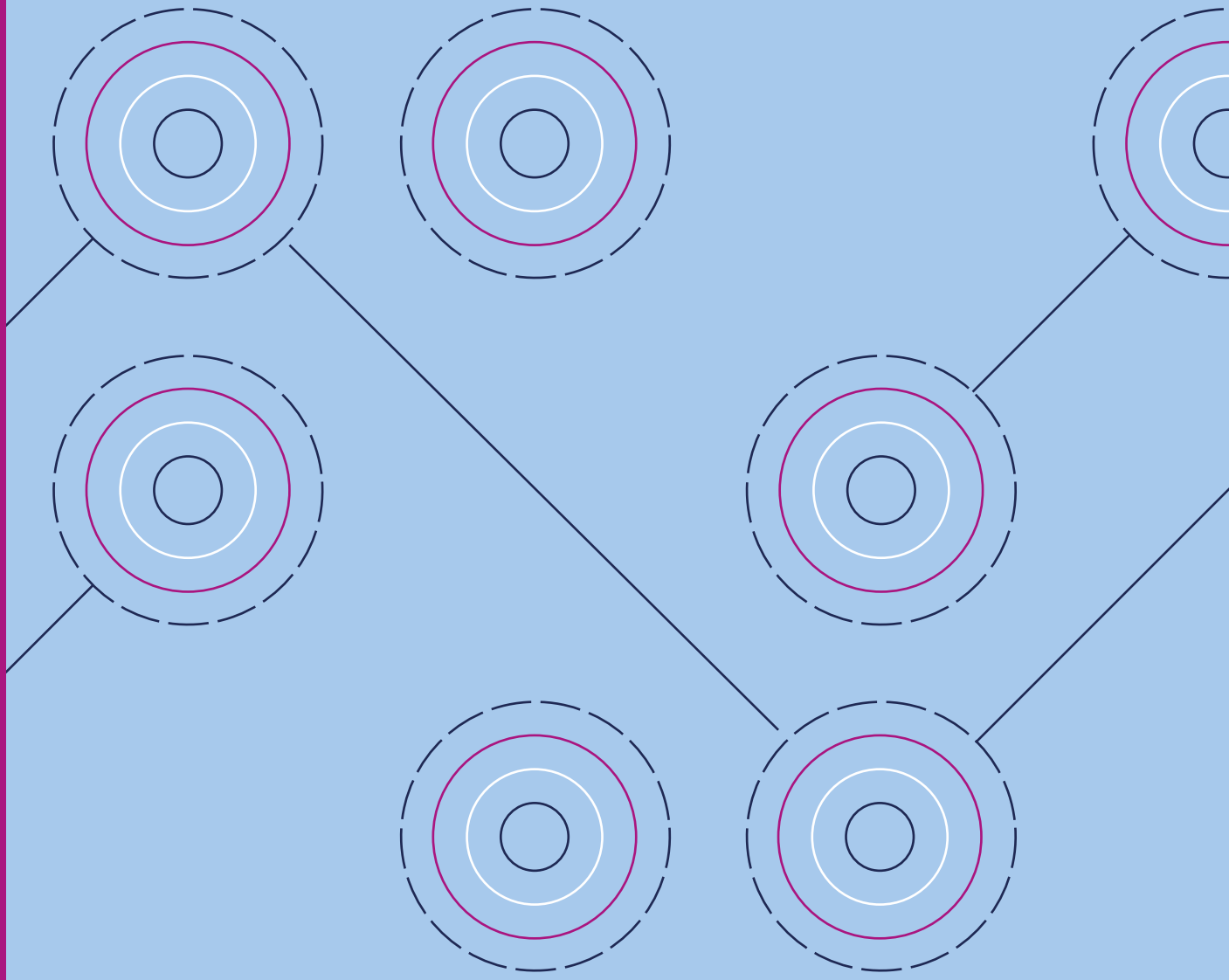
Chapter 10. Bacterial vaginosis (BV)



Chapter 10. Bacterial vaginosis (BV)

Catriona Bradshaw, Catherine Ison, Janet Wilson and Jörgen Skov Jensen

10.1 Introduction

Bacterial vaginosis (BV) is a polymicrobial dysbiosis of the vaginal microbiome, which can be asymptomatic, or present as a clinical syndrome with an abnormal vaginal discharge and malodour. It is the most common cause of vaginal discharge among women of childbearing age. BV prevalence varies across countries and population groups, but a recent systematic review and meta-analysis reported the global prevalence of BV among women of reproductive age as being in the range of 23–29% *(1)*. BV is characterized by an increase in diversity and load of facultative and anaerobic bacteria, a reduction in beneficial lactobacilli, and an increase in vaginal pH and volatile amines *(2–4)*. This contrasts with the optimal vaginal microbiome in reproductive aged women, which is typically dominated by lactic acid producing lactobacilli and a vaginal pH < 4.5 *(5,6)*. BV is associated with an increased risk of gynaecological and obstetric sequelae, including preterm delivery, spontaneous abortion, and increased risk of HIV acquisition and transmission and acquisition of STIs *(7–9)*.

Molecular studies have shown a large number of bacterial species are associated with BV. BV-associated bacteria include species such as *Gardnerella* spp., *Prevotella* spp., *Fannyhessea vaginae*, *Mobiluncus* spp., *Megasphaera* spp., and *Sneathia* spp., with many species having synergistic interdependent relationships *(2,3)*. The bacterial nomenclature is in rapid evolution with renaming of some recently cultured species such as *Megasphaera* type 1 and 2 to *Megasphaera lornae* and *Megasphaera hutchinsoni*, respectively *(10)*. *Atopobium vaginae* has been recently renamed as *Fannyhessea vaginae (11)*, and *Gardnerella vaginalis* has been subdivided into four separate species with the addition of *Gardnerella leopoldii sp. nov.*, *Gardnerella piotii sp. nov.*, and *Gardnerella swidsinskii sp. nov. (12)*.

While the exact aetiological agent (s) responsible for BV are not known, *Gardnerella* spp. are considered likely to be key founder organisms, displaying the greatest propensity to adhere to vaginal epithelial cells and initiate biofilm formation *(13,14)*. *Gardnerella* spp. and *Prevotella* spp. produce virulence factors such as sialidases, which degrade the cervicovaginal mucus, enhancing biofilm formation and facilitating attachment of other BV-associated bacteria *(15,16)*. This pathogenic process results in the symptoms and signs of BV, which form the criteria for one of the commonly used diagnostic methods, Amsel's criteria (outlined below) *(17)*. The mucinases and sialidases released by BV-associated bacteria cause the characteristic homogenous vaginal discharge of BV, and cause exfoliation of vaginal epithelial cells that are visible on microscopy as "clue cells". Some BV-associated bacteria produce volatile amines, which are responsible for the malodour and are the basis of the "amine test" *(18–20)*, and reduction and/or loss of lactic-acid producing lactobacilli cause the elevation in vaginal pH.

While the pathogenesis of BV is still not well understood, epidemiological and molecular data collectively indicate sexual transmission is likely to be involved in acquisition and recurrence. BV is associated with inconsistent condom use and new/increased number of sexual partners and has an epidemiological profile consistent with an STI by meta-analysis *(21,22)*. Molecular data support exchange of the genital microbiota between sexual partners, with the urethral and penile microbiota of males being more similar to the vaginal microbiota of a female partner than a non-partner *(23)*. Men with a high prevalence and abundance of penile BV-associated bacteria are more likely to have a female partner with microscopically confirmed BV (Nugent's criteria) *(24,25)*, and male circumcision reduces the prevalence and abundance of BV-associated bacteria in men *(26)*, and risk of BV in female partners *(27)*. Studies of female couples show high concordance for BV by microscopy, high concordance for specific BV-associated bacteria, and female couples without BV have high concordance for lactobacilli strains *(28–30)*. Among a cohort of women with female partners, incident BV occurred at a median of four days following sexual activity, with an increase in the relative abundance of *Gardnerella* spp., followed by *P. bivia*, and *F. vaginae* (formerly *A. vaginae*), suggesting an incubation period analogous to that of other bacterial STIs (i.e. chlamydia, gonorrhoea) *(31)*. Collectively, these data support exchange of both optimal and nonoptimal bacterial species between sexually active individuals,

but whether specific organisms can be targeted in partner treatment strategies to improve BV cure remains an area of ongoing research *(22,32)*.

10.2. Diagnosis

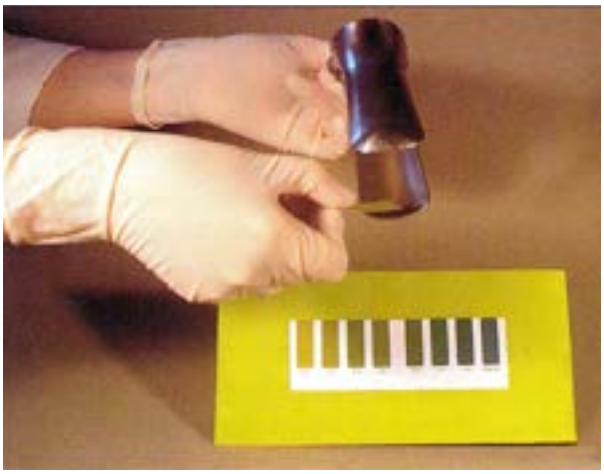
As BV lacks a single aetiology and is polymicrobial in nature, the laboratory diagnosis of BV has been fraught with difficulty, with multiple methods described in the literature and well summarized in recent reviews *(33–36)*. The Amsel criteria are a well-established and commonly used method to diagnose BV *(17)*. They require a clinical examination, but the diagnosis of BV can be made without access to a microscope if all the three clinical criteria are present. BV diagnosis is based on the presence of at least three of the following four criteria (Amsel's criteria):

- homogeneous white-grey adherent discharge;
- a vaginal fluid pH of > 4.5;
- the release of a fishy amine odour from the vaginal fluid when mixed with 10% potassium hydroxide (KOH) solution;
- "clue cells" visible on microscopic examination.

Discharge. The evaluation of this clinical sign is the most subjective. While the typical BV discharge is a homogenous thin white-grey discharge that adheres to the vaginal walls, vaginal discharge may not be markedly greater than that seen in healthy women. Moreover, the application of vaginal douches and products, and presence of blood, can reduce and/or alter the amount and appearance of discharge, impacting on this criterion.

Vaginal pH. The pH of vaginal fluid should be measured using pH indicator paper strips of appropriate range (3.8–6.0) and increments of no greater than 0.5, such as Whatman narrow range pH paper. A specimen is collected with a swab from the lateral and posterior fornices of the vagina and the swab is then rolled directly on to the paper strip. Alternatively, the pH paper can be touched to the tip of the speculum after it has been withdrawn from the vagina (Fig. 10.1). However, contact with cervical mucus or blood must be avoided as this falsely elevates the pH. The normal mature vagina has an acid pH of ≤ 4.0. In BV, the pH is generally elevated to > 4.5. The vaginal pH test has the highest sensitivity of the four characteristics, but the lowest specificity; an elevated pH also is observed if the vaginal fluid is contaminated with menstrual blood, cervical mucus, or semen, and in women with a *T. vaginalis* infection.

Fig. 10.1: pH testing of vaginal fluid, comparing to a standardized colour scale



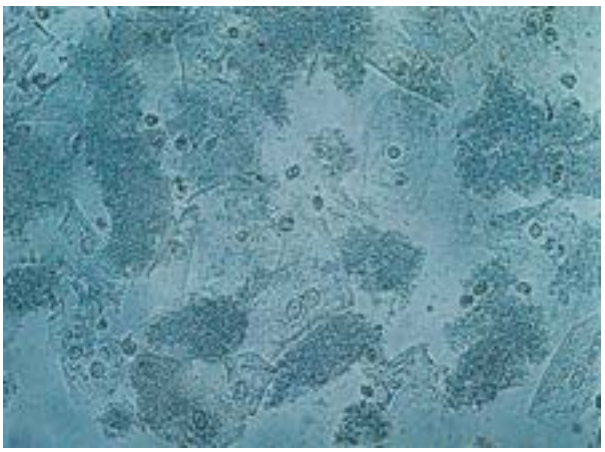
Source: WHO, 2013 *(37)*, reproduced by permission of the Division of Microbiology, Institute of Tropical Medicine, Antwerp, Belgium.

Odour. Women with BV often complain of an unpleasant vaginal smell. This odour is due to the release of amines, produced by decarboxylation of the amino acids lysine (to cadaverine) and arginine (to putrescine) by anaerobic bacteria. When KOH is added to the vaginal fluid, these amines immediately become volatile, producing the typical fishy odour. Place a drop of vaginal fluid on a glass slide and add a drop of 10% KOH. Hold the slide close to the nose to detect the amine odour. After a positive reaction, a specimen will quickly become odourless upon standing because the amines will be rapidly and completely volatilized. In some parts of the world, KOH is not available due to its caustic nature, and so if only three of the four criteria are performed, the sensitivity of the Amsel criteria is reduced. However, KOH is only used to increase our ability to smell the amines, so if a fishy odour is detectable during clinical examination, then the amine criterion can be considered positive. This criterion is somewhat subjective as there can be a marked difference between individuals' ability to smell the odour.

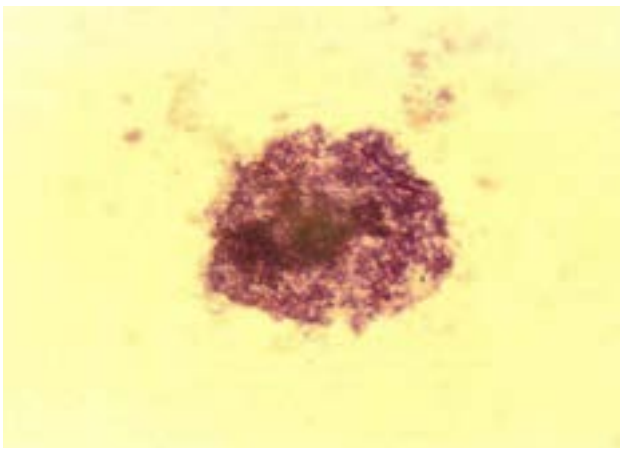
Clue cells. Mix a drop of vaginal fluid with a drop of saline on a glass slide. Place a coverslip over the suspension and examine microscopically at 400× magnification. Clue cells are squamous epithelial cells covered with many small cocco-bacillary organisms, giving a stippled, granular appearance; the edges of these epithelial cells are not clearly defined, owing to the large number of bacteria present and the apparent disintegration of the cells (Figs 10.2A and 10.2B). In most patients with BV, a mixture of normal exfoliated vaginal epithelial cells and 20% or more clue cells will be seen. The adhering bacteria on the cells are *G. vaginalis* mixed with anaerobes and are likely to represent BV-biofilm.

Fig. 10.2: Microscopy of vaginal smears

A. Clue cells in vaginal wet mount (400×)



B. Clue cell in Gram stain of vaginal smear



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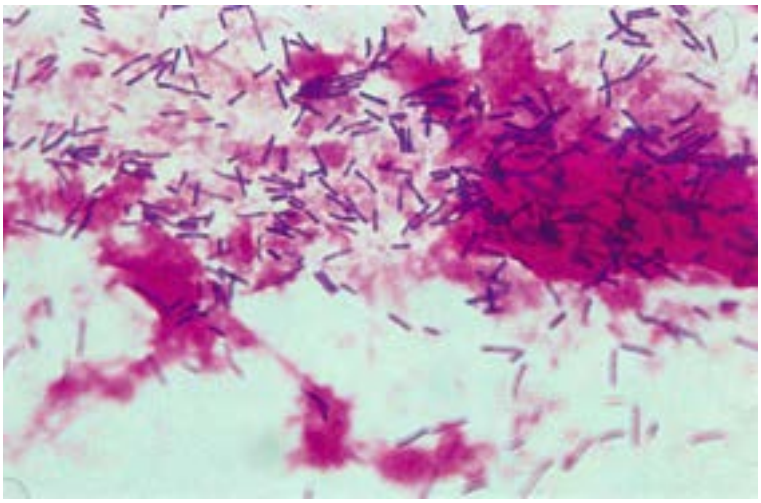
BV is recognizable on a Gram-stained vaginal smear. The slides can be examined in the clinic, if facilities are available, or stored for examination in the laboratory for later independent verification. Grades of vaginal flora ranging from normal, through intermediate, to BV morphotypes can be seen in the smear (Fig. 10.3). In the smear of a woman with BV, lactobacilli are either absent or reduced in number and are replaced by a mixed microbial flora. Two methods are commonly used for reading smears: Ison-Hay criteria and Nugent's criteria. The Ison-Hay criteria provide a relatively simple scoring method of vaginal bacterial morphotypes *(38)* and are suitable for use in routine clinical practice. Nugent's criteria *(39)* are slightly more complex and score the individual bacterial morphotypes and counts, thus providing a quantitative analysis that is particularly useful for research purposes, but is also used in clinical settings with expertise in vaginal microscopy. Inter-observer variability is reportedly the same whether using Nugent's criteriaor the Ison-Hay criteria *(40)*. Importantly, both methods require a microscope, skills in microscopy and an understanding of an optimal and non-optimal vaginal microbiota. However, unlike the Amsel's criteria method, both Ison-Hay and Nugent's criteria have the advantage of not being dependent on clinical examination, if this is not possible.

Fig. 10.3: Gram stain of normal vaginal smear showing lactobacilli (1000×)



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10.3 Ison-Hay criteria

This method assesses the relative proportions of the different bacterial morphotypes and grades them as follows *(38)*:

- Grade I (normal flora), lactobacilli morphotypes only or predominant (Fig. 10.4A)
- Grade II (intermediate flora), reduced lactobacilli morphotypes with mixed bacterial morphotypes (Fig. 10.4B)
- Grade III (BV), mixed bacterial morphotypes with few or absent lactobacilli morphotypes (Fig. 10.4C)

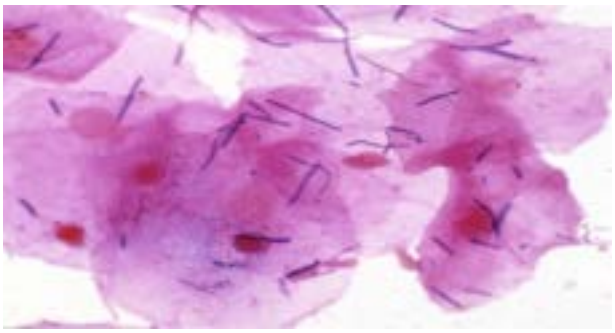
Two additional grades in the Ison-Hay system are:

- Grade 0, epithelial cells with no bacteria seen (Fig. 10.4D)
- Grade IV, epithelial cells covered with Gram-positive cocci only (Fig. 10.4E)

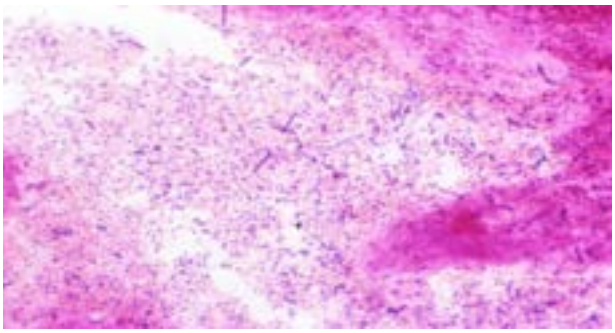
Both Grade 0 and Grade IV are found in normal women. Grade 0 predominantly follows intravaginal antimicrobial treatment and Grade IV is found in a small number of women who are longitudinally colonized with Gram-positive cocci, usually streptococci, often with no or reduced lactobacilli.

Fig. 10.4: Ison-Hay grades

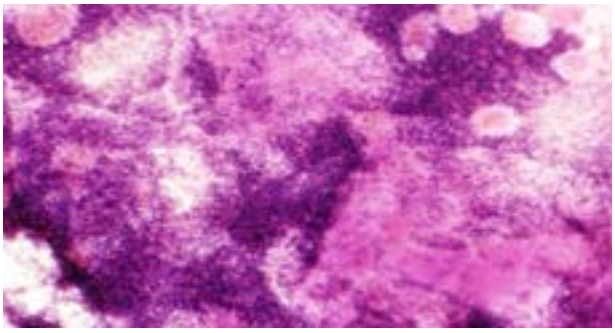
A. Grade I



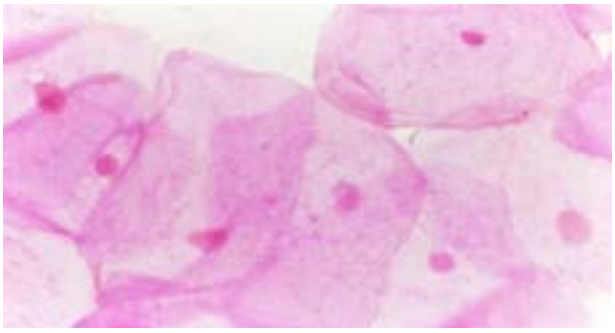
B. Grade II



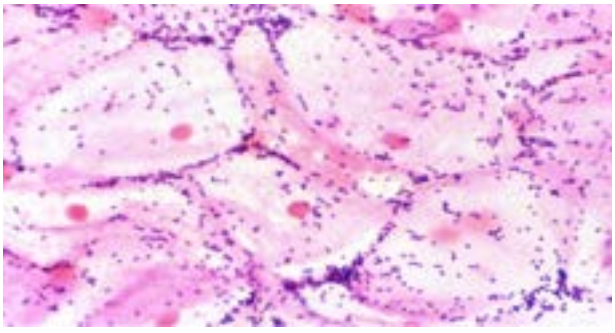
C. Grade III



D. Grade 0



E. Grade IV



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10.4 Nugent's criteria

This method relies on scoring of individual types of organisms. A score of 0–10 is derived from a weighted combination of the following: large Gram-positive rods (lactobacilli); small Gram-negative or Gram-variable rods (e.g. *G. vaginalis* or other anaerobes); and curved Gram-negative or Gram-variable rods (e.g. *Mobiluncus* spp.). Each of these three groups is quantitatively weighted as outlined in Table 10.1 *(39)*.

Table 10.1: Scoring system for number of morphotypes per high power field

	Average number of morphotypes per high-power-field (from 2–3 representative fields)		
Score	Gram-positive rods *(Lactobacillus)*	Gram-variable rods (e.g. *Gardnerella vaginalis*)	Curved rods (e.g. *Mobiluncus* spp.)
0	> 30	0	0
1	5–30	< 1	1–5
2	1–4	1–4	> 5
3	< 1	5–30	
4	0	> 30	
	Sum of the three scores: 0–10		

Based on this scoring of vaginal morphotypes, according to Nugent's criteria, a total score of:

- 7–10 (the sum of the rating scores of the three groups described above) is diagnostic of BV
- 4–6 is considered an intermediate microbiota
- 0–3 is considered an optimal or normal microbiota.

For example, a diagnosis of "severe BV" scores 10 (i.e. 4 for absence of *Lactobacillus* morphotypes, 4 for 4+ *Gardnerella* morphotypes and 2 for 4+ *Mobiluncus* morphotypes). Meanwhile, a "normal" vaginal Gram smear scores 0 (i.e. 0 for 4+ *Lactobacillus* morphotypes, 0 for 0 *Gardnerella* morphotypes and 0 for 0 *Mobiluncus* morphotypes).

- BV diagnosis can be made using Amsel's criteria by demonstrating the presence of at least three of the following four criteria: homogenous adherent whitish-grey vaginal discharge, pH > 4.5, amine smell and clue cells.
- Culture of organisms associated with BV is of no diagnostic value.
- Where facilities are available, a Gram stain of the vaginal discharge showing grades of vaginal flora from normal through intermediate to BV morphotypes can be scored by the Ison-Hay and Nugent's criteria for BV diagnosis.

10.5 Point-of-care (POC) tests

Microscopy of a Gram-stained vaginal smear (or wet preparation) currently remains the preferred laboratory method for the diagnosis of BV as it is cheap, rapid and can perform as a point-of-care (POC) test. However, as outlined above, microscopy requires training, expertise and access to a microscope.

A number of other POC tests have been described which may be useful, particularly if there is no microscope available. However, a series of parameters should be considered before using these tests, such as sensitivity, specificity, comparators used and the prevalence of BV, which will affect the predictive values.

Other POC tests:

1. Combination of amine test and pH > 4.5. In the absence of laboratory diagnostic facilities, the combination of a positive amine test and vaginal pH > 4.5 has been evaluated for BV diagnosis against the Nugent's criteria and Amsel's criteria. In a population of predominately symptomatic women in India, the combination of a positive amine test and pH > 4.5 was 83% sensitive and 47% specific compared to the Nugent's criteria *(41)*. In another study in tertiary health centres in India, a positive amine test and pH > 4.5 was 88% sensitive and 93% specific compared to using all Amsel's criteria *(42)*.

2. OSOM BV Blue (Genzyme Diagnostics). This is a commercially available POC test that measures sialidase, an enzyme produced by some BV-associated bacteria including *Gardnerella* spp. It is a chromogenic test, and on addition of vaginal secretions a blue/green colour indicates BV. The test takes 10 minutes and compared with the use of Nugent's and Amsel's criteria, it has a sensitivity of 88–94% and specificity of 91–98% *(43)*.
3. FemExam (Cooper Surgical). This POC test consists of two cards with indicators that measure vaginal pH, amines and proline aminopeptidase activity. Indicators on FemExam card 1 measure pH ≥ 4.7, and trimethylamine with concentration > 0.5 mmols. Card 2 measures proline aminopeptidase activity and takes 2 minutes. Compared to the Nugent's criteria, the combined sensitivity of cards 1 and 2 is 91% and specificity is 62% *(44)*.
4. BD Affirm VP (Becton Dickinson). This oligonucleotide probe test can be used as a POC test, but performance is better in a laboratory. It detects high concentrations of *Gardnerella* spp. (> 5×10^5 colony forming units per ml/vaginal fluid) and has a 30- to 45-minute turnaround time. It has a 94% sensitivity and 81% specificity compared to the Nugent method for BV diagnosis in symptomatic patients *(45)*. Addition of vaginal pH and the amine test increase sensitivity and specificity for BV diagnosis. The test is also available as Affirm VPIII, which includes detection of *Candida* and *T. vaginalis (46)*.
5. VGTest ion motility spectrometry (3QBD Ltd). This is a portable desktop ion mobility spectrometer (VGTest) used to detect malodorous biogenic amines (trimethylamine, puretrescine and cadaverine). The test showed 83% sensitivity and 92% specificity compared with the use of Amsel's criteria *(47)*.

10.6 Molecular diagnostic assays

Molecular assays are based on detection of specific bacterial targets, are generally more objective than microscopy, and have the advantages that they can detect non-cultivatable bacteria, often provide quantitation, and can be used on clinician-collected or self-collected swabs in symptomatic women. Commercial assays include nucleic acid amplification tests (NAATs) and direct DNA probe assays, and have been summarized in recent reviews *(33–36)*. Some assays have been approved by regulatory bodies (e.g. the U.S. Food and Drug Administration [FDA], CE-marked), and others are laboratory-developed tests that require internal validation before use. A number of commercially available assays are listed below with published sensitivities and specificities. Few comparisons between assays have been performed, and their listing in this chapter is not intended as an endorsement of any specific assay.

NAATs amplify bacterial DNA or RNA allowing sensitive detection of target molecules. As BV is a polymicrobial condition, most NAATs detect combinations of bacterial targets that have been selected to optimize sensitivity and specificity for BV compared to established diagnostic methods, such as Nugent's and Amsel's criteria. They use algorithms and quantitative targets that include varying numbers of BV-associated bacteria such as *Gardnerella* spp., *F. vaginae* (formerly *A. vaginae*), *M. lornae* (formerly *Megasphaera* type 1) and/or BVAB2, and often include one or more *Lactobacillus* species associated with an optimal vaginal microbiota (*L. crispatus*, *L. jensenii* and/or *L. gasseri*).

Examples of nucleic acid amplification tests (NAATs)

1. Hologic Aptima BV (Hologic). This real-time transcription-mediated amplification (TMA) NAAT for use on the automated Panther system detects RNA markers from *Gardnerella* spp.and *F. vaginae* (formerly *A. vaginae*), *L. crispatus*, *L. jensenii*, and/or *L. gasseri* in clinician- and patient-collected specimens. It uses an algorithm to report a qualitative result. Compared to Nugent's criteria (plus Amsel for intermediate Nugent), sensitivity and specificity in clinician-collected samples were 95.0% and 89.6%, respectively for BV. Sensitivities and specificities were similar in patient-collected samples. TMA also had higher sensitivity and specificity than clinicians' diagnoses and in-clinic assessments using Amsel criteria *(48)*.
2. Seegene Allplex Vaginitis Assay (Seegene). This multiplex quantitative polymerase chain reaction (PCR) assay detects and quantitates *Gardnerella* spp., *F. vaginae*, *Mobiluncus* spp., *Lactobacillus* spp. (plus seven *Candida* spp. and *T. vaginalis*). Compared to the Nugent method, sensitivity was 91.7% and specificity was 86.6% *(49)*.
3. Lab Corp NuSwab (Lab Corp of America Holdings). This multiplex quantitative PCR assay detects four bacterial species: *F. vaginae*, BVAB2, *M. lornae*, and *L. crispatus*. Compared to Nugent and Amsel criteria in symptomatic BV, it has 97% sensitivity and 92% specificity. The test reports a score: 0–1 = BV negative, 2 = intermediate and 3-6 = BV *(50)*. An extended version can detect *Candida albicans*, *C. glabrata*, *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*.
4. Quest Diagnostics SureSwab BV DNA quantitative real-time multiplex PCR (Quest Diagnostics) detects three lactobacilli (*L. crispatus*, *L. jensenii* and *L. acidophilus*) as negative predictors of BV and three BV-associated bacteria (*Gardnerella* spp., *F. vaginae* and *Megasphaera* spp.) as positive predictors of BV. No published, peer-reviewed efficacy data are available to assess its performance.

5. BD Max Vaginal Panel (Becton Dickinson). This quantitative real-time multiplex PCR detects two lactobacillus spp. (*L. crispatus* and *L. jensenii*) and four BV-associated bacteria (*Gardnerella* spp., *F. vaginae*, *M. lornae* and BVAB2). Compared to combined Nugent and Amsel criteria, sensitivity was 91% and specificity 86%. It also detects *T. vaginalis* and *Candida* spp. *(51)*.
6. MDL OneSwab BV Panel (Medical Diagnostic Lab). This quantitative real-time multiplex PCR uses quantitation of *Gardnerella* spp. and detection of *F. vaginae*, *M. lornae*, *M. hutchinsoni*, BVAB2 and *Lactobacillus* profiling. Compared to combined Nugent and Amsel criteria, sensitivity was 99% and specificity 94% *(52)*.
7. ATRiDA BV test (ATRiDA B.V.). This multiplex quantitative PCR targets *Gardnerella* spp., *F. vaginae*, *Lactobacillus* spp. and total bacteria. Sensitivity and specificity are reported to be 97% and 70%, respectively, relative to the Amsel criteria. Performance was higher in symptomatic women (sensitivity 98% and specificity 77%) *(53)*.
8. Amplisens Florocenosis/Bacterial vaginosis-FRT PCR kit (InterLab Service). This PCR assay measures relative concentrations of *Lactobacillus* spp., *Gardnerella* spp. clades 1 and 2, *F. vaginae* and total bacteria. Compared to vaginal microbiota analysis (16S ribosomal RNA gene sequencing), the assay was 81–98% sensitive and > 91% specific in two studies *(54)*.

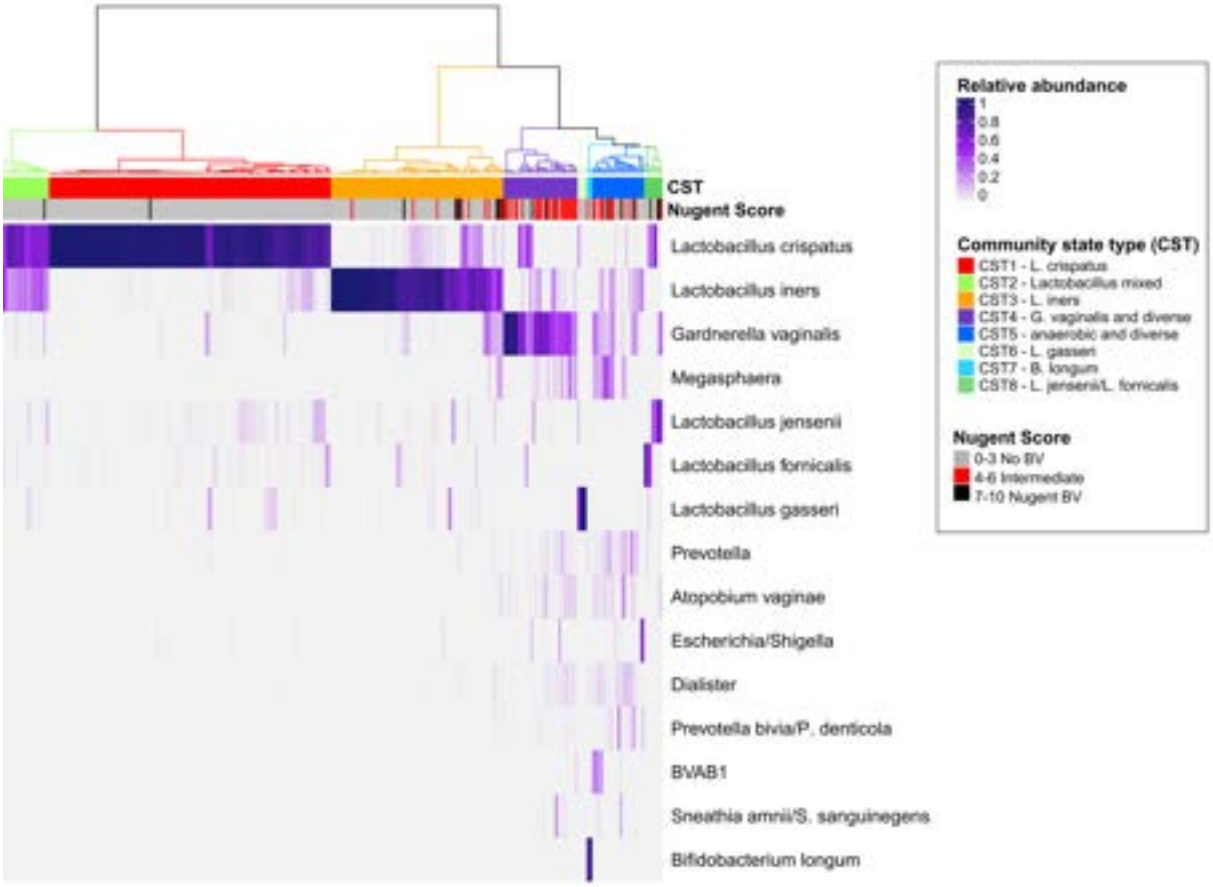
10.7 Future diagnostic approaches

Molecular methods such as high-throughput amplicon sequencing and metagenome sequencing can be used to determine the composition of the vaginal microbiota. Due to sequencing costs, procedural challenges, complexity of analysis and hands-on time, these methods have been more suited to research than diagnostics *(33)*.

Amplicon sequencing involves amplification of short regions of the 16S rRNA gene followed by sequencing. It is one of the most common methods used for characterizing the bacterial composition of the vaginal microbiota, although the *cpn60* gene can also be used. The 16S rRNA gene encodes the 16S rRNA and comprises nine hypervariable regions (V1–V9) flanked by highly conserved regions. The hypervariable sequence regions vary between bacteria, while conserved regions are identical or highly similar across bacteria. Universal PCR primers, which are complementary to conserved regions, can be used to amplify the hypervariable regions, allowing taxonomic identification of bacteria using bioinformatic methods that match sequences to a database of classified sequences. Primers targeting the V3–V4 regions are commonly used to characterize the composition of the vaginal microbiota, but there is currently no universal consensus, and different regions (V1–V3 or V1–V4) of the 16S rRNA gene have also been used.

Amplicon-based sequencing and bioinformatics methods have enabled researchers to group vaginal bacteria into clusters or community-state types (CSTs) that provide a deeper understanding of the diversity of the vaginal microbiota between women and across populations, but also correlate well with the diagnosis of BV by clinical and/or microscopic criteria. An early publication using pyrosequencing of the V1–V2 regions of the 16S rRNA gene described five CSTs, four of which were dominated by *Lactobacillus* species (CST I: *L. crispatus* dominated; CST II: *L. gasseri* dominated; CST III: *L. iners* dominated; CST V: *L. jensenii* dominated), and one CST that was deficient in *Lactobacillus* (CST IV) *(3)*. CST IV was a highly diverse group with low relative abundance of lactic acid bacteria and high abundance of anaerobic bacteria, which correlated with the syndrome of BV. Next-generation sequencing methods have the advantage of providing high resolution and very detailed information, being high throughput and able to detect small variations in genes, but they require highly resourced environments and are more complex and costly than NAATs used in diagnostics. Algorithms specifically for the diagnosis of BV are also not yet commercially available. However, attempts to standardize the classification of BV CSTs have been developed *(55)* and could potentially improve comparability between studies.

Fig. 10.5: Example of heatmap showing diversity of vaginal microbiota, based on sequencing of V3-V4 regions of the 16S rRNA gene



Source: Adapted with permission from Plummer et al., 2019 *(56)*.

Similarly, metagenomic sequencing has not been used for diagnostics. It provides more detailed information than 16S rRNA sequencing, with greater resolution for species and strain types. It has been more widely used in epidemiological studies and surveillance, including antimicrobial resistance surveillance. Other future methods that may be employed in diagnostic assays include tests that detect bacterial metabolites (based on metabolomics), proteins (based on proteomics) or immune markers, as biomarkers of BV *(33)*.

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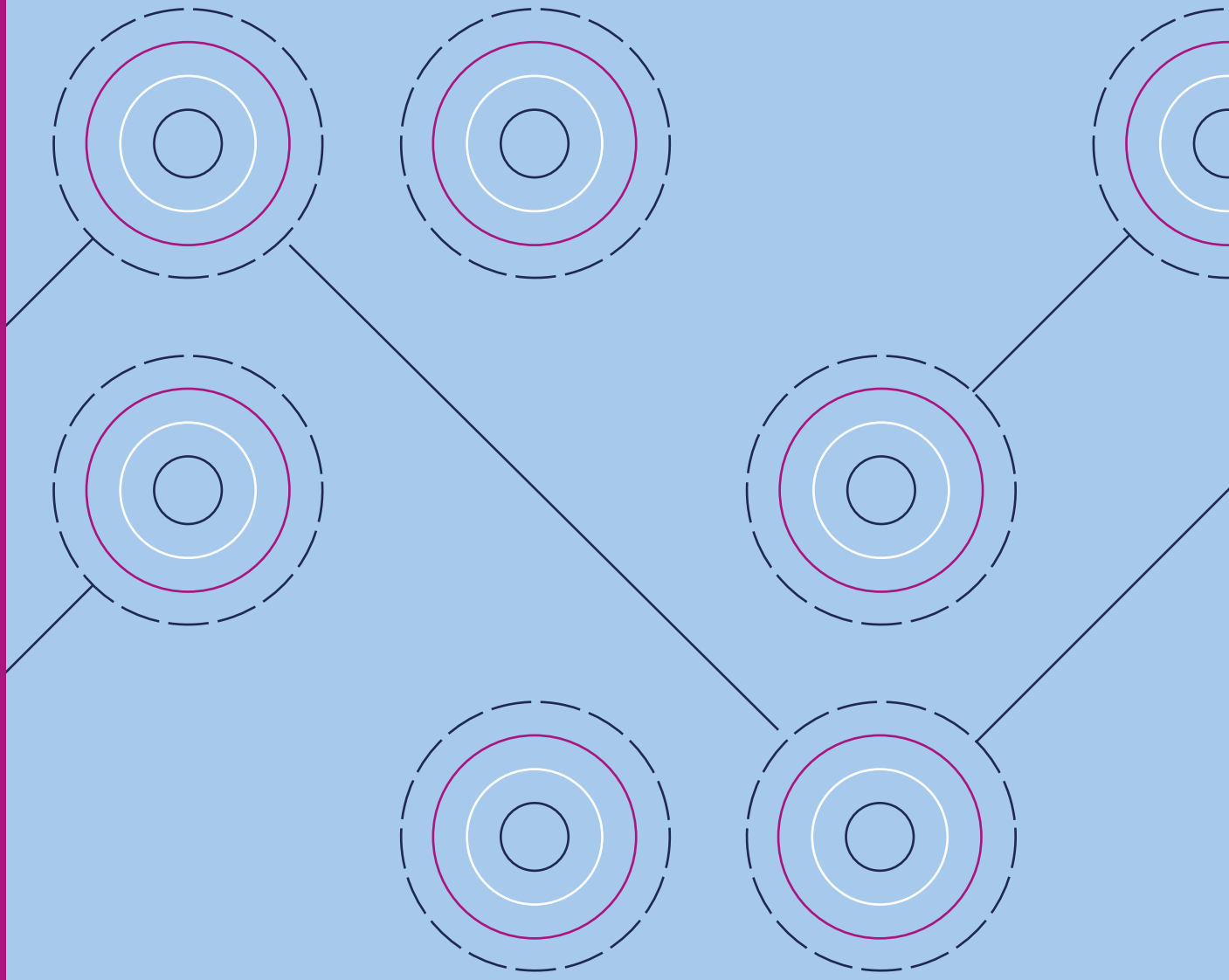
Chapter 11. Candidiasis



Chapter 11. Candidiasis

Catriona Bradshaw, Catherine Ison, Janet Wilson and Jörgen Skov Jensen

11.1 Introduction

Vulvovaginal candidiasis (VVC) is one of the commonest causes of vulvovaginitis and affects 70–75% of women in their lifetime *(1)*. VVC is caused by the fungus *Candida albicans* in approximately 85% of cases *(2)*, with the remaining 15% being caused by non-*albicans* species *(3)*. *C. glabrata* is the most common of the non-albicans species, with other species such as *C. krusei, C. tropicalis, C. parapsilosis, C. dubliniensis* and *Saccharomyces cerevisiae* less commonly causing vaginitis *(4)*. *Candida* spp. are usually of endogenous origin and can be isolated from the genital tract in up to 25% of asymptomatic healthy women of reproductive age. For *Candida* spp. to colonize the vagina, they must first adhere to the vaginal epithelial cells and then grow, proliferate and germinate, before finally invading the vulvovaginal epithelium and causing symptomatic inflammation. In addition to the damage caused by the fungus itself, the host response plays an important role in the symptoms of VVC. Some women develop a local mucosal overreaction of innate immunity with the recruited neutrophils, contributing to inflammatory symptom *(5,6)*.

Important predisposing factors for colonization and inflammation include:

- changes in oestrogen levels associated with the menstrual cycle, pregnancy, hormone replacement therapy and oral contraceptives;
- the use of antibiotics, which disrupts the *Lactobacillus*-dominated microbiota;
- diabetes mellitus (especially if taking sodium-glucose cotransporter 2 [SGLT2] inhibitors *[7]*);
- immunosuppression;
- atopy *(8)*.

Candidiasis is usually of endogenous origin and not considered to be an STI, although *Candida* spp. may be transmitted between partners. Symptomatic VVC is associated with vulvovaginal itch, soreness, swelling, and increased vaginal discharge, which is often thick and white with cheese-like curds; however, asymptomatic colonization with *Candida* spp. is also very common *(2)*. Recurrent VVC is defined as three or more episodes of symptomatic VVC over 12 months, and affects approximately 5% of women, although recent self-reported surveys indicate a prevalence as high as 9% in European and North American women *(9)*. Recurrent VVC is associated with the risk factors described above *(10)*, but host factors including genetic predisposition and atopy, and drug resistance and sub-therapeutic levels of antifungals, can also play a role *(11)*. Use of irritant chemical products and local allergy may also contribute to the induction of symptomatic vaginitis and vulvitis.

In men, the significance of *Candida* spp. is unclear, although it may be transmitted between sexual partners and can cause balanitis or balanoposthitis and rarely urethritis. Typically, men develop an allergic response to candidal antigen, although fulminant infection may be seen more frequently in patients with the risks described above and especially if taking SGLT2 inhibitors *(7)*.

11.2 Diagnosis

The diagnosis of VVC is usually established using the combination of clinical manifestations and microscopy of a wet mount preparation or Gram-stain. As mentioned, the classic symptoms and signs of VVC include vaginal itching, an odourless curdy white discharge (“cottage cheese”), a burning sensation in the vulva, dysuria, and erythema of the labia and vulva. Symptoms and signs, however, are often more equivocal. Detection of budding yeast cells and/or pseudohyphae by wet mount or potassium hydroxide (KOH) microscopy or Gram-stain can be performed in the laboratory or the clinic and has a very high predictive value for the diagnosis of VVC. Note that non-*albicans Candida* species generally do not form any traditional hyphae. While microscopy is generally used, in women with classic signs it is often reasonable to give therapy based on a presumptive clinical diagnosis without further confirmation by microscopy, if this is not available. More detail on microscopy is outlined in section 11.4.

In women with abnormal vaginal discharge and irritation, and in the absence of a microscope, the detection of a pH of < 4.5 is a good indicator of VVC and

can help to differentiate it from bacterial vaginosis and trichomoniasis, both of which typically produce a pH of > 4.5. A narrow range pH paper is an inexpensive, sensitive and simple method to use and is available in most settings.

Culture is a highly sensitive method for the detection of *Candida* spp., but it must be interpreted with caution, as colonizing *Candida* spp. can also be cultured from women without symptomatic VVC. Therefore, culture should be performed only if symptoms are considered likely to be due to VVC and microscopy is negative. Although as mentioned, presumptive treatment based on symptoms of VVC is often appropriate, as culture results take 48–72 hours. Culture is recommended if presumptive treatment has failed to resolve the symptoms and signs, and in recurrent VVC where long-term treatment is being considered. Culture is essential if antimicrobial susceptibility testing is required. More detail on culture and antimicrobial susceptibility testing are outlined in sections 11.5 and 11.6, respectively.

Molecular detection of *Candida* spp. using nucleic acid amplification tests (NAATs) is a new emerging area in diagnostics for VVC and is outlined in section 11.7.

11.3 Collection of specimens

Obtain a sample of discharge from the lateral vaginal wall with a swab (the type of fibre is not important). In patients who have only a slight vaginal discharge and extensive involvement of the vulva or labia, it is better to collect a specimen from the irritated mucosa. Direct microscopy can be done immediately at the clinic site, or the specimen may be transported to the laboratory. The use of a transport medium, such as Amies or a liquid-based medium, is not necessary for yeast identification, but is to be preferred to maintain viability and motility of trichomonads that may be present in the sample.

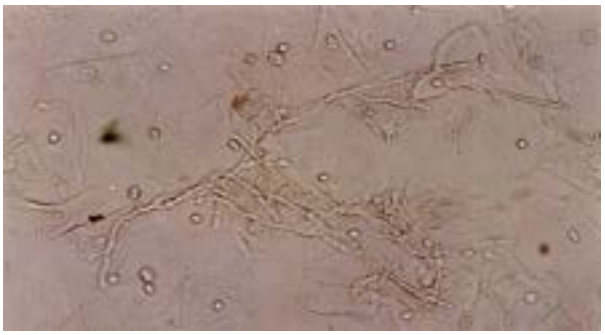
In males with balanitis, use a swab pre-moistened in saline to collect the sample from the glans penis.

11.4 Direct microscopy

Place the specimen on a glass slide and, if necessary, depending on its fluidity, mix with a drop of saline. Cover the preparation with a cover slip and examine microscopically at 400× magnification not only to detect yeast cells, but also to assess the presence of trichomonads and clue cells. Yeasts are round to ovoid cells, 4 µm in diameter and showing typical budding (blastoconidia) (Fig. 11.1). The addition of 10% KOH to the preparation increases the detection sensitivity of yeasts slightly, making the recognition of mycelia (pseudohyphae) much easier, but an additional sample will be needed to look for trichomonads as they will be killed by the KOH. Yeasts can easily be recognized on a Gram-stained smear as they are Gram-positive cells (Fig. 11.2).

Fig. 11.1: Potassium hydroxide (KOH) preparations showing yeasts

A. Budding yeasts and pseudo-hyphae (400×)



B. Yeasts without pseudo-hyphae

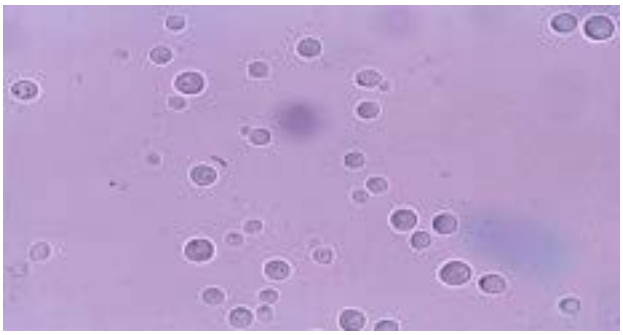


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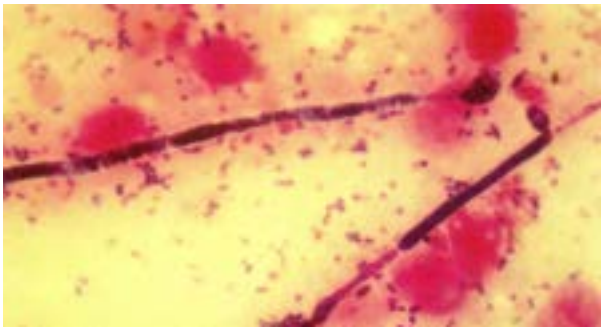
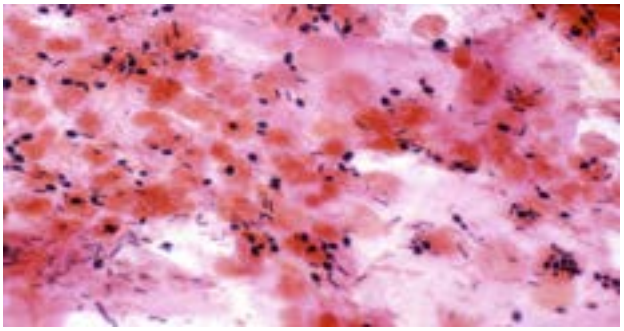
Fig. 11.2: Gram-stained smears of vaginal discharge

A. Gram-positive yeasts and pseudo-hyphae (1000×)



B. Budding yeasts without hyphae, likely to be a non-*albicans Candida* species



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11.5 Culture

Sabouraud dextrose agar with chloramphenicol or chromogenic agar are excellent growth media for the isolation of *Candida* spp. After inoculation of the clinical specimen, the plates are incubated at 36 °C for two days. Colonies of yeast cells are opaque and white to creamy in colour on non-chromogenic agar. Microscopy can be used to confirm the presence of yeast cells.

Further identification of yeasts is not necessary for routine diagnosis of uncomplicated VVC, however this is recommended if treatment has failed to resolve the symptoms and signs, and in recurrent VVC where long-term treatment is being considered, as many of the non-*albicans* yeasts are inherently less susceptible to azoles. Use of a chromogenic agar will provide a simple detection of non-*albicans Candida* spp.

11.5.1 Identification of species

Cultivation on commercially available chromogenic medium can be used for the isolation and presumptive identification of clinically important yeast *(12)*. This identifies *C. albicans* and non-albicans species based on a colour change of the colonies and is a convenient screening method. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is a rapid and reliable procedure for the accurate identification of pathogenic *Candida* strains that requires minimal hands-on time or time for the interpretation of the result *(13)*.

The germ tube test is a simple and cheap test for the presumptive identification of *C. albicans*. A colony is emulsified in 0.5 ml of bovine or horse serum and incubated at 36 °C for 4 hours. *C. albicans* will show short lateral hyphal filaments without any constrictions.

A complete identification of yeasts to species level can be obtained by means of auxanographic methods for carbohydrate and nitrate assimilation, or through carbohydrate fermentation tests. These methods are time-consuming and require expertise. Commercial kits are available.

- The diagnosis of candidiasis is established with a combination of clinical features and microscopy of a sample of an appropriate specimen.
- Culture of the genital discharge should be considered for negative microscopy in the presence of clinical symptoms. Different methods of identification of the species are available.
- Antimicrobial susceptibility testing of *Candida* is warranted when treatment has failed to resolve the symptoms and signs and in recurrent candidiasis. This should be performed in specialist centres.
- An increasing number of molecular tests for *Candida* spp. are entering use, although more data are needed to clearly define their role in the diagnosis of VVC.

11.6 Antifungal susceptibility testing

Antimicrobial resistance in *C. albicans* is an increasing problem, probably due to indiscriminate prescription of azole drugs. Furthermore, several non-*albicans Candida* spp. are inherently resistant to azoles. Consequently, antimicrobial susceptibility testing is important for the clinical management of patients with treatment failure, or in patients requiring long-term suppression therapy. Most of the methods used for susceptibility testing are designed for invasive yeast infection. However, the vaginal pH is significantly lower than that used for standard testing, and this may lead to minimum inhibitory concentration results that are misleading, as most antimicrobials have decreasing activity at acidic pH which is the norm for the vaginal niche. Consequently, it has been suggested that genital tract yeast isolates should be tested at pH 4.5 *(14,15)*. Most susceptibility testing is carried out as broth dilution testing (e.g. according to the Clinical and Laboratory Standards Institute guidelines, M27 *[16]*), but other methods exist.

11.7 Molecular diagnostics

An increasing number of molecular tests for *Candida* spp. are entering use, although more data are needed to clearly define their role in the diagnosis of VVC. Some commercially available assays are yet to publish their performance characteristics, and performance characteristics of three molecular assays have been outlined below. Importantly, exclusive use of NAATs for diagnosis of VVC and absence of culture precludes antimicrobial susceptibility testing *(11)*.

- **BD MAX Vaginal Panel** (Becton Dickinson). This quantitative real time multiplex PCR detects *Candida* spp. (defined as the *Candida* group [comprising *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. dubliniensis*], *C. glabrata* and *C. krusei*). The assay also detects *T. vaginalis*, two *Lactobacillus* spp. (*L. crispatus* and *L. jensenii*) and four BV-associated bacteria (*G. vaginalis*, *A. vaginae* (renamed to *Fannyhessea vaginae*) and *Megasphaera* type 1 (renamed to *Megasphaera lornae*) and BVAB2). On clinician-collected swabs, compared to *Candida* culture and sequencing for species identification, BD MAX was 90.9% sensitive and 94.1% specific for detection of *Candida* group and 75.9% sensitive and 99.7% specific for detection of *C. glabrata*. On self-collected swabs, compared to *Candida* culture and sequencing for species identification, BD MAX was 92.2% sensitive and 91.9% specific for *Candida* group and 86.7% sensitive and 99.6% specific for *C. glabrata* *(17)*. Another study using the same population comparing BD MAX Vaginal Panel to culture, found that for the *Candida* group, the assay had higher sensitivity (90.7% vs 57.5%) and specificity (93.6% vs 89.4%) than a clinical diagnosis of VVC *(18)*.
- **Seegene Allplex Vaginitis Test** (Seegene). This multiplex PCR detects and quantifies seven *Candida* spp. (*C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. lusitaniae*, *C. dubliniensis*). The assay also detects *T. vaginalis*, three BV-associated organisms (*A. vaginae* [renamed to *Fannyhessea vaginae*], *G. vaginalis*, *Mobiluncus* spp.) and *Lactobacillus* spp. Compared to culture for *Candida* spp., it was 91.1% sensitive and 95.6% specific for detection of any *Candida* spp. In a subset of symptomatic women, sensitivity for *C. albicans* was reduced (66.7%) *(19)*.
- **BD Affirm VPIII Candida Test** (Becton Dickinson). This nucleic acid probe-based assay detects *C. albicans* and *C. glabrata*. Compared to a reference group of culture for *Candida* spp. and the absence of other causes of vaginitis, the assay had 58.1% sensitivity and 100% specificity *(20)*. When evaluated as a point of care assay and compared to BD MAX Vaginal Panel it had excellent specificity for *Candida* spp. (100%) but low sensitivity (52.9% *(21)*.

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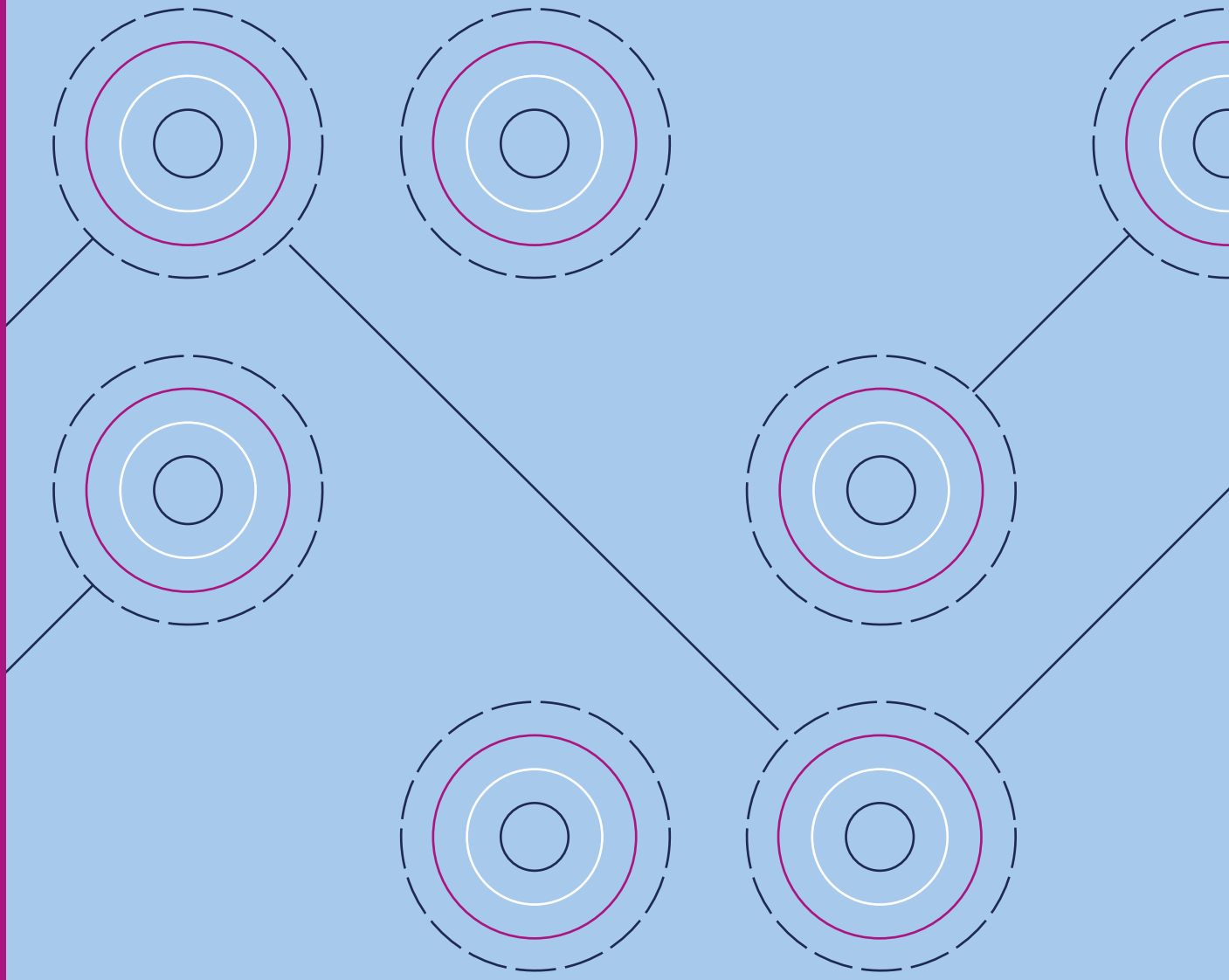
Chapter 12. Herpes simplex virus (HSV) infections



Chapter 12. Herpes simplex virus (HSV) infections

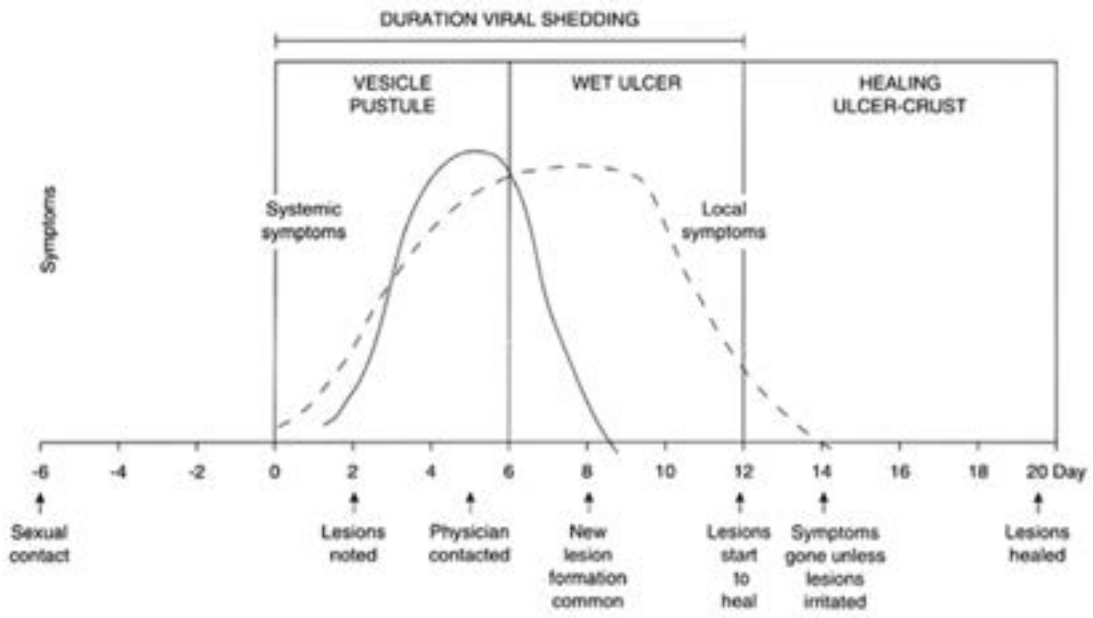
David Lewis and Anna Wald

12.1 Introduction

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are large double-stranded DNA viruses. HSV-1 and HSV-2 share a similar genome structure, with 40% sequence homology and 83% homology of their protein-coding regions, which explains the many biological similarities and antigenic cross-reactivity between the two serotypes.

Genital herpes is a common sexually transmitted infection (STI), affecting more than 400 million people worldwide *(1)*. The incubation period of both HSV-1 and HSV-2 is usually 2–10 days (Fig. 12.1). During primary infection, HSV particles enter through breaks in the skin or mucosa, attaching to and entering epithelial cells in which they replicate. Viral particles are then taken up by free sensory nerve endings and transported to the sensory ganglion serving that area of the skin. Subsequent replication and spread within the ganglion establish lifelong latency. Antegrade spread to the skin and mucosa results in clinical symptoms and signs *(2)*. Skin and mucosa manifestations may include vesicular lesions leading to shallow ulcerations that crust and heal spontaneously within 2–3 weeks without scarring (Fig. 12.1). Lesions lead to the focal destruction of the epithelial layer and an infiltration of inflammatory cells develops in the surrounding edge and in the underlying dermal layer. However, only 10–30% of new infections are symptomatic. After initial infection, the virus reactivates intermittently from the latent state and travels back down the sensory nerves to the skin or mucosal surface. Viral shedding can occur either in the presence of lesions (clinical reactivation) or with very mild or no symptoms (subclinical reactivation). Shedding from mucosal surfaces leads to transmission to sexual partners.

Fig. 12.1: Clinical course of primary genital herpes



Source: Corey et al., 1983 *(2)*,

The clinical severity of initial genital herpes depends on whether the host is immunologically naive. Primary infection, defined as infection in a person without previous HSV-1 or HSV-1, is clinically most severe and most likely to have systemic manifestations. Initial non-primary infection is defined as new acquisition of HSV-2 in someone with pre-existing HSV-1 infection. While previous HSV-1 infection does not protect against HSV-2 acquisition, HSV-2 infection in a person with previous HSV-1 is generally milder and more likely to be unnoticed. About a quarter of people with a first episode of HSV-2 have a first recognized recurrence, as the infection was acquired previously but was not symptomatic.

Recurrent episodes tend to be milder than initial episodes, normally healing within seven days, but occasionally can be severe, particularly in immunocompromised individuals. Approximately 70–90% of people with symptomatic genital HSV-2 and 20–50% with symptomatic genital HSV-1 will have a recurrence within the first year. The average number of recurrences for genital HSV-2 is four in the first year of infection. After the first year of infection, people with HSV-2 continue to have recurrences while persons with genital HSV-1 only rarely have additional recurrences. Similarly, viral shedding continues to be substantially higher in individuals with genital HSV-2 than genital HSV-1. As such, diagnosis requires identification of the type of HSV to inform prognosis and counselling. Immunocompetent people with genital herpes can have frequent, painful and recurrent genital lesions and the diagnosis itself is associated with much psychosocial distress.

The classical pattern of HSV-1 and HSV-2 infections associated with oral or genital diseases, respectively, remains in resource-poor countries where HSV-1 infection is almost universally a disease of childhood and HSV-2 infection is sexually transmitted. In contrast, the differentiation of HSV-1 from HSV-2 based on the anatomical site of infection is far from absolute in resource-rich countries because genital herpes may frequently be caused by HSV-1. The delay in acquisition of oral HSV-1 infection in early life makes a significant proportion of young adults susceptible to genital HSV-1 infection through initiation of sexual activity, including orogenital sexual contact. Genital HSV-1 infections now account for at least half of all first-episode genital herpes in young adults in resource-rich countries. HSV-2 seropositivity increases after sexual debut and steadily increases with age. HSV-2 seroprevalence varies according to global region, ranging from approximately 10–40% in adults and reaching 60–95% in some key populations, for example, people living with HIV and female sex workers. Patients who acquire HSV-2 share similar risk factors to patients acquiring other STIs, that is, a high number of lifetime sexual partners, previous history of STIs and early sexual debut.

The most serious consequence of genital herpetic infection is the potential for transmission from an infected woman to the neonate during vaginal delivery. This can result in disseminated infection, central nervous system involvement and possibly neonatal death. Even in the era of potent antiretroviral therapy, HSV-2 infection has been linked to a three-times higher risk of HIV acquisition, probably resulting from mucosal infiltration with activated CD4-bearing lymphocytes, the target cells for HIV-1 attachment. Similarly, the risk for HIV transmission to an uninfected sexual partner is higher if the source partner also has HSV-2.

12.2 Overview of diagnostic procedures

Genital herpes is often diagnosed on clinical grounds due to the presence of a cluster of vesicular lesions or shallow painful ulcers. However, as genital ulceration can be due to other STIs, non-STI infections or non-infectious causes, and given that mixed infections may occur, laboratory confirmation of HSV in lesion material is best practice to establish a correct diagnosis. The choice of test and how to interpret the results are important considerations for both clinicians and microbiologists *(3)*. Laboratory methods used for the diagnosis of HSV infection usually require direct detection of HSV in material from lesions (Table 12.1). Detection of HSV DNA in clinical specimens using amplified molecular tests is the method of choice due to their high sensitivity and specificity, minimal collection and transport requirements, and the fact that same-day results can be delivered to clinicians, if required. Older methodologies, such antigen detection and viral culture, may still be occasionally used in some settings. Cytology (Tzanck smears) is not recommended as a diagnostic technique. Indirect serological methods may be of occasional assistance in patient management and are required for HSV seroprevalence surveys (Table 12.2). Serological assays can be used to screen for infection with HSV by detecting HSV-type-specific antibodies; however, interpretation requires care due to the challenges of suboptimal sensitivity and specificity of some assays, particularly in younger populations.

Table 12.1: Methods to detect herpes simplex virus (HSV) in lesions

Diagnostic technique	Sample type(s)	Sensitivity	Specificity	Advantage	Disadvantage	Reference(s)
Polymerase chain reaction (PCR)	Swab of skin lesions Swab of vesicular fluid/exudate from vesicle base Swab of mucosal surface Aqueous or vitreous humour Cerebrospinal fluid Blood	93–100%	93–100%	Allows virus detection and typing in the same test Result usually within 24–48 hour Automation (labour efficiency) Resistance genotyping possible Real-time PCR allows quantitative analysis	Risk of intra-laboratory contamination Cost Resistance genotyping rarely available	*(4–6)*
Loop-mediated isothermal amplification (LAMP)	As for PCR	—	—	Temperature-independent mechanism Reduced hardware requirements	Nonspecific amplification may occur Labour-intensive optimization of primer sets	*(7)*
Helicase-dependent amplification (HDA)	As for PCR	97–100%	93–100%	Rapid results (< 1.5 hour) Assay works at one temperature	—	*(8–11)*
Immunofluores-cence	Lesional smear	61%	90–100%	Rapid result (3 hours) Allows virus detection and typing in the same test	Requires fresh vesicles Suboptimal sensitivity Requires expensive microscope and special slides	*(12,13)*
Latex agglutination	Swab of skin lesions Swab of vesicular fluid/exudate from vesicle base	50–73%	89–93%	Rapid result (3 hours)	Suboptimal specificity	*(14,15)*

Table 12.1 (continued): Methods to detect herpes simplex virus (HSV) in lesions

Diagnostic technique	Sample type(s)	Sensitivity	Specificity	Advantage	Disadvantage	Reference(s)
Viral culture	Swab of skin lesions Swab of vesicular fluid/exudate from vesicle base Biopsy material Conjunctival or corneal smear	100%	100%	Resistance phenotyping possible	Slow process (5–14 days) Sensitivity affected by cell line type and inoculum size	*(16)*
Tzanck smear	Scraping skin/ mucosal lesional base Biopsy material Conjunctival or corneal smear	33%	97%	Simple and low-cost	Suboptimal sensitivity Large amount of material required Cannot differentiate between HSV-1 and HSV-2	*(13)*

Table 12.2: Methods to detect antibodies against herpes simplex virus (HSV) in blood

Diagnostic technique	Sample type(s)	Sensitivity	Specificity	Advantage	Disadvantage	Reference(s)
Haemagglutination	Serum	97%	84%	Rapid result (3 hours)	Requires high concentration of HSV particles. Assay has several manual steps	*(17)*
Enzyme-linked immunosorbent assay (ELISA)	Serum	92–100%	61–85%	Easier to perform (semi-automated) Can determine the specificity of antibodies against HSV-1 and HSV-2 Specificity may be suboptimal but can be improved using purified or recombinant gG1 or gG2 protein	Seroreversion of gG-specific antibodies can occur Some ELISAs perform suboptimally when testing sera from adolescents; sensitivity can be improved by raising the cut-off value	*(16,18)*
Western blot	Serum	98%	65–100%	Considered the "gold standard" to detect type-specific HSV antibodies	Time-consuming; expensive	*(19)*
Multiplex flow immunoassay (MFI)	Serum	93–99%	85–99%	Rapid result (< 4 hours) Fully automated; can detect multiple analytes in a single reaction tube	—	*(20)*
Luciferase immunoprecipitation assay	Serum	92–100%	96–100%	Rapid result (< 2.5 hours) Can be adapted to different formats (e.g. microfluidic devices, rapid tests, 96-well plates, point-of-care devices)	—	*(21)*
Microfluidic-based point-of-care device	Whole-blood Serum	94%	99%	Very rapid results (15 minutes)	—	*(22)*

gG: glycoprotein G.

12.3 Specimen collection and transport

HSV-1 and HSV-2 can be recovered by swabbing anogenital or oral lesions. A Dacron swab on a wire or plastic shaft should be used for collecting samples for nucleic acid amplification tests (NAATs) or viral culture. Calcium alginate swabs inhibit NAAT-based detection methods and reduce viral recovery. Because HSV genital shedding is intermittent, testing asymptomatic patients is not recommended as this approach is unlikely to provide confirmation of infection status.

For fresh lesions, a sample of vesicular fluid or ulcer exudate should be tested for the presence of herpesviruses with a NAAT. For older healing lesions, the diagnostic yield may be lower but molecular assays are still likely to remain positive. In the event that herpesviruses are not detected, individuals with older lesions should be advised to return when new lesions appear for repeat sampling.

Careful attention should be given to the conditions for transport and storage of clinical specimens. After sampling, specimens for viral detection should be sent as a dry swab (for NAAT only), placed in a universal transport medium (for NAAT or culture) or placed immediately into vials containing 1 ml of appropriate viral transport medium (for cell culture). In those rare instances where HSV culture is still requested, culture specimens should be sent to the laboratory within 4 hours; failing this, specimens may be placed at 4 °C overnight and sent the next day. The transport time from specimen collection to laboratory should be no more than 48 hours and, in such situations, it is essential to send the samples on ice in a cooler box. Virus recovery is substantially reduced after a freeze–thaw cycle; freezing at −20 °C pending transport to the laboratory is not advised.

12.4 Direct diagnosis of HSV from clinical specimens

12.4.1 Virus detection and quantification using molecular techniques

Several molecular procedures have been proposed to detect or quantify HSV genomes in clinical samples, including in-house competitive polymerase chain reaction (PCR), PCR detection followed by DNA enzyme immunoassay hybridization, real-time PCR assay and several commercially available kits *(3–6)*. Real-time NAATs allow both the detection and quantification of HSV DNA in clinical samples. Compared with traditional NAATs, real-time PCR allows HSV DNA amplification in a single reaction tube; it is thus faster, allows simplified conditions of performance and lowers the risk of cross-contamination. Primers from HSV DNA sequences common to both HSV-1 and HSV-2 (HSV DNA polymerase [*pol F* gene], HSV thymidine kinase or glycoprotein B domain) may identify HSV DNA. Primers and probes for HSV DNA sequences specific for HSV-1 or HSV-2, including the glycoprotein G, D or I genes, permit amplification of just one HSV type.

The use of NAATs for HSV diagnosis also allows less stringent sample transport conditions than those required for diagnosis by culture. Strict validation of in-house PCR or commercially available NAATs in individual settings is important. In each DNA extraction and subsequent analysis, an internal positive control, which permits the detection of amplification inhibitor samples and controls the quality of sample preparation, and a negative control should be included. Certified and registered reference panels comprising coded control specimens ideally should be used as intra- and inter-laboratory quality controls (see also Chapter 5).

Molecular biology has evolved over time and improved techniques for diagnosis have been developed that avoid the traditional requirement of a series of temperatures to facilitate denaturation, annealing and elongation, as seen with traditional NAATs, such as PCR. A loop-mediated isothermal amplification (LAMP) assay has been developed to enable simultaneous detection of herpesviruses, including HSV-1 and HSV-2 *(7)*. LAMP assays operate independently of temperature, require less hardware and can offer high specificity once optimization of the amplification procedure is achieved. Helicase-dependent amplification (HDA) techniques use a thermostable helicase enzyme that enables amplification to occur at a single temperature *(8–11)*. Both LAMP and HDA technologies may have a role in the development of future point-of-care (POC) tests for the detection of herpesviruses.

NAATs are the most sensitive test currently available to detect HSV infections. The detection rates for NAATs substantially exceed that of viral culture. Furthermore, while viral culture and NAATs both allow detection of asymptomatic HSV shedding, NAATs are much more sensitive in this setting. However, as mentioned earlier, it is not recommended to test asymptomatic patients using NAATs because failure to detect HSV does not indicate the absence of HSV infection; this is because viral shedding is intermittent.

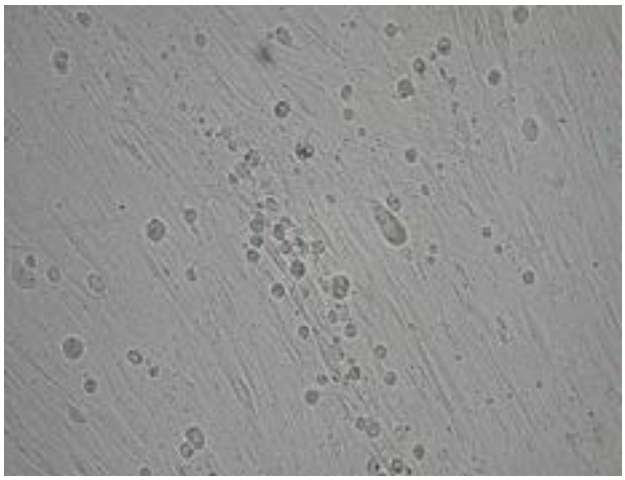
12.4.2 Virus isolation and typing in cell culture

Virus isolation in tissue culture roller tubes, as described below, is slow, labour-intensive and expensive. More rapid culture of HSV can be achieved by using shell vials or multi-well plates, and centrifuging the specimen onto cell monolayers on coverslips in a similar manner to chlamydia culture. A key advantage of culture is that it has high specificity and facilitates phenotypic sensitivity testing. However, its substantially lower sensitivity for detecting herpesviruses, compared to NAATs, means that this technique is now rarely used outside of specialized laboratories. Importantly, failure to detect HSV by culture does not indicate the absence of HSV infection.

The most frequently used cells for HSV isolation from clinical specimens include primary human diploid fibroblasts, as well as cell lines such as MRC-5 cells (human fibroblasts), Vero cells (monkey kidney), HEp-2 cells (laryngeal squamous cell carcinoma), baby hamster kidney and rabbit kidney cells. Cultured cells are grown to a confluent monolayer in a tissue culture tube flattened on one side. The culture growth medium is removed and culture tubes are then inoculated with 0.25-ml aliquots of the vortexed specimens. The tubes are then incubated at 36 °C for 1 hour to enhance cell absorption of the viral particles. After absorption, 2 ml of herpes cell maintenance medium are added and the culture tubes are further incubated at 36 °C in an atmosphere containing 5% CO_2 for 7 days. The culture tubes should be examined daily using a stereoscopic microscope to check for the appearance of the characteristic cytopathic effect of HSV, which usually develops 24–72 hours after inoculation. The cytopathic effect caused by HSV is characterized by the transformation from elongated, scattered cells (Fig. 12.2A) to enlarged, refractile, rounded cells, ballooning, increasing in number and developing a granular appearance (Fig. 12.2B). Focal necrosis of cells may occur and syncytia and multinucleated giant cells may be found at the edge of the foci in HSV-infected cell cultures.

Fig. 12.2: Herpesvirus isolation on cell culture (50×)

A. Confluent monolayer of uninfected MRC-5 human diploid fibroblasts



B. Typical cytopathic effect of HSV on MRC-5 cells obtained after 24–48 hours of viral culture

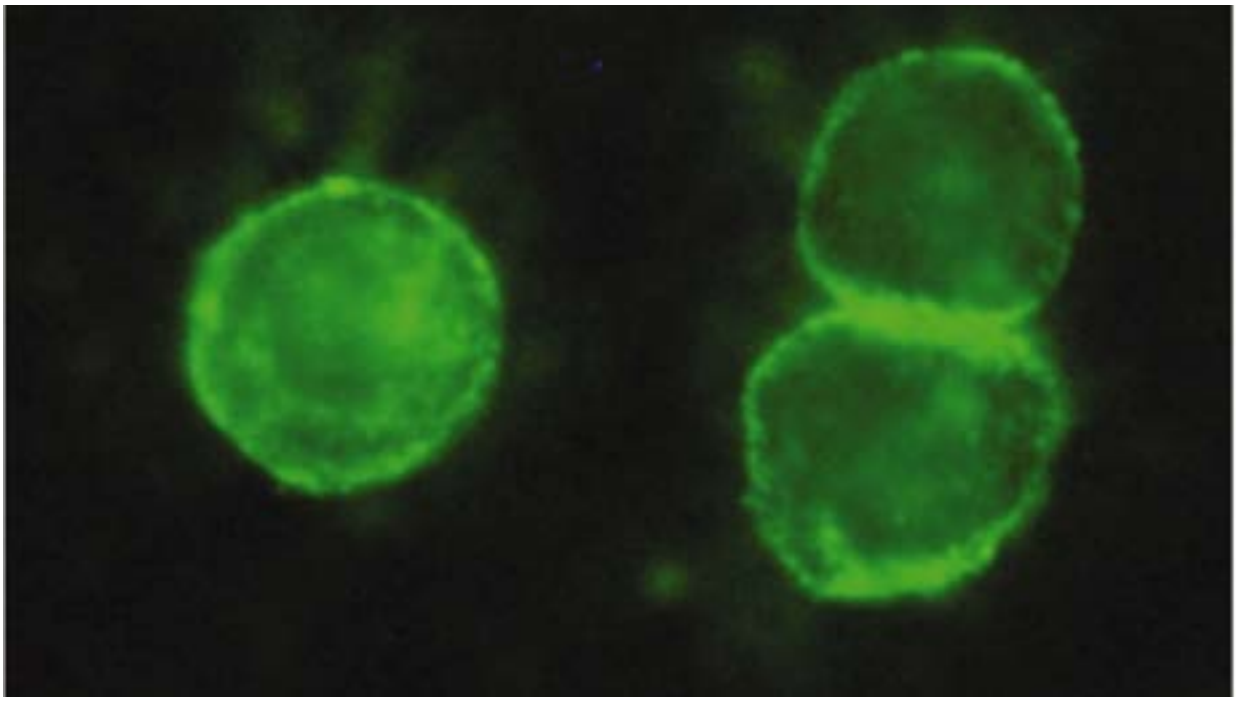
Source: Laurent Bélec, from WHO, 2013 *(23)*.

Confirmation of HSV in cell cultures demonstrating a cytopathic effect is recommended because other viruses may cause a similar cytopathic effect. Confirmation and HSV typing can be performed directly on infected cell cultures using fluorescein isothiocyanate (FITC)-labelled or immunoperoxidase (IP)-labelled, type-specific monoclonal antibodies directly on a smear of cultured material or by testing the cell supernatant using a NAAT. Identification and typing by direct immunofluorescence (IF) is the most practical approach. The culture medium is removed from the infected monolayer and 1 ml of 5% fetal calf serum in phosphate-buffered saline (PBS) solution is added. The cells of the flat monolayer side are then scraped off the coverslip and homogenized and centrifuged at 500*g* for 5 minutes; one drop of the vortexed pellet is then spotted onto each of two wells of a polytetrafluoroethylene-coated glass slide, air-dried and fixed with cold (2–8 °C) acetone for 10 minutes. One well is then stained with fluorescein-labelled, HSV-1-specific monoclonal antibody and the other with fluorescein-labelled, HSV-2-specific monoclonal antibody. The slide is incubated for 30 minutes at ambient temperature in a moist chamber and then washed three times for 5 minutes with PBS, using a mechanical stirrer. Coverslips are subsequently placed on the slides using a glycerol : PBS (50 : 50) solution, and the slides are examined under a fluorescence microscope at 400× magnification. When one of the spots shows apple-green fluorescent particles while the second does not, the identification of HSV is confirmed and the virus type is determined. Isolates of cultured HSV may be stored in 0.2 M sucrose in 0.02 M PBS, pH 7.2 (2-sucrose-phosphate-based transport medium), at −80 °C or in liquid nitrogen.

12.4.3 Viral antigen detection

When mucocutaneous lesions are present, viral antigen can be detected in lesional material using IF, IP staining or enzyme-linked immunoassays (EIAs). Although IF may be classified as a rapid diagnostic test in the laboratory, it does not allow for near-patient diagnosis. Ideally, cells should first be concentrated in the laboratory before preparing smears. This will improve sensitivity to detect HSV-1 or HSV-2 antigens using type-specific, fluorescein-labelled monoclonal antibodies, as visualized under a fluorescence microscope (Fig. 12.3). The main limitations of direct IF are that it is time-consuming, relatively expensive in terms of reagents and capital, and less sensitive than modern molecular methods. Direct IF has a low sensitivity of 61% compared to culture in cases of symptomatic disease *(12)*; sensitivity is much lower in asymptomatic cases, precluding its use in such situations.

Fig. 12.3: Intracellular immunofluorescence of herpes simplex virus (HSV) in a clinical specimen (400×)



Source: Laurent Bélec, from WHO, 2013 *(23)*.

Indirect IP staining has the advantage of using a normal light microscope, making it more suitable for intermediate-level laboratories. Specimens are prepared as for the direct IF test and incubated with rabbit or mouse HSV-specific polyclonal, or HSV-1-specific or HSV-2-specific monoclonal antibodies. Binding is detected using horseradish peroxidase-labelled antibody directed to the rabbit or mouse immunoglobulins using standard IP methods. The sensitivity of direct IF and IP methods are similar.

Herpetic proteins can also be detected in clinical samples by using a classical capture EIA using HSV-specific polyclonal or monoclonal antibody. The sensitivity of commercially available capture enzyme-linked immunosorbent assays, compared with that of viral culture, is greater than or equal to 95% with specificities ranging from 62% to 100% in symptomatic patients. The sensitivity of antigen capture EIA may be higher than that of virus culture for typical presentations but lower for cervical and urethral swabs. However, most commercially available assays do not differentiate between HSV-1 and HSV-2.

A rapid POC latex agglutination assay for HSV antigen detection was evaluated in the pre-NAAT era but its performance was poor compared with culture when testing lesion swab eluants *(14, 15)*. Provided that 25% or more of the cell culture monolayer exhibited visible cytopathic changes, the latex agglutination assay had a high sensitivity when used to confirm the presence of herpesviruses in cell cultures *(15)*.

12.4.4 Cytological examination

Direct examination of specimens and cytological examination using conventional staining procedures (Tzanck smear, Papanicolaou or Romanowsky stains) have low sensitivity and specificity and should not be relied on for diagnosis of herpesvirus infection *(13)*.

12.5 Indirect serological diagnosis of herpetic infections

12.5.1 Laboratory-based serological tests

Table 12.2 summarizes various methods used to detect HSV infections and enable HSV type-specific antibody testing. Although the accuracy of HSV serological assays might not match that of HIV antibody tests, these tests are an improvement on clinical diagnosis of genital herpes, which has a sensitivity of 39% at best and yields a false-positive diagnosis in approximately 20% of patients.

Serological screening for HSV-1 or HSV-2 in the general population is not recommended. However, serological testing can sometimes be useful to diagnose individuals without lesions, those with atypical symptoms or those with healing lesions and negative HSV cultures. Serological investigations may also assist in managing the sex partners of people with genital herpes, particularly where concerns are raised about transmission. Serodiscordant couples can be counselled about strategies to reduce the risk of infection and disease.

HSV serological testing of pregnant women is not routinely recommended; however, it may be helpful in situations where the woman's partner has a history of genital herpes and the couple have concerns related to HSV transmission during the pregnancy. HSV-1 or HSV-2 seronegative women should be counselled about strategies to prevent a new infection with either virus type during pregnancy.

Testing of people living with HIV is not routinely recommended. Although HSV-2 seropositivity increases the risk of HIV transmission and frequent HSV recurrences augment HIV replication, there is limited evidence to inform the management of HSV-2 coinfection in this population without symptoms of genital herpes. There are inconsistent data suggesting that HSV-2 increases the risk of perinatal HIV transmission among HSV-2 seropositive, HIV-infected women; at present, serological testing of pregnant women living with HIV is also not recommended.

Unfortunately, the accuracy of commercially available HSV serological assays is suboptimal, with HSV-2 assays lacking specificity and HSV-1 assays lacking sensitivity. In deciding whom to test, clinicians should keep in mind that individuals with undiagnosed HSV infection account for most new transmissions and that studies in many settings show that most patients welcome the opportunity to learn their serological status.

Serology may be helpful in managing patients with first-episode genital herpes, where differentiating between primary and established infection guides counselling and management. Immunoglobulin G (IgG) antibodies are negative in primary genital herpes and only become detectable two weeks to three months after the onset of symptoms and then persist indefinitely. Therefore, directly after infection, there is a window in which testing for HSV antibodies will give a negative result even through HSV particles may be detected with molecular assays; this may be helpful to indicate newly acquired herpetic infection. Primary HSV infections can also be documented by demonstrating seroconversion with paired sera using any serological method. Because immunoglobulin M testing can also be positive during reactivation of the disease, it cannot be used to distinguish primary from recurrent infection; therefore, it is of limited use for routine diagnostic purposes. If mucocutaneous lesions are present, type-specific serology and direct virus testing can help to establish if the episode is new acquisition of HSV infection or reactivation (Table 12.3). The distinction between newly acquired HSV and reactivated HSV is helpful for epidemiological studies; sometimes, it is helpful to manage psychosocial issues.

Table 12.3: Virological and serological approach to herpes simplex virus type 2 (HSV-2) diagnosis in the presence and absence of anogenital lesions

	HSV-2 detection by direct method	HSV-1-specific IgG	HSV-2-specific IgG	Interpretation
First assessment of anogenital lesions	Positive	Positive or negative	Negative	Acute HSV-2 infection
	Positive	Positive or negative	Positive	Recurrent HSV-2 infection with HSV-2 infection acquired at least 6 weeks ago
No lesions	NA	Negative	Negative	Patients at risk for acquiring orolabial or anogenital HSV-1 infection or HSV-2 infection
	NA	Positive	Negative	Patients with previous HSV-1 infection at risk for acquiring orolabial or anogenital HSV-2 infection
	NA	Negative	Positive	Patients with previous HSV-2 infection at risk for acquiring orolabial or anogenital HSV-1 infection
	NA	Positive	Positive	Patients previously infected with both HSV-1 and HSV-2
Recurrent anogenital lesions	Positive	Positive or negative	Positive	Recurrent HSV-2 infection
	Negative	Negative	Positive	Possible recurrent HSV-2 infection. Other potential causes of anogenital ulcerative disease should be considered.

NA: not applicable.

Source: adapted from Gupta et al. *(24)*.

Because nearly all HSV-2 infections are sexually acquired, the presence of type-specific HSV-2 antibody usually implies anogenital infection; thus, education and counselling appropriate for individuals with anogenital herpes should be provided. The presence of HSV-1 antibody alone is more difficult to interpret. Most individuals with HSV-1 antibody acquired oral HSV infection during childhood, which might be asymptomatic. However, acquisition of anogenital HSV-1 is increasing and anogenital HSV-1 may also be asymptomatic. Lack of symptoms in an HSV-1 seropositive individual does not distinguish anogenital from orolabial infection. Persons with HSV-1 infection, regardless of the site of infection, remain at risk for HSV-2 acquisition. Counselling of HSV-1 IgG-positive patients, who are negative for HSV-2 IgG antibodies, should take into account that HSV-1 is an uncommon cause of recurrent genital disease.

The HSV-1 and HSV-2 genomes each encode at least 80 different structural and non-structural polypeptides, including at least 10 glycosylated proteins, designated glycoproteins A (gA) through I (gA–gI). Most antibodies produced during HSV infection are raised against these surface glycoproteins. gB–gE trigger potent immune responses. Some epitopes that are present on these glycoproteins are shared by HSV-1 and HSV-2; therefore, they cause a significant degree of cross-reactivity when testing with certain commercial assays. However, no cross-reactivity between gG1 in HSV-1 and gG2 in HSV-2 has been detected *(25)*. Modern type-specific HSV serological assays are based on the detection of HSV-

specific gG1 (HSV-1) and gG2 (HSV-2) antibodies using native, purified or recombinant gG1 or gG2 as antigens because these perform better than other HSV serological assays. These type-specific gG-based serological tests are preferred for patient testing.

Type-specific HSV gG-based serological EIAs first became commercially available in 1999; however, older assays that do not accurately distinguish HSV-1 from HSV-2 antibody (despite claims to the contrary) remain on the market. The sensitivities and specificities of these gG type-specific tests for the detection of HSV-2 antibody vary (Table 12.2). In one study where the performance of U.S. Food and Drug Administration-approved type-specific EIAs was compared to a non-commercial western blot, the EIAs had lower sensitivity for detecting HSV-1 antibodies compared to HSV-2 antibodies, and lower specificity for detecting HSV-2 antibodies compared to HSV-1 antibodies *(26)*. False-positive results may occur, especially in patients with a low likelihood of HSV infection. Repeat or confirmatory testing might be indicated in some settings, especially if recent acquisition of genital herpes is suspected.

Comparisons of EIA-based tests to western blot in sub-Saharan Africa demonstrated a lower specificity than previously observed, particularly in HIV-infected individuals *(27)*. More generally, the poor specificity of certain herpes type-specific, gG-based tests has been reported on samples from sub-Saharan Africa *(18, 28)*. The concordance between two modern type-specific EIAs for HSV-2 antibody detection was assessed in a study of African women with genital ulceration *(18)*. The authors reported that concordance may be substantially improved by using higher index values to define positive results in one of the assays (HerpeSelect, Focus Technologies). The reason for this observation is not well understood but there are concerns that the use of higher index values for diagnosis will miss those cases of HSV-2 with low HSV-2 antibody levels, that is, newly infected patients or patients infected for prolonged periods with low HSV-2 recurrence rates.

New HSV type-specific serological technologies have emerged, which have improved features over conventional EIAs, including rapid result generation, increased convenience and, in some cases, full automation. These include fluorescence immunoassays, multiplex flow immunoassays and luciferase immunoprecipitation assays (Table 12.2).

12.5.2 POC serological tests

Microfluidic-based POC devices have been developed to detect HSV-2-specific antibodies, although most are not available commercially *(3, 22, 29)*. These immunoassays are designed to use capillary blood from a finger stick (or serum) and typically use the lateral flow of serum through a membrane containing either a dot or line of gG1 or gG2 antigen. These POC tests perform relatively well with sensitivities of 94–100% and specificities of 97–100% *(22)*. In common with EIA-based systems, the performance of these POC systems depends in part on herpesvirus prevalence within the population. Although more expensive that EIA-based systems, the major benefit of such near-patient assays is that they provide a rapid result, allowing for more timely patient education and counselling while the patient is still on site.

12.6 Therapeutic monitoring: drug resistance testing

Repeated courses of episodic therapy, and to a lesser extent long-term prophylaxis, with antiherpetic drugs such as valaciclovir or acyclovir can result in the development of resistance, especially in immunocompromised patients *(30)*. The relative prevalence of acyclovir-resistant HSV isolates differs between immunocompetent and immunocompromised individuals on account of prolonged viral replication and impaired host response that may favour the survival of less fit drug-resistant HSV strains. The persistence of lesions for more than one week after the beginning of therapy without appreciable improvement, atypical appearance of the lesions or emergence of new satellite lesions despite antiviral administration is suggestive of treatment failure. Acyclovir resistance can be diagnosed by testing a virus against antiviral agents (phenotypic assays) or by identifying specific thymidine kinase (*uL23*) and DNA polymerase (*uL30*) gene mutations conferring resistance to antiviral drugs (genotypic assays). For genotypic methods to be helpful in clinical practice, it is essential to be able to discriminate between random variations (polymorphisms) and true drug-resistant mutations.

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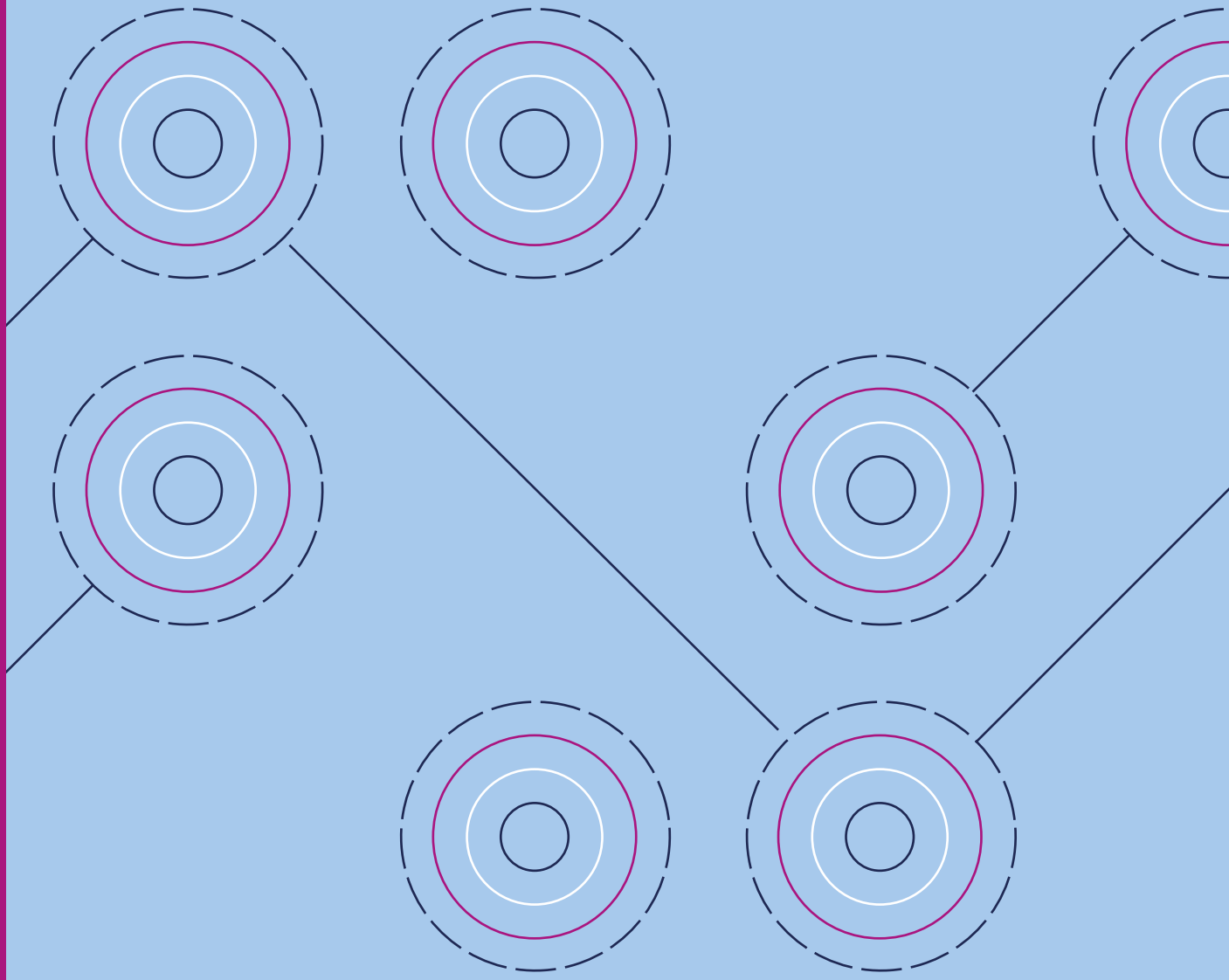
Chapter 13. Syphilis



Chapter 13. Syphilis

John R. Papp, Pingyu Zhou, Rosanna Peeling and Edward W. Hook III

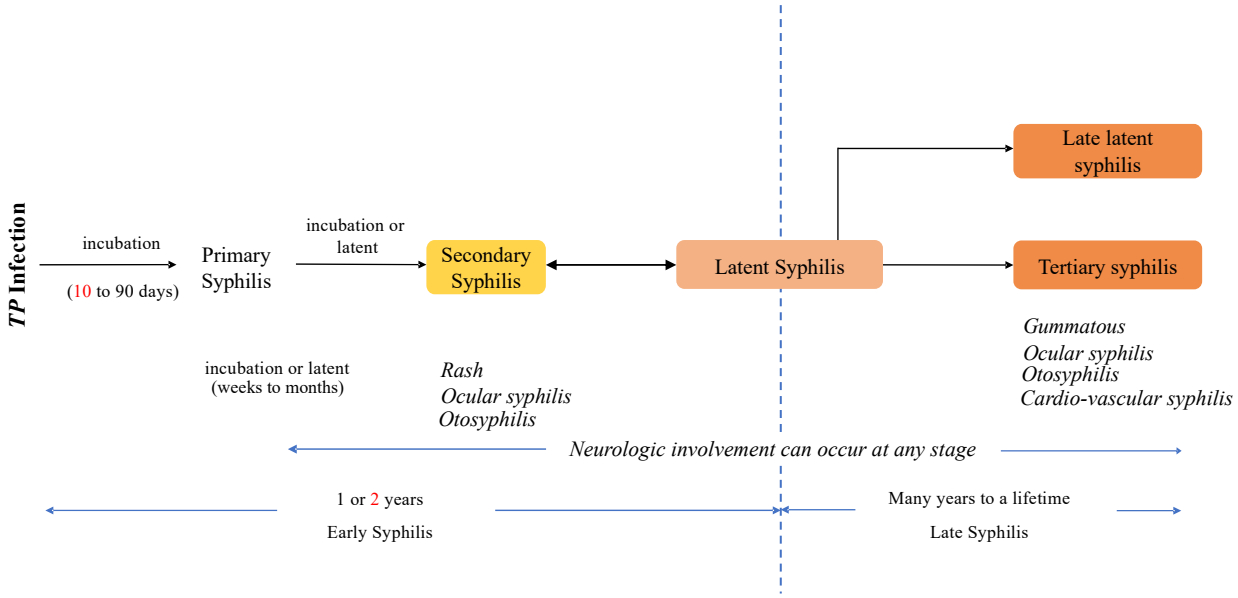
13.1 Introduction

Syphilis is a sexually transmitted disease caused by the obligate microaerophilic spirochete bacteria, *Treponema pallidum* subsp. *pallidum* (referred to as *T. pallidum* below) that can also be transmitted vertically from a pregnant woman to her fetus. The organism is closely related to three other pathogenic *Treponema* species associated with skin, bone or mucosal infections that are mostly transmitted by direct skin/mucosa-to-skin/mucosa contact. *T. pallidum* subsp. *pertenue*, the causative agent of yaws, is found in tropical areas in Africa, Asia and Latin America. Pinta is a rare disease caused by *T. carateum* and is found in tropical areas of Latin America. Endemic syphilis, or bejel, may occur following infection with *T. pallidum* subsp. *endemicum* mostly in children in Cuba and in the eastern Mediterranean and West Africa. These four pathogens are distinguished by their mode of transmission, epidemiology and clinical manifestations *(1)*. Due to morphologic and antigenic similarities between the organisms, they cannot be differentiated by routine testing.

T. pallidum enters the body through the mucosa or micro-abrasions in the skin where it replicates quickly and causes a systemic infection that may lead to serious sequelae in multiple organ systems, including the central nervous system and the ocular and otic systems. Congenital syphilis can be acquired by vertical transmission and may result in spontaneous abortions, miscarriages or stillbirths. Infants with congenital syphilis may have clinical signs of infection at birth or months to years after birth. The clinical features in the adult progress through different stages from primary syphilis to late latent or possibly tertiary syphilis (Fig. 13.1). The stage of syphilis may not progress in a linear manner in all cases. For example, people can move back and forth between early latent syphilis and secondary syphilis for the first couple of years if untreated. Involvement of the central nervous system can occur at any stage.

The first manifestation of syphilis in adults is the appearance of single or multiple small nodules, which in turn ulcerate and present about three weeks after exposure, though the incubation period ranges from 10 to 90 days *(2)*. Classically, a single, painless lesion with a clean base is present, though lesions may be multiple, typical or painful. The lesion is commonly found in the coronal sulcus, on the glans or penile shaft in men, and on the vulva, vaginal walls or cervix in women. Oral, perianal and/or rectal lesions may develop following oral or anal sex and can be difficult to detect by both patients and clinicians. Since they are frequently painless, primary lesions may remain unnoticed. If left untreated, the ulcer will resolve spontaneously within 3–8 weeks. Genital primary chancres are usually associated with bilateral inguinal lymphadenopathy, which is classically discrete and non-tender.

Fig. 13.1: Schematic representation of the general course of untreated syphilis



Source: Courtesy of Dr Pingyu Zhou, STD Institute, Shanghai Skin Disease Hospital, Shanghai, China.

In the untreated patient, the onset of the secondary stage of disease may occur some six weeks to six months after initial infection *(3,4)*. The primary chancre may disappear or still be present when clinically apparent secondary lesions occur. The main dermatologic feature of secondary syphilis is rash, which can mimic many skin disorders, but mostly presents as an evenly distributed skin rash that may be macular, papular or papulo-squamous and frequently, but not always, involves the palms and/or soles of the feet. Condylomata lata is characterized by large and raised wart-like lesions that are usually whitish or grey in coloration and are typically found in warm, moist areas such as the vulva or the perianal region. The rash may be accompanied by generalized lymphadenopathy and fever, headache, malaise and other organ system disorders, such as neurosyphilis.

If secondary syphilis remains undiagnosed or untreated, all visible manifestations of the disease resolve spontaneously within several months *(3,4)* and the patient will pass into a period of latency. Signs and symptoms of secondary syphilis may recrudesce several times during the first couple of years post-infection. Latent syphilis is divided into early latent infections thought to have occurred within the past year, late latent infections that are longer than a year in duration and latent syphilis of unknown duration. Recommended therapy for early latent syphilis is shorter than that for late latent, latent syphilis of unknown duration and tertiary stages. To reduce onward transmission, partner notification and treatment are more strongly emphasized for persons with early latent syphilis than those with late latent or syphilis of unknown duration (Fig. 13.1). However, it may be impossible to determine the exact duration of an untreated infection and such cases should be classified and managed as late latent disease by default. During the latent stages of the disease, there are no signs or symptoms; therefore, a diagnosis must be based on the results of serological testing.

The interval between secondary and tertiary syphilis may be years or decades. The disease may remain latent for life and never progress to tertiary syphilis in up to two thirds of patients *(5,7)*. Manifestations of tertiary syphilis include cardiovascular syphilis, with aneurysms or stenosis resulting from multiplication of treponemal spirochetes in the thoracic aorta or coronary arteries; syphilitic gummas, with soft granulomatous growths that can cause tissue destruction in any organ system including bones and cartilage; and neurosyphilis, with late neurologic manifestations including tabes dorsalis and general paresis. Neurosyphilis can also occur during any stage of syphilis and may be asymptomatic or symptomatic during early stages of infection of the central nervous system *(8)*.

Syphilis is diagnosed based on the identification of risk factors for exposure to *T. pallidum*, signs and symptoms of syphilis, known sexual contact with a person diagnosed with syphilis, and laboratory test results. Testing for syphilis includes the direct detection of the organism or detection of reactive antibodies, typically in serum or whole blood, that are suggestive of exposure to *T. pallidum.* Serology is the mainstay for syphilis testing and blood should be collected for serology even if direct detection testing is performed. The spirochete cannot be visualized by conventional microscopy because of its slender morphology and poor uptake of aniline dyes used for Gram stains *(9)*. The detection of reactive antibodies in cerebrospinal fluid (CSF) is used for diagnosis of neurosyphilis.

13.2 Direct detection methods for the diagnosis of syphilis

Direct observation of the organism by microscopy or amplification of *T. pallidum*-specific nucleic acid sequences can be performed using specimens obtained from skin lesions, body fluids (e.g. bullous rash or nasal discharge) or tissue sections obtained by biopsy (Table 13.1). A recent study suggested that saliva is a non-invasive, readily available sample for *T. pallidum*-specific nucleic acid testing *(10)*. The rabbit infectivity test was a historic direct detection test for *T. pallidum* but is no longer performed outside research laboratories, as it is time-consuming (requiring approximately 1–2 months to complete) and requires access to a suitable animal facility *(11)*. Recent advances have been described for the propagation of *T. pallidum* on artificial media, but these would not be considered as a diagnostic test *(12)*.

Table 13.1: Direct detection tests for *Treponema pallidum*

Method	Sample	Advantages	Disadvantages
Darkfield microscopy	Chancres or other moist cutaneous lesions of primary, secondary or congenital syphilis collected within 20 minutes	Diagnosis possible during a single clinical visit by visualization of characteristic motile organisms	Syphilis cannot be ruled out based on a negative test result Should not be used with oral or rectal specimens due to commensal treponemes that may be misclassified as *T. pallidum* (false positive) Requires specialized equipment Labour-intensive Technical expertise required Highly subjective interpretation No longer in common use
Direct fluorescent antibody staining for *T. pallidum*	Chancres or other moist cutaneous lesions of primary, secondary or congenital syphilis	Specimens from oral lesions are acceptable Specific for *T. pallidum* Storage and shipment of specimens possible	Syphilis cannot be ruled out based on a negative test result Requires specialized equipment Antibody conjugates may be difficult to find Labour-intensive Technical expertise required Highly subjective interpretation Not commercially available in many countries
Immunohistochemistry	Biopsies from skin, mucosal or tissue lesions that have been frozen or formalin-fixed and paraffin-embedded tissues	Tissue samples from placenta and umbilical cord are acceptable Tissue biopsies may be useful in unusual cases of syphilis Storage and shipment of specimens possible	Syphilis cannot be ruled out based on a negative test result Requires specialized equipment and reagents Technical expertise required Highly subjective interpretation
Nucleic acid amplification test	Skin, mucosal, tissue lesions and body fluids	Specimens from oral or rectal lesions, either fresh or frozen, are acceptable	Not commercially available in all countries Syphilis cannot be ruled out based on a negative test result Requires specialized equipment Technical expertise required Limited data availability for all specimen types, including blood and cerebrospinal fluid (CSF), so results should be interpreted with caution.

Source: Adapted from Peeling et al., 2017 *(4)*, with permission from *Nature Reviews Disease Primers*.

13.2.1 Darkfield microscopy

Darkfield microscopy can be used as a point-of-care (POC) method for the direct detection of *T. pallidum* in cases of primary or secondary or early congenital syphilis. Dedicated sexually transmitted infection (STI) clinics and hospital laboratories close to a clinical site should consider darkfield microscopy if they have the resources for a specialized microscope fitted with a darkfield condenser and well-trained personnel who can adhere to strict technical conditions and ongoing proficiency testing in order to produce reliable results.

In darkfield microscopy, only light rays striking organisms or particles at an oblique angle enter the microscope objective, resulting in the appearance of bright, white luminescent bodies against a black background. Darkfield microscopy must be performed by well trained and experienced personnel who are able to adjust the microscope correctly. However, darkfield microscopy should not be used with material from oral lesions because *Treponema denticola* found in the oral cavity can be easily confused as *T. pallidum*.

Both primary and secondary lesions of syphilis can be examined by darkfield microscopy. The ideal specimen is a serous exudate from active moist lesions, which is free of red blood cells. Active moist lesions should be cleansed carefully with a sterile gauze swab and sterile saline. The lesion then should be gently abraded with a sterile dry swab and squeezed to produce a serous exudate. If bleeding occurs, the drops of blood should be wiped away and the serous liquid that appears transferred to a glass slide using a thin stainless steel or platinum spatula or bacteriological loop, or by pressing the slide directly onto the fluid. The material may be mixed with a drop of saline to give a homogeneous suspension that then can be covered with a coverslip. The slide should be examined immediately, as the characteristic motility of the organisms is an important factor for identification. Any delay in examination rapidly reduces this motility. The chance of visualizing treponemes also decreases if the lesion is dry or is healing.

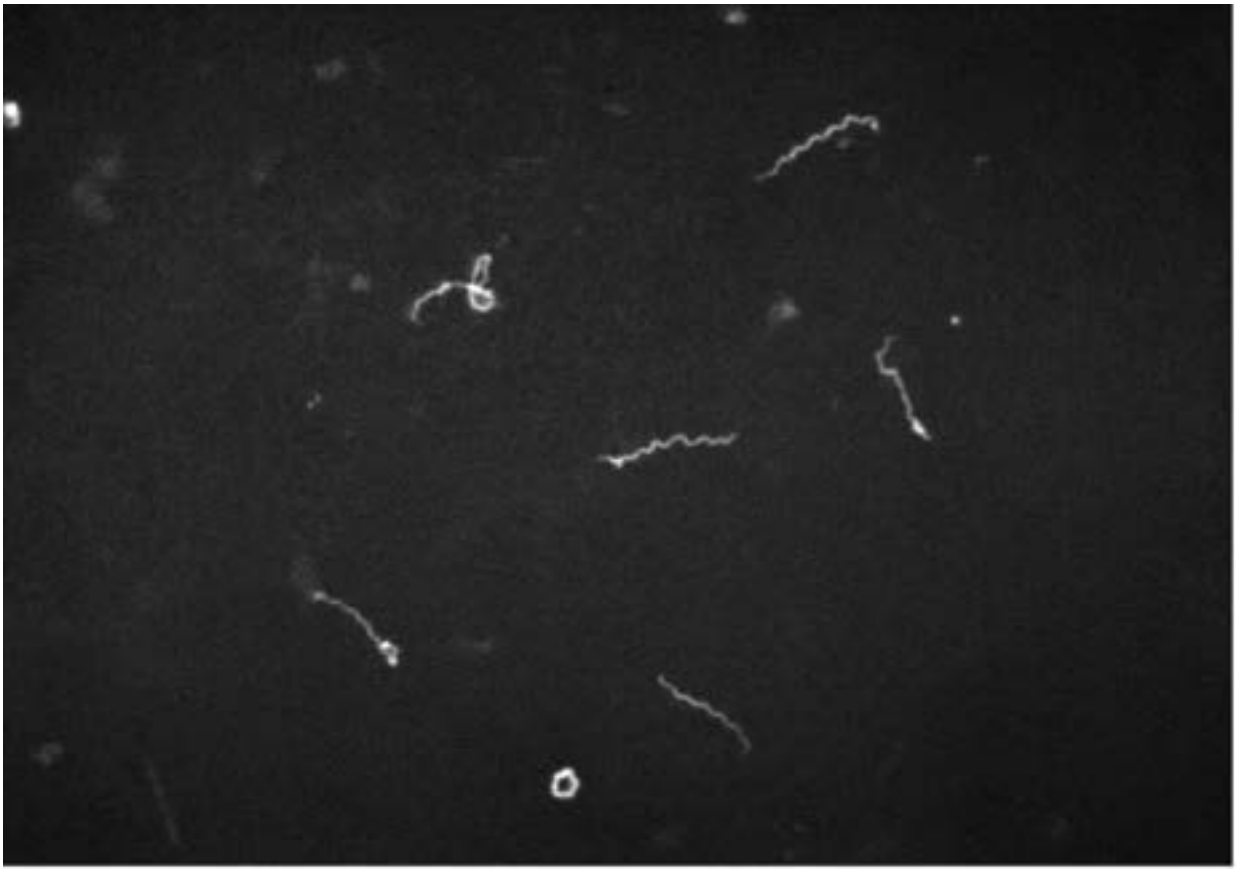
Correct optical alignment of the darkfield microscope is critical for successful darkfield microscopy. A few drops of immersion oil should be placed on the condenser of a previously aligned microscope. The condenser should then be lowered slightly so that the oil is below the level of the stage. The specimen to be examined should then be placed on the stage and the condenser elevated until there is good contact between the oil and the underside of the slide. It is important to avoid trapping air bubbles in the oil. The specimen should be initially examined using a low-power objective (10×). After focusing the objective, the light should be centred in the middle of the field by adjusting the centring screws located on the condenser, and the condenser focused by raising and lowering the condenser until the smallest possible diameter of light is obtained. The light should then be re-centred if necessary. Using the dry 40× objective, the specimen should be brought into focus, and the slide examined carefully. Darkfield microscopy is best conducted in a darkened room.

T. pallidum appears as bright, white spiral bodies illuminated against a black background. The organism is identified by its typical morphology, size and movement. It is a thin (0.10–0.18 µm wide) organism, 6–20 µm long, with 8–14 regular, tightly wound deep spirals (Fig. 13.2). It exhibits quick and rather abrupt movements. It rotates relatively slowly about its longitudinal axis. This rotation is accompanied by syncopated bending or twisting in the middle of the organism. Lengthening and shortening (like an expanding spring) may be observed. Distortion may occur in tortuous convolutions. Other, usually oral, spirochaetes may be seen. However, they are often more loosely coiled or thicker and coarse with different movements, including a writhing motion with marked flexion and relaxation of the coils.

The demonstration of treponemes with the characteristic morphology and motility of *T. pallidum* provides strong evidence for diagnosis for primary and secondary syphilis *(13)*. Patients with a primary chancre that is darkfield-positive may be serologically negative, but normally would be expected to seroconvert within a few days. However, failure to visualize the organism on darkfield examination does not exclude a diagnosis of syphilis. When compared to clinically defined syphilis and laboratory findings such as serology, the sensitivity and specificity of darkfield microscopy varies based on the stage of disease, between 75–100% and 94–100% for primary lesions, and 58–71% and 100% on secondary lesions, respectively *(14–19)*. The performance of darkfield microscopy depends on the stage of the disease, condition of the lesion from which the specimen is collected, and the competency of the microscopist.

NOTE: The darkfield technique can be practised and the microscope optics optimized by using specimens obtained along the gum margin, inside the mouth. Gingival epithelial cells and oral bacteria, including spiral organisms, can be seen as bright, white bodies against a black background. However, oral specimens should not be solely used for training because proficiency requires the ability to distinguish *T. pallidum* from other *Treponema* species.

Fig. 13.2: *Treponema pallidum*, darkfield microscopy



Note: The motility and morphology varies among different *Treponema* species.[34]

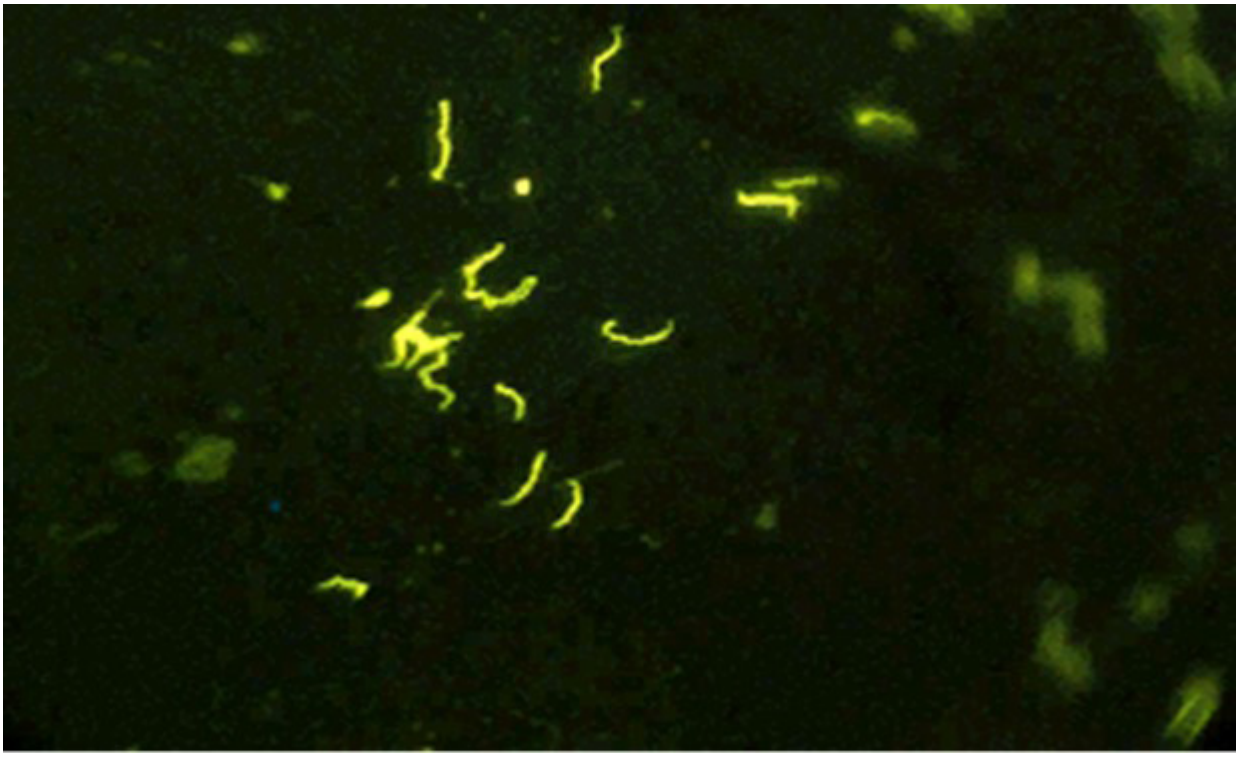
Source: Public Health Image Library (https://phil.cdc.gov/Details.aspx?pid=10179), courtesy of the Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA.

13.2.2 Direct fluorescent antibody (DFA) test

The method used to collect lesion material for the DFA test is identical to that used for darkfield microscopy. Specimens should be smeared onto a 1 cm^2 area of a microscope slide, allowed to air dry, and fixed with acetone or methanol, after which they can be packed for transport to the laboratory. After adding commercially obtained fluorescein-labelled anti-*T. pallidum* globulin, incubation and washing, the slides should be examined with a fluorescence microscope. Any *T. pallidum* spirochaetes in the specimen appear as apple-green-stained organisms with the typical *T. pallidum* morphology against a black background (Fig. 13.3). The DFA test has similar performance to darkfield microscopy when early primary lesions are present, but it is more sensitive in older primary lesions where performance of darkfield microscopy begins to decline. The specificity of DFA is higher than darkfield microscopy, especially if monoclonal antibody is used to make the fluorescein conjugate, because the DFA technique eliminates confusion with other spiral organisms *(18)*. Unfortunately, the specific fluorescein conjugate is not commercially available in many countries.

[34] For further information, see: https://www.youtube.com/watch?v=KlsfI50IrMU

Fig. 13.3: *Treponema pallidum*-positive direct immunofluorescence test



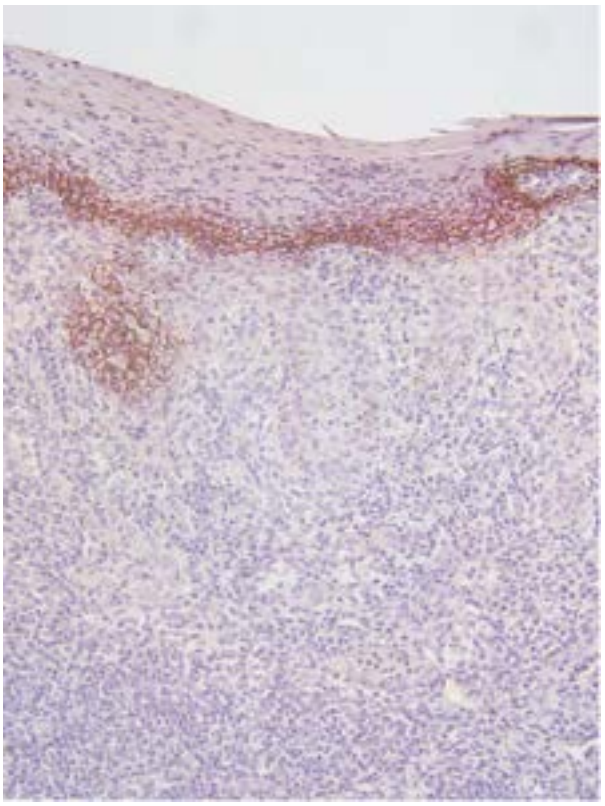
Source: Courtesy of David Cox, Centers for Disease Control and Prevention, Atlanta, GA, USA.

NOTE: The fluorescein conjugate can be conveniently titrated by applying two-fold dilutions of the conjugate to commercial slides that can be purchased to perform the fluorescent treponemal antibody absorption (FTA-Abs) test, and incubating, washing and examining the specimen as in the direct immunofluorescence test procedure above. The dilution to be used for the direct immunofluorescence test is the highest dilution of conjugate that shows clear, specific fluorescence in the absence of background staining.

13.2.3 Immunohistochemistry

Immunohistochemistry (IHC) is used for the direct detection of formalin-fixed paraffin-embedded tissue biopsies from the skin, brain, placenta, umbilical cord or other tissues. The most frequently evaluated method for tissue sections, the avidin-biotin peroxidase complex (ABC) technique, involves heat-induced epitope exposure and incubation with rabbit anti-*T. pallidum* immunoglobulin antibodies. Specific binding is detected using biotinylated anti-rabbit immunoglobulin antibodies followed by incubation with peroxidase-conjugated avidin-biotin complex for visualization of the stained treponemal spirochetes (Fig. 13.4). When compared to a clinical and/or serological diagnosis of secondary and tertiary syphilis, the sensitivity of the IHC ABC method ranged from 64% to 94% and was 100% specific *(15,19-21)*. IHC ABC should be used for evaluating atypical lesions and tissue biopsies in the evaluation of suspected syphilis (primary, secondary, congenital and gummatous) when the diagnosis remains uncertain.

Fig. 13.4: *Treponema pallidum*-positive in immunohistochemistry test (100×)



Source: Courtesy of Dr Pingyu Zhou, Shanghai Skin Diseases Hospital, Tongji University, Shanghai, China.

13.2.4 Nucleic acid amplification tests (NAATs) for *T. pallidum*

Nucleic acid amplification tests (NAATs) have been primarily developed by research and/or diagnostic laboratories over the past few decades for the detection of specific *T. pallidum* nucleic acid sequences and have only recently become commercially available in Europe and Australia. As of 2022, commercial NAATs for syphilis are not available in the USA because none have been cleared by the U.S. Food and Drug Administration. However, some large commercial laboratories in the USA offer clinical testing for syphilis using NAATs developed in-house. For direct detection, NAATs can be used to examine specimens from any lesion exudate, body fluids, and fresh, frozen or fixed and paraffin-embedded tissue specimens *(22)*.

Most NAATs target the *tpp47* (*tp074*) and/or *polA* (*tp0105*) *T. pallidum* genes and have different sensitivities depending on the stage of syphilis and specimen type *(23–26)*. Estimating the sensitivity of these NAATs based on published literature can be challenging due to small sample sizes and different reference standards that include combinations of clinical diagnosis, serology and darkfield microscopy. For example, the sensitivity of NAATs can range from 72% to 95% when testing lesion exudate from primary syphilis and from 20% to 86% on secondary lesion swabs based on whether the lesion sampled was skin rash or condylomata lata *(17,25–30)*. The sensitivity of a NAAT that targets the *polA* gene was reported to be 84% when maculopapular lesions of patients with secondary syphilis were scraped with the non-cutting edge of a sterilized blade *(31)*. NAATs can be used to test whole blood, serum, plasma and CSF for *T. pallidum*. However, studies estimating the performance of these specimen types are limited by small sample sizes. A recent study reported that saliva can be used as a non-invasive and convenient diagnostic fluid sample for different stage of syphilis *(10)*. In the study of 234 syphilis patients, the sensitivity of NAATs in saliva and plasma were 31.0% (9/29) and 51.7% *(15/29)*, respectively, in primary syphilis, 87.5% *(63/72)* and 61.1% *(44/72)* in secondary syphilis, 25.6% *(21/82)* and 8.5% (7/82) in latent syphilis, and 21.6% *(11/51)* and 5.9% (3/51) in symptomatic neurosyphilis. Robust studies with larger sample sizes and standardized specimen collection would be required to better estimate the true performance of NAATs for the detection of *T. pallidum*.

A NAAT that targets the 23S rRNA of *T. pallidum* was used to screen for rectal and pharyngeal infections in 24 men who have sex with men to determine if the test would enhance the sensitivity of detecting early syphilis when combined with serologic results *(32)*. The 23S rRNA NAAT had a higher sensitivity for rectal swabs (41.6% versus 37.5%) and higher sensitivity for pharyngeal swabs (29.2% versus 12.5%) compared to a reference NAAT targeting the *tpp47* gene. However, further research involving larger sample sizes would be required before recommending NAATs as a screening test for *T. pallidum*.

NAATs can also be multiplexed to detect multiple pathogens that may be present in a single specimen and can be tailored to aid in the differential diagnosis of genital ulcer disease. The SpeeDx PlexPCR VHS (SpeeDx Pty Ltd), is commercially available in Europe and Australia, can detect *T. pallidum*, varicella zoster virus, and herpes simplex virus (HSV) 1 and 2 from one ulcerative lesion specimen. In a retrospective study involving a panel of 250 previously tested and stored specimens, the positive and negative agreement varied between 95% and 100% among all three pathogens *(33)*. The clinical implementation of this multiplex NAAT requires further investigation. The development of multiplex NAATs to include emerging pathogens such as mpox should be made in consultation with clinicians, epidemiologists and laboratorians to ensure that the test is clinically useful.

13.3 Serological testing for syphilis

Serological tests for syphilis are divided into two categories based on whether they measure antibodies to lipoidal or *T. pallidum*-specific antigens. Non-treponemal (lipoidal antigen) serologic tests include the Wassermann reaction, rapid plasma reagin (RPR), Venereal Disease Research Laboratory (VDRL) and toluidine red unheated serum test (TRUST) tests. Treponemal serologic tests include the FTA-Abs, *T. pallidum* haemagglutination assay (TPHA), microhaemagglutination assay for *T. pallidum* (MHA-TP), *T. pallidum* passive particle agglutination assay (TPPA), enzyme immunoassay (EIA), chemiluminescence immunoassay (CIA), and the vast majority of POC or rapid tests that are commercially available (Table 13.2). None of the non-treponemal (lipoidal antigen) or treponemal serologic tests can distinguish infections caused by other *T. pallidum* subspecies.

To address the problem of falsely positive tests, reactive serologic tests should be confirmed with a second unrelated test to support a diagnosis of syphilis. The use of only one serologic test without a second test may misclassify a patient's syphilis status. However, in some settings that lack resources, patient management decisions may need to be based on only one test.

Table 13.2: Serologic tests for *Treponema pallidum*

Method	Sample	Advantages	Disadvantages
Non-treponemal (lipoidal antigen) serologic tests			
Venereal Disease Research Laboratory (VDRL) slide test	Serum, plasma or cerebrospinal fluid (CSF)	Quantitative and can be used to monitor treatment efficacy Can be used with CSF in suspect cases of neurosyphilis Inexpensive Relatively simple Results available in < 15 minutes	False positives due to cross reactivity with acute and chronic conditions Manual test Subjective interpretation False negatives can occur due to the prozone effect Requires a microscope and centrifuge Antigen suspension must be freshly prepared Cannot be used on whole blood Occasional biological false-positive results during pregnancy or in patients with autoimmune disease
Rapid plasma reagin (RPR) or toluidine red unheated serum test (TRUST)	Serum, plasma or CSF	Quantitative and can be used to monitor treatment efficacy Inexpensive Relatively simple Does not require a microscope Stable antigen (does not require fresh preparation) May be used with CSF in suspect cases of neurosyphilis Results available in < 15 minutes	False positives may occur in dusty environments and due to cross reactivity with acute and chronic conditions False negatives can occur due to the prozone effect Manual test Subjective interpretation Requires a centrifuge Cards cannot be reused Cannot be used on whole blood Occasional biological false-positive results during pregnancy or in patients with autoimmune disease
Treponemal serologic tests			
Fluorescent treponemal antibody absorption (FTA-Abs) test	Serum, plasma or CSF	May be available in some reference laboratories capable of sustaining the test	Not recommended for the resolution of discordant treponemal and non-treponemal (lipoidal antigen) tests due to potentially lower sensitivity and specificity Time consuming Expensive Requires specialized reagents and a microscope Manual test Highly subjective interpretation
T. pallidum passive particle agglutination (TPPA)	Serum or plasma	Inexpensive	Manual test Subjective interpretation Has been withdrawn from markets in the European Union and United Kingdom
T. pallidum haemagglutination (TPHA) and microhaemagglutination assays (MHATP)	Serum or plasma	Inexpensive	Manual test Subjective interpretation
Treponemal enzyme immunoassay (EIA)	Serum	Can be automated (suitable for high-throughput screening of asymptomatic populations and blood or plasma donors)	Expensive Requires specialized laboratory equipment

Table 13.2 (continued): Serologic tests for *Treponema pallidum*

Method	Sample	Advantages	Disadvantages
Chemiluminescence immunoassay (CIA)	Serum	Can be automated (suitable for high-throughput screening of asymptomatic populations and blood or plasma donors)	Expensive Requires specialized laboratory equipment
Treponemal rapid tests	Whole blood, plasma or serum	Allows for presumptive diagnosis and treatment during a single clinical visit Easy to use; results in < 20 minutes Relatively inexpensive	Cannot distinguish between new and prior, previously treated syphilis
Dual treponemal and non-treponemal (lipoidal antigen) rapid tests	Whole blood, plasma or serum	Allows for diagnosis and treatment during a single clinical visit Rapid and easy to use Can distinguish between new and previously treated infections	Can be more costly than traditional treponemal tests on a per-test basis
Dual syphilis and HIV rapid tests	Whole blood, plasma or serum	Can detect treponemal and HIV antibodies in the same test using a single finger-prick specimen	Cannot distinguish between new and prior, previously treated syphilis

Source: Adapted from Peeling et al., 2017 *(4)*, with permission from *Nature Reviews Disease Primers*.

13.3.1 Non-treponemal (lipoidal antigen) serological tests

The first described non-treponemal (lipoidal antigen) serologic test, the Wassermann test, measures antibodies to antigens from liver extracts, initially from congenitally infected fetuses and subsequently from heart tissue of patients with syphilis, by complement fixation. Wassermann tests are now rarely used. Subsequent tests target the same antigens as the Wassermann tests but are based on flocculation or agglutination as a measure of reactivity. These tests detect IgG and IgM antibodies to lipid antigens from a combination of cardiolipin, phosphatidylcholine (lecithin) and cholesterol released from damaged host and bacterial cell walls during syphilis infection, which results in a detectable humoral immune response *(9,34)*. Non-treponemal (lipoid antigen) serologic tests can provide quantitative results through serial two-fold dilutions and have been generally used to test for syphilis when patients present with signs and symptoms of syphilis or have a known sexual contact. Using increases or decreases in titres, they can also be used to assess patients for reinfection and monitor treatment outcome, respectively.

Following treatment, titres from non-treponemal (lipoidal antigen) serologic tests usually decrease four-fold over about 12 months in up to 80% of patients *(35–37)*. The non-treponemal (lipoidal antigen) serologic test eventually becomes non-reactive, especially in patients with primary syphilis *(35,38,39)*. For patients treated for latent syphilis, a four-fold decrease in the non-treponemal (lipoidal antigen) test titre may take up to 24 months *(40)*. However, titres in some patients may not serorevert to become non-reactive after treatment and may remain persistently reactive *(41)*. This is known as a serofast state and is most common in persons treated one or more years after infection *(41,42)*.

A false negative non-treponemal (lipoidal antigen) serologic test may result when the specimen has an excess of anti-lipoidal antibodies that interfere with the lattice formation of antibody-antigen complexes that clump cells together for agglutination of flocculation. This phenomenon, which rarely occurs, is referred to as a prozone and can be avoided by diluting the serum sample prior to testing. When testing serum from patients with syphilis, false negative RPR tests due to a prozone occurred in less than 0.85% of samples tested *(43)*.

Reactive non-treponemal (lipoidal antigen) serologic tests may be associated with other infections, immunizations, injection drug use, underlying autoimmune or other chronic inflammatory conditions, or pregnancy. A non-treponemal (lipoidal antigen) serologic test that is reactive for conditions other than syphilis is referred to as a biological false positive and has been estimated to occur in 0.2–0.8% of tests *(44,45)*.

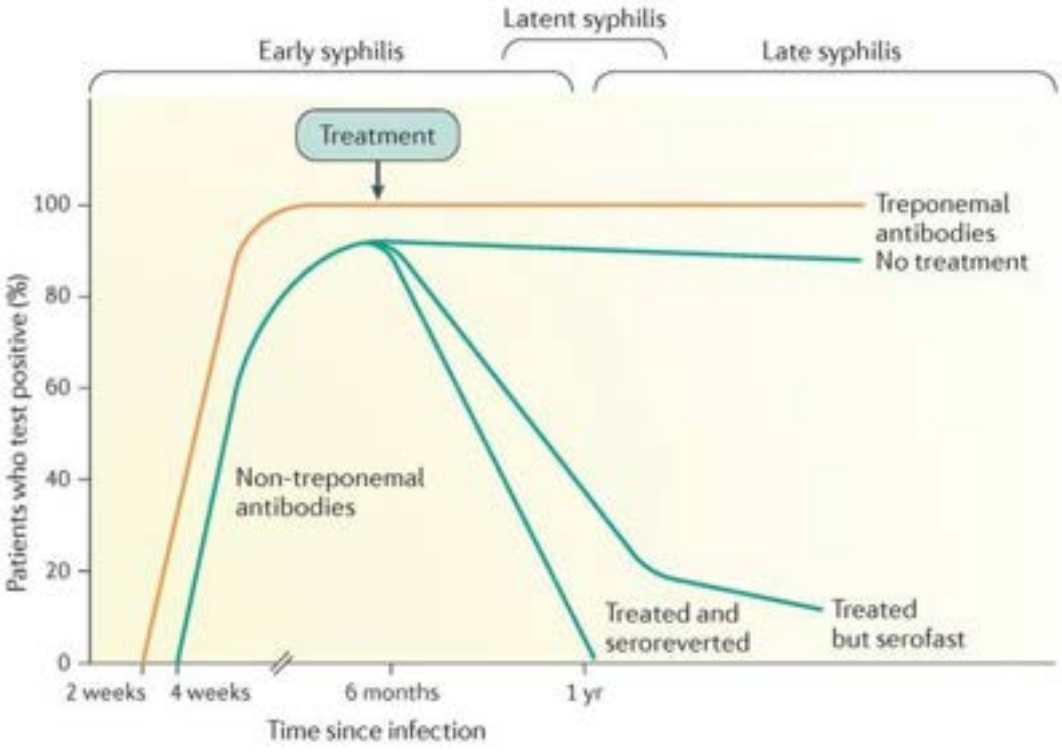
Non-treponemal (lipoidal antigen) serologic tests become reactive after primary lesions have been present for several days and may be non-reactive during early primary syphilis, when *T. pallidum* can be detected in lesions by darkfield microscopy or NAATs. Following seroconversion, non-treponemal antibody titres rise to reach a peak between 1–2 years following infection in patients who have not been treated. Thereafter, during the late latent and tertiary stages of the disease, the titre will decline slowly or remain unchanged. The outcome of treatment can be monitored by determining antibody titres using non-treponemal (lipoidal antigen) tests (Fig. 13.5).

i. Rapid plasma reagin (RPR) test

The main advantages of the RPR over the VDRL include the use of a stabilized antigen, the use of cards instead of slides, and the addition of charcoal particles to the antigen as a visual indicator of flocculation. The antigen is not coated onto these particles, but the charcoal is trapped in the lattice formed by the antigen-antibody complex in reactive samples, causing the reaction to become visible to the naked eye. The test can be performed on heated to inactivate complement or unheated serum or plasma and is performed within 18 mm circles on plasticized cards. The test requires a refrigerator to store the reagents, a centrifuge for processing blood and a shaker for mixing the reaction. The RPR is most widely available as a macroscopic non-treponemal test and is used around the world. Fig. 13.6 shows the procedure for performing the RPR test.

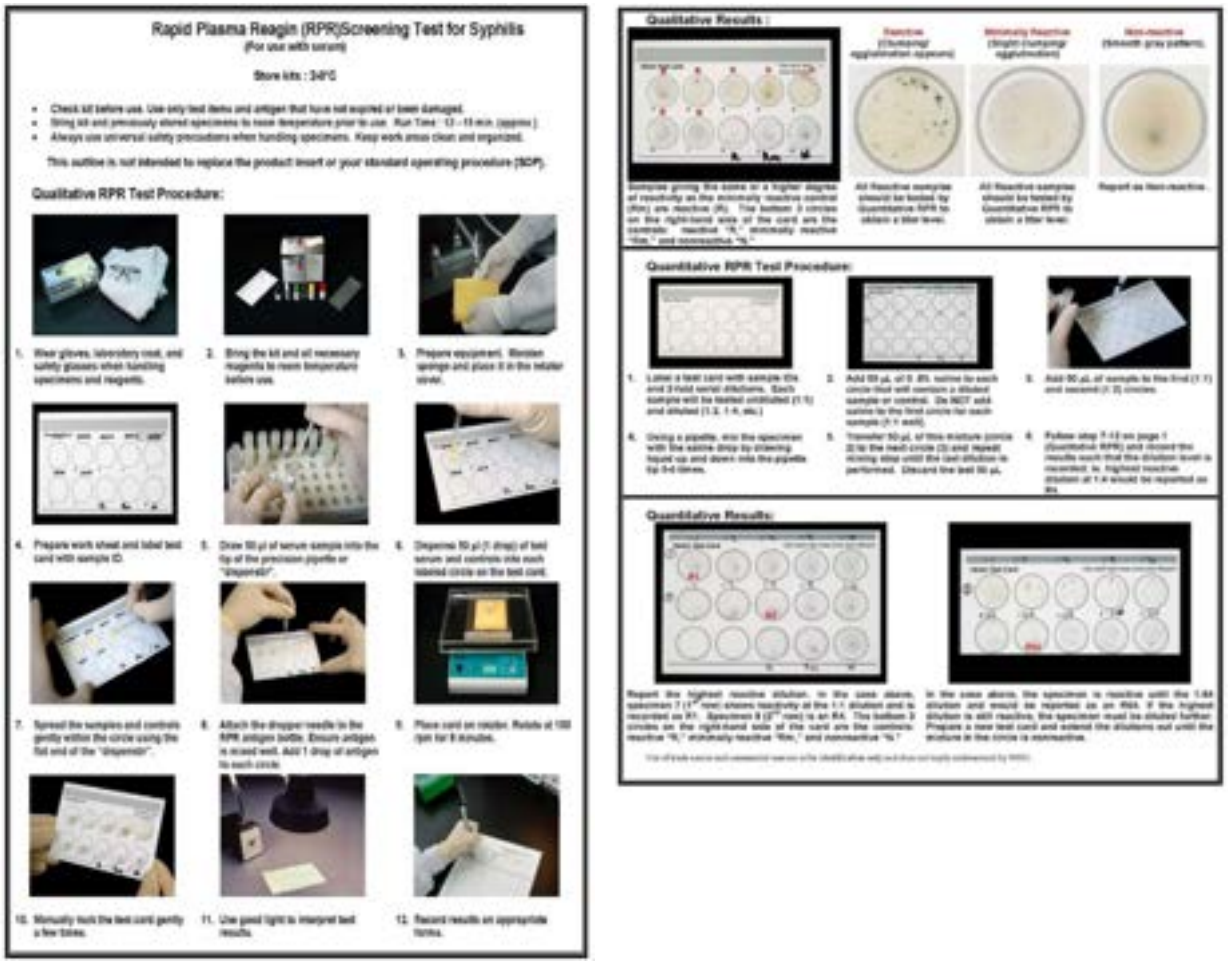
The procedure for performing the TRUST is the same as the RPR but uses toluidine red instead of charcoal to visualize the flocculation reaction. Unlike those used in the RPR test, TRUST reagents do not require refrigerated storage. Both the RPR and TRUST tests can be used with CSF specimens in suspected cases of neurosyphilis *(46,47)*.

Fig. 13.5 Antibody response to syphilis *(4)*



Source: Reproduced from Peeling et al., 2017 *(4)*, with permission from *Nature Reviews Disease Primers*.

Fig. 13.6: Procedures for performing the rapid plasma reagin (RPR) test



Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

ii. VDRL test (adapted from Larsen et al., 1998 *[11]*)

In the VDRL test, the antigen is not stable, and a suspension must be freshly prepared on the day of use. The test should be performed on serum that has been heat inactivated at 56 °C for 10 minutes and the results read with a microscope at 100× magnification. The VDRL test should be performed on CSF specimens obtained from patients with suspected neurosyphilis *(46,47)*.

a. Reagents required

- VDRL antigen. A colourless, alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol, and 0.21% ± 0.01% lecithin. The antigen should be stored either in the dark at room temperature (23–29 °C) or refrigerated at 2–8 °C, but not frozen. At these temperatures, the antigen components remain in solution. Bottles or vials that contain a precipitate should be discarded.
- VDRL-buffered saline, pH 6.0 ± 0.1 (1.0% NaCl). VDRL-buffered saline may be purchased or prepared in the laboratory. VDRL-buffered saline comprises:
  - Formaldehyde, neutral (ACS), 0.5 ml
  - Na_2HPO_4, anhydrous, 0.037 g
  - KH_2PO_4, 0.170 g
  - NaCl, 10.0 g
  - Distilled water, 1000.0 ml

The pH of the solution should be measured and the solution stored in screw-capped bottles.

Note: When an unexplained change in reactivity of the controls occurs, the pH of the saline should be measured to determine whether this is a factor. Buffered saline that is outside the range of pH 6.0 ± 0.1 should be discarded.

- Control serum samples. Reactive (R), weakly reactive (W) and non-reactive (N) sera in lyophilized or liquid form are used as controls in the test. If quantitative tests are to be performed, a control serum that can be titrated to at least a 1:4 dilution should be used.
- Acetone
- Alcohol, 95% ethanol
- Paraffin
- 0.9% saline. Add 0.9 g of dry sodium chloride to each 100 ml of distilled water.
- 10.0% saline. Add 10 g of dry sodium chloride to each 100 ml of distilled water.

b. Equipment

- Bottles: 30 ml with flat inner-bottom surface.
- Safety pipetting device with disposable tips delivering 50 µl.
- Pipettes: 1.0 ml, 5.0 ml and 10.0 ml.
- Microscope slides, measuring 5 × 7.5 cm, with 12 paraffin or ceramic rings approximately 14 mm in diameter. Note: the rings must be high enough to prevent spillage during rotation.
- Slide holder, for 5 × 7.5 cm slides.
- Ringmaker, to make paraffin rings approximately 14 mm in diameter
- Mechanical rotator adjustable to 180 ± 2 rpm, circumscribing a circle 19 mm in diameter on a horizontal plane.
- Binocular microscope with 10× eyepieces, 10× objective.
- Discard containers; disinfectants.
- Disposable latex gloves, safety glasses and protective clothing.
- Cover for slides while on rotator to maintain humidity and prevent drying.
- Syringes: 2 ml or 5 ml.

c. Test procedures for serum specimens

Preparing the antigen suspension

1. Prepare fresh VDRL antigen suspension each day. Maintain the temperature of the buffered saline, antigen and equipment between 23–29 °C at the time the antigen suspension is prepared.
2. Dispense 0.4 ml of VDRL-buffered saline into the bottom of a round, 30 ml, stoppered bottle with a flat inner-bottom surface or a 25 ml stoppered flask.
3. Add 0.5 ml of VDRL antigen suspension directly onto the saline, while rotating the bottle continuously but gently on a flat surface. Antigen should be added drop by drop at a rate allowing approximately 6 seconds for each 0.5 ml of antigen.
4. The last drop of antigen should be expelled from the pipette without touching the pipette to the saline and rotation of the bottle continued for 10 seconds.
5. Add 4.1 ml of buffered saline.
6. Cap the bottle and shake approximately 30 times in 10 seconds. The antigen suspension is then ready for use and may be used during that working day.
7. Mix the VDRL antigen suspension by gently swirling it each time it is used. (The suspension should not be mixed by forcing it back and forth through a syringe and needle, since this may cause breakdown of particles and loss of reactivity.)

Qualitative test (serum)

1. Slide flocculation tests for syphilis are affected by room temperature. For reliable and reproducible test results, the VDRL antigen suspension, controls and test specimens must be kept at room temperature, 23–29 °C, when tests are performed.
2. Add 50 µl of the sera to be tested into each ring of a paraffin or ceramic-ringed slide.
3. Holding the VDRL antigen suspension-dispensing needle and syringe in a vertical position, dispense several drops of antigen to clear the needle of air. Then add one free-falling drop (17 µl) of antigen suspension to each ring containing serum.
4. Place the slide on the mechanical rotator and rotate the slide for 4 minutes at 180 ± 2 rpm under a cover to maintain a humid atmosphere and prevent excessive evaporation.
5. Immediately after rotation, read the slide and record the test results.
6. All serum specimens that produce reactive, weakly reactive or “rough” non-reactive results in the qualitative VDRL slide test should be tested quantitatively and the end-point titre recorded.

d. Reading and reporting results

1. Read slides microscopically, using 10× eyepieces and a 10× objective.
2. Report results as follows:

Reading	Report
Medium or large clumps	Reactive (R)
Small clumps	Weakly reactive (W)
No clumping or very slight roughness	Non-reactive (N)

Quantitative test

1. Prepare two-fold dilutions of the serum to be titrated. Quantitative tests for three serum specimens up to the 1 : 8 dilution may be performed on one slide.
2. Perform the test on the two-fold dilutions of serum in the same manner as for the qualitative test.
3. Read the results microscopically using 10× eyepieces and a 10× objective as for the qualitative test.
4. Record titres as the highest dilution that yields a reactive (not weakly reactive) result.
5. After completing the day’s tests, discard the antigen suspension and clean the dispensing needle and syringe by rinsing with water, alcohol and acetone, in that order. Remove the needle from the syringe after cleaning.

13.3.2 Treponemal serologic tests

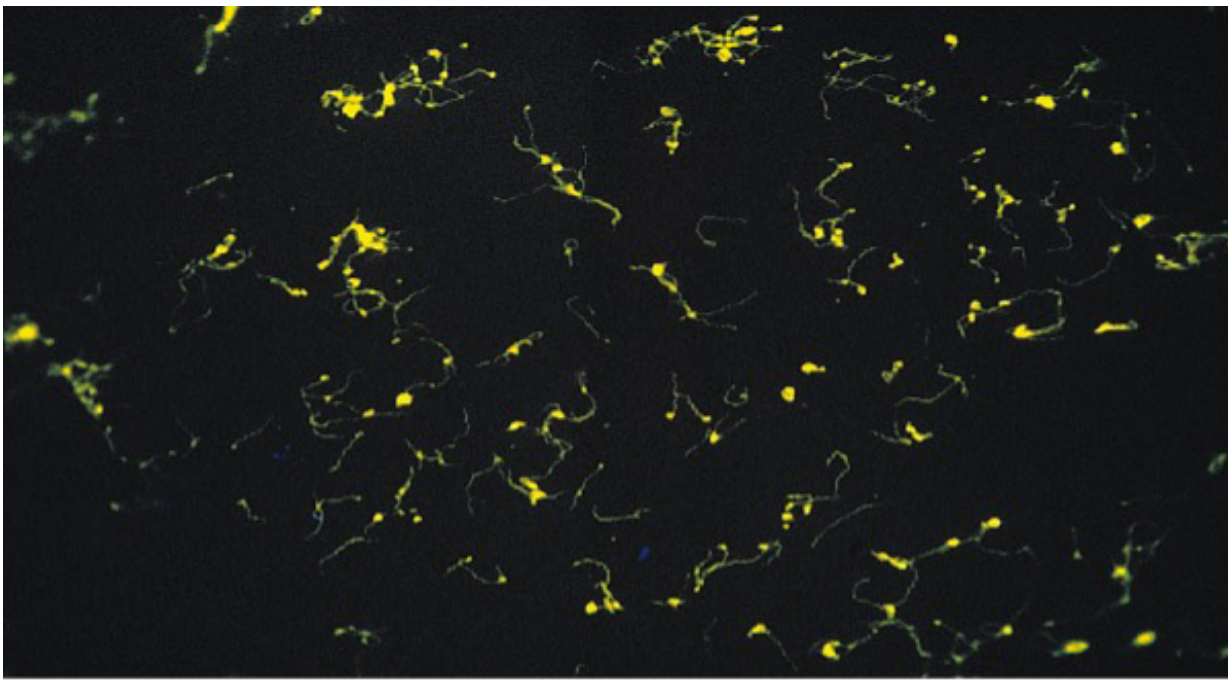
Treponemal serologic tests quantitatively detect antibodies to *T. pallidum*-specific antigens from either intact *T. pallidum*, sonicated *T. pallidum* or defined recombinant proteins. These tests were traditionally used to confirm that a reactive non-treponemal (lipoidal antigen) serologic test was due to *T. pallidum* infection and to evaluate patients with signs and symptoms suggestive of syphilis in early primary infection when non-treponemal (lipoidal antigen) tests are not yet reactive *(4,48)*. Antibodies detected by treponemal serologic tests generally persist after treatment and cannot be used to distinguish between current and previously treated infections (see Fig. 13.5). However, approximately 15–20% of patients treated for primary syphilis can become non-reactive in some treponemal tests (FTA-Abs, MHA-TP) within two to three years after treatment *(38,39)*. As with all diagnostic tests, false positives can occur with treponemal serologic tests, but the prevalence has not been well established.

All the current treponemal tests use whole cell lysates of *T. pallidum* or single or a mixture of recombinant treponemal antigens to detect antibodies against specific treponemal cellular components. There are several different test platforms used for treponemal serological testing, including indirect immunofluorescence, agglutination testing using sensitized erythrocytes or gelatin particles, EIAs, including variants that use chemiluminescence technology and immunochromatographic (lateral flow), or flow-through POC tests.

i. The fluorescent treponemal antibody absorption (FTA-Abs) test

The FTA-Abs test uses a fluorescein isothiocyanate (FITC)-labelled antihuman immunoglobulin to detect antibody binding to whole *T. pallidum* that has been fixed on a glass slide and read under a florescent microscope. The performance of the FTA-Abs test can be limited by laboratory expertise and quality control practices. A fluorescence microscope that has been properly evaluated with suitable control reagents and maintained, technical experience, quality assured reagents and an appropriate dilution of the conjugate are all critical to the reliability of the FTA-Abs test (Fig. 13.7). Unfortunately, both false-positive and false-negative FTA-Abs results are common due to laboratory error and subjective interpretation of the test *(17,28)*.

Fig. 13.7: A positive FTA-Abs test showing fluorescing *Treponema pallidum* spirochaetes



Source: Larsen et al., 1998 *(11)*.

a. Reagents required

- *T. pallidum* antigen slides can be obtained commercially. Alternatively, the antigen can be purchased as a suspension or prepared from *T. pallidum* (Nichols strain) extracted from rabbit testicular tissue and washed in phosphate-buffered saline (PBS) to remove rabbit globulin. Store unopened vials at 2–8 °C.
- FITC-labelled antihuman immunoglobulin.
- Prepare sorbent from cultures of non-pathogenic Reiter treponemes, usually with no preservative added. It is frequently dispensed in 5 ml amounts and lyophilized or as a suspension.
- Reactive control serum. Obtain a pool of human serum from seropositive donors that are 4+ reactive. Dispense the pooled serum into aliquots and store frozen, preferably at –70 °C or below. The highly reactive serum can be diluted appropriately with non-reactive sera to produce a minimally reactive 1+ control. The 1+ control displays the least degree of fluorescence reported as reactive and is used as a reading standard.
- Non-specific control serum. The non-specific control serum is a serum pool obtained from individuals without syphilis. No preservative is added. This control shows a > 2+ nonspecific reactivity at a 1 : 5 dilution in PBS and essentially no staining when diluted 1 : 5 in sorbent.
- A low fluorescence, non-drying immersion oil.
- Acetone.

Reagents to be prepared

- Phosphate-buffered saline (PBS). Prepare by adding the following to 1 L distilled water:
  - NaCl, 7.65 g
  - Na_2HP0_4, 0.724 g
  - KH_2P0_4, 0.21 g

Note: The pH should be determined and adjusted to pH 7.2 ± 0.1 using 1N NaOH. Store large volumes of PBS.

- 2.0% Tween 80 in PBS. Prepare as follows:
  - Warm the reagents in a 56°C water-bath.
  - To 49 ml of sterile PBS, add 1 ml of Tween 80.
  - Adjust the pH to 7.2 with 1N NaOH.
  - Discard the reagent if a precipitate develops or the pH changes.
- Mounting medium. Prepare as follows:
  - Add 1 part PBS (pH 7.2) to 9 parts glycerine (reagent grade).

b. Equipment

- Incubator, 35–37 °C
- Waterbath, adjustable to 56 °C
- Centrifuge
- Safety pipetting devices
- Micropipettes delivering 10–200 µl
- Loop, bacteriological, standard, 2 mm diameter, 26 gauge, platinum
- Absorbent paper
- Slide board with moist chamber and paper towels
- Staining dishes, glass or plastic, with removable slide carriers
- Microscope slides, 1 x 3 inches, with a frosted end, 1 mm thick, with two circles, 1 cm inside diameter
- Coverslips, no. 1, 22 mm square
- Test tubes (12 × 75 mm) and holders
- Discard containers and disinfectants
- Disposable latex gloves, safety glasses and protective clothing
- Fluorescence microscope with 10× eyepieces and 10× and 40× objectives
- Vortex mixer

If antigen slides are not acquired commercially, they can be prepared from treponemal suspensions as follows:

1. Wipe slides with clean gauze following storage of clean slides in alcohol.
2. If necessary, rehydrate the antigen according to manufacturer's instructions. Store opened vials at 2–8 °C. These should be stable for a week.
3. Thoroughly mix antigen suspensions on a vortex mixer for 10 seconds and examine samples by darkfield microscopy to ensure that treponemes are adequately dispensed (single organisms rather than clumps) before making slides for the FTA-Abs test.
4. Prepare very thin *T. pallidum* antigen smears within each circle using a 2 mm wire loop. Place one loop-full of antigen within the two 1 cm circles and allow to air dry for at least 15 minutes.
5. Fix slides in acetone for 10 minutes and allow to air dry. Store acetone-fixed smears at –20 °C. Smears should not be thawed and refrozen.

Sorbent

- Rehydrate freeze-dried sorbent with sterile distilled water or according to manufacturer's instructions. Store the rehydrated sorbent at 2–8 °C or at –20 °C. It is usable as long as acceptable reactivity is obtained and the product is not contaminated.

Fluorescein-labelled antihuman immunoglobulin (conjugate)

1. Rehydrate the FITC-labelled conjugate according to manufacturer's instructions. If cloudiness is observed, centrifuge at 500*g* for 10 minutes. Aliquot in small volumes and store at –20 °C. The thawed conjugate should not be refrozen, but store at 2–8 °C.
2. Prepare serial doubling dilutions of the new conjugate in PBS pH 7.2, containing 2% Tween 80 so that the dilutions include the titre suggested by the manufacturer.

3. Test each conjugate dilution with the reactive 4+ control serum diluted 1 : 5 in PBS, and with the appropriate minimally reactive 1+ control dilution using the FTA-Abs procedure described below.
4. Include a non-specific staining control with each conjugate dilution.
5. Prepare a previously tested conjugate at its working dilution and perform tests with a reactive 4+ control serum, a minimally reactive 1+ control serum, and a non-specific staining control with PBS to act as controls when first testing a new conjugate batch.
6. Read slides in the following order:
    i. Examine the three control slides (see step 5, above) to ensure that reagents and testing conditions are satisfactory.
    ii. Examine the new conjugate slides, starting with the lowest dilution of conjugate and record readings as 1+, 2+, 3+ or 4+.
    iii. The endpoint of the titration is the highest dilution, giving maximum 4+ fluorescence with the reactive serum control and a 1+ reading with the 1+ dilution. The working titre of the new conjugate is one doubling dilution below the endpoint and should be the endpoint of the minimally reactive control.
    iv. The new conjugate should not stain non-specifically at three doubling dilutions below the working titre of the conjugate.
    v. Store the conjugate as directed by the manufacturer and dispense in not less than 0.3 ml aliquots < –20 °C. A conjugate with a working dilution of 1 : 1000 or higher may be diluted 1 : 10 with sterile PBS containing 0.5% bovine serum albumin and 0.1% sodium azide prior to freezing.
    vi. Verify the titre of the conjugate after storage for several days in the freezer.

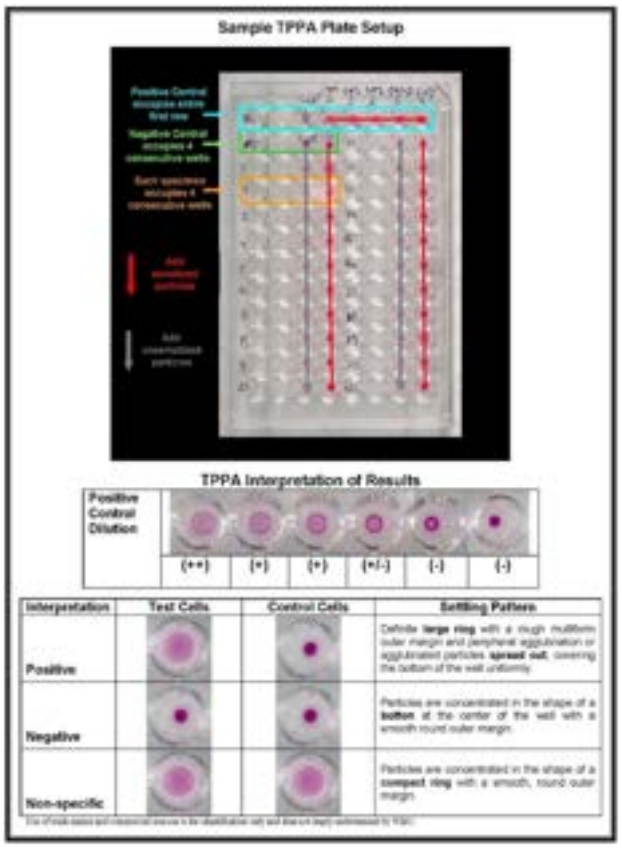
c. Test procedure

1. Identify previously prepared antigen slides by numbering the frosted end.
2. Number each tube and slide to correspond to the test serum and the control serum to be tested.
3. Prepare reactive (4+), minimally reactive (1+) and non-specific control serum dilutions in sorbent or PBS according to the directions.
4. Pipette 200 µl of sorbent into a test tube for each test serum.
5. Add 50 µl of the heated test serum to the appropriate tube and mix.
6. Cover the appropriate antigen smears with 30 µl of the reactive (4+), minimally reactive (1+) and non-specific control serum dilutions.
7. Cover the appropriate antigen smears with 30 µl of the PBS and 30 µl of the sorbent for non-specific staining controls.
8. Cover the appropriate antigen smears with 30 µl of the test serum dilutions.
9. Prevent evaporation by placing slides in a moist chamber and incubate at 35–37°C for 30 minutes.
10. Place slides in slide carriers and rinse for 5 seconds with running PBS. Then place slides in a staining dish containing PBS for 5 minutes and agitate slides by dipping them in and out of the PBS at least 20 times. Using fresh PBS, repeat the rinsing procedure. Finally, rinse slides for 5 seconds in running distilled water and gently blot with absorbent paper.
11. Dilute FITC-labelled antihuman IgG to its working titre in PBS containing 2% Tween 80 and layer approximately 30 µl of the diluted conjugate onto each smear.
12. Repeat steps 9 and 10.
13. Mount slides immediately by placing a small drop of mounting medium on each smear and applying a cover glass.
14. Place slides in a darkened room and read within 4 hours.
15. Check smear by darkfield microscopy, using the tungsten lamp first, to verify the presence of treponemes on the smear, then read using the fluorescence microscope with the appropriate FITC filters.

ii. Treponemal agglutination assays

The TPHA, MHA-TP and TPPA are indirect agglutination assays, but differ in that the TPHA and MHA-TP have *T. pallidum* antigens bound to avian or ovine erythrocytes, respectively, and the TPPA has *T. pallidum* antigens bound to gelatin particles. They are easier to perform than the FTA-Abs test and are less technically demanding to obtain accurate results. Based on a few studies with 400–500 patients, the sensitivity of TPPA is slightly better than TPHA and results are more consistent because TPHA can be affected by batch-to-batch variation of the avian or ovine erythrocytes *(49,50)*. Fig. 13.8 shows the procedure for performing the TPPA test. The method for performing the TPHA and MHA-TP are similar.

Fig. 13.8: Procedures for *Treponema pallidum* passive particle agglutination assay (TPPA)



Source: Centers for Disease Control and Prevention, Atlanta, GA, USA.

iii. Treponemal enzyme immunoassays (EIAs) and chemiluminescence immunoassays (CIAs)

EIAs and CIAs have several advantages: they are automated with high throughput and lower labour costs per specimen; create fewer laboratory occupational hazards from pipetting; do not produce false negatives due to the prozone; and allow more objective result interpretations by calibrated machines that measure either optical density or fluorescence, depending on the system design. The read-out is typically an index value calculated as a signal to cut-off ratio or fluorescence ratio based on values between the specimen and calibrator controls. The raw reading outputs and index values can be stored for future retrieval. All EIAs and CIAs may be performed on serum, and some can also be performed on plasma, including heparinized, EDTA and citrate plasma.

The majority of treponemal EIAs employ either sonicated *T. pallidum* antigen, a single recombinant treponemal antigen, or a mixture of recombinants coated on to the wells of microtitre plates. A dilution of patient's serum is added to each well. If specific antibodies to *T. pallidum* are present in the serum, they will bind to the treponemal antigens. After washing off any excess antibody, a conjugate comprising biotinylated anti-human IgG labelled with streptavidin-peroxidase is added to detect bound-specific antibody. After a further washing step to remove any excess conjugate, an enzyme substrate is added to detect the antigen-antibody-conjugate complex. A colour reaction takes place if the patient has antibodies to the *T. pallidum* antigen(s). The intensity of the colour development is directly proportional to the concentration of antibodies present. The colour change is read using a plate reader.

CIAs to detect treponemal antibody are most widely used in high-throughput clinical laboratories. These assays either use the principle of attachment of specific antibody to antigen-coated beads and subsequent detection of beads that have been tagged with phycoerythrin conjugated to goat anti-human IgG, or detect antibodies using an isoluminol-antigen conjugate to generate flashes of chemiluminescence that are detected by a sophisticated photomultiplier system.

Due to the large number of EIAs and CIAs commercially available worldwide, a detailed description of the test procedure for each manufacturer's test is beyond the scope of this chapter. Therefore, the reader is requested to follow the instructions included in the package insert provided in each kit by the manufacturer.

iv. Rapid POC syphilis tests

There are several POC syphilis serologic tests or dual POC serologic tests for HIV and syphilis that are widely available and have been pre-qualified by WHO *(51)*. These tests are generally formatted either as lateral flow strip tests or as flow-through devices. In the lateral flow format, one or more recombinant antigens are striped onto a nitrocellulose immunochromatographic strip to capture specific treponemal antibodies, and a coloured line is produced upon binding either anti-human immunoglobulin linked to an enzyme, colloidal gold or coloured latex particles. A separate control line is also incorporated into the test, which either acts as a procedural control (in some tests) or indicates specimen adequacy (by indicating the presence of non-specific human immunoglobulin in the patient's specimen). In the flow-through format, antigen spots replace lines and binding of specific antibody to the antigens occurs during passage through the membrane rather than when passing laterally along a membrane strip. All these tests provide a rapid indication of previous exposure to treponemal infection and are most suited in locations that lack access to quality laboratory diagnostics. These tests can be performed using whole blood (obtained by finger prick), plasma or serum; and do not require special equipment (including a refrigerator or centrifuge). Although the tests are easy to perform, strict adherence to the manufacturer's instructions is necessary to ensure ongoing accuracy of the tests.

Antibodies detected by treponemal rapid POC tests likely persist for life, despite treatment, and both current and past infection will cause a positive test. Sole reliance on POC tests in populations with a high prevalence of past syphilis may result in overtreatment. Ideally, a quantitative non-treponemal test should be performed to increase the specificity of a diagnosis of active infection, and to serve as a baseline to monitor treatment. For the use of rapid syphilis tests for prenatal screening, WHO recommends giving any pregnant woman who tests positive on a rapid treponemal test a single dose of penicillin to protect the fetus from being infected while awaiting confirmation of active infection with a non-treponemal test. Given the serious adverse effects of syphilis in pregnancy and the serious risks of congenital syphilis and fetal death, overtreatment is considered acceptable for most prenatal care programmes *(52)*.

v. Serologic test algorithms for syphilis

The traditional approach to serological testing for syphilis involves the screening of sera with a relatively inexpensive, sensitive, but relatively less specific and labour-intensive non-treponemal test and, if found to be reactive, confirmation with a more specific, but more expensive treponemal test (Fig. 13.9A). This approach, known as the traditional algorithm, proved to be effective, particularly in those settings where the disease had been frequently encountered. The subsequent titration of confirmed-reactive sera using a quantitative non-treponemal test also provides a more precise interpretation of these results together with a baseline measurement (titre) against which the efficacy of therapy can be evaluated.

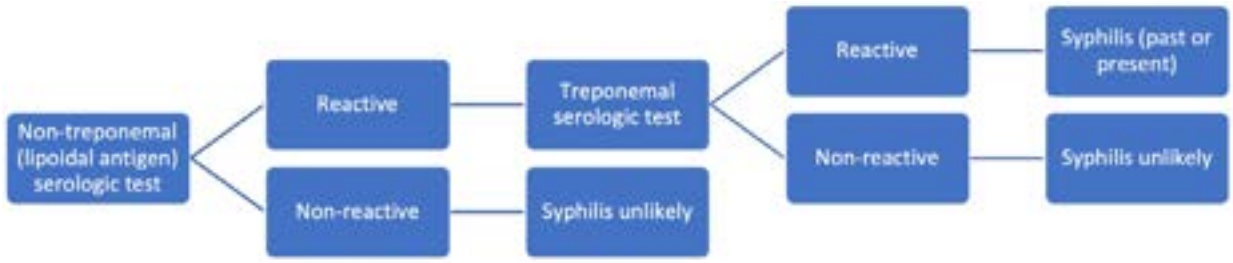
The development of treponemal enzyme-linked immunosorbent assays (ELISAs) and CIAs that can be automated has led many laboratories to change the approach to a so-called "reverse" algorithm, where a treponemal serologic test is the first test in the sequence (Fig. 13.9B). The reverse algorithm was adopted in many high-volume laboratories in industrialized countries where labour costs are high and the seroprevalence is low. Sera are screened using treponemal serologic tests, and if reactive, reflex to a non-treponemal serologic test. If both treponemal and non-treponemal serologic tests are reactive, then a quantitative non-treponemal serologic test should be performed.

The sensitivity of the traditional algorithm may be lower than the reverse algorithms because serum from some patients with early or late syphilis may not be reactive in non-treponemal serologic tests *(53)*. In contrast, patients with past syphilis may be misdiagnosed using the reverse algorithms. Serologic test results, regardless of the algorithm, should be considered along with findings from a physical examination of the patient, previous history of disease and recent sexual risk before initiating treatment and partner notification activities.

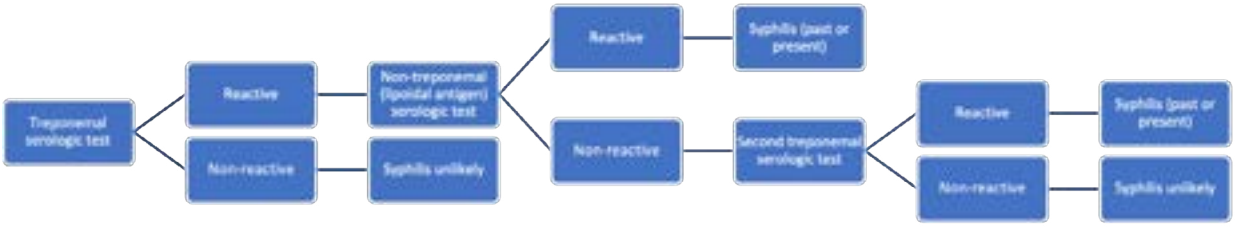
Considerations for test/algorithm selection include cost, labour, volume of specimen test requests, throughput, laboratory space and turnaround time and the likelihood of patients returning for their results. In addition, clinicians need all test results for timely clinical management. If the reflex confirmatory test is delayed, clinical management can be delayed. The laboratory processing the initial screening test should ensure sufficient resources for reflex testing.

Fig. 13.9: Syphilis serologic testing algorithms

A. Traditional algorithm



B. Reverse algorithm



Source: John R. Papp, Pingyu Zhou, Rosanna Peeling and Edward W. Hook III.

Table 13.3: Sensitivity estimates of selected serological tests for syphilis by stage of disease

Test	Sensitivity estimate by stage of syphilis				
	Primary	Secondary	Early latent	Late latent	References
Non-treponemal (lipoidal antigen) serologic tests					
RPR	48.7–92.7%	91.0–100%	No estimate	No estimate	(48,54–63)
VDRL	50.0–78.4%	100%	82.1–100%	63.6–100%	(48,54–58,60, 64–71)
Treponemal serologic tests					
FTA-Abs	78.2–90.0%	92.8–100%	100%	92.6–100%	(50,72,73)
MHA-TP	45.9–88.6%	90.0–100%	99.0–100%	90.3%	(49,54,74–76)
TPPA	94.5–96.0%	100%	94.4–100%	86.8–100%	(50,75,77)

RPR: rapid plasma reagin; VDRL: Venereal Disease Research Laboratory; FTA-Abs: fluorescent treponemal antibody absorption; MHA-TP: microhaemagglutination assay for *T. pallidum* assay; TPPA: *T. pallidum* passive particle agglutination assay.

vi. Serologic test performance for syphilis

The performance of serologic tests for syphilis depends on the stage of syphilis, specimen type and quality, patient population, autoimmune disease and infections or co-infections with agents other than *T. pallidum*. Studies that estimate the performance of non-treponemal (lipoidal antigen) and treponemal serologic tests are limited by small sample sizes, the use of different reference standards, and lack of reported stage of syphilis. Most studies estimate sensitivity and not specificity of these tests. Table 13.3 highlights the wide variation of sensitivity estimates of serologic tests for syphilis.

In cases where indeterminate reactions have been detected in any test, the test should be repeated, if possible, on a new serum specimen. If initial assays are non-reactive and primary syphilis is suspected, repeating the tests after 2–3 weeks on a new serum sample will allow sufficient time for the development of a detectable antibody response *(4,48)*. Finally, some patients given adequate treatment that was initiated very early in primary syphilis may remain non-reactive.

NOTE: Serological testing remains a useful tool in helping to establish a diagnosis of syphilis, but the infection status of a person cannot be determined solely by serology. A diagnosis is made by a medical professional based on current clinical signs and symptoms, physical examination, previous history of disease, comprehensive sexual risk assessment and biomedical test results.

13.4 Laboratory considerations for specimens from cases of suspected congenital syphilis

Any skin and mucous membrane lesions present in a newborn born to a seropositive mother should be examined by darkfield microscopy, DFA or NAAT for direct evidence of infection with *T. pallidum*. Oral treponemes do not appear in the mouth until approximately six weeks after birth, so there is little chance of false-positive results being obtained from oral specimens.

The finding of a reactive serological test in a neonate may be the result of passive transfer of maternal antibody across the placenta during pregnancy and, therefore, cannot be considered diagnostic. Some infants with congenital syphilis have an RPR titre that is the same or one or two dilutions less than the maternal titre. In particular, if a mother acquires syphilis and seroconverts late in pregnancy the baby may be delivered prior to a mature antibody response. However, the finding of a significantly higher RPR/VDRL titre (i.e. ≥ 4-fold higher) in the neonate's serum compared to the maternal titre or the detection of a significant rise in RPR/VDRL titre (≥ 4-fold) during a three-month period have been considered indicators of congenital infection.

Modifications of the FTA-Abs test (FTA-IgM), specific EIAs and line immunoassays that detect only IgM may be used to detect specific anti-treponemal IgM, which are unable to cross the placental barrier. The finding of such IgM antibody in the baby's circulation is an indication of congenital infection, but specific IgM cannot be detected in all cases of congenital disease *(78)*. Two older studies reported good correlation between FTA-IgM or EIAs with clinical findings or other reactive serology *(79,80)*, but these studies were not performed with commercial IgM assays and recent published clinical data are lacking.

In IgM-specific EIAs, anti-human IgM (μ chain specific) antibody is coated onto the wells of microtitre plates. A measured dilution of the patient's serum is added to the wells of the plate. The anti-human IgM captures any available IgM antibody in the patient's serum. Purified *T. pallidum* antigen is added to the wells of the plate and excess antigen washed away. A mixture of biotinylated human or rabbit anti-*T. pallidum* antiserum and streptavidin horseradish peroxidase is added to the wells of the plate. After rinsing off the unbound complexes, enzyme substrate and chromogen indicators are added. If the serum contains specific IgM antibodies to *T. pallidum,* a colour reaction takes place with the intensity of the colour proportional of the concentration of antibodies present.

13.5 Laboratory considerations for specimens from cases of suspected neurosyphilis

The diagnosis of neurosyphilis is challenged by a lack of consensus on the clinical implications of abnormal CSF findings in patients with no neurological symptoms or signs but with serologic evidence of syphilis. While a high proportion of these have treponemes in their central nervous systems, they do not require the enhanced treatment required for neurosyphilis *(35)*. As a result, lumbar puncture is not performed routinely in early syphilis unless clinically indicated. However, when indicated, CSF specimens should be examined for total protein and leukocyte counts and a VDRL-CSF test performed. The VDRL-CSF test has high specificity (99–100%) but low sensitivity for neurosyphilis *(81,82)*. Thus, while a reactive VDRL-CSF test is an indicator of neurosyphilis, a non-reactive test cannot be used to exclude neurosyphilis. The VDRL test is not widely available, and some studies have used RPR to test CSF *(46,82,83)*. The sensitivity of RPR-CSF ranged from 75–79% and 60–69% in patients with symptomatic and asymptomatic neurosyphilis, respectively, in two studies *(82,83)*. The specificity of RPR-CSF in these studies ranged from 93–99% and 83–100% in symptomatic and asymptomatic neurosyphilis patients. RPR-CSF had a sensitivity ranging from 52–59% and specificity ranging from 85–100% in symptomatic and asymptomatic neurosyphilis patients *(46)*. In contrast, the FTA-Abs CSF test has high sensitivity but low specificity for neurosyphilis owing to passive transfer of specific IgG antibodies across the blood-brain barrier. The FTA-Abs CSF test has been used in unique clinical circumstances such as in patients with non-specific neurologic signs or symptoms, reactive serologic tests and a non-reactive VDRL-CSF, even if CSF lymphocytic pleocytosis and elevated CSF protein are present, because of its negative predictive value. For TPPA-CSF, the sensitivity ranges from 75.6–95.0%, with the highest sensitivities reported when reactive VDRL-CSF was the criteria for neurosyphilis *(47,84–86)*. Specificity ranged from 85.5–100% and was highest if a TPPA titre of ≥ 1 : 640 was used to define neurosyphilis *(47)*. Based on these limited data, TPPA-CSF appears to have similar performance to FTA-Abs CSF in studies of patients with definitive/presumptive neurosyphilis *(40)*.

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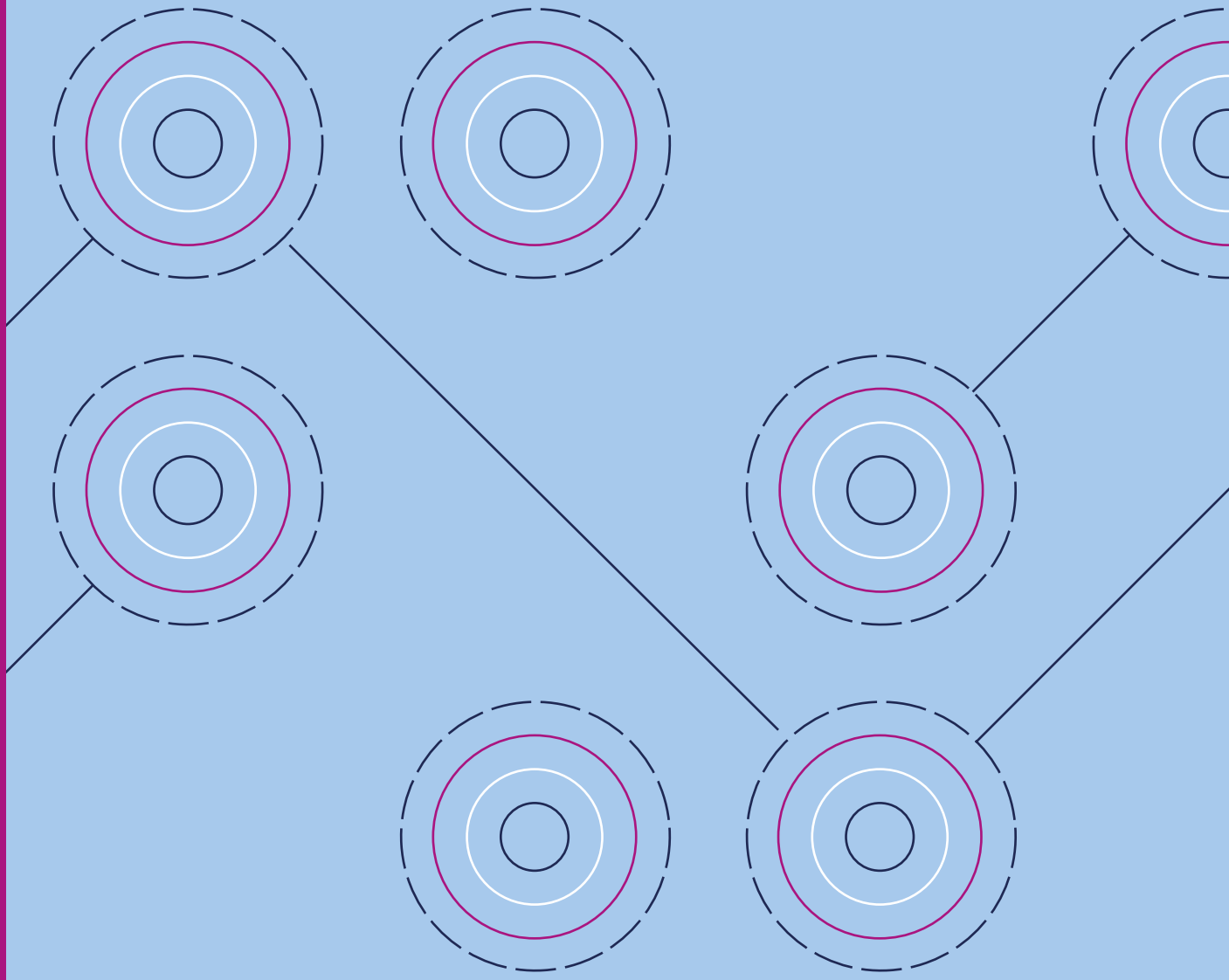
Chapter 14. Lymphogranuloma venereum (LGV)



Chapter 14. Lymphogranuloma venereum (LGV)

Michelle Cole and Olivia Peuchant

14.1 Introduction

Lymphogranuloma venereum (LGV) is caused by the distinct "L" biovar of *Chlamydia trachomatis,* which contains genovars L1, L2, subvariants such as L2a and L2b, and L3. The L genovars are more invasive than those responsible for the eye disease, trachoma (genovars A–C) and those causing non-gonococcal urethritis and associated infections of the genital tract (genovars D–K). The traditional disease course is divided into three stages *(1)*. In the primary stage, a painless primary lesion (papules, ulcers or herpetiform erosions) appears at the site of inoculation and usually heals in a few days. The lesion may therefore pass unnoticed. The secondary stage is characterized by the spread of infection through the lymphatic system to regional lymph nodes, resulting in inguinal lymphadenopathy, known as "buboes". The lymph node can abscess, rupture spontaneously and form a fistula. In the current LGV endemic among men who have sex with men (MSM), the secondary stage manifests as erosive, ulcerative proctitis, with tenesmus, discharge and pain *(2)*. If untreated, the disease reaches the tertiary stage with chronic lymphadenitis that can lead to elephantiasis *(1)*.

LGV that presents as genital ulcer disease (GUD) occurs worldwide, but it is most common in tropical and subtropical regions *(1)*. However, recent prevalence data are lacking for many regions. A recent South African study detected LGV in 20% of *C. trachomatis*-positive vaginal specimens from women living with HIV who attended antenatal services *(3)*. In 2003, a cluster of rectal LGV infections, with characteristic lymph node involvement and/or proctitis or proctocolitis, was reported in MSM in the Netherlands *(4)*. LGV in MSM has since been reported globally, mainly from high-income regions such as North America, Australia and Europe *(5)*. Where available, epidemiological surveillance in these regions has shown that LGV is now endemic and is almost exclusively in MSM. Infection is often associated with symptomatic proctitis, in those living with HIV and in individuals who are part of dense sexual networks with high rates of partner change *(6–8)*. In some regions, the epidemiology of LGV is shifting, with increasing LGV diagnoses in those who are HIV negative *(9–12)*. This change is probably due to the availability of pre-exposure prophylaxis (PrEP), the administration of which often requires regular testing for sexually transmitted infections (STIs), which will provide more opportunities for LGV detection *(9,11)*. In addition, PrEP usage has enabled increased mixing of sexual networks in respect to HIV status *(9,13).* Heterosexual transmission of LGV variants that are most common in MSM continue to occur rarely *(7,14,15)*. In addition to LGV diagnoses in those with symptoms, a high prevalence of asymptomatic LGV infection (27–49%) has been detected *(10,16–18)*. Therefore, the testing of all anorectal *C. trachomatis* positive samples from MSM for LGV is performed in some settings *(10,18,19)*. The scarcity of LGV data in some countries is probably due to undertesting and/or underreporting, rather than lack of cases *(8)*. Limited data are available on the number of rectal *C. trachomatis* that belong to a LGV genovar due to limited systematic screening and testing for LGV globally. Changes in the indications for LGV testing over time complicate the interpretation of LGV positivity trends (there is confounding in the data due to the use of indications for testing that can be selective for individuals with symptoms of proctitis or living with HIV, or the use of universal testing regardless of symptoms or HIV status) *(10, 18)*.

Sequencing of the *C. trachomatis omp*A gene revealed that the LGV outbreaks among MSM were predominately attributable to the genovariant L2b. This was previously identified in 1981 among MSM in San Francisco, USA *(20,21)*. However, recent evidence has shown a change towards an L2/434-Bu predominance in some countries, as well as the emergence of other L2b genovariants *(8,22–24)*. Recombination between L and non-L *C. trachomatis* strains can occur, leading to the emergence of new recombinant strains. Notably, an L2b/D-Da

hybrid variant was identified in Portugal *(25)* and was subsequently reported in other countries *(22,23,26,27)*. While some recombinant variants have been identified via *omp*A sequencing, whole genome sequencing (WGS) allows better characterization. WGS studies have shown some clonality of the LGV population in MSM *(21)*.

14.2 Laboratory tests

Until the early 1980s, isolation of *C. trachomatis* in cell culture remained the main method for diagnosis of chlamydial infection (see Chapter 8). Isolates from cases of LGV were observed to grow more rapidly in tissue culture cells than non-L chlamydial isolates. Since the mid-1990s, nucleic acid amplification tests (NAATs) have become the tests of choice for diagnosis of chlamydial infections, due to their greater sensitivity (see Chapter 8). However, NAATs for *C. trachomatis* detection cannot discriminate between non-LGV and LGV strains.

The identification of L genovars of *C. trachomatis* is of both epidemiological and therapeutic interest. For patient therapeutic management, LGV testing should ideally be performed within one week. This is because LGV treatment requires three weeks of doxycycline, while an infection with a non-L genovar requires only one week of treatment *(19)*. For the specific detection of LGV in suspected clinical specimens, a commercial *C. trachomatis* NAAT (see Chapter 8) should first be used. If *C. trachomatis* positive, reflex testing for LGV genovars should be carried out using a NAAT targeting the unique LGV-specific 36-bp deletion of the *pmp*H gene, from the same specimen *(19,28–30)*. "In-house" LGV assays are often utilized in reference laboratory settings or in laboratories that serve larger MSM cohorts. Commercial assays are now available – including multiplex NAATs for GUD or simplex polymerase chain reaction (PCR) targeting the *pmp*H gene – which demonstrate very good performance in the context of rectal LGV detection *(31,32)*.

Because the major outer membrane protein (encoded by *ompA*) is subject to recombination, the diagnosis of LGV should not be made by *omp*A genotyping alone. Genotyping is appropriate for epidemiological studies to survey the dynamics and evolution of LGV variants in the MSM population.

The specimens for LGV NAATs include:

- anal swabs (self- or clinician-collected);
- rectal biopsies under anuscopy or rectoscopy;
- swabs of ulcer base exudate from primary anogenital lesions;
- lymph node/bubo aspirates; and
- pharyngeal swabs and urogenital specimens from men and women, only when indicated by a clinical or epidemiological context that is strongly suggestive of LGV *(19, 33)*.

Where LGV genovar-specific *C. trachomatis* NAATs are unavailable, *C. trachomatis*-specific serological assays could be used for a presumptive LGV diagnosis for individuals with a high antibody titer and LGV clinical symptoms *(19)*. Serological testing for LGV diagnostics should, however, be used with care, due to the lack of standardization of interpretation criteria, and the inability of serology to distinguish between past and current chlamydial infection *(19)*.

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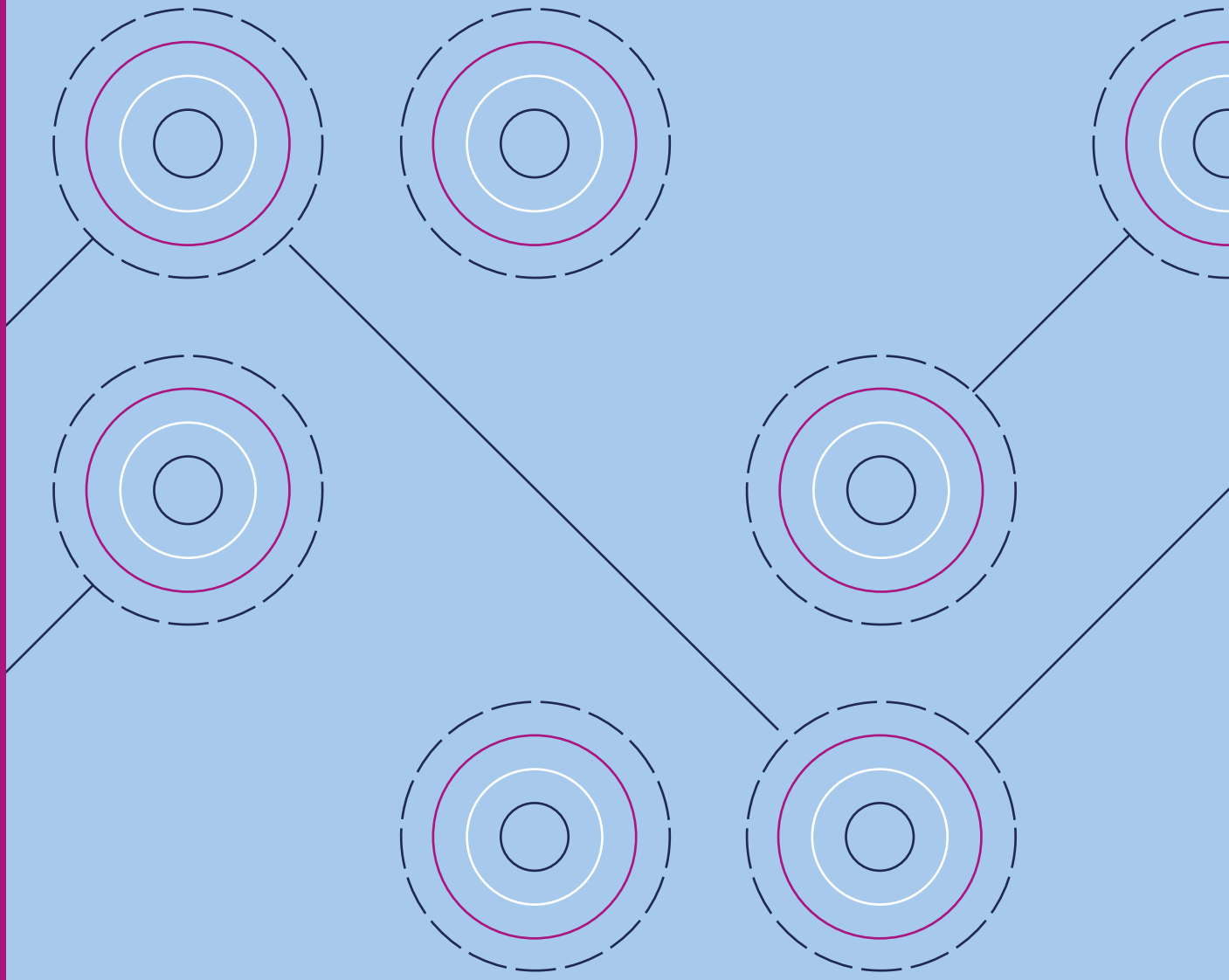

Chapter 15. Chancroid

David Lewis and Magnus Unemo

15.1 Introduction

Chancroid, also known as soft chancre or ulcus molle, is caused by *Haemophilus ducreyi.* The infection is transmitted exclusively by sexual contact, and the bacterium can invade through healthy or abraded skin. Chancroid produces ulcers on the genitalia, typically the penile coronal sulcus in men, and the vulva in women. Perianal chancroid may occur in individuals who engage in anally receptive sexual intercourse. Chancroid may be associated with suppurative inguinal lymphadenitis, particularly if there has been a delay in the patient presenting to health services, or the correct diagnosis being made. Asymptomatic genital carriage of *H. ducreyi* has been postulated to occur in a very small number of cross-sectional (non-randomized) studies but the presence of a true carriage status remains unproven.

The incubation period is usually between 4–10 days. The genital ulcer starts as a tender papule that becomes pustular and ulcerates within 2 days. The ulcer is frequently soft and painful, irregular in shape, with undetermined edges, and is usually not indurated. These are the classical features that differentiate chancroid from syphilitic ulcers. However, it is important to note that the sensitivity of diagnosis based simply on the clinical appearance of the ulceration is poor. The base of the ulcer often is covered by a purulent and necrotic exudate, and the ulcer base bleeds easily when scraped or swabbed. Multiple lesions are common, and they may merge to form very large ulcers. Painful, unilateral inguinal adenitis also may occur. If not adequately and promptly treated, this may lead to the spontaneous rupture of suppurating lymph nodes (buboes). Atypical presentations of chancroid are common, and the infection can be easily confused with other types of genital ulcer disease (GUD), particularly genital herpes. Some genital ulcers may be infected with more than one GUD pathogen, but this is less common nowadays as a result of the decline in relative prevalence of bacterial GUDs.

While chancroid was previously very common in certain parts of the world, the prevalence of the infection has waned dramatically since the 1990s. In part, this is due to increased access to antimicrobial agents, the roll-out of syndromic management, improved health care for sex workers, and sexual behavioural change in the era of HIV infection *(1).* Importantly, in recent years, *H. ducreyi* has been reported as an important cause of chronic leg ulceration in yaws-related surveys conducted in yaws-endemic countries *(2).*

H. ducreyi is a short, non-motile, Gram-negative bacillus. It is a fastidious organism, and because of its complex nutritional requirements, highly enriched selective culture media are needed for its isolation. The growth of *H. ducreyi* isolates may be either aerobic or anaerobic. Growth is optimal at 32–33 °C in a humidified atmosphere. Most strains, particularly on primary isolation, require carbon dioxide (CO_2) and a humidified atmosphere. *H. ducreyi* has few distinguishing biochemical characteristics: all strains reduce nitrate to nitrite, are positive for both oxidase and alkaline phosphatase, and require haemin (X factor) for growth.

15.2 Overview of diagnostic procedures

The laboratory diagnosis of chancroid traditionally has been based on recovery of *H. ducreyi* in culture, which is a technically demanding procedure with low yield outside of highly skilled laboratories used to working with the pathogen *(3).* Initially, several laboratory-developed nucleic acid amplification tests (NAATs) have been used to enhance diagnostic sensitivity *(3).* Although modern commercial multiplex NAATs for genital ulceration are able to detect *H. ducreyi* as part of the panel of infectious agents targets, it has not proven possible to evaluate assay performance in clinical settings among patients with GUD, due to the lack of chancroid cases. In addition, several laboratory-developed techniques have been described, including the use of monoclonal antibody-based antigen detection and DNA probes *(3).* Direct microscopy has very low sensitivity and specificity and, therefore, is of little use as a diagnostic tool for chancroid. The currently available research-based serological assays are only useful for sero-epidemiological purposes.

15.3 Specimen collection and transport

Specimens for *H. ducreyi* culture should be obtained from the base of the ulcer. Clean the ulcer with a dry gauze or a swab to remove crusts and superficial debris. Extensive cleaning is not required, as it may cause bleeding, as well as being painful for the patient. Collect the exudate from the base with a swab; the type of fibre used on the swab does not seem to affect culture sensitivity. Isolation of *H. ducreyi* from inguinal bubo pus has been much less successful than that from genital

ulcer material and, therefore, is rarely performed. For optimal results, inoculate the specimens immediately on to the isolation media and keep in either a candle jar or a clinic-based incubator in a humidified 5% CO_2-enriched atmosphere at a temperature no higher than 35 °C, until final incubation. When culture media are not available at the clinical site, specimens may be transported at 4 °C in a transport medium such as Amies or Stuart's medium. A thioglycolate-haemin-based medium containing L-glutamine and bovine albumin seems to maintain the viability of *H. ducreyi* for several days at 4 °C *(4)*.

15.4 Isolation and identification of *H. ducreyi*

For many years, bacteriological culture for *H. ducreyi* was the main tool for diagnosis of chancroid in the clinical setting, and the "gold standard" for evaluating other diagnostic methods. Successful culture is critically dependent on using freshly made media (i.e. ideally less than 7 days old) and on attention to correct incubation conditions. However, with the advent of more sensitive laboratory-developed NAATs, it is now appreciated that culture may detect, at best, only 75% of *H. ducreyi* infections *(5)*.

Initial attempts to cultivate *H. ducreyi* used fresh clotted rabbit blood heated to 55 °C, fresh clotted human blood, and heat-inactivated human serum, but these methods were subject to recurrent microbial contamination *(3)*. This problem of contamination was subsequently addressed by adding vancomycin to semisolid chocolate agar at a concentration of 3 µg/ml *(6)*. It should be noted that growth of some *H. ducreyi* strains may be inhibited at this concentration of vancomycin and such strains would require isolation on non-antibiotic-containing culture media.

Several selective artificial media have been developed and are reviewed in detail elsewhere *(7)*. Nsanze et al. demonstrated that the yield of positive cultures may be increased when more than one type of culture medium was used to isolate *H. ducreyi* from genital ulcer material *(8)*. Differences in nutritional requirements between *H. ducreyi* strains may account partially for these observations. A medium containing GC agar base, 1–2% of haemoglobin, 5% fetal calf serum, 10% cofactors-vitamins-amino acids (CVA) enrichment and vancomycin (3 µg/ml) appears to have the highest sensitivity for the isolation of *H. ducreyi* from clinical specimens, with positive cultures being reported in up to approximately 80% of clinically defined chancroid cases (Fig. 15.1) *(7)*. It has been noted, however, that some *H. ducreyi* isolates will not grow on this medium, but may instead be isolated on a different medium made of Mueller-Hinton (MH) agar, 5% chocolated horse blood, 1% CVA enrichment, and vancomycin (3 µg/ml) *(5)*. To assist with optimizing *H. ducreyi* culture in clinical settings, two different media may be incorporated into a single biplate. A charcoal-based medium has been developed that avoids the need to add costly fetal calf serum, which makes this medium more cost-effective in resource-poor settings (Fig. 15.2) *(9)*. Annex 1 lists recipes for suitable media. In setting up facilities for *H. ducreyi* culture, it is advisable to use at least two of the above-mentioned culture media and to define their sensitivities during pilot studies.

Fig. 15.1: *Haemophilus ducreyi* growing on an enriched GC agar-based chocolate plate

Source: WHO, 2013 *(10)*.

Fig. 15.2: Clumps of *Haemophilus ducreyi* on a charcoal-based medium

Source: WHO, 2013 *(10)*.

After inoculation, incubate the culture plates at 32–34 °C in either a humidified 5% CO_2-enriched atmosphere or, preferably, in microaerophilic conditions. Incubate the cultures for 48 hours prior to the initial reading and keep for 5 days before concluding they are negative. *H. ducreyi* colonies may vary in size, depending on the individual strain, as well as the time and temperature of incubation, the atmosphere and the growth medium. Colonies are non-mucoid, raised and granular, have a greyish yellow colour, and can be pushed intact in clumps across the surface of the agar with a bacteriological loop. Colonies are either translucent or opaque, and this variability may give the impression of a mixed, impure culture. Gram staining of smears from colonies shows Gram-negative coccobacilli in short chains, clumps or whorls. The bacteria are pleiomorphic in approximately 50% of the cultures. Individual bacteria may appear to have bipolar staining. In many areas of the world, almost all *H. ducreyi* isolates produce β-lactamase. This characteristic can contribute to a presumptive identification. For species confirmation, a combination of the following methods can be performed: oxidase test, nitrate reduction, porphyrin test and detection of alkaline phosphatase.

15.4.1 Oxidase test

The production of cytochrome oxidase can be demonstrated by placing a few drops of tetramethyl-*p*-phenylenediamine hydrochloride on a strip of filter paper and by rubbing the growth from several colonies on to the impregnated area using a bacteriological loop. A colour change to bluish purple within 1 minute indicates a positive result.

15.4.2 Nitrate reduction

Prepare a dense bacterial suspension (McFarland standard 3, 10^9 colony-forming units [CFU]/ml) and transfer 0.04 ml to a small tube. Add 0.04 ml of 0.5 g/L sodium nitrate solution and 0.04 ml of 0.025 mol/L phosphate buffer, pH 6.8 and incubate in a water-bath at 37 °C for 1 hour. Then add 0.06 ml of 8 g/L sulfanilic acid in 5 mol/L acetic acid and 0.06 ml of 5 g/L of α-naphthylamine in 5 mol/L acetic acid. The tube is shaken and, if a pink colour is observed, the test is positive.

15.4.3 Requirement for haemin (X factor): the porphyrin test

The classical growth test with haemin-impregnated discs or strips cannot be used to detect *H. ducreyi.* The only reliable way of demonstrating haemin requirement is the porphyrin test. Make a dense bacterial suspension (McFarland standard 3, 10^9 CFU/ml) in 0.5 ml of a solution of 2 mmol/L δ-aminolevulinic acid hydrochloride in 0.1 mol/L phosphate buffer, pH 6.9 containing 0.8 mmol/L magnesium sulfate solution. Incubate in a water bath at 37 °C for 4 hours. Expose the substrate to Wood's light (wavelength 360 nm) in a dark room. A red fluorescence indicates the presence of porphyrins, i.e. no requirement forhaemin. Thus, *H. ducreyi* should give a negative result and not exhibit a red fluorescence.

15.4.4 Detection of alkaline phosphatase

Make a dense bacterial suspension (McFarland standard 3, 10^9 CFU/ml) in a tube containing 0.5 ml of 0.3 g/L phenol-free disodium phosphate in 0.01 mol/L Sörensen's citrate-sodium hydroxide buffer, pH 5.6 and incubate the tube in a water bath at 37 °C for 4 hours. Add 4 drops of 5 g/L 2,6-dibromoquinone-4-chlorimide in methanol, shake, and keep the tube at room temperature for 15 minutes. Add 0.3 ml of *n*-butanol, shake, and stand for 5 minutes. A bluish purple colour in the butanol layer indicates a positive result.

15.4.5 Other characteristics of *H. ducreyi*

Catalase, indole and urease tests are negative. *H. ducreyi* is not considered to be saccharolytic. However, positive reactions for different carbohydrates have been reported. *H. ducreyi* possesses a wide range of aminopeptidase activity, and all tested isolates have shown activity with β-naphthylamide derivatives of L-lysine, L-arginine, L-alanine, L-glycine, glycyl-glycine, glycyl-L-alanine and L-leucine.

15.5 Nucleic acid amplification tests (NAATs) for detection of *H. ducreyi*

At the time of writing, no commercially available NAAT has been approved by the U.S. Food and Drug Administration for the detection of *H. ducreyi*. Several laboratory-developed NAATs have been described in the literature, and utilize a number of different molecular targets, including the *H. ducreyi* 16S rRNA gene, the *rrs*(16S)-rrl (23S) ribosomal intergenic spacer region, and the *gro* EL gene *(3)*. Several multiplex laboratory-developed and commercial polymerase chain reaction assays also have been developed for the detection of GUD pathogens, including *H. ducreyi (11,12)*. A recombinase polymerase amplification assay has recently been designed to detect both *H. ducreyi* and *Treponema pallidum* in yaws-like lesions *(13).* Finally, a loop-mediated isothermal amplification (LAMP) assay detecting *H. ducreyi* and *T. pallidum* was recently published *(14)*.

15.6 Nucleic acid probe technologies

DNA-DNA and DNA-RNA hybridization assays have been investigated as potential means to detect *H. ducreyi* in the laboratory, where they have proved to be 100% sensitive and 100% specific compared with culture, but their role as clinical diagnostic tools are limited compared with the more sensitive NAATs *(3)*.

15.7 Microscopy

Direct examination of clinical material on Gram-stained smears occasionally can be useful for the diagnosis of chancroid if typical small Gram-negative bacilli grouped in chains resembling "schools of fish", "railway tracks", or "thumb prints" are visualized. However, these classical morphological appearances are rarely seen in clinical practice. In addition, most genital ulcers harbour polymicrobial flora due to secondary contamination. The presence of other Gram-negative bacilli on a smear can be misleading and contributes to the poor performance of microscopy as a diagnostic tool. Consequently, because of its low sensitivity and low specificity, Gram staining of smears is not recommended for the diagnosis of chancroid.

15.8 Antigen detection of *H. ducreyi*

A number of monoclonal antibodies against prominent *H. ducreyi* antigens, including the 29 kDa outer membrane protein and lipo-oligosaccharide, have been utilized to detect *H. ducreyi* infection in several diagnostic formats, including immunofluorescence and immunolimulus assays *(3)*. These assays are laboratory-developed for research and have not been used as a diagnostic tool in clinical practice.

15.9 Serology

Serological tests for the detection of antibodies against *H. ducreyi* currently are not commercially available. Human and rabbit serological responses to *H. ducreyi* infection have been detected by a number of technologies; for example, enzyme immunoassays, precipitation, agglutination and dot immunobinding *(3)*. A humoral response to *H. ducreyi* infection develops during the ulcerative stage of chancroid, although it appears, based on clinical experience and human experimental inoculation studies, that there is probably no acquired

immunity to *H. ducreyi.* Serological tests offer little in terms of diagnostic assistance but would be a useful tool for those undertaking sero-epidemiological surveys for past infection with chancroid within communities.

15.10 Antimicrobial susceptibility testing

High-level plasmid-mediated and/or chromosomally mediated resistance to sulfonamides, penicillin, kanamycin, streptomycin, tetracycline, chloramphenicol and trimethoprim has been observed and described in *H. ducreyi* isolates. Plasmid-mediated and chromosomal resistance patterns may vary greatly among geographically diverse areas. A large number of *H. ducreyi* isolates exhibit resistance to several antimicrobial agents. As yet, there are no standard procedures for antimicrobial susceptibility testing for this organism.

Most of the published studies have used the agar dilution technique to determine minimum inhibitory concentrations (MICs). One of the most suitable media is MH agar enriched with 1% haemoglobin, 5% fetal calf serum, and 1% IsoVitaleX enrichment supplement (Annex 1). Alternatively, MH agar can be replaced by GC agar base. The determination of antimicrobial MICs for *H. ducreyi* isolates is usually only performed in specialized reference laboratories.

The list of antimicrobials to be tested should include those drugs locally recommended for treatment of chancroid, as well as alternative therapeutic agents, those antimicrobials that are useful for the epidemiological study of *H. ducreyi* and, finally, newly developed drugs requiring microbiological assessment. In the past, commonly tested antimicrobials have included sulfamethoxazole and trimethoprim (used alone and in combination), tetracycline, chloramphenicol, erythromycin, kanamycin (or streptomycin), ciprofloxacin (or fleroxacin) and ceftriaxone (or cefotaxime).

The preparation of antimicrobial stock solutions and dilutions for use in MIC testing is described in detail in Chapter 7, section 7.8.4(ii).

To prepare the medium, dissolve dehydrated MH agar and haemoglobin separately in distilled water. The volume of water used should be 16% less than the normal medium formula (to allow for the volume of the supplements and antimicrobial solution, which will be added later). Boil and dispense the MH and haemoglobin separately into containers, in volumes appropriate for the number of plates to be prepared for each antimicrobial dilution, which will depend on the number of isolates to be tested. Autoclave in tightly closed containers, allow to cool down to a temperature of 50–55 °C in a water bath, then mix together in one container and add 5% fetal calf serum, 1% *H. ducreyi* supplement, and 10% of the required concentration of the antimicrobial solution. Mix gently and pour approximately 20 ml into petri dishes with an internal diameter of 9 cm. Once the agar has solidified, the plates may be stored for up to one week in sealed plastic bags, at 4 °C.

To prepare the inoculum, suspend the growth from a 24-hour subculture on enriched GC or MH agar (similar to isolation medium but without vancomycin) into tryptic soy broth (TSB) to a density of 10^8 CFU per ml. *H. ducreyi* colonies often are so cohesive that a homogenous suspension cannot be obtained, even after vigorous shaking on a vortex mixer. The use of an orange 25G needle and syringe to break up the clumps, through repetitive drawing up and squirting out of the suspension, may assist with the creation of a more homogenous suspension, as well as being less destructive to the bacilli. Centrifugation at low speed (500 g) may be helpful to sediment large clumps. The density of the supernatant then is compared to McFarland 0.5 standard (10^8 CFU/ml). Dilute the suspension in TSB (1 : 10) to obtain 10^7 CFU/ml, and place 0.5 ml of this dilution into the corresponding well of a replicator seed block.

Warm the MIC test plates to room temperature and, if required, dry by placing in an incubator in an inverted position, with the lids ajar. Transfer the prepared bacterial inocula to the test plates using a multipoint replicator, to produce spots containing approximately 10^4 CFU. Inoculate a control plate containing no antimicrobials first, followed by the plates containing the different antimicrobials, starting with the lowest concentration for each agent. Finally, inoculate a second control plate. Allow the inocula to dry, invert the plates and incubate at 33 °C in a humidified 5% CO_2-enriched atmosphere for 24 hours.

The MIC is the lowest concentration of antimicrobial that yields no growth, very few single colonies, or a fine, barely visible haze. The growth on both control plates should be confluent and without contamination. The determination of the MIC for sulfonamides is somewhat difficult since the end-points are less sharp than for other antimicrobials. A standardized reading and reproducible results are obtained if the second dilution on which there is a marked decrease of growth is taken as the MIC. It may be helpful to compare this growth to that on the control plates.

15.11 Conservation of isolates

To maintain the viability *of H. ducreyi* isolates in the laboratory, they should be subcultured every four days. Strains also can be preserved for up to four weeks by inoculating them on enriched medium, such as chocolate agar stabs. For maintenance periods of several months, suspensions in skim milk can be stored frozen at –70 °C. For long-term preservation, isolates can be suspended in a cryoprotective medium (such as fetal calf serum + 10% dimethylsulfoxide or skim milk + 20% glycerol) and stored in liquid nitrogen. Lyophilization may offer an alternative approach.

15.12 References

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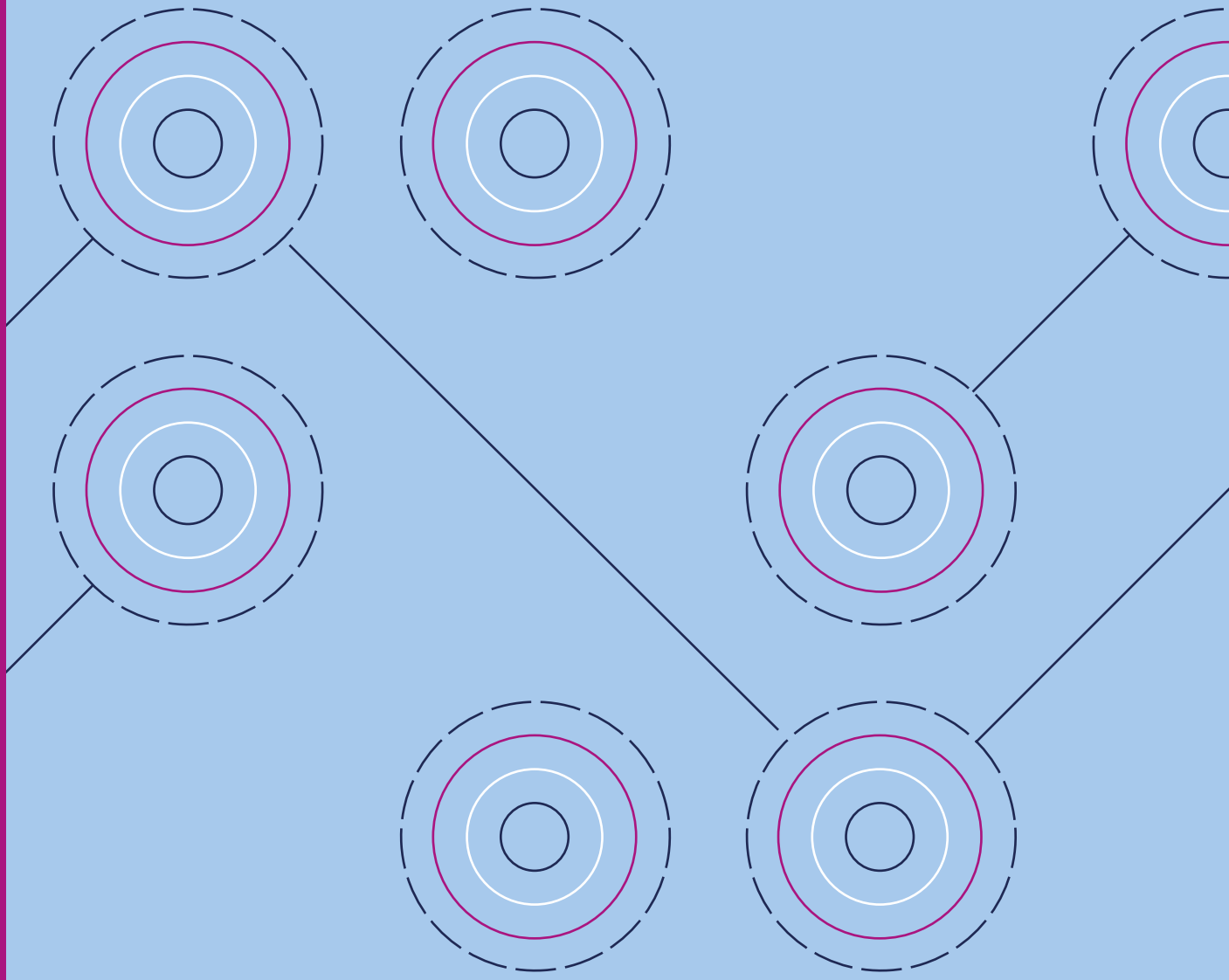
Chapter 16. Donovanosis (granuloma inguinale)



Chapter 16. Donovanosis (granuloma inguinale)

David Lewis and Magnus Unemo

16.1 Introduction

Donovanosis, which is also referred to as granuloma inguinale, is a chronic, rare infection affecting the skin, the mucous membranes, and the lymphatic system of the genitalia and perineal area *(1)*. The occurrence of donovanosis is geographically limited to Brazil, India and Papua New Guinea, and also the Caribbean and Southern Africa subregions, and only sporadic cases are seen now. The infection, which is of low infectivity, is transmitted between humans principally by sexual contact. The incubation time may be prolonged, varying between 1–12 weeks, but typically about 7 weeks. The infection starts as an indurated subcutaneous nodule that erodes the skin surface to form a beefy red, hypertrophic, granulomatous ulcer with a well defined border. The lesion bleeds easily on contact. The ulcer progresses slowly and may become painful when a secondary bacterial infection develops. Such secondary infection with other organisms may contribute to necrotic debris in the ulcer. New lesions may be formed by auto-inoculation, and inguinal lymph nodes may become enlarged as a result of secondary infection (pseudobuboes). Donovanosis may spread haematogenously to bones, joints, and the liver; dissemination also may result in cutaneous lesions at extragenital body sites. Genital and perianal lesions at various stages may resemble lesions formed by other conditions, such as syphilis, chancroid, carcinoma and amoebiasis.

Donovanosis is caused by *Klebsiella* (formerly *Calymmatobacterium*) *granulomatis combinatio nova*, a Gram-negative bacterium (1.5 × 0.7 µm), that can be observed enclosed in vacuoles in large histiocytic cells, where it is referred to as the "Donovan body" *(1,2)*.

16.2 Overview of laboratory diagnosis

Laboratory diagnosis depends on the microscopic visualization of Donovan bodies in stained smears obtained from clinical lesions or in stained histological sections of tissue biopsies. The bacterium can only be cultured with difficulty in specialist centres, using monocyte/HEp-2 cell cultures. It is not yet possible to grow the bacterium on artificial media. Laboratory-developed, that is, in-house, nucleic acid amplification tests (NAATs) have been reported in the literature, but such assays are not available in most countries for routine diagnostic purposes *(3)*.

16.3 Collection of specimens and preparation of smears

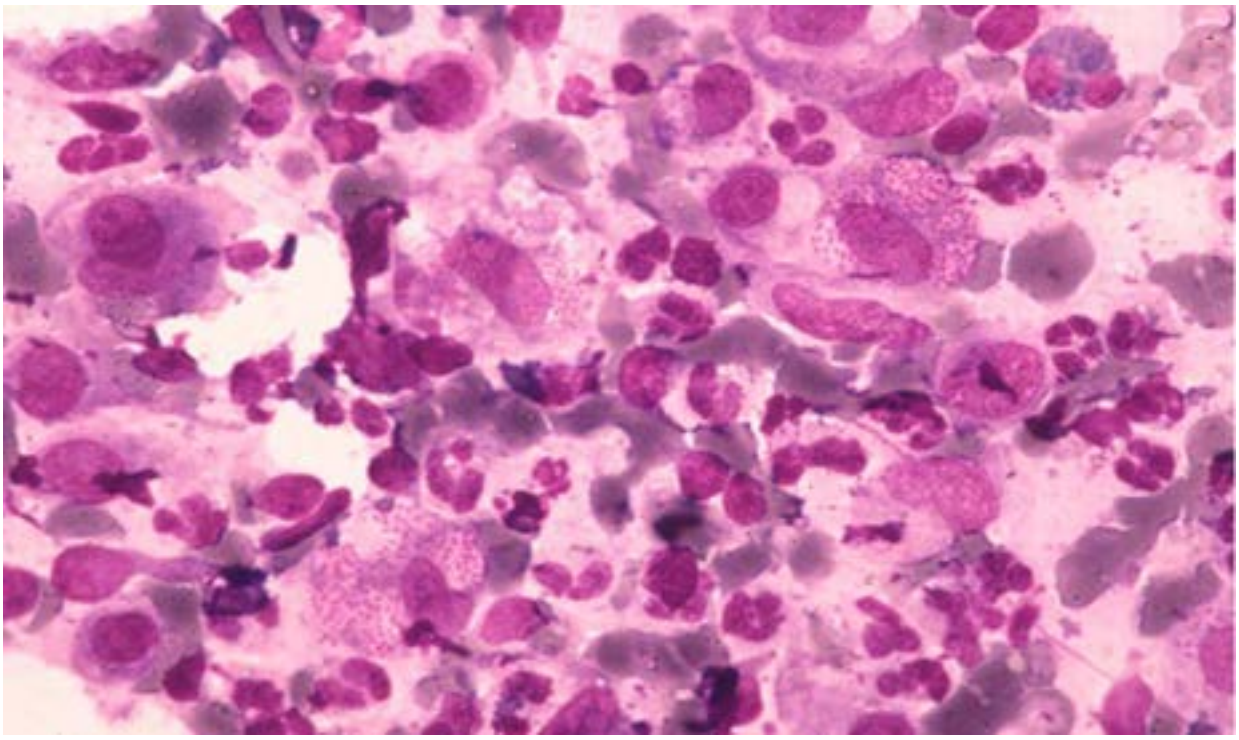
Prior to taking the smear of the ulcer material, roll a cotton-tipped swab across the lesion gently to remove exudates due to secondary infection and/or debris in a manner that minimizes bleeding. A second swab should be rolled across the ulcer base, ensuring good sampling of the ulcer edges, where Donovan bodies are most likely to be found, and then rolled evenly on a glass slide. Air dry the slide prior to transport to the laboratory. It should be noted that some practitioners prefer to use punch biopsy forceps to remove a small piece of tissue, which is then crushed, spread on a glass slide, and then air dried. A crush preparation facilitates the microscopic interpretation and enhances diagnostic value. Swabs for NAATs should ideally be placed in a transport medium including nucleic acid preservatives. If no transport medium is available, a dry swab can be taken and placed in +2–8 °C prior to transport and/or testing.

16.4 Staining and microscopy

A simple, rapid (1 minute) method "RapiDiff", has been described to visualize Donovan bodies *(4)*. With this staining method, the slide is dipped five times in a fixative, six times in eosin solution, six times in a thiazine dye mixture and then rinsed with phosphate buffer, with a pH of 6.8. Use of a 10% Giemsa or similarly diluted Leishman stain can be employed as an alternative following fixation of the material on the slide with methanol for 2–3 minutes. Cover the slide with diluted stain for 10 minutes (Leishman's stain) or up to 30 minutes (Giemsa stain) and then rinse the slide in a stream of buffered water or phosphate-buffered saline

(pH 7.0–7.2). Subsequently, leave the slide to dry in air and then examine it with a light microscope using oil immersion (1000× magnification). The Donovan bodies (0.5–0.7 × 1–1.5 µm) appear as coccobacilli within large vacuoles (25–90 µm in diameter) in the cytoplasm of large histiocytes and occasionally in plasma cells and polymorphonuclear leukocytes. The organisms are bluish purple in colour and often are surrounded by a prominent clear to acidophilic pink capsule (Fig. 16.1). Typical bacteria resemble closed safety pins. Contamination with other bacteria will often be observed. Although microscopy of ulcer smears is the conventional way to diagnose donovanosis, Donovan bodies also have been identified from Papanicolaou smears used in routine cervical cytology screening *(5)*.

Fig. 16.1: Giemsa-stained smear of ulcer material containing monocytes and Donovan bodies (1000×)



Source: WHO, 2013 *(6)*.

16.5 Histopathology

Histological examination of a biopsy may be helpful in the differential diagnosis between donovanosis and other conditions. An ulcer with a mixed inflammatory infiltrate of plasma cells, neutrophils and histiocytes, with a conspicuous absence of lymphocytes, suggests donovanosis. Take a piece of tissue (3–5 mm thick) from the edge of the lesion with a punch biopsy forceps and place in a container with formaldehyde-saline fixative. The demonstration of Donovan bodies using Warthin–Starry silver impregnation reagent constitutes a diagnosis *(7)*. Paraffinized biopsies should be cut in sections of 6 µm. After de-paraffination and hydration with distilled water, fix the sections on a glass slide with glycerol, dry and treat with the acid silver nitrate solution at 43 °C for 30 minutes. Wash with hot water, rinse with distilled water, dehydrate in 95% ethanol and clear in xylene.

16.6 Culture

Following a report describing the culture of *K. granulomatis* from faeces in 1962, it has subsequently proven very difficult to culture the bacterium from clinical specimens *(1,8)*. In 1997, two groups reported successful culture of *K. granulomatis* using different culture systems, a monocyte co-culture system and a modified chlamydial culture technique *(9,10)*. Apart from these isolated reports, there are no other in vitro culture techniques for the isolation of *K. granulomatis.* However, the bacterium can be cultured by inoculation of clinical specimens into the yolk sac of five-day-old embryonated chicken eggs *(11)*. The organism is detectable after 72 hours of incubation.

16.7 Nucleic acid amplification tests (NAATs)

An in-house diagnostic polymerase chain reaction (PCR) test has been developed, which targets the *phoE* gene and incorporates post-amplification *Hae*III restriction enzyme-based digestion of amplicons *(3)*. This method has been refined further into a colorimetric PCR test *(12)*. Additionally, an in-house genital ulcer disease multiplex PCR (GUMP) test has been developed, which targets donovanosis, as well as other causes of genital ulcer disease such as herpes simplex viruses, *Haemphilus ducreyi* and *Treponema pallidum (13)*. No commercial NAATs for diagnosis of donovanosis are available.

16.8 Serological tests

No reliable serological assays are available to assist with diagnosis.

16.9 References

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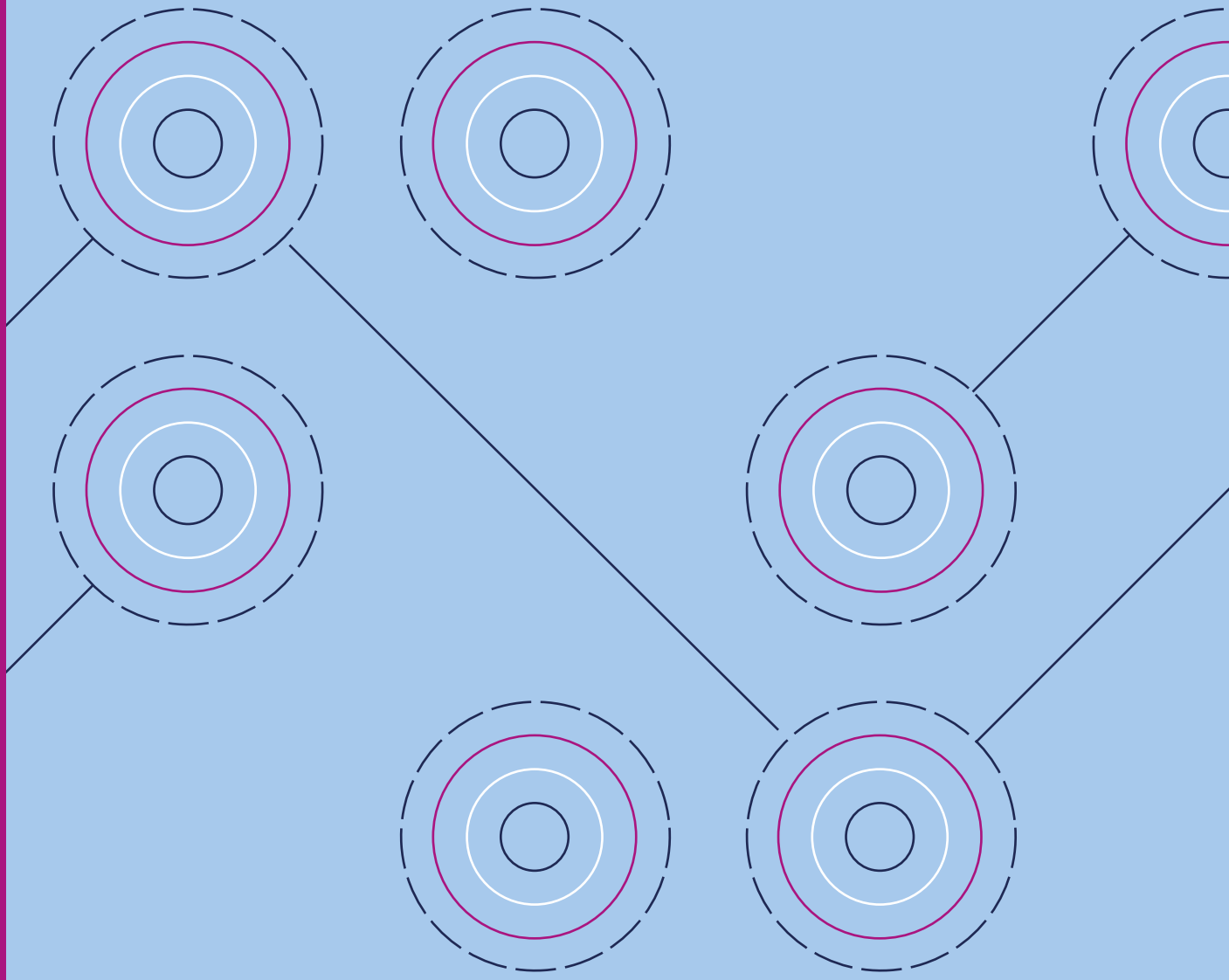
Chapter 17. Human papillomavirus (HPV) infections



Chapter 17. Human papillomavirus (HPV) infections

Suzanne Marie Garland and Anna-Lise Williamson

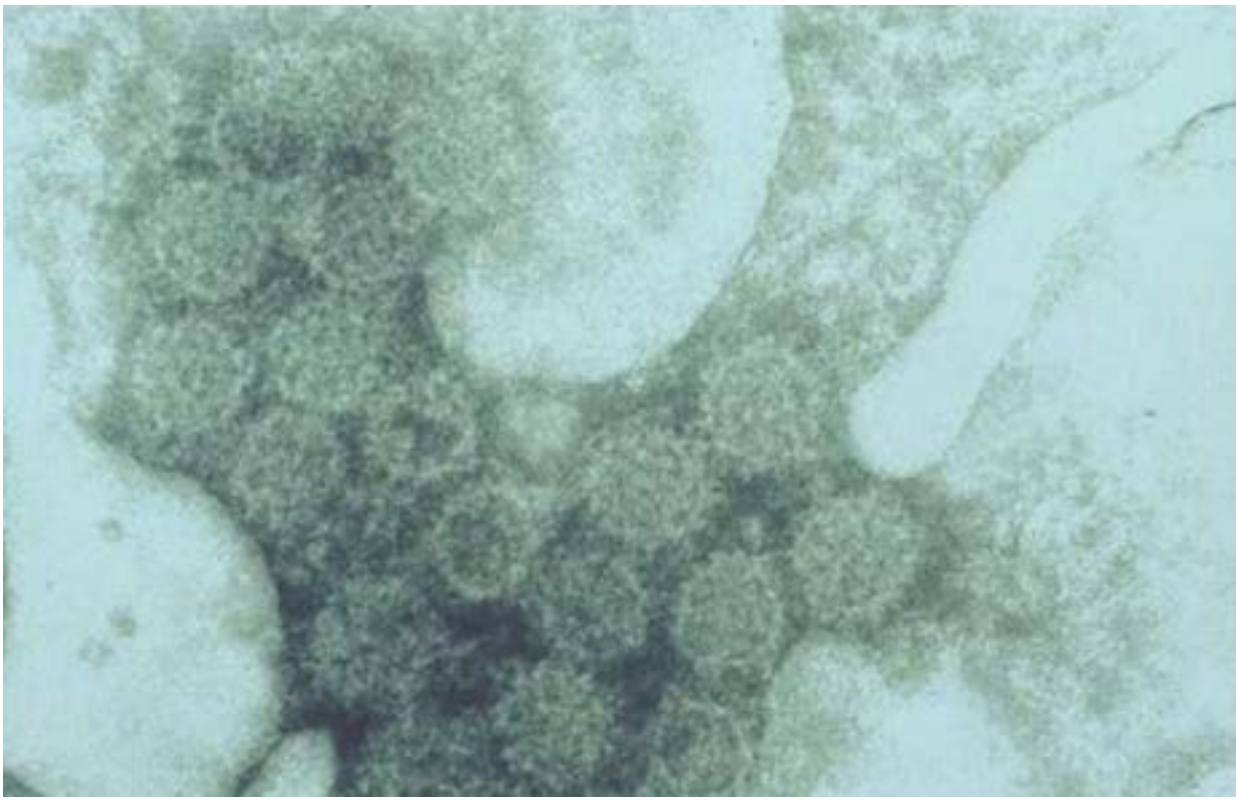
17.1 Human papillomaviruses (HPVs)

Human papillomaviruses (HPVs) are small (approximately 55 nm diameter), icosahedral, non-enveloped viruses with a circular and supercoiled double-stranded DNA genome of approximately 8 kb (Fig. 17.1). These viruses typically infect the skin and mucosal surfaces of humans *(1)*. Unlike most viruses infecting humans, papillomaviruses cannot be propagated by conventional in vitro culture; therefore, a classic antigenic and serotyping classification cannot be used for typing. Instead, a genotyping approach is used to identify and classify these viruses. Individual HPVs are referred to as types or genotypes, distinguished on their genomic sequence, and numbered in the order of the discovery. The L1 gene that encodes for the major component of the viral capsid (Fig. 17.2) is the region most conserved between individual types and used to form phylogenetic trees in taxonomy (Fig. 17.3) *(2)*. Those HPVs with L1 sequence divergence of 2–10% are known as subtypes and less than 2%, variants (Fig. 17.4).

The term "genus" is used for the higher order clusters, named using the Greek alphabet, and within genus, small clusters are referred to as species and given a number. The *Firstpapillomavirinae* is one of two subfamilies of the family *Papillomaviridae*. They infect a range of hosts and there are more than 50 genera and 130 species *(3)*. HPVs fall into five genera, named according to the Greek alphabet with Alpha-, Beta- and Gamma-papillomavirus being the largest groups, with the Alpha papillomaviruses being important in mucosal infections and the remainder for cutaneous infections *(4)*. For example, the genus α papillomaviruses contain species α 9 and α 7, which contain, respectively, the two most common causes of cervical cancer, HPV genotype 16 and 18 *(5)*. In order for an HPV type to be assigned a number, the viral DNA must be cloned sequenced and submitted to the International HPV Reference Centre. There are 221 different HPV types described *(6)* and in total 800 unique putative novel HPV types when comparing all correct HPV sequences in GenBank *(6)*. Forty are known specifically to infect the anogenital mucosa of humans (mucosotrophic HPVs).

HPV types frequently detected in the anogenital tract are subdivided into low-risk (LR) and high-risk or oncogenic (HR) types, based on their relative risk for the rare complication of neoplasia *(7)*. LR HPV types are typically found in low-grade intraepithelial lesions (non-precancerous lesions), as well as anogenital warts and recurrent respiratory papillomatosis (RRP) *(8)*. HPV types 6 and 11 account for approximately 90% of anogenital warts *(9,10)* and juvenile onset RRP *(11)*. The HR HPVs are found in low- and high-grade lesions, as well as most cancers of cervix and anal canal and a substantial proportion of other anogenital sites (vulva, vagina, and oropharyngeal cancers) *(12)*. The most oncogenic HPV is HPV type 16: collectively, HPV 16 and 18 are consistently responsible for approximately 70% of all cervical cancer cases worldwide *(13–16)*. Apart from anogenital related cancers, oncogenic HPVs, type 16 especially, have a causal role in some oropharyngeal cancers, particularly those of the tonsil and back of tongue *(17–19)*. In the USA, oropharyngeal cancer is the number one cause of HPV-related cancer exceeding cervical cancer *(18)*.

Fig. 17.1: Electron microscopy of native human papillomavirus (HPV) showing the virus particles in selected cells from a Pap smear



Source: Courtesy of Colin Laverty, Sydney, NSW, Australia, 1978.

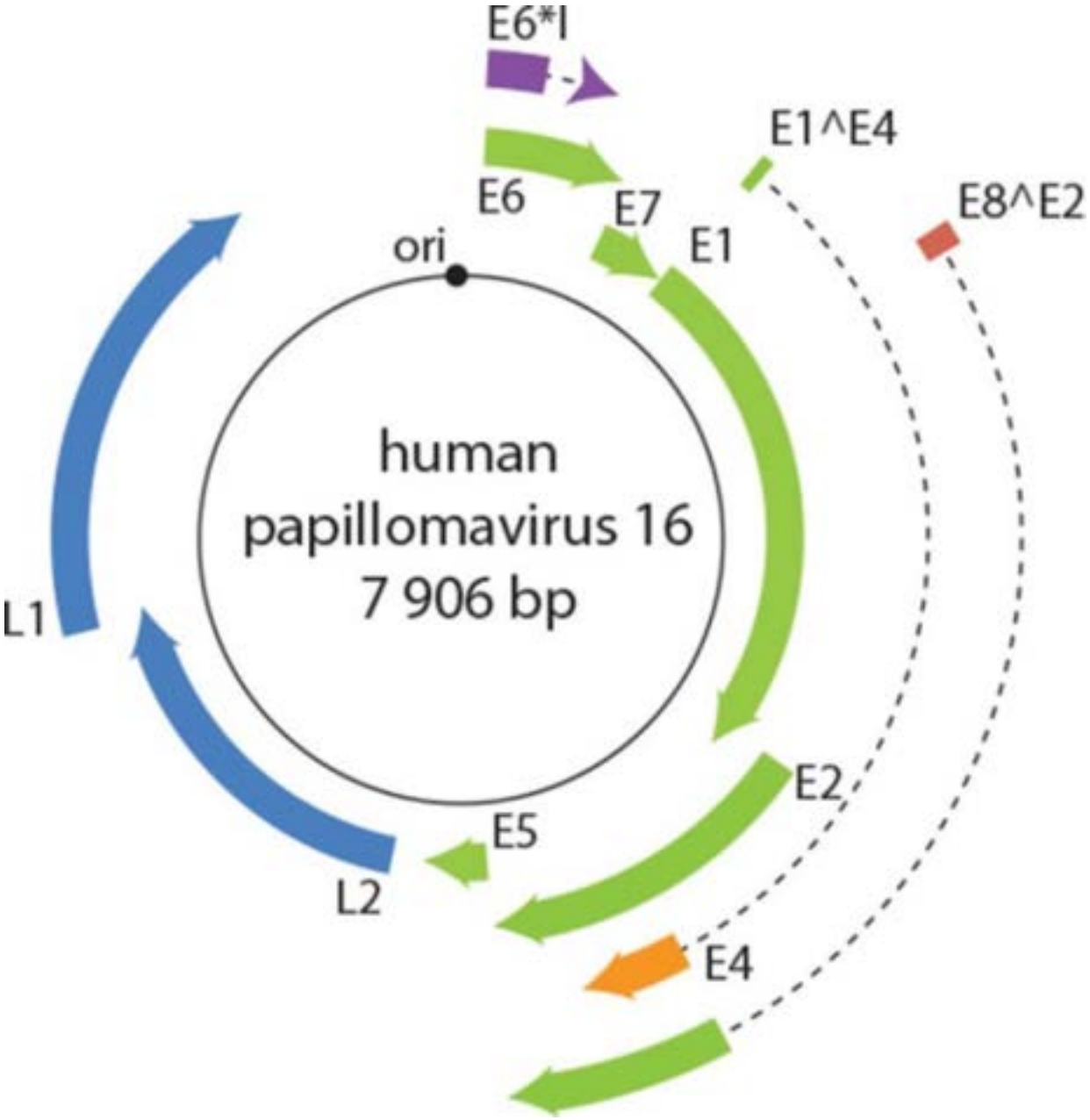
17.2 Natural history of genital HPV infection

The majority of genital HPV infections are sexually acquired, transient, and cleared slowly by the host immune system *(20)*. More uncommonly, in approximately 5–10% of cases, HPV infections may become persistent. Persistent HR HPV is a prerequisite for development of precursor lesions to cancers, which in turn have the risk of progressing to cancer over many years if not treated *(21,22)*. Established cofactors for the development of persistent infection and cervical cancer are cigarette smoking, long-term oral contraceptive use, high parity, early age of first infection, and immunosuppressive states such as HIV coinfection *(23,24)*. HIV coinfection increases both incidence of HPV and persistence of HPV resulting in higher incidence of HPV associated cancers *(25–27)*. It is estimated that the inherited genetic contribution to cervical cancer risk is around 30% *(28)*.

Transmission of genital HPV largely occurs via direct sexual contact (genital skin with genital skin) with an infected individual, resulting in estimated lifelong HPV acquisition rates as high as 60–80% *(29–32)*. The median age of first sexual intercourse in many western countries, such as Australia, the United Kingdom, and the USA is 16 years old *(33,34)*. Therefore, it follows that the highest rates of newly acquired HPV infections are observed in young women, peaking at an age soon after the start of sexual activity, with estimates of up to 75% of HPV infections occurring in 15- to 24-year-old females. Recent data suggest that many infections in young HPV-naive women are multiple infections (multiple HPV types detected) *(35,36)*.

Less information is known about the natural history of HPV infection in males. Again, infection (penile, genital skin and/or anal) is very common, occurring soon after the start of sexual activity, with rapid rates of acquisition as well as clearance *(26,37,38)*. In contrast to females where HPV prevalence increases rapidly with age and then declines to a lower plateau, in male studies HPV acquisition (as detected by HPV DNA) with age does not decline, but rather stays as a flat line *(30,39)*. Sexual behaviour with increased numbers of sexual partners increases the risk, whilst circumcision has a protective effect *(39,40)*.

Fig. 17.2: Diagram of the human papillomavirus (HPV) type 16 genome

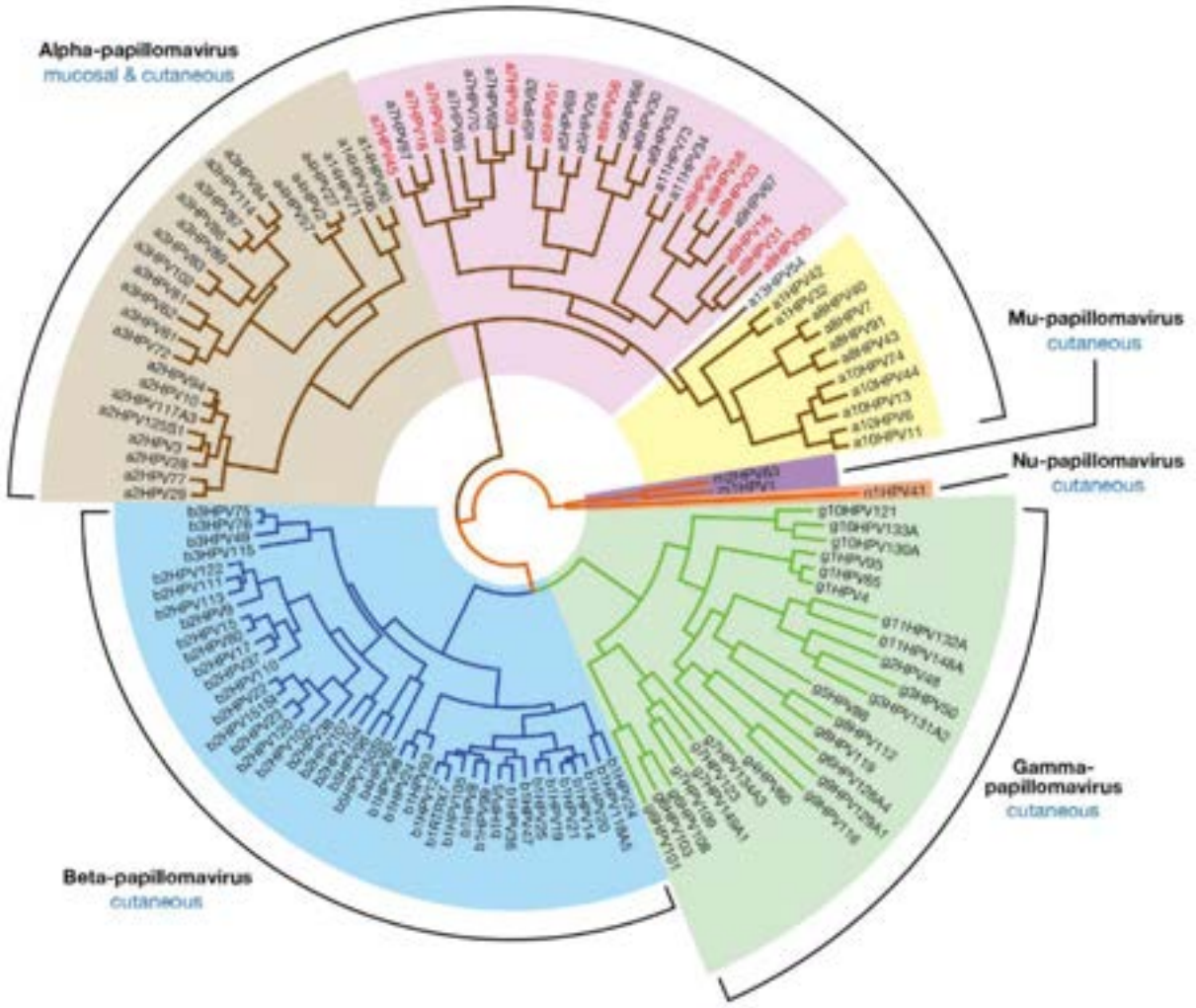


Key:

The central circle represents the viral dsDNA, and includes the location of the origin of replication (ori).
The outer boxes indicate the protein-coding open reading frames.
The dotted lines represent intron sequences.

Source: Van Doorslaer et al., 2018 *(3)*, reprinted with permission from the *Journal of General Virology.*

Fig. 17.3: Evolutionary relationship between human papillomaviruses (HPVs)

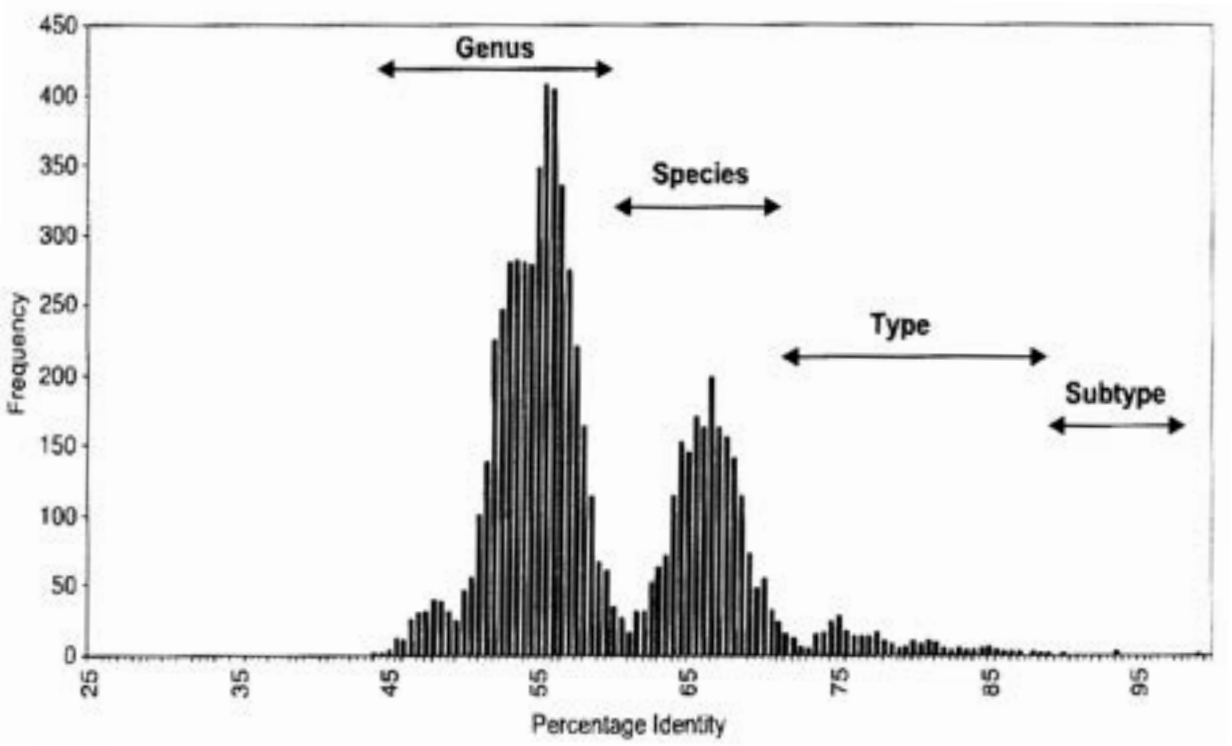


Key:

HPV types fall into five genera, with the Alpha-, Beta- and Gamma-papillomavirus representing the three largest groups; HPV types from the Alpha-papillomavirus genus are often classified as low-risk cutaneous, low-risk mucosal, or high-risk according to their association with the development of cancer. The high-risk types are highlighted with red text.

Source: Egawa et al., 2015 *(4)*, reprinted with permission from *Viruses.*

Fig. 17.4: Frequency distribution of pairwise identity percentages from nucleotide sequence comparison of the human papillomavirus (HPV) L1 open reading frames (ORFs)



Source: de Villiers et al., 2004 *(5)*, reprinted from *Virology*, © 2023, with permission from Elsevier.

17.3 Immunology of HPV

The host immune response to HPV involves both humoral and cell-mediated responses *(41)*. Following natural infections, the humoral antibody response is type-specific and first detected 6–18 months after infection *(42)*. The response is weak and only approximately 50–60% of women who are HPV DNA-positive mount a measurable antibody response *(42)*. Following natural infections, women may remain HPV DNA-positive despite development of specific antibodies; such individuals are not fully protected from developing subsequent disease from the identical HPV type. Clearance of HPV infection and resolution of clinical lesions such as genital warts is characterized by an effective cell-mediated immune response *(43)*.

HPV serology is not used diagnostically. Sero-surveillance is useful to estimate on a population basis the age and prevalence of exposure in pre-vaccine settings *(42,44)*. Post-vaccination, serology is used as a measure of vaccine effectiveness *(45,46)*. Protection from vaccination (as the vaccines are administered intramuscularly, the host immune response systemically to the L1 in the VLPs is rapid and strong being many times greater than that seen post natural infection) corresponds to detectable neutralizing antibodies, although the actual mechanism of protection is not completely understood. Animal studies with host-specific papillomaviruses show neutralizing antibodies from primary infection that are protective, even following challenge with the respective virus *(47)*. Passive transfer of antibodies from immunized dogs also protects from challenge with canine oral papillomavirus *(48)*. It is noteworthy that although durability of vaccine-induced antibodies in human trials is now documented beyond 12–15 years, an immune correlate of protection is yet to be defined *(49,50)*. It is thought that only very low antibody titres are required to maintain protection *(51,52)*.

17.4 Disease manifestations

17.4.1 Anogenital warts

Anogenital warts, also known as condylomata acuminata, are benign exophytic, papular or flat growths that may occur anywhere in the anogenital area. They are extremely common, particularly in young people commencing sexual activity, and largely diagnosed clinically. Lesions rarely may cause problems because of size and obstruction, but the biggest problems are cosmetic, treatment is crude often requiring repetition due to recurrences and psychosocial. Lesions have a tendency to recur after treatment. HPV types 6 and 11 cause the majority (85–90%) *(10,53–55)*. In countries where HPV vaccination against HPV 6 and 11 has been implemented there is a significant decrease in genital warts *(56–58)*.

17.4.2 Recurrent respiratory papillomatosis (RRP)

RRP is a very rare condition characterized by recurrent wart-like growths (papillomas) in the upper respiratory tract, although it can involve the lower respiratory tract, resulting in significant morbidity and mortality. HPV types 6 and 11 largely cause these lesions. The larynx is most commonly affected, resulting in voice changes. In young children, airway obstruction may occur. The diagnosis is made clinically by observation of characteristic warty lesions at laryngoscopy or bronchoscopy. Lesions are benign but recur frequently after treatment. There are two patterns of onset: the juvenile onset form (JoRRP) in children (mean age 2 years) as well as the late onset form seen in adults *(8)*. Since the introduction of HPV vaccination of young girls, it has been shown with high coverage of 4vHPV vaccination that JoRRP has significantly declined in their offspring *(11)*.

17.4.3 Anogenital precancers and cancers

Persistent infection with oncogenic or HR HPV genotypes is a prerequisite for the development of precursor lesions which if not treated have an increased risk of becoming anogenital cancers. This has been best studied in the cervix, where criteria for cytological and histological identification of slowly progressing precancers were first defined. Nomenclature has changed throughout the years and continues to evolve. Cancer precursors have been termed dysplasias (mild, moderate, severe) or cervical intraepithelial neoplasia (CIN1, 2, 3), and most recently high- or low-grade squamous intraepithelial lesions (HSILs, LSIL, respectively). Terminology in other anatomic sites follows the same pattern; anal (AIN), vulvar (VIN), vaginal (VaIN) and penile (PIN). In each case, precursor lesions (high-grade or grade 3) are asymptomatic, slowly progressing, and diagnosed with histology. Screening is currently recommended only for cervical lesions, although approaches to anal screening are being evaluated *(59)* and guidelines being developed following the recent findings from the Anal Cancer–HSIL Outcomes Research (ANCHOR) study *(60)*. Currently treatment is only recommended for precursor (high grade) lesions.

Strong laboratory and epidemiologic evidence links HPV to cervical cancer and persistent HR HPV is considered a necessary, but not sufficient causal factor for cervical cancer. In the absence of HPV infection, cervical cancer is extremely unlikely, and this tight link is the reason why vaccine aims to prevent the most frequent oncogenic HPV infections to prevent cancer, as well as the reason why HPV testing is being recommended as a more sensitive and objective test for cervical screening. Current HPV vaccines target HR types HPV 16 and 18, which account for 70% of cervical cancer worldwide *(13–16)* with the 9vHPV vaccine targeting the next most frequent five HR HPVs and being used now in some national immunization programmes (NIPs) given the extra 20% protection *(61)*. The epidemiologic evidence is less established for other HPV-associated cancers, but HPV-attributable cancers include those in other anogenital sites as well as oropharynx (base of tongue and tonsil), resulting in a significant burden of disease (Table 17.1). The proportion of cancers with HPV differs at each site, and HPV 16 is particularly prominent in non-cervical cancers.

Table 17.1: Number of cancer cases attributable to human papillomavirus (HPV) and corresponding attributable fraction (%) by cancer site, and sex; global, 2018

HPV-related cancer site	Number of incident cases	Number attributable to HPV	(%) attributable to HPV	Number attributable by gender	
				Males	Females
Cervix uteri	570 000	570 000	100.0	-	570 000
Anus squamous cell carcinoma	29 000	29 000	100.0	9 900	19 000
Vulva	44 000	11 000	25.0	-	11 000
Vagina	18 000	14 000	78.0	-	14 000
Penis	34 000	18 000	53.0	18 000	-
Oropharynx	140 000	42 000	30.0	34 000	8 100
Oral cavity	280 000	5 900	2.1	3 900	2 000
Larynx	180 000	4 100	2.3	3 600	500
Total HPV-related sites	1 295 000	694 000	53.6	69 400	624 600

Source: de Martel et al., 2020 *(62)*.

17.5 Laboratory procedures for HPV detection

HPV testing relies on molecular methods. This is because, as noted earlier, conventional diagnostic viral methods cannot culture HPV. Testing methods for epidemiologic surveillance differ from those used in clinical applications. Epidemiologic methods require type-specific assays with high analytic sensitivity. Because clinical assays are using HPV as a marker of underlying disease or as a predictor of incipient disease, they are matched to disease endpoints *(63,64)*. There are no specific antiviral treatments for HPV per se; treatments are directed to the HPV-associated cancer precursors or other HPV-related lesions. A plethora of HPV assays (> 400) are available commercially, as well as laboratory-developed tests *(65)*. Use in clinical settings requires that laboratories use clinically validated assay. Because cancer precursors are associated only with HR HPV types, there are no clinical indications for LR HPV testing *(66)*. There is some variation in which HR HPV types are clinically relevant, but most assays target 14 HR types, although the most recent International Agency for Research on Cancer (IARC) evaluation classifies 12 HPV types as oncogenic (i.e. HPV 16,18,31,33,35,39,45,5 1,52,56,58 and 59), and HPV 68 being probably and HPV 66 possibly oncogenic *(67)*.

17.6 Clinical application of HPV detection assays

This section should be read in conjunction with *WHO guideline for screening and treatment of cervical pre-cancer lesions for cervical cancer prevention, second edition* – both the initial publication focusing on HPV DNA testing *(68)* and the subsequent version focusing on HPV mRNA testing *(69)*, as well as the policy brief on HPV nucleic acid amplification tests (NAATs) – comparing HPV DNA and HPV mRNA testing – to screen for cervical pre-cancer lesions and prevent cervical cancer *(70)*.

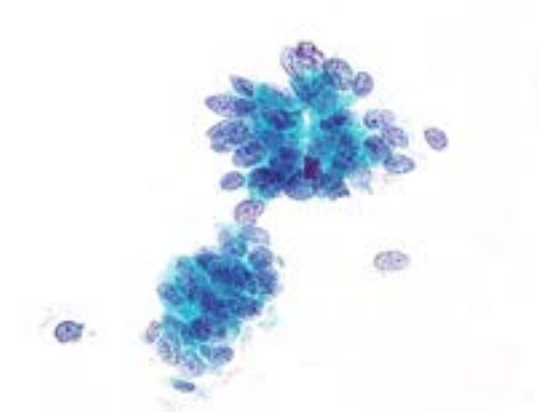
Clinical application of HPV detection assays include primary cervical screening alone or in combination with cervical cytology (Fig. 17.5) in women ≥ 30 years of age. Longitudinal studies and randomized controlled trials (RCTs) show that HPV NAATs have a higher sensitivity and are more objective for predicting prevalent or incipient high-grade dysplasia *(71)*. Moreover, the negative predictive value (NPV) is extremely high and superior to cytology. Therefore, it is proposed that those who are negative for HPV DNA on two occasions can have screening at longer intervals, for example, every 10 years instead of every 5 years. In addition, the combination of cytology and HPV DNA testing is not cost-effective and does not add sensitivity in primary screening programmes *(72–78)*. WHO, in its Global strategy to accelerate the elimination of cervical cancer as a public health issue (i.e. to make this preventable disease a rare disease at < 4/100 000 by 2030), has a three pillars approach (90% of girls fully vaccinated with HPV vaccine by 15 years of age, 70% of all women screened at least twice in a lifetime at 35 and 45 years of age with a high precision HPV test and 90% of women identified with cervical disease receive treatment and care) *(79)*.

The WHO screening strategy recommends, particularly for those countries with limited resources, a screen-and-treat approach, whereby HR HPV DNA detection is used as the primary screening test alone – rather than visual inspection with acetic acid (VIA) or cytology *(68,69)*– and those who test positive are offered immediate treatment without further testing *(68,70)*. Studies have shown that this is a feasible approach and implementation studies are currently under way *(80–82)*. Alternatively, a screen, triage and treat approach can be applied, where the decision to treat is based on a positive primary screening test followed by a positive second test (i.e. a triage test, which could be an HPV DNA test, cytology or VIA), with or without histologically confirmed diagnosis. The screening algorithms for HPV as a primary screening test for women living with HIV differ from those for HIV-negative women, with screening preferably starting earlier in women living with HIV, at the age of 25, and with shorter screening intervals. WHO suggests using an HPV DNA primary screening test with triage rather than without triage *(68)*.

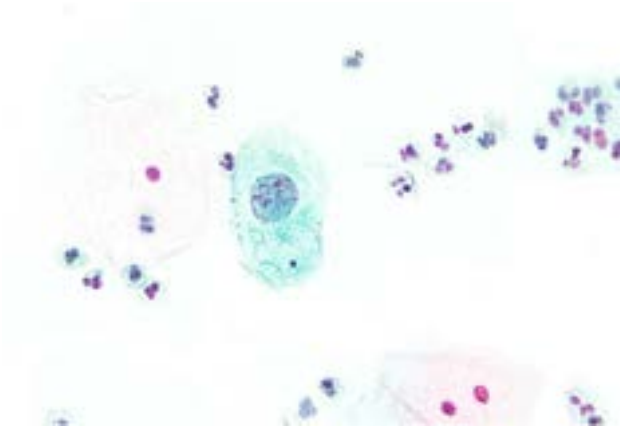
HPV DNA assays have also been recommended for triage of women with minimal cytology abnormalities (inconclusive, equivocal, or borderline) where Pap cytology is used for cervical screening, to discriminate those truly HPV-related and requiring follow-up *(83)*.

In addition, in some populations, HPV NAATs have been recommended for use after ablative therapy for high-grade dysplasia and for monitoring women for evidence of persistent or recurrent disease and as a test-of-cure *(84)*.

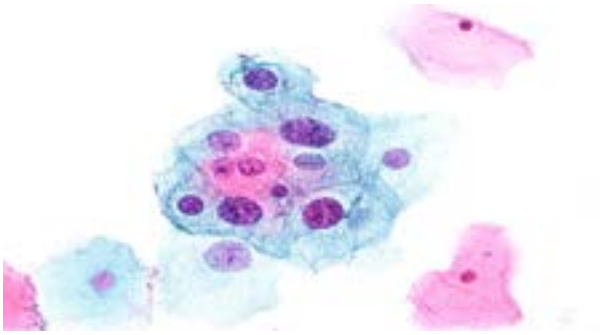
Fig. 17.5: Papanicolaou (Pap) stain original magnification x400



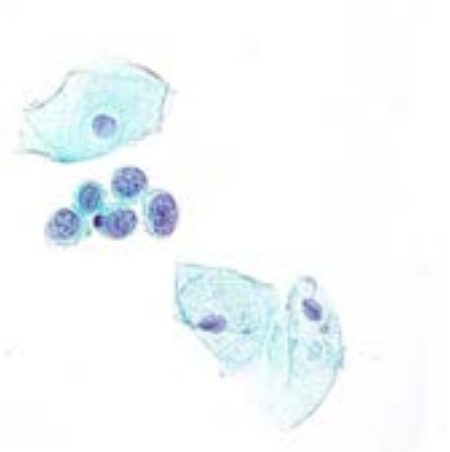
A. Adenocarcinoma in situ (AIS) clusters of ovoid nuclei with irregularly clumped chromatin – the superior clumped cluster shows protrusion of the peripheral cells resembling the feathers of a bird in flight



B. Atypical squamous cells of unknown significance (ASCUS) a single squamous cell with a mildly enlarged, hyperchromatic nucleus with coarseness of chromatin and a moderate amount of cytoplasm – no evidence of koilocytosis



C. Low-grade squamous intraepithelial lesion (LSIL), numerous single and clustered squamous cells with moderately enlarged, irregular, hyperchromatic nuclei



D. High-grade squamous intraepithelial lesion (HSIL) a few immature squamous cells with high nuclear cytoplasmic ratio and hyperchromatic irregular nuclei (cervical intraepithelial neoplasia [CIN], grade 3 [CIN 3])

Source: Courtesy of James Scurry.

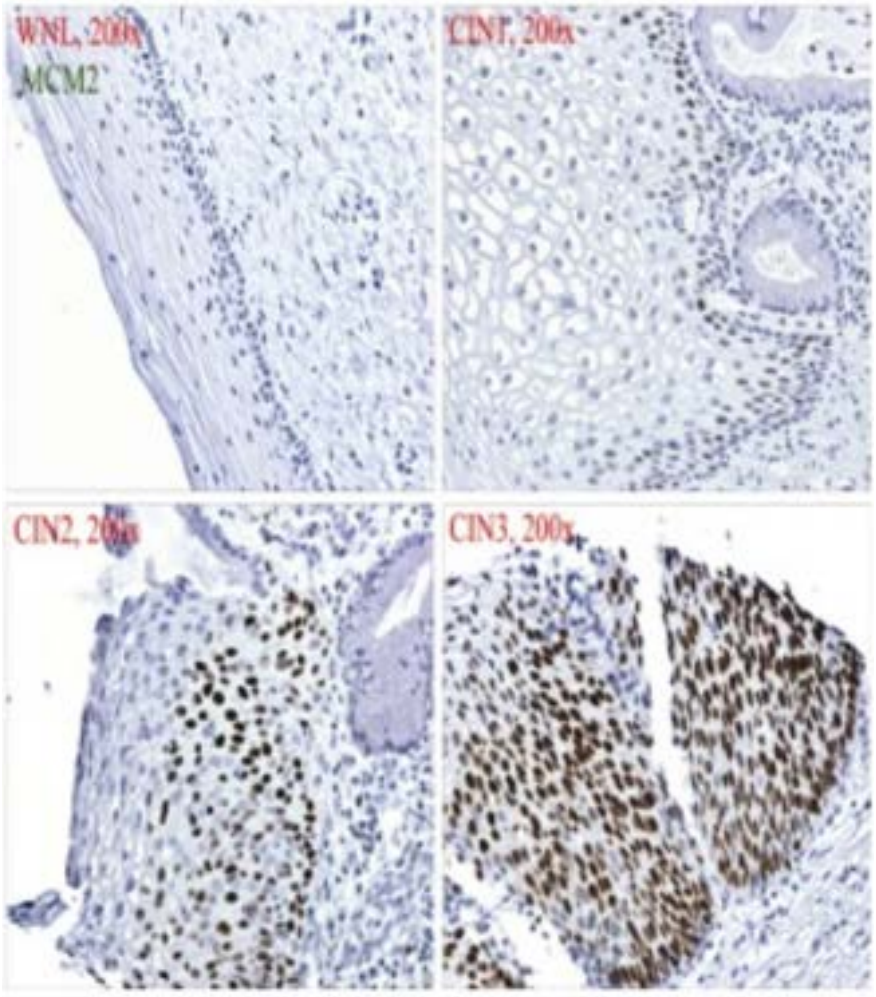
Use of HPV as a primary cervical cancer screen has already been adopted in some high-income countries (HIC) *(85–87)* and implementation is being investigated in some low- and middle-income countries (LMICS) *(88,89)*. It is advised as at least twice in a woman's lifetime, at 35 and 45 years age and as per the second pillar of the WHO strategy to eliminate cervical cancer as a public health concern *(90)*. HPV testing has an excellent negative predictor of disease but lacks specificity for direct treatment. Triage of those found +HR HPV (some with limited genotyping such as 16/18/45) utilize cervical cytology as a second test.

Two HPV assays used in major RCTs comparing cytology and HPV testing (Hybrid Capture 2 [signal amplification assays using clinician-collected samples] and PCR-EIA GP5+/6+) are now used as standard comparator tests for clinical validation of various HR HPV DNA tests following international recommendations known as the Meijer's criteria *(91)*.

Other molecular progression markers are being evaluated, such as the host factor $p16^{INK4a}$ (p16), a cyclin-dependent kinase inhibitor. Elevated p16 indicates removal of the negative feedback control supplied by the retinoblastoma protein (pRB). When oncogenic HPV E7 proteins bind to pRB, p16 is overexpressed and elevated, representing active expression of HPV oncogenes (Fig. 17.6) *(92)*.

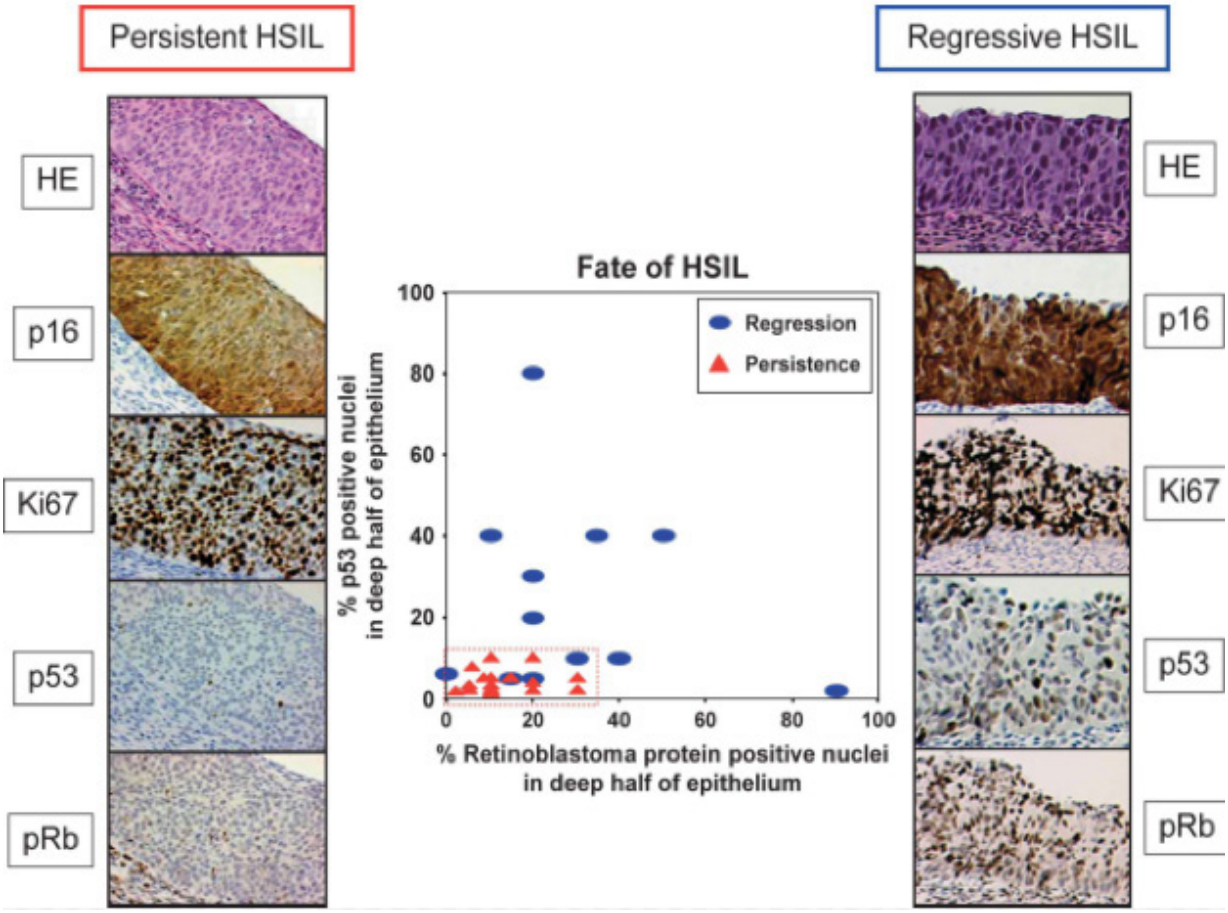
Moreover, in combination with increased expression of other host factors such as Ki67, overexpression of p16 correlates well with transforming viral infections (Fig. 17.7) *(93)*. Other host and/or viral progression markers such as methylation markers are being researched to better predict which of those women with HR HPV types that need clinical follow up *(94)*.

Fig. 17.6: P16 is not expressed in normal tissues (top left image), but is expressed as increasing abnormality in cervical intraepithelial neoplasia 1 (CIN1), CIN2 and CIN3



Source: Courtesy of Magnus von Knebel Doeberitz, Department of Applied Tumour Biology, Institute of Pathology, University of Heidelberg, Heidelberg, Germany.

Fig. 17.7: Various host and viral risk factor markers in high-grade squamous intraepithelial lesion (HSIL)



Key:

Centre: Scatterplot of retinoblastoma protein (pRb) and p53 detection in the lower half of the epithelium of HSIL lesions that on follow-up persist (red triangles) or regress (blue circles)

Left: persistent HSIL

Right: HSIL with regression

Source: Baak et al., 2005 *(93)*. Reproduced with permission from the *American Journal of Surgical Pathology*.

17.7 HPV DNA detection methods

Historically, methods used to detect HPV DNA were direct-probe hybridization assays such as dot blot and Southern blot. These were labour-intensive and time-consuming, and also had low sensitivity, requiring large amounts of DNA in clinical samples. Clinical applications now rely on highly standardized assays that streamline sample handling and rely on some form of amplification for testing. The list in Box 17.1 shows the current clinically validated tests *(95,96)*, including four NAATs (the four at the end of the list) that have been clinically validated for cervical screening *(97–101)* since publication of the 2020 list of human papillomavirus assays suitable for primary cervical cancer screening *(95)*.

Box 17.1: Current clinically validated HPV DNA NAATs

- Hybrid Capture 2 HPV DNA Test (Qiagen, USA)
- GP5+/6+ PCR-EIA (Diassay, the Netherlands)
- Abbott RealTime High Risk HPV Test[a] (Abbott Molecular, USA)
- Anyplex II HPV HR Detection (Seegene, Republic of Korea)
- BD Onclarity HPV Assay[b] (BD Diagnostics, USA)
- cobas 4800 HPV Test[b] (Roche Molecular Diagnostics, USA)
- HPV-Risk Assay (Self-Screen BV, the Netherlands)
- PapilloCheck HPV-Screening Test (Greiner Bio-One, Germany)
- Xpert HPV[a] (Cepheid, USA)
- Alinity m HR HPV Assay (Abbott Molecular, USA)
- cobas 6800/8800 HPV Test[b] (Roche Molecular Diagnostics, USA)
- APTIMA HPV Assay[c] (Hologic, USA)
- CLART HPV 4S (GENOMICA S.A.U., Spain)*
- NeuMoDx (NeuMoDx Molecular, a QIAGEN company, USA)*
- OncoPredict SCR (Hiantissrl, Italy)*
- APTIMA HPV (mRNA) (Hologic, USA)*

Notes:

[a] WHO prequalification of in vitro diagnostics

[b] FDA approved for HPV primary screening

[c] FDA approved only with cotesting with cytology

Source: 2020 list of human papillomavirus assays suitable for primary cervical cancer screening (Arbyn et al., 2021 [95]) except for items labelled *, which were more recently clinically validated (97–101).

WHO also has a policy brief on HPV NAATs for cervical pre-cancer screening, comparing HPV DNA-based and HPV mRNA-based molecular NAATs *(70)* and a guideline focusing on HPV mRNA-based NAATs *(69)*.

The Hybrid Capture test (HC2), originally by Digene (Qiagen), was widely used in early studies and was the first FDA-approved assay. HC2 is a semiquantitative assay with a very good inter-laboratory comparison and high NPVs for CIN2/3 lesions *(102)*. In addition, a low-cost version was designed to be performed in low-resource settings requiring minimal equipment and training and operating as a rapid assay *(103)*. Although for a long time only a research-based assay, this *care*HPV test became available for use clinically. Its sensitivity for CIN2+ in a large trial in China was 90%, being not significantly different from HC2 with which it was compared; hence, promising as a primary screening method for cervical-cancer prevention in low-resource regions *(103)*. Results do not differentiate which of the HR HPV types are present, but report results as indicating the presence of one or more types included in the assay.

There are many HPV tests on the market. A recently published updated review in 2020 revealed an almost doubling of distinct HPV assays, with 254 assays with 425 variants available on the global market: yet most had no analytical or clinical evaluation published in peer reviewed literature. Furthermore, more than 90% had no regulatory evaluation, nor were evaluated following a stringent clinical validation protocol *(65)*. In a recent comprehensive systematic review of HR HPV assays for primary cervical screening that fulfil international validation criteria in predicting underlying/incipient cervical precursor disease (CIN2+), only a small number successfully passed all requirements. Reproducibility of screening assays and non-inferior accuracy for detecting CIN2+ is fundamental in choice of screening assays. This review by Arbyn et al. also included a meta-analysis of the relative sensitivity and specificity of the index assay compared to the comparator tests *(95)*.

Moreover, as the original comparator tests did not provide genotyping alternative comparator assays are required. Thus, the birth of the validation protocol known as VALGENT (acronym for VALidation of HPV GENotyping tests) that allows validation of tests for use in cervical cancer screening *(98)*. This involves the collation of fresh or archived cervical cell specimens from women attending routine screening supplemented with cytologically abnormal samples. Each VALGENT panel includes an assay already validated for screening *(97, 104–120)*. As more changes occur such as self-collected sampling more validation protocols are required.

For all details of major HPV assays, the reader should refer to the WHO's HPV manual, which details each step along the way, including description of primers, appropriate controls, prevention of contamination, and interpretation of data, etc. *(121)*.

17.8 HPV mRNA assays

In addition to DNA assays, others detect mRNA transcripts as expression of E6/E7 oncogenes *(69,101)*. The APTIMA HPV Test (Hologic), which detects E6 and E7 mRNA of 14 HR HPV types in aggregate, is the most evaluated mRNA-based HPV test and it has been approved by the U.S. Food and Drug Administration (FDA) for cervical cancer screening in combination with cytology. It has been accepted for primary cervical cancer screening on clinician collected cervical samples at intervals of around five years. APTIMA is less sensitive on self-collected samples than clinician-collected samples *(118)*. There is no evidence indicating that APTIMA is less safe in screening with five year or shorter intervals. However, longitudinal relative performance indicators are imprecise, heterogeneous, and based on few studies *(101)*.

17.9 Principles of various molecular assays for HPV detection

17.9.1 Preanalytics

Suitable specimens and appropriate handling of clinical samples are essential in obtaining an accurate result. In the detection of HPV, suitable samples include swabs, brushes, lavage devices, first-void urine (preferably with DNA preservative), scrapes and tissue, including fresh, fresh-frozen and formalin-fixed, paraffin-embedded tissue *(122)*. Appropriate handling for swabs includes transporting dry or in a viral transport medium, whereas scrapings and biopsy samples should be collected in viral transport medium. Transport of swabs is appropriate at room temperature within 24 hours, or at 4°C up to four days *(122)*. Approved assays with specified collection and storage methods, and results would not be considered valid if collection and storage deviate.

17.9.2 Analytics

Following specimen collection, extraction or release of nucleic acid from samples must occur *(122)*. Results would not be considered valid if processing or extraction deviate. Use of commercial assays does not obviate the need for laboratories to maintain diligent quality assurance (QA) and quality control (QC) methods. Inclusion of positive and negative cell line controls can be useful in monitoring results. Processing water blanks through all steps of the assay is particularly crucial for monitoring false-positive results that may occur through cross-sample contamination.

Some assays include an endogenous host cell target, such as β-globin, to monitor the presence of amplifiable DNA. Samples negative for the endogenous target and HPV cannot be interpreted. Clinically approved assays include guidelines for monitoring and reporting assays. Although well verified, in-house, laboratory-developed assays can be used in routine diagnostics. Where an approved commercial assay is available, it is recommended by accrediting authorities that the latter should be utilized. Some of the advantages of doing so are that commercial assays usually include quality-controlled reagents, as well as appropriate controls.

17.9.3 Laboratory quality assurance

Good microbiological practices should be followed, including appropriate positive and negative controls. Participation in proficiency panels and external quality assessment (EQA) programmes is essential to determine assay performance and every effort must be made to participate in such programmes for each analyte *(123)*. The International HPV Reference Center provides two types of proficiency panels: global HPV LabNet TYPING proficiency panels which are designed for the verification of genotyping accuracy of HPV tests (the panel consists of 41 samples containing purified whole genomic plasmids of HPV 6, 11, 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68a and 68b in a background of human cellular DNA), and global HPV LabNet SCREENING proficiency panels which are designed for the screening needs (the panels consist of 12 samples containing purified whole genomic plasmids of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68a and 68b in a background of human cellular DNA). Participant laboratories are asked to perform HPV screening using one or more of their usual HPV screening assays *(124)*. More detailed QA and QC can be found in the recently published International Papillomavirus Society (IPVS) policy statement on HPV nucleic acid testing guidance *(96)* for those utilizing/considering HPV as primary precancer screening.

17.10 Development of a WHO HPV LabNet

A group of experts, convened by WHO, met in Geneva in August 2005 and recommended the establishment of a global HPV LabNet, to contribute to improving the quality of laboratory services for effective surveillance and HPV vaccination impact monitoring, and to conduct training *(125–127)*. It was envisaged that the HPV LabNet would speed up the introduction of HPV vaccines by facilitating the implementation of validated, standardized laboratory procedures; by developing QA system and proficiency testing; by training personnel; and by providing a network for surveillance. The WHO HPV LabNet was initially funded by a grant from the Gates Foundation and was in operation under the auspices of WHO from 2006–2010. During these four years of operation, it focused on core activities of developing international standards for HPV DNA and serology assays, standardizing assays and developing a laboratory manual and training programme emphasizing QA and QC. It collaborated with the National Institute for Biological Standards and Control (NIBSC) in developing the international standards for HPV types 16 and 18 DNA and HPV type 16 antibodies, which are available in the NIBSC catalogue. The first WHO HPV laboratory manual was published in 2009 based on knowledge and experience gained through WHO's international collaborative studies *(121)*. The manual aims to assist in establishing the laboratory support required for implementation and monitoring of HPV vaccination programmes, and focuses on epidemiologic assays, not clinical assays. In conjunction with NIBSC there is a collection of WHO International Standards now available for HPV DNA genotypes HPV 31, 33, 45, 52 and 58.

The HPV LabNet member laboratories continue to operate as a group to maintain a network of experts for optimal HPV testing, meeting at the International Papillomavirus Conference.

17.11 The future: progression markers

Not all women or men with persistent HR HPV, or for that matter all CIN3 or AIN3, progress to cervical or anal cancer respectively if untreated; therefore, other markers for predictors of progression are being investigated, such as p16, Ki67 and viral and host methylation markers. These progression markers hold promise together with HR HPV detection to more accurately diagnose high-grade dysplasia which require treatment without overwhelming colposcopy/anoscopy clinics *(92–94,128–130)*.

Self-sampling for genital HPV testing is becoming more acceptable for cervical screening, with evidence confirming HPV self-sampling can increase cervical cancer screening uptake in some communities *(131–133)*. When using clinically validated HR HPV DNA assays based on PCR, testing on self-collected samples is similarly accurate as on clinician-collected samples *(131,134–139)*. In Sweden and piloted in South Africa, self-collected specimens are collected on FTA cards which can be stored at room temperature for extended periods of time *(140)*. A new validation protocol for emerging HPV tests is being developed which incorporates the contemporaneous collection of a self-sample as well as a clinician taken sample. Known as the VALHUDES protocol, it generates evidence to confirm similar clinical accuracy on self-sampled versus clinician-sampled samples utilizing particular HR HPV assay(s) on vaginal self-samples and first-void urine with standardized protocols *(136)*.

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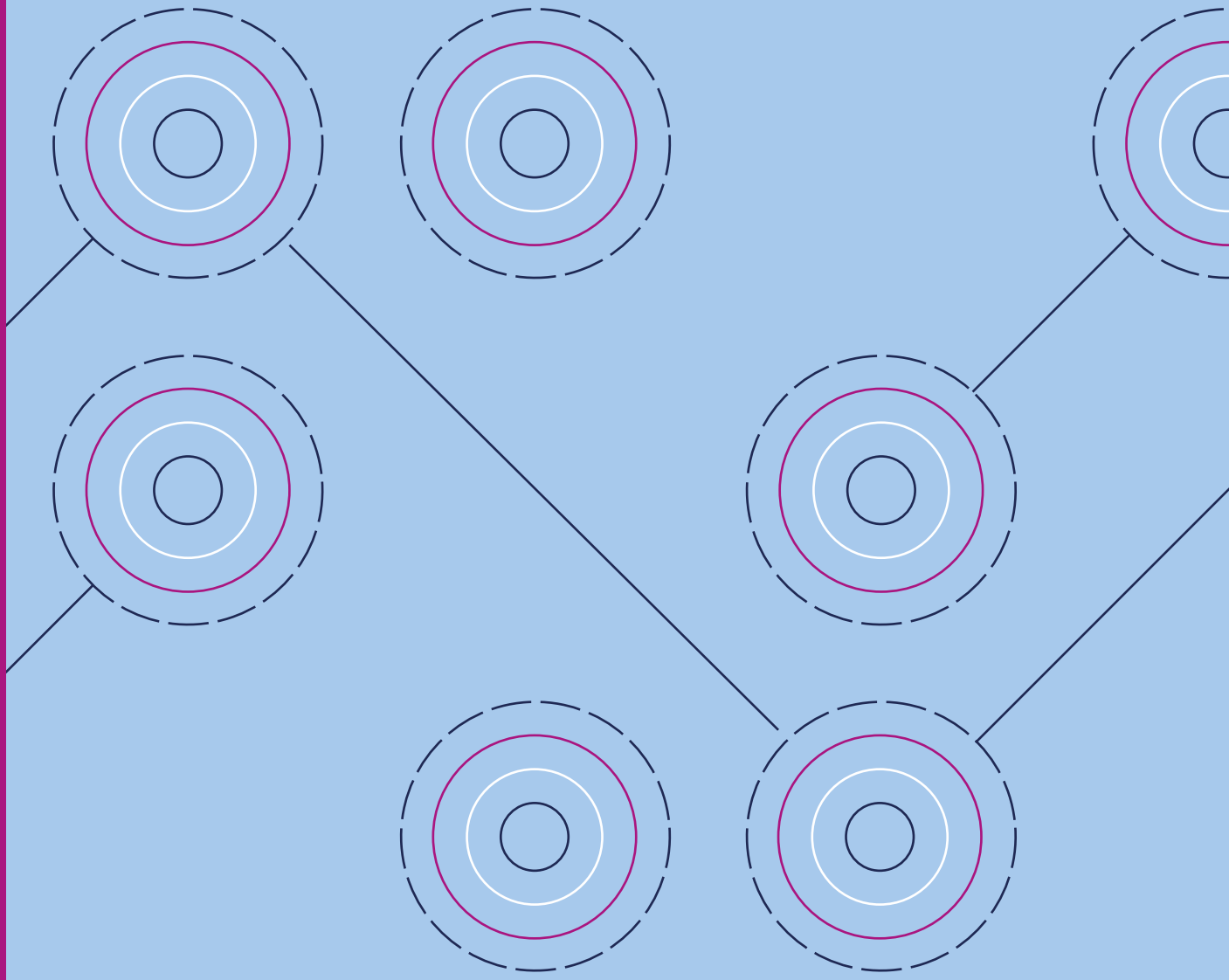
Chapter 18. Human immunodeficiency virus (HIV) infections



Chapter 18. Human immunodeficiency virus (HIV) infections

Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang

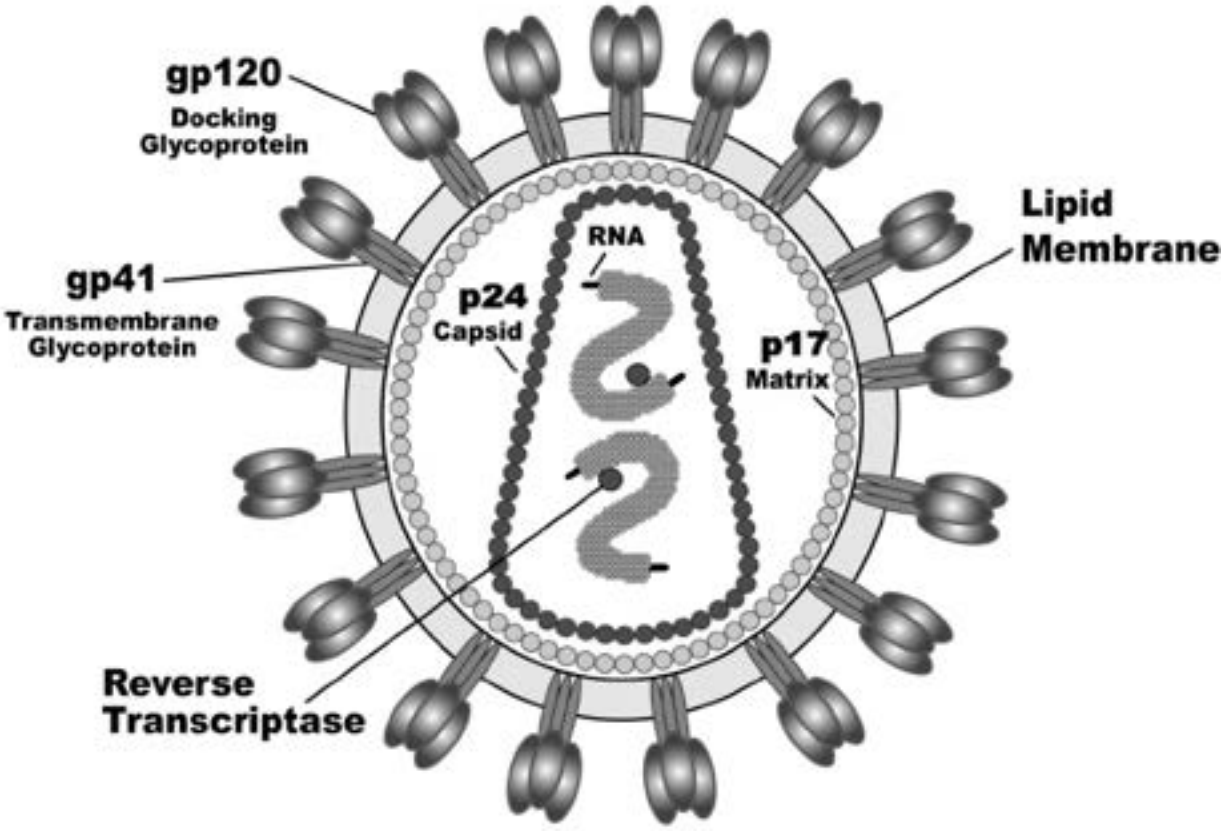
18.1 Introduction

The first cases of acquired immunodeficiency syndrome (AIDS) were described in 1981, and the causative virus was first isolated in 1983. Since then, the virus has spread worldwide, with an estimated > 38 million people living with HIV worldwide and 1.5 million new infections in 2021 alone *(1)*. Sub-Saharan Africa has been impacted the most, with 26 million people living with HIV and an estimated 860 000 new infections in 2021. The prevalence of HIV has remained > 10% among adults in some countries. Although effective antiretroviral treatments have been developed and treatment coverage has expanded to many resource-poor countries, most countries are striving to reach UNAIDS 95-95-95 goals *(2)*. Therefore, in the absence of an effective vaccine, accessible HIV diagnostics with counselling for prevention, pre-exposure prophylaxis (PrEP) for high-risk negative individuals, and referral of HIV-infected people to appropriate care and immediate treatment (i.e. test-and-treat) are important strategies to achieving elimination.

HIV is transmitted primarily through exchange of body fluids which can occur through the sexual route, sharing needles among injecting drug users and via contaminated blood or blood products. HIV-infected pregnant women can transmit the virus to the infant during pregnancy, during delivery (perinatal transmission) or through breastfeeding (post-natal transmission). HIV transmission also is reported following organ transplantation when the donor is later found to be HIV-positive. To ensure safety, blood, blood products, and organ donors are now routinely tested for the presence of HIV or HIV antibodies to eliminate the possibility of HIV transmission.

HIV belongs to the family *Retroviridae* and genus *Lentivirus* along with similar viruses, such as visna virus, caprine arthritisencephalitis virus, equine infectious anaemia virus, and simian immunodeficiency virus. The virion is approximately 100 nm in diameter with a conical capsid that contains two copies of genomic RNA (Fig. 18.1). The capsid protein, p24, is the major component of the virus and is further covered by a lipid envelope containing two glycoproteins, gp41 and gp120. These surface glycoproteins are important for binding to CD4 T-cells, a first step to the infection process, as well as generating host immune responses to the virus. HIV is highly divergent, and several different subtypes or clades exist worldwide. Different subtypes are more prevalent in specific areas of world. In addition, there are two major HIV types, HIV-1 and HIV-2. HIV-1 is subdivided into three groups, including M (subtypes A–K), N and O. HIV-1 is the most common virus dominating the epidemic, with multitudes of subtypes and recombinant viruses. HIV-2 is mainly in West Africa, with occasional cases reported in many countries throughout the world. There are some differences among HIV-1 subtypes but for diagnostic purposes they cross-react heavily; therefore, antigens/proteins derived from a single subtype are adequate to diagnose infection irrespective of prevalent subtypes. Since there is some level of cross-reactivity among HIV-1 and HIV-2, additional specific tests are required to accurately diagnose HIV-2 infection or dual infections.

Fig. 18.1: Structure and organization of HIV virus particle



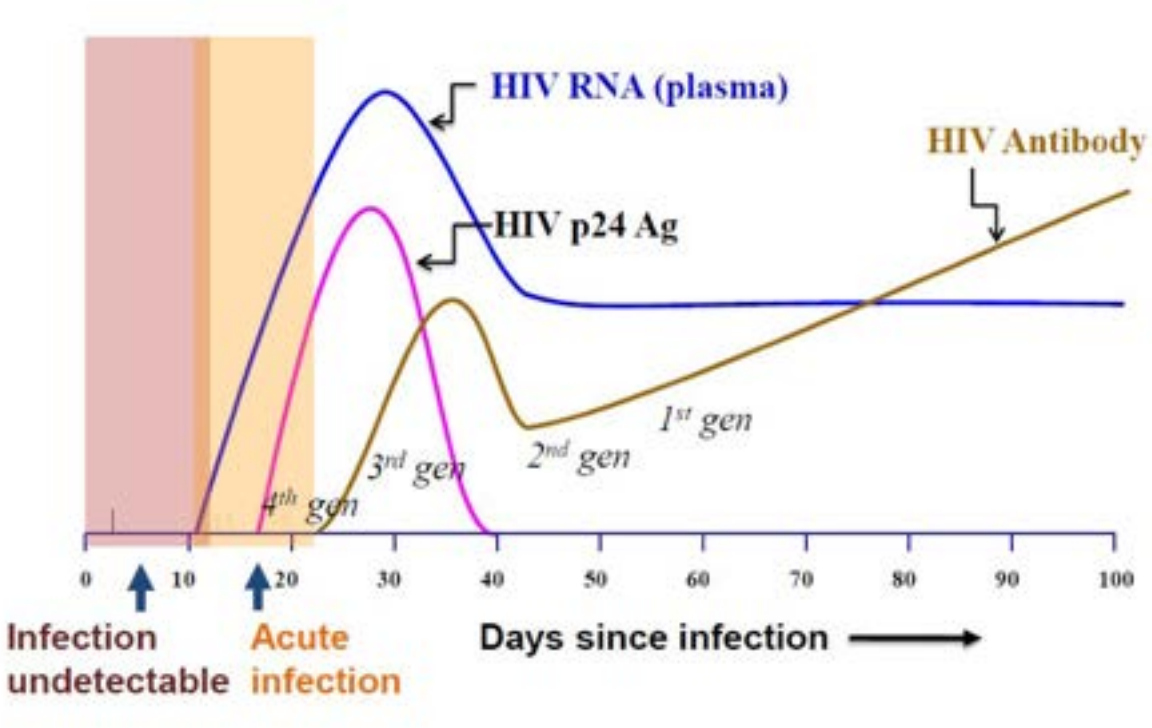
Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

18.2 Diagnostic testing

18.2.1 Serological diagnosis of HIV infection

A serological diagnosis of HIV infection is obtained routinely by detection of HIV antibodies in the blood or other body fluids of children over 18 months of age, adolescents, and adults. Antibodies are elicited on average approximately 3–6 weeks after infection, although in rare cases, development of detectable antibodies may take up to 3 months following infection. Therefore, HIV infection cannot be excluded on the basis of a negative test 3–6 weeks after documented exposure. During the initial period of virus replication, antibodies are absent and HIV diagnosis may not be made accurately using antibody-only tests. This so called window period can be shortened by using methods that can directly detect one or more components (p24 antigen or RNA) of HIV. Virus replication in the body elicits both humoral and cell-mediated immune responses, which reduces the virus level to a set point. The presence of detectable virus in most untreated individuals continues to stimulate B-cell responses and antibody levels remain high throughout the subsequent period, unless the patient is highly immunocompromised, as in the later stages of the disease. Therefore, detection of HIV-specific antibodies is a highly reliable marker for diagnosis of HIV infection (Fig. 18.2).

Fig. 18.2: Early dynamics of virus replication and antibody development with markers of HIV infection – approximate times (days since infection) for reactivity using first-, second-, third- and fourth-generation enzyme immunoassays



Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

There are three main uses for HIV testing. HIV testing is performed to: (i) ensure safety of blood, blood products or organ transplant, (ii) perform individual/patient diagnosis and (iii) conduct surveillance. Testing for blood safety usually is performed using the most sensitive and sophisticated testing procedures that detect both HIV antibody and p24 antigen and HIV-1 RNA to reduce the window period. Diagnostic testing usually requires a combination of tests in an algorithm as part of the testing strategy to ensure accuracy of HIV diagnosis. Testing strategies for surveillance may depend on the population being tested but, just as for individual diagnosis, use of two or more tests, including a more specific test, is highly recommended to increase the positive predictive value and accuracy of HIV prevalence estimates.

i. Enzyme immunoassays (EIAs)

Soon after discovery of HIV, EIAs were developed to diagnose HIV infection. These EIAs detected HIV-specific antibodies, a proxy for HIV infection, and used virus lysate as antigens. The first-generation assays were later replaced with second-generation assays that employ more specific antigens in the form of synthetic peptides or recombinant proteins. These assays were more sensitive and specific in detecting HIV antibodies; however, they still did not detect very early antibody responses in the form of IgM, owing to their format and design. The third-generation EIAs utilize a sandwich format that includes antigens labelled with enzyme and are able to detect early IgM responses, thus reducing the window period. New fourth-generation EIAs were developed to reduce the window period further by combining detection of viral antigen (p24) in addition to HIV antibodies (Fig. 18.2). These combination antigen-antibody EIAs are very sensitive in detecting acute HIV infection prior to development of antibodies and are now used routinely for blood/blood products screening in many countries. Although molecular assays can detect viral nucleic acid a few days earlier than the p24 antigen detected by fourth-generation EIAs, the cost and complexity of nucleic acid detection may outweigh the benefits in routine testing except in sophisticated blood banks.

Although EIAs are qualitative assays, a close review of optical density values may be informative. Those with high signal/cut-off ratios are highly likely to be HIV-positive compared with those that have low signal/cut-off ratios. Therefore, the positive predictive value (PPV) of one or two reactive EIA results will be much higher if the results are interpreted in the context of signal. Those with a low signal, even when reactive by two EIAs, should be further tested with a more specific test or a follow-up sample to confirm infection.

ii. Rapid tests

In the last 15 years, HIV diagnosis has moved increasingly from laboratory to non-laboratory settings as a result of availability of dozens of HIV rapid tests. Worldwide, more than 100 million people were tested with HIV rapid tests in 2020 alone. HIV rapid tests come mainly in two different formats, which include immunoconcentration devices or lateral flow cassettes or strips (Fig. 18.3).

Fig. 18.3: Example of HIV rapid tests, showing negative and positive results

A. Lateral flow device

B. Flow-through immunoconcentration device that includes both HIV and syphilis diagnosis

Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

Because rapid tests are developed to detect HIV antibodies within minutes (1–15 minutes), compared with EIAs, which may take up to 2–4 hours, the devices are optimized to accelerate antigen-antibody interaction. This requires the use of a high concentration of antigen and detection of antigen-antibody complexes with sensitive colour reagents, such as colloidal gold. Rapid tests are ideal for providing same-day results in a variety of situations such as voluntary counselling and testing, testing hard-to-reach populations, home-based counselling and testing, provider-initiated testing, testing of pregnant women, mobile testing and now self-testing. HIV rapid tests are commonly used in all settings to provide more cost-effective and rapid diagnosis. They can be performed using serum, plasma, or whole blood, facilitating the use of finger pricks specimens. Rapid tests are simple to perform and can be used outside laboratory environments by trained lay workers and counsellors, thus expanding access to HIV testing. WHO and the Centers for Disease Control and Prevention (CDC) in the USA have developed an extensive training package that addresses various issues, including quality, accuracy and safety. In addition, several simple and practical approaches have been developed to ensure the quality of rapid testing *(3–5)*. With expanded availability of care and treatment, there is an increased drive to provide counselling and testing to millions of people worldwide as an important component of prevention. Further, use of rapid testing now allows for same day/same hour diagnosis of HIV-positive individuals and rapid linkage to antiretroviral therapy (ART) initiation.

Owing to high demand and an expanded market, there are more than 50 HIV rapid test kits available worldwide, not all with desired performance characteristics. It is important to use rapid tests that are manufactured to the highest quality according to good manufacturing practices and have performance characteristics equivalent to other diagnostic methods. WHO's prequalification programme for in vitro diagnostics *(6)* assesses the quality of new rapid test kits and have recently released a new tool kit for optimal selection of test kits and testing algorithm *(7)*. Additionally, some of the new rapid tests that combine diagnosis of HIV and syphilis in a single device are approved by the WHO Prequalification Team *(8)*. They can be very useful in sexually transmitted infection (STI) clinics or in antenatal care (ANC) clinics where diagnosis of both HIV and syphilis are important and would otherwise require two different tests *(8,9)*.

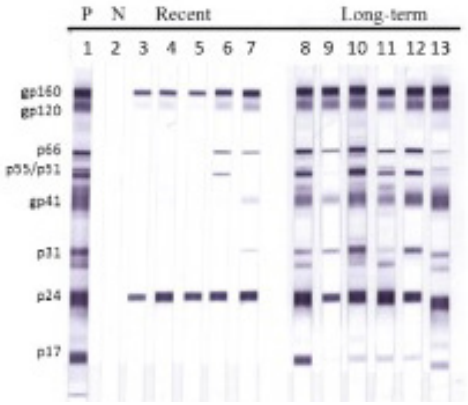
Fourth-generation rapid tests capable of diagnosing acute HIV infection by detection of p24 antigen, in addition to detecting HIV antibodies, have been developed. Detection of p24 antigen can reduce the window period by a few days and identify people who are in the acute phase of infection. However, because detection of acute infection is a rare event even in high prevalence/incidence settings, a positive antigen test should be followed up by testing at four weeks or later to confirm seroconversion. A field evaluation of such a rapid test demonstrated that sensitivity and specificity of Ag detection are not yet acceptable *(10)*. Accordingly, WHO does not recommend detection of acute infection as part of routine HIV testing services. As with any other testing, hands-on training and robust quality assurance (QA) measures are essential to ensure the accuracy of rapid testing.

Several rapid tests have been developed that use oral fluid (OF) specimens collected using a swab. OF contains 1/500–1/1000 times less IgG than in blood specimens but sufficient to diagnose HIV infection. Most OF antibodies are transferred passively from the blood rather than locally elicited and contain a total complement of serum IgG antibodies, albeit at a lower concentration *(11)*. OF tests can simplify HIV testing further, making them more accessible while reducing biohazard risk associated with blood-based testing. Currently, at least three oral fluid based rapid test kits are commercially available.

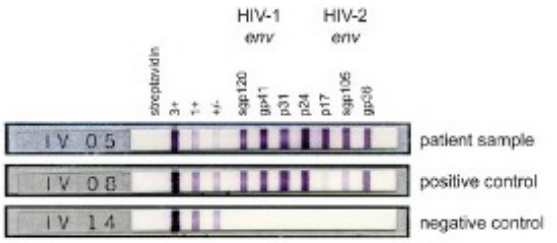
iii. Supplementary assays

Although the individual assays listed above are very sensitive and specific, false-positive and false-negative results do occur. False-positive results can have major consequences at the individual level and can adversely impact HIV programmes. Therefore, supplementary assays were developed that can confirm initial positive results and/or confirm type-specific diagnosis (HIV-1 or HIV-2). These assays, based on different formats and principles, include immunofluorescence assays (IFAs), western blot (WB), dot or line immunoassays (LIAs) (Fig. 18.4). IFA involves use of slides with HIV-infected fixed cells. HIV antibodies, if present, bind the HIV antigens in fixed cells and are detected by fluorescein-tagged secondary antibodies and observed under a fluorescence microscope. IFA is no longer a commonly used method in most settings and has been replaced by other methods, such as WB or LIA. Both of these involve the use of membrane strips with separated HIV-specific proteins or recombinant proteins or peptides. WB strips are probed with serum specimens, and the bound HIV-specific antibodies are detected by secondary antibodies that are conjugated with an enzyme followed by a substrate that yields a coloured product. Fig. 18.4A shows typical banding patterns on WB indicating presence of antibodies to specific viral proteins, including a positive control showing all the viral protein specific antibodies that can be detected with their characteristic pattern (Lane P). Individuals with new/recent infections have weak antibodies and directed to fewer proteins, typically p24 and gp120/gp160. As the infection progresses, antibodies develop to additional viral proteins and get stronger over time as shown for those with long-term infections (see Fig. 18.4A). LIAs are similar to the WB assay but use selected purified recombinant proteins or peptides, instead of viral lysate, that are immunologically important for diagnosis. WBs and LIAs are expensive and require complex interpretation to establish a diagnosis based on a combination of antigen-specific antibodies (to p24 and one or more envelope proteins) present in the blood. These assays detect only HIV-specific IgG. Therefore, they cannot be used to confirm presence of HIV-specific IgM or virus detected by more sensitive third- and fourth-generation EIAs, respectively.

Fig. 18.4: Supplementary serological HIV assays

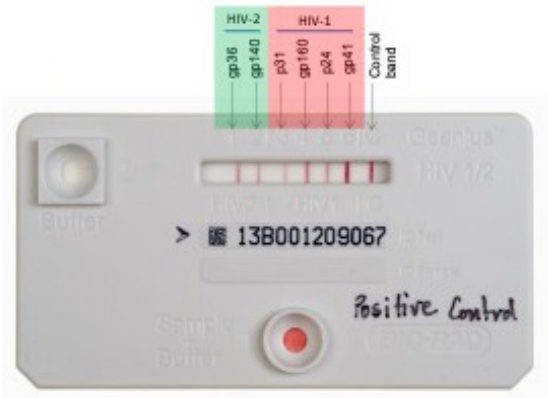


A. Western blot assay and antibody banding pattern that may be observed during recent infections (strips 3–7) and long-term infections (strips 8–13)



B. Inno-LIA line immunoassay

Key: P: positive control specimen; N: negative control specimen
Note: Various virus specific proteins are shown next to positive control

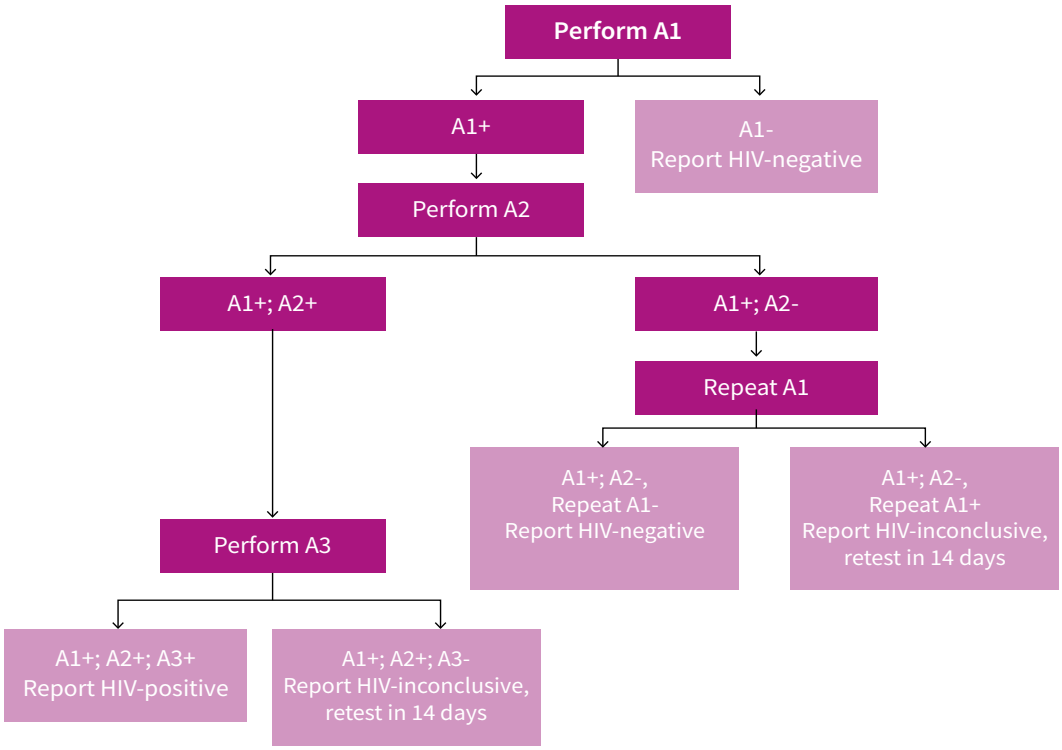


C. Geenius HIV-1/2 rapid supplementary test that include various HIV-1 or HIV-2 recombinant or peptide antigens to detect specific antibodies

Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

Although WB was widely used for over three decades, it can take several days to weeks to complete the testing; thus, delaying diagnosis and linkage to care. Moreover, indeterminate results do occur that do not meet positive interpretation criteria and require additional follow-up testing. These issues, combined with the high cost and complexity of manufacturing, testing and interpretating with WB has led WHO's recommendation to move away from WB. A testing algorithm that involves the use of three rapid tests in a serial algorithm can provide results that are quick and nearly as reliable as confirmatory WB or LIA assays but at a much-reduced cost. New WHO guidance recommends the use of three test strategies and three consecutive positive test results to increase positive predictive value of HIV diagnosis, especially in the context of decreasing undiagnosed HIV prevalence among individuals presenting in HIV testing services (Fig. 18.5). Tests should be selected carefully with the first test having high sensitivity and subsequent tests having high specificity to ensure accurate diagnosis and reduce common false positives. Use of all highly sensitive third- or fourth-generation EIAs in an algorithm can lead to a high proportion of false positives *(12)*.

Fig. 18.5: HIV diagnostic strategy requiring three consecutive HIV-reactive results on different tests



A1: Assay 1 (first test); A2: Assay 2 (second test); A3: Assay 3 (third test).

Note: Assays (tests) are HIV rapid diagnostic tests (RDTs) or enzyme immunoassays (EIAs).

Source: WHO, 2020 *(13)*.

18.2.2 Detection of HIV RNA, DNA, or p24

HIV also can be diagnosed by direct detection of virus or virus components (p24 antigen, RNA, or proviral DNA). Detection of virus by culture or other methods is not commonly performed because of poor sensitivity compared to standard immunological or molecular methods and the complexity of virus culture techniques. Detection of p24 antigen or HIV-1 RNA or DNA play an important role when antibody-based diagnosis is not possible, such as in perinatally exposed infants under 18 months of age or detecting acute infection in adults prior to development of HIV antibodies *(14–18)*. Molecular detection of a nucleic acid target is more sensitive and specific than p24 detection which requires further confirmation either by neutralization or seroconversion.

Depending on the stage of infection, HIV may be found primarily as pro-viral DNA in infected cells or as RNA in the blood (as a component of free virus particles and intracellular RNA). Commercial kits are available that can detect DNA and/or RNA either qualitatively or quantitatively. For diagnostic purposes, qualitative assays are sufficient and have applications for early detection of HIV infections in adults and infants. Acute infection detection by nucleic acid amplification tests (NAATs) has application in increasing safety of donor blood and blood products and possibly in high-risk populations. Many blood banks in developed and

developing countries routinely use NAATs as part of their testing algorithms. Acutely infected individuals have high viral loads and are at high risk of transmission of infection to partners. Therefore, detection of acutely infected individuals has been promoted as part of overall HIV prevention strategy. However, detection of acute cases in most settings is low yield and very expensive considering the short window period of approximately two weeks *(19)*. Therefore, WHO does not currently recommend it.

Another important application for molecular tests is early diagnosis of HIV infection in perinatally exposed infants. Because all infants born to seropositive mothers have acquired HIV antibodies passively, routine antibody-based assays cannot be used to confirm or rule out HIV infection. Residual antibodies in uninfected infants persist and can be detected up to 18 months of age. Furthermore, HIV antibodies may be delayed in development and detection in HIV-infected infants. Therefore, detecting the presence or absence of HIV RNA, DNA or TNA in infants up to 18 months of age is the most definitive way of diagnosing HIV infection in infants. For infant diagnosis in resource-limited settings, blood is spotted onto filter papers and dried (dried blood spot [DBS]) as a means to ensure proper specimen collection, processing, transport and storage of blood specimens. With a major focus on prevention of mother-to-child transmission, molecular assays for infant diagnosis have been widely implemented. There are several commercial assays that are used for HIV diagnosis in infants. These assays include manual extraction of nucleic acids or state-of-the-art molecular platforms that offer advanced automation for the extraction of nucleic acids and qualitative detection of HIV-1 RNA, DNA or threose nucleic acid (TNA). Manual nucleic acid extraction requires a significant number of steps and there is a potential for human error and polymerase chain reaction (PCR) contamination. With the need for higher quality and accuracy of molecular testing, platforms have been designed to minimize user interventions and improve sample throughput. The qualitative detection of HIV-1 infection in infants has been reported to be very sensitive depending on the specimen source (whole blood or DBS). Specimen pooling, which is often performed to detect acute infections in adults to increase efficiency and reduce cost, is not recommended for infant diagnosis. However, a quantitative HIV NAAT assay on whole blood specimens may be used as an aid to diagnosis if validated by the manufacturer.

While performance of quality rapid serological testing for HIV at peripheral sites has become routine, point-of-care (POC) PCR testing is also advancing. Newer POC testing modalities that can be performed in resource-constrained settings are based on isothermal amplification with rapid detection of target sequences. Some POC test assays utilize small portable instruments and generally perform nucleic acid extraction and amplification, signal amplification, and detection. They are strongly recommended for high burden settings based on the significant clinical benefits such as same-day ART initiation of HIV-positive infants *(20)*.

18.2.3 Testing algorithms

For HIV diagnosis, three tests are combined into an algorithm to increase the PPV of an initial positive test result. In most situations, the most commonly used algorithm includes serial or sequential use of tests (serial algorithm). Although a negative result is usually given to a client based on a single test, a positive result is further confirmed with second and third follow-up tests, using different tests each time. The serial algorithm is logical and cost-effective and includes a more sensitive test for the first, followed by more specific tests to eliminate false-positive results. It is important to select the correct combination of tests to provide an accurate diagnosis *(7)*. Due to the success of HIV programmes globally, undiagnosed HIV prevalence has declined substantially in most countries. Therefore, WHO now recommends the use of a three-test strategy in all settings to increase the PPV of HIV diagnoses irrespective of HIV prevalence (Fig. 18.5) *(13)*. This is particularly important as we expand test-and-treat, initiating ART immediately after diagnosis irrespective of CD4 level.

18.3 Clinical laboratory monitoring of HIV infection

18.3.1 Role of CD4 testing in HIV clinical monitoring

Infection with HIV leads to the development of AIDS, which is characterized by loss of the CD4 T-cells that are required for a person's immune system to properly function. When a CD4 count declines, HIV-positive individuals are more likely to become infected with opportunistic pathogens. CD4 testing is used in the clinical monitoring of HIV-infected people to identify people living with advanced HIV disease who are eligible to receive the advanced disease package of care that includes diagnosis, prophylaxis and/or treatment for opportunistic infections, such as *Cryptococcus* and tuberculosis.

CD4 T cell count was the primary laboratory-based test used to guide the initiation of patients on ART monitor treatment response. WHO global ART guidelines for adults and adolescents in 2002 recommended that ART be initiated in adults with clinical stage 4 disease or with a CD4 cell count of < 200 cells/µl *(21)*. In 2013, HIV viral load testing was recommended as the preferred monitoring approach to diagnose and confirm treatment failure, with CD4 testing being recommended in areas with an insufficient HIV viral load testing capacity *(22)*. WHO released new guidelines in 2015 recommending the initiation of ART for all HIV-infected individuals irrespective of CD4 counts *(23)*. In 2017, for adults, adolescents and children over 5 years old, advanced HIV disease was defined as a CD4 cell count < 200 cells/mm^3 or WHO clinical stage 3 or 4 at presentation for care *(24)*. Relying on clinical staging alone may miss substantial numbers of HIV-infected individuals with serve immune suppression. Identifying advanced HIV disease and determining eligibility for elements of a package of care still requires CD4 cell count testing. In addition, determining the immune status of HIV-infected individuals with treatment failure by HIV viral load criteria can help in guiding clinical management decisions.

i. Basics of the CD4 assay

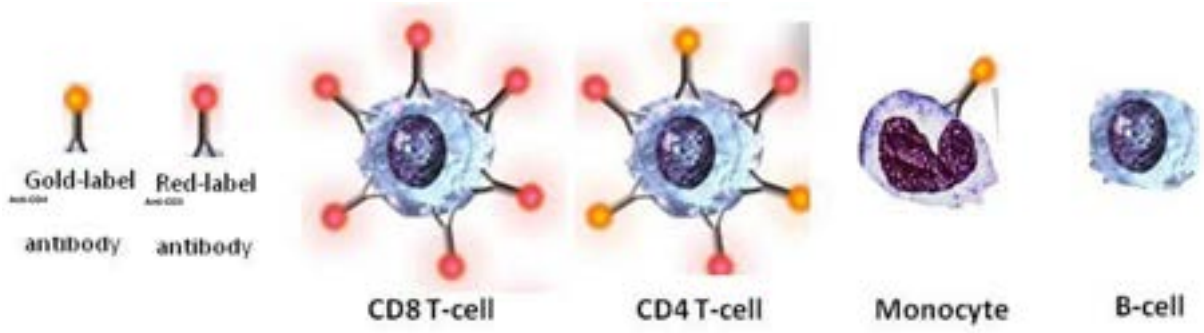
In general, CD4 counts refer to the enumeration of CD4 T-cells, also called CD4-lymphocytes, or Helper T-cells. CD4 assays are used to determine the whole blood absolute CD4 cell concentration and/or the percentage of CD4 cells in the lymphocyte population. Normal CD4 counts are between 400–1600 cells/mm^3 with the normal CD4 percentage of lymphocytes between 35–55% *(25)*. The normal CD4 count in children younger than 5 years of age is higher than in adults and declines with age. This makes it difficult to determine the immune status for children using absolute CD4 counts; therefore, CD4 percentages have been used in monitoring HIV-infected children.

The standard method for CD4 counts uses labelled monoclonal antibodies to identify blood cell surface molecules such as CD4, CD3, CD8 and CD45. The major white cells with labelled anti-CD4 and CD3 antibodies are depicted in Fig. 18.6.

Blood cells that express the surface molecules of interest are identified with flow cytometers that detect the specific label of each of the monoclonal antibodies. Flow cytometers also differentiate blood cells by the cell's light-scatter properties. The recommended gating strategy for CD4 count identifies the lymphocyte population by CD45 markers and side-scattering properties. There are also several dedicated CD4 assays that use specific fixed gating strategies, which may be manually modified, that are unique to the assay.

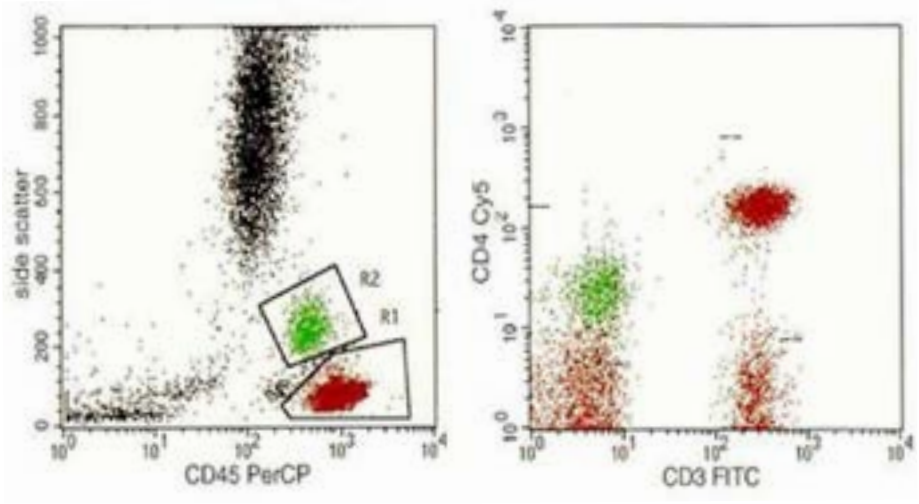
As an example of CD4 testing, a flow cytometric analysis with two-parameter dot plots is shown in Fig. 18.7. The first dot plot identifies the white cell population by the light-scattering property of the cells and CD45 surface molecules. The box represented by R1 identifies the lymphocyte population and R2 the monocyte population. The second dot plot only looks at the cells gated in R1 and R2 boxes from the first dot plot. The CD4 cells are the upper right cluster of cells having both CD4 and CD3 expression. CD8 T-cells are just below the CD4 cluster expressing CD3 but not CD4. B-cells are clustered in the lower left corner not expressing CD4 or CD3. Monocytes are the green cluster of cells above the B-cells, expressing low CD4 and not CD3. The flow cytometer can count the number of cells in each of the clusters to help determine the CD4 counts.

Fig. 18.6: CD4 testing flow cytometry gating strategies



Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

Fig. 18.7: Example of flow cytometer dot plots from a CD4 assay



Source: Bharat Parekh, Larry Westerman, Lara Vojnov and Chunfu Yang.

ii. CD4 platforms

CD4 counts were traditionally determined using a dual-platform or single platform technique with a flow cytometer. A dual-platforms flow cytometer provides the percentage of CD4 cells in the white blood cell or lymphocyte population and a haematology analyser provides the absolute white blood cell or lymphocyte counts. Single-platform methods are preferred and have been shown to improve the precision of the CD4 assay *(26)*. The single-platform method determines the CD4 count on flow cytometers from a precisely determined volume of whole blood sample. The single-platform methods are based either on a volumetric principle by counting CD4 cells in a unit volume of processed sample or based on comparison of the counts of a known number of microbeads added to the processed sample of the CD4 cells counts.

Newer CD4 platforms involve multicolour fluorescence imaging with portable analysers and single-use cartridges. Also, a lateral-flow cartridge CD4 assay is available which estimates the specimens CD4 counts to be above or below a set standard CD4 count, including the threshold for advanced HIV disease (200 cells/mm^3). These newer CD4 platforms can be deployed at point-of-care or near-care settings to support faster clinical decisions for this population, who are most vulnerable to high morbidity and mortality. For further information, refer to the WHO list of prequalified in vitro diagnostic products for available CD4 technologies, including laboratory-based, near point-of-care, and point-of-care products *(6)*. Prior to the implementation of CD4 assays, consideration should be given to in-country, or WHO, policies on the use of these CD4 tests with regard to: patient need for CD4 testing access, infrastructure and personnel needs for proper placement, supply and service logistics, and evaluation and monitoring of testing quality *(21,26)*.

18.3.2 Viral load testing

Although CD4 is an important clinical parameter to understand the clinical status of patients in identifying those with advanced HIV, with the recommendation of rapid ART initiation for all people living with HIV regardless of their CD4 status, viral load testing has become the preferred method of monitoring treatment and viral suppression in accordance with the updated WHO guidelines *(27)*. The WHO prequalification programme for in vitro diagnostics has been assessing commercially available HIV viral load (VL) testing platforms, including both laboratory-based and point-of-care (POC) testing platforms *(6)*. Countries in resource-limited areas could refer to this list when making procurement decisions. Most VL testing platforms were designed to use in vitro nucleic acid amplification and real-time detection technologies to quantify HIV RNA in plasma/DBS specimens with comparable detection sensitivity and dynamic ranges. Plasma specimens are preferred for VL testing while DBS specimens are recommended in settings where logistical, infrastructural or operational barriers prevent routine VL monitoring using plasma specimens *(27)*. Since POC VL testing platforms became commercially available, and those assessed have been placed on the WHO list of prequalified in vitro diagnostic products *(6)*, many resource-limited countries have been expanding their VL testing and monitoring using POC VL platforms for populations with challenges in accessing routine VL testing or needing more rapid testing results for clinical decision-making, including pregnant and breastfeeding women and infants, children and adolescents. Studies have reported that POC VL testing shortened the time of clinical decision making and switching ART-patients with unsuppressed VL to new regimens *(28,29)* resulting in improved retention in care and VL suppression rates when compared with a standard-care-of-testing *(27,29)*.

18.3.3 Drug resistance testing

HIV drug resistance (HIVDR) refers to the ability of HIV to continue replicating in the presence of antiretroviral (ARV) drugs that usually suppress its replication. HIVDR is caused by mutations or changes in the relevant portions of the viral RNA genome that are targeted by ARV drugs, which ultimately can lead to changes in enzymatic protein structures essential for viral replication and enable HIV to replicate in the presence of the relevant ARV drugs. With the current WHO recommended Dolutegravir (DTG)-based preferred first- and second-line regimens in resource-limited settings *(27)*, the most commonly prescribed ARV drugs are against protease (PR), reverse transcriptase (RT) and integrase (IN) regions of the HIV polymerase (*pol*) gene; thus, HIVDR mutations that are commonly detected are in the PR, RT and IN regions. In general, the evolution of drug-resistant HIV subpopulations can significantly compromise the ability of ARV drugs to suppress viral replication. Once resistant viral strains emerge and replicate, they can persist indefinitely either as circulating viruses or integrated into memory T-lymphocyte genomes as proviral DNA. Not only can these drug-resistant viral strains cause HIVDR in patients who acquired them during ART or preventing new HIV infections in PrEP, but they also can be transmitted to naive individuals, potentially leading to compromised ART efficacy in newly infected individuals when they are receiving ART. With the higher genetic barrier to develop HIVDR in the currently preferred DTG-based regimens *(30)*, however, HIVDR development and transmission in resource-limited settings appears to be levelling off or decreasing *(31)*. Two established methods are available for HIVDR testing: genotypic and phenotypic testing. Both tests are complex and expensive. In resource-rich countries, monitoring patients on ART for acquired HIVDR and detection of transmitted HIVDR in recently HIV-infected individuals have become the standard of care *(32)*. Owing to the limited and/or lack of infrastructure, insufficient numbers of trained personnel and high cost, individual HIVDR testing is not performed routinely in most resource-limited countries. Furthermore, with new, optimized treatment regimens that include DTG, the clinical value of individual HIVDR testing remains unclear. Core activities of the 2021 updated WHO HIVDR strategy included developing and implementing a national action plan for HIVDR surveillance, monitoring the quality-of-care indicators associated with and predicting HIVDR (also known as early warning indicators of HIVDR) and implementing HIVDR surveys. To prevent the emergence and transmission of HIVDR in countries rapidly scaling-up ART and PrEP programmes and to ensure that the ART regimens selected for inclusion in national ART guidelines continue to be effective, WHO recommends surveillance of acquired HIVDR in populations receiving ART, of pretreatment HIVDR among adults initiating first-line ART, of pretreatment HIVDR among treatment-naive infants newly diagnosed with HIV and of HIVDR among PrEP users diagnosed with HIV *(33)*.

i. Genotypic HIVDR testing

The conventional genotypic HIVDR testing uses Sanger sequencing-based technology to detect nucleotide sequence changes from which the amino acids of HIV PR, RT and IN enzymes are deduced. The amino acid sequences of the PR, RT and IN regions of the HIV *pol* gene are compared to those of a wild-type reference viral stain or a subtype-specific consensus reference sequence and any amino acid changes are recorded as a change in the amino acid position at a specific codon. Mutations/amino acid changes are described in a standard format based on the numeric position of the mutant codon in the amino acid sequence of PR, RT or IN. For instance, a change from methionine (M) to valine (V) at position 184 of RT is described as M184V. The letter to the left of the number represents the amino acid in that position in the reference RT and the letter to the right of the number displays the comparable amino acid associated with the mutation in the tested HIV strain. Conventional genotypic testing has been shown to be highly reproducible and sensitive, and it provides a complete and accurate genetic sequence of the domains of interest. Studies have indicated that conventional genotypic testing can identify HIVDR mutations presented at about 20% in circulating quasispecies *(34,35)*. There are two commercially available HIV-1 genotyping kits: ViroSeq HIV-1 Genotyping Systemand Thermo Fisher Scientific HIV-1 resistance testing kits. In addition, laboratories around the world have developed and validated conventional genotypic tests that were designed for genotyping diverse HIV-1 group M subtypes and circulating recombinant forms *(36–40)*.

While conventional genotypic HIVDR testing has been widely used for population-based HIVDR surveillance in resource-limited countries, its limited capability to quantitatively detect HIVDR below 20% of the circulating viral quasispecies in the clinical management of ART patients has been well documented *(41)*. The demand for genome-wide sequencing has made next generation sequencing (NGS)-based genotypic HIVDR technologies commercially available since 2005 when the 454 pyrosequencing technique was launched by 454 Life Sciences *(42)*. Since then, many NGS technologies became available, but they have mainly been adopted for HIVDR genotyping in research and clinical settings in resource-rich countries *(43)*. Although NGS technologies apply different mechanisms, they hold the characteristics of being massive, parallel, clonal sequencing of input DNA without the need of molecular cloning or even without need of PCR amplification or DNA synthesis among the third-generation of platforms *(44)*. Some of the WHO ResNet laboratory members have developed their own NGS-based HIVDR genotypic assays *(45–47)*, but their adoption for routinely supporting surveillance of HIVDR in resource-limited settings is still in question.

ii. Phenotypic HIVDR testing

In vitro phenotypic testing uses the HIV-1 PR, RT and IN regions of the *pol* gene derived from an HIV-infected individual and incorporates these gene regions to generate a recombinant virus. The susceptibility of the recombinant virus is then determined by susceptibility testing in the presence of various concentrations of a relevant ARV drug. Results are expressed as susceptibility fold changes in the 50% inhibitory concentrations (IC_{50}) compared to cut-off values generated by reference wild-type virus. However, there are several limitations of phenotypic testing. Notably, phenotypic testing may not predict the clinical outcome adequately if there is a mixed population of wild-type and mutant viral strains present in the viral quasispecies. In addition, phenotypic testing is available only in a limited number of laboratories where different methods are being used which may lead to discordant results because there is no standardized approach for phenotyping drug resistance testing. More importantly, the cost of phenotypic testing is currently much higher than genotypic testing. Because of these limitations, phenotypic testing is not recommended for HIVDR surveillance in resource-limited countries.

iii. Specimen types for HIVDR testing

Specimen types routinely used for HIVDR genotypic testing are plasma and DBS, however the former is the gold standard for HIVDR testing. Due to the requirement of laboratory infrastructure, trained personnel and cold chain for storage and transportation of plasma specimens, DBS can be considered a suitable specimen type for HIVDR surveillance if they are collected, stored and transported following the WHO-recommended methodology *(40)*. There have been many studies evaluating and validating DBS as a specimen type for HIVDR testing for surveillance in resource-limited settings *(48–54)*. While there are important advantages to using DBS in HIVDR testing *(37,38,55–57)*, there are also disadvantages; the foremost of which is the reduced genotyping sensitivity due to the inherent nature of lower specimen input volume. Several laboratories have developed more sensitive in-house (laboratory-developed tests) genotyping assays and adopted them for use with DBS for HIVDR genotyping with the genotyping sensitivity at 1000 copies/ml of plasma VL and one of the assays with an enhanced capability of genotyping the IN region in a partially multiplexed format has become commercially available recently *(36)*. When DBS specimens are used for HIVDR surveillance, WHO recommends using two of the assay methodologies *(36,39)* for HIVDR genotyping analysis.

iv. Quality-assured genotyping results

As with any molecular technique, HIVDR genotypic testing is prone to cross-contamination if the test is not performed in the manner and laboratory facilities for which they are designed. To standardize HIVDR genotypic testing and ensure the quality-assured genotyping data in resource-limited settings for WHO recommended HIVDR surveillance activities, the second edition of WHO HIVResNet HIVDR laboratory operational framework lays out the detailed requirements for (i) Samples collection, handling transportation and storage; and (ii) Quality-assured WHO-designed genotyping laboratory services for generating reliable and comparable results. Additionally, the framework specifies post testing quality assurance procedures to ensure that the genotyping results generated are quality-assured *(58)*. Furthermore, since 2007, WHO-designed HIVDR genotyping laboratories at the national, regional and specialized levels around the globe have been participating in the external quality assurance programme provided by the Virology Quality Assurance programme *(46)*.

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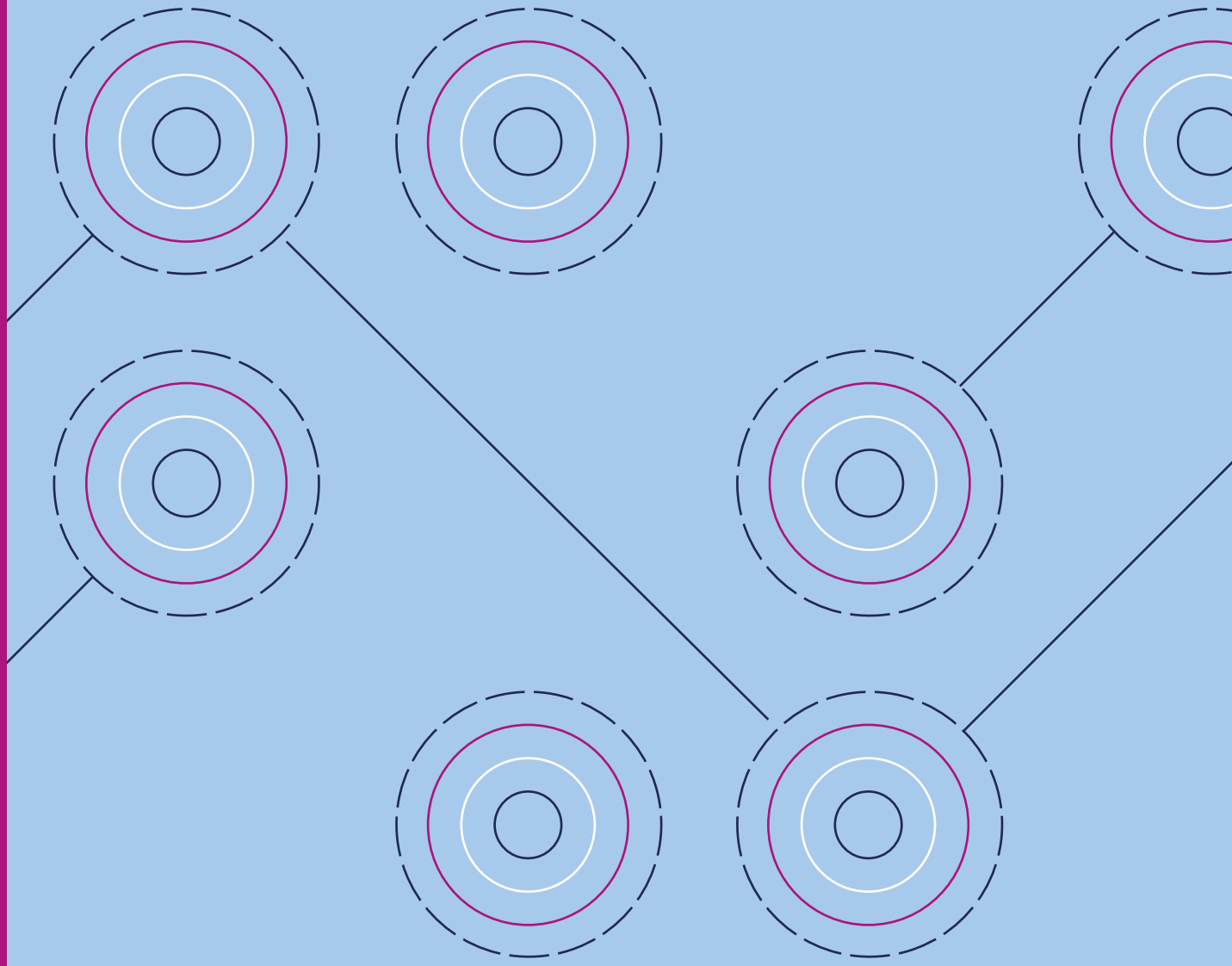
Annex 1. Media, reagents, diagnostic tests and stains (recipes)



Annex 1. Media, reagents, diagnostic tests and stains (recipes)

Sumudu Perera and Jo-Anne Dillon

Note: The reagents for media and stain preparation are listed in alphabetical order by category. Some recipes appear in the chapters and are not repeated in this annex.

Transport, storage and culture media

Amies transport medium *(1)* (non-nutritive media)[1]

Charcoal, pharmaceutical neutral	10 g
Sodium chloride	3.0 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Potassium chloride	0.2 g
Sodium thioglycolate	1.0 g
Calcium chloride	0.1 g
Magnesium chloride	0.1 g
Agar	4.0 g
Distilled water	1 L

1. Suspend all ingredients in 1 L of distilled water.
2. Boil to dissolve the agar completely.
3. Distribute into small, screw-capped tubes or bottles, stirring meanwhile to keep the charcoal evenly suspended.
4. Sterilize by autoclaving at 120 °C for 15 minutes.
5. Immediately cool in cold water to keep the charcoal uniformly suspended.
6. Store at 2–8 °C for up to six months.

Pre-prepared media are commercially available.

Storage medium for microorganisms

Brainheart infusion (BHI) + 20% glycerol

BHI	3.7 g
Glycerol	20 ml
Distilled water	80 ml

1. Mix the ingredients in a 150-ml screw-capped bottle.
2. Autoclave and cool to room temperature.
3. Using a sterile pipette, aseptically dispense 1.0 ml of the solution into 1.5-ml screw-capped polypropylene cryotubes. (It is essential to use proper tubes to avoid breakage.)
4. Store the medium at 4 °C until use.

Skimmed milk + 20% glycerol

1. Suspend 10 g skimmed milk powder in 100 ml distilled water.
2. Aliquot 80 ml of this skimmed milk solution to a new screw-capped bottle and add 20 ml glycerol.
3. Autoclave for 15 minutes at 112–115 °C.
4. Using a sterile pipette, aseptically dispense 1.0 ml of the solution into 1.5-ml screw-capped polypropylene cryotubes. (It is essential to use proper tubes to avoid breakage.)
5. Store the medium at 4 °C until use.

Fetal calf serum (FCS) + 10% dimethylsulfoxide (DMSO)

1. Filter-sterilize DMSO.
2. Combine 90 ml of sterile FCS and 10 ml of filter-sterilized DMSO.

[1] Other commercially available non-nutritive transport systems include ESwab (aerobes, anaerobes and fastidious bacteria) and VCM medium (virus, *Chlamydia* and *Mycoplasma*).

3. Using a sterile pipette, aseptically dispense 1.0 ml of the solution into 1.5-ml screw-capped polypropylene cryotubes. (It is essential to use proper tubes to avoid breakage.)
4. Store the medium at 4 °C until use.

Chocolate agar (1)

Gonococcal (GC) agar base composition per litre (commercially available)

Peptone	15 g
Corn starch	1 g
Dipotassium hydrogen phosphate	4 g
Potassium dihydrogen phosphate	1 g
Sodium chloride	5 g
Agar	10 g

1. Dissolve 36 g of GC agar base in 500 ml of distilled water.
2. Mix and boil.
3. Dissolve 10 g haemoglobin (a dried powder of bovine haemoglobin) in 500 ml of distilled water.
4. Mix and boil.
5. Autoclave at 121 °C for 15 minutes.
6. Cool to 50–55 °C.
7. Add 10 ml of IsoVitaleX or cofactors-vitamins-amino acids (CVA) enrichment and prepared haemoglobin suspension to GC medium.
8. Mix and pour into Petri dishes.

To prevent contamination during *Haemophilus ducreyi* culturing, vancomycin can be added to semisolid chocolate agar at 3 µg/ml.

Note: Some *H. ducreyi* strains can be inhibited at this concentration and would require non-antibiotic-containing culture media.

Diamond's medium (1,2)

Ingredients refer to the commercially available product.

Trypticase	20.0 g
Yeast extract	10.0 g
Maltose	5.0 g
L-cysteine hydrochloride	1.0 g
L-ascorbic acid	0.2 g
Dipotassium hydrogen phosphate	0.8 g
Potassium dihydrogen phosphate	0.5 g
Agar	0.5 g

1. Dissolve 38 g of the Diamond's medium powder in 900 ml of distilled water.
2. Autoclave and cool the medium to 50 °C.
3. Add 100 ml of sheep or bovine serum and 0.1 g/L chloramphenicol.
4. Store at 4 °C and consume within three months.

Modified Diamond's medium

Ingredients in the commercial preparation (it comes in liquid form, pH 6.5 ± 0.2 at 25 °C)

Casein peptone	24.0 g
Yeast extract	12.0 g
Maltose	6.0 g
Streptomycin	1.5 g
Cysteine L-hydrocholoride	1.2 g
L-ascorbic acid	0.24 g
Amphotericin B	2.0 mg
Penicillin	1 000 000 U
Horse serum	120.0 ml
Demineralized water	ml

Another commercial culture system is the InPouch TV culture system.

Diagnostic sensitivity agar (DST) supplemented with 5% lysed horse blood and 1% IsoVitaleX or 1% Kellogg's supplement (used as a substitute medium for gonococcal agar dilution)

Ingredients in commercial DST powder

Proteose peptone	10.0 g
Veal infusion solids	10.0 g
Glucose	2.0 g
Sodium chloride	3.0 g
Disodium hydrogen phosphate	2.0 g
Sodium acetate	1.0 g
Adenine sulfate	0.01 g
Guanine hydrochloride	0.01 g
Uracil	0.01 g
Xanthine	0.01 g
Aneurine	20 µg
Agar	12 g
Distilled water	Add up to 1 L

Horse blood (lysed by freeze–thawing)

Kellogg's supplement (see recipe for GC medium agar base [GCMB] agar)

1. Dissolve 40 g of powder in 1 L of distilled or deionized water.
2. Boil until powder is completely dissolved.
3. Autoclave for 15 minutes at 121 °C.
4. Cool to 50–55 °C, add 50 ml of lysed horse blood and 10 ml of IsoVitaleX or Kellogg's supplement.
5. Mix and aseptically pour into Petri dishes.

GCVIT (i.e. GCMB supplemented with 1% defined growth supplement [Kellogg's defined supplement or IsoVitaleX/Vitox]) *(3,4)*

GCMB with supplements is recommended for agar dilution, disk diffusion and ETEST (bio Mérieux) methods for *N. gonorrhoeae* antimicrobial susceptibility testing.

GCMB	36 g
IsoVitaleX/Vitox/Kellogg's defined supplement	10 ml
Distilled water	Add up to 1 L

1. Dissolve 36 g of GCMB in 1 L distilled water and autoclave at 121 °C for 15 minutes.
2. Allow the agar media to cool to 50 °C in a water bath, add 10 ml of dissolved IsoVitaleX, Vitox or Kellogg's defined supplement and mix well.
3. Dispense 20–25 ml per 9-cm Petri dish and 60 ml per 14-cm Petri dish.
4. Allow the plates to solidify at room temperature and store inverted at 2–8 °C in sealed plastic bags for up to four weeks.

IsoVitaleX and Vitox can be purchased commercially while Kellogg's defined supplement is prepared in-house.

Kellogg's defined supplement (4,5)

Glucose	40 g
Glutamine	1.0 g
0.5% ferric nitrate solution	10 ml
Distilled water	90 ml

1. Mix all ingredients in a 150-ml flask.
2. Sterilize by autoclaving.
3. Cool in a 50 °C water bath.
4. Add 1 ml sterile 20% cocarboxylase solution (filter-sterilized but not autoclaved).
5. Aseptically dispense the solution in a 100 ml screw-capped storage bottle and store at 4 °C (stable for several months).

IsoVitaleX supplement or CVA enrichment
(commercially available)

Ingredients in commercial preparations – formula per 10 ml

Diphosphopyridine nucleotide (coenzyme I)	2.5 mg
Cocarboxylase	1.0 mg
p-Aminobenzoic acid	0.13 mg
Thiamine hydrocholoride	0.03 mg
Vitamin B_{12}	0.1 mg
L-glutamine	100.0 mg
L-cystine dihydrochloride	11.0 mg
L-cysteine hydrochloride dehydrate	259.0 mg
Adenine	10.0 mg
Guanine hydrochloride	0.3 mg
Iron (III) nitrate nonahydrate	0.2 mg
Glucose	1.0 g

Note: a *cysteine*-free growth supplement is required for agar dilution tests with carbapenems and clavulanate.

GCMB + 2% haemoglobin + 5% FCS + 1% IsoVitaleX enrichment + vancomycin (3 µg/ml) *(3)*

GCMB	36 g
Bovine haemoglobin	20 g
FCS	50 ml
IsoVitaleX enrichment	10 ml
Vancomycin solution	3 mg in 5–10 ml of fluid
Distilled water	1 L

1. In a 2-L flask (A), add the bovine haemoglobin to 500 ml distilled water and stir well to mix.
2. In another 2 L flask (B), add the GCMB to a further 500 ml distilled water and heat on a stir plate until boiling.
3. Autoclave both flasks at 121 °C for 30 minutes then cool to 56 °C.
4. With an aseptic technique in a laminar flow hood, slowly pour the sterilized bovine haemoglobin solution (flask A) carefully into flask B containing the GCMB, pouring onto the internal side of the flask to avoid creating bubbles.
5. Add 50 ml (5%) sterile FCS, 10 ml (1%) IsoVitaleX enrichment and a sterile volume of fluid containing 3 mg vancomycin (filter-sterilized if required).
6. Mix the medium with a careful swirling movement.
7. Pour the medium aseptically into 20-ml Petri dishes and let the medium set before placing in plastic bags and storing in the fridge at 4 °C until needed.
8. Agar plates should be used within 1–2 weeks of preparation.

The following are different media types that can be used for *H. ducreyi culturing* (see Chapter 15)

1. GC agar base, 1–2% haemoglobin, 5% FCS, 10% CVA and 3 µg/ml vancomycin.
2. Mueller-Hinton agar, 5% chocolate horse blood, 1% CVA enrichment and 3 µg/ml vancomycin.
3. Two different media types can be combined into a single split plate *(6,7)*. These can include:
    d. GC agar, 2% bovine haemoglobin, 5% FCS, 1% IsoVitaleX supplement and 3 µg/ml vancomycin (GC agar, 2% bovine haemoglobin, 5% FCS).
    e. Mueller-Hinton agar, 5% chocolate horse blood, 1% IsoVitaleX supplement and 3 µg/ml vancomycin (Mueller-Hinton agar supplemented with 5% chocolate horse blood).

Note: FCS can be substituted by adding 0.2% activated charcoal.

Note: for *H. ducreyi* minimal inhibitory concentration testing, Mueller-Hinton agar (or GCMB) supplemented with 1% haemoglobin, 5% FCS and 1% IsoVitaleX supplement can be used.

Gonochek-II

The Gonochek-II (TCS Biosciences) is a growth-independent test used to distinguish *Neisseria* species by their ability to hydrolyse three enzymes specifically produced by a species: proline iminopeptidase/prolyliminopeptidase (PIP) for *Neisseria gonorrhoeae*, gammaglutamylaminopeptidase for *Neisseria meningitidis* and beta-galactosidase for *Neisseria lactamica*.

The Gonochek-II kit consists of a single tube containing three chromogenic substrates. The hydrolysis of these substrates produces a distinct colour. The specific colour produced relates to the enzyme present and therefore indicates the presence of *N. gonorrhoeae*, *N. meningitidis* or *N. lactamica*, respectively.

Other commensal *Neisseria* species may produce PIP, so enzymatic tests should be performed on strains grown on selective media. PIP-negative *N. gonorrhoeae* strains have also been reported (*8–10*); therefore, specimens should be confirmed using an alternative method *(11)*.

Gonogen II

Gonogen II (New Horizons Diagnostics Corporation) is a monoclonal antibody-based colorimetric test in which monoclonal antibodies against the PorB protein (PorB1a [IA] and PorB1b [IB]) of *N. gonorrhoeae* have been pooled and adsorbed to suspended metal-sol particles. Gonogen II uses a solubilizing buffer to strip the cell wall from the test microorganism, thereby exposing the PorB protein. A pool of monoclonal antibodies linked to a red metal-sol carrier is used to detect the antigens specific to *N. gonorrhoeae*. The subsequent antigen–antibody complex is detected by a filtration device giving rise to a clear-cut red dot end-point *(11)*.

1. Label a separate test tube for each isolate and the control strains.
2. Dispense 0.5 ml of solubilizing buffer and adjust McFarland turbidity to 1.0 by collecting colonies from selective or enriched agar.
3. Vigorously shake or vortex the Gonogen II reagent.
4. Add one drop of Gonogen II reagent into each of the tubes to be tested. Mix well.
5. Allow tubes to stand for at least 5–15 minutes (longer reaction times increase the clarity of the reaction).

Iscove's modified Dulbecco's medium (IMDM) *(12)* *(commercially available)*
Ingredients in commercial preparations

Amino acids	Concentration (mg/L)
Glycine	30.0
L-alanine	25.0
L-arginine hydrochloride	84.0
L-asparagine (free base)	25.0
L-aspartic acid	30.0
L-cystine 2HCl	91.4
L-glutamic acid	75.0
L-glutamine	584.0
L-histidine hydrochloride-H_2O	42.0
L-isoleucine	105.0
L-leucine	105.0
L-lysine hydrochloride	146.0
L-methionine	30.0
L-phenylalanine	66.0
L-proline	40.0
L-serine	42.0
L-threonine	95.0
L-tryptophan	16.0
L-tyrosine disodium salt	104.0
L-valine	94.0

Vitamins	Concentration (mg/l)
Biotin	0.013
Choline chloride	4.0
D-Calcium pantothenate	4.0
Folic acid	4.0
Niacinamide	4.0
Pyridoxal hydrochloride	4.0
Riboflavin	0.4
Thiamine hydrochloride	4.0
Vitamin B_{12}	0.013
i-Inositol	7.2
Inorganic salts	
Calcium chloride ($CaCl_2$) anhydride	165.0
Magnesium sulfate ($MgSO_4$) anhydride	97.67
Potassium chloride (KCl)	330.0
Potassium nitrate (KNO_3)	0.076
Sodium bicarbonate ($NaHCO_3$)	3024.0
Sodium chloride (NaCl)	4505.0
Sodium phosphate monobasic (NaH_2PO_4-H_2O)	125.0
Sodium selenite (Na_2SeO_3-$5H_2O$)	0.017
Other components	
D-Glucose (Dextrose)	4500.0
HEPES	5958.0
Phenol red	15.0
Sodium pyruvate	110.0

Jembec medium *(1)* (nutritive media)

Modified Thayer–Martin medium containing 0.25% glucose (see Thayer–Martin medium recipe).

Other commercially available nutritive transport systems include Transgrow (see recipe below), Gono-Pak and InTray GC system (commercially available).

Liquid cytology medium (commercially available)

Modified Eagle's medium (MEM-VG) (commercially available)

Modified Thayer–Martin medium (nutritious selective culture medium)

1. Prepare GC agar with 1% haemoglobin and 1% IsoVitaleX or CVA (see Chocolate agar recipe)
2. Add selective antimicrobial mixtures: VCAT (vancomycin, colistin, amphotericin B or anisomycin, trimethoprim lactate), VCNT (vancomycin, colistin, nystatin, trimethoprim lactate) or LCAT (lincomycin, colistin, amphotericin B or anisomycin, trimethoprim lactate). Antimicrobial mixtures are commercially available.

These supplements should be hydrated as recommended by the manufacturer and added to the medium *(11,13)*. Final concentrations per litre should be:

- vancomycin 2.0–4.0 mg
- lincomycin 1.0 mg
- colistin 300 000 IU–7.5 mg
- trimethoprim 3.0–6.5 mg.

Mueller-Hinton broth (commercially available)

Ingredients in commercial preparations:

beef extract	2.0 g
acid digest of casein	17.5 g
soluble starch	1.5 g

1. Suspend 21 g of dehydrated medium in 1 L distilled water.
2. Mix and dispense in small aliquots (1.5 or 2 ml) and autoclave.

Mueller-Hinton agar + 5% chocolate horse blood + 1% IsoVitaleX enrichment + vancomycin (3 µg/ml) *(3)*

This is recommended for the culture of *H. ducreyi*:

Mueller-Hinton agar base	36 g
Horse blood	50 ml
FCS	50 ml
IsoVitaleX enrichment	10 ml
Vancomycin solution	3 mg in 5–10 ml of fluid
Distilled water	1 L

1. Weigh out 38 g of Mueller-Hinton agar base and make up to 1 L with distilled water, autoclave and cool to 56 °C.
2. Add 50 ml (5%) of sterile horse blood when the dissolved agar base reaches 56 °C and mix.
3. Chocolatize the suspension by placing the flask in a 70 °C waterbath for approximately 15 minutes.
4. Cool to 56 °C and then add 50 ml (5%) sterile FCS, add 10 ml (1%) IsoVitaleX enrichment and 3 mg sterile vancomycin (filter-sterilized if required).
5. Mix the medium with a careful swirling movement.
6. Pour the medium aseptically into 20-ml Petri dishes and let the medium set before placing in plastic bags.
7. Store in the fridge at 4 °C until needed.
8. Agar plates should be used within 1–2 weeks of preparation.

New York City (NYC) agar *(1)* (nutritious selective culture medium, commercially available)

1. Suspend 18 g of GCMB in 430 ml distilled water, mix and boil.
2. Autoclave and cool to 50–55 °C.
3. Add 50 ml of laked horse blood, yeast autolysate and VCAT inhibitor (according to the manufacturer's instructions).
4. Mix and pour into Petri dishes.

Yeast autolysate contains:

Yeast autolysate	5.0 g
Glucose	0.5 g
Sodium bicarbonate	0.075 g

Laked horse blood: lyse by freeze–thawing or by adding 5 ml/L saponin to GCMB medium.

VCAT inhibitor: see modified Thayer–Martin medium.

Sabouraud dextrose agar

Glucose	40 g
Neopeptone or polypeptone (commercially available)	10 g
Agar	15–20 g
Demineralized water	1 L

1. Mix the ingredients.
2. Heat the mixture to dissolve completely.
3. Adjust to pH 5.6.
4. Dispense into tubes (18–25 mm in diameter).
5. Autoclave at 121 °C for 15 minutes.

If chloramphenicol is to be added to the medium, add appropriate volume and concentration.

Candida species can also be cultured on chromogenic agar, available commercially.

Skimmed milk medium *(1)*

Dried skimmed milk powder	100 g
Distilled water	1 L

1. Suspend 100 g of dried milk powder in 1 L distilled water.
2. Autoclave for 15 minutes at 112–115 °C.
3. Store at 4–8 °C.

Note: It is important to avoid overheating, otherwise caramelization will occur.

Stuart transport medium (non-nutritive medium)

Sodium thioglycolate	1.0 g
Sodium glycerophosphate	10.0 g
Calcium chloride	0.1 g
Methylene blue (MB)	0.002 g
Cysteine hydrochloride	0.5 g
Agar	3–5.0 g
Distilled water	1 L

1. Mix the ingredients in distilled water and bring to the boil.
2. Adjust the pH to 7.3–7.4 and distribute into small screw-capped bottles.
3. Autoclave at 121 °C for 15 minutes and immediately tighten the cap.
4. The medium should be colourless when cooled.
5. Store in a refrigerator at 4–8 °C.
6. If the colour changes to blue on storage, the medium is aerated and unfit for use. Trapped air can be removed by loosening the screwcap and reheating the medium.

Pre-prepared media are commercially available.

Sucrose phosphate transport medium (2SP) with antibiotics *(1)*

Dipotassium hydrogen phosphate	2.1 g
Potassium dihydrogen phosphate	1.1 g
Sucrose	68.5 g
Distilled water	1 L

1. Combine the ingredients, bring the pH to 7.2 and filter-sterilize.
2. Aseptically add supplements (see below) to 90-ml aliquots.

Supplements:

FCS	10 ml
Gentamicin	10 mg
Vancomycin	10 mg
Amphotericin B	0.5 mg

Sucrose-phosphate-glutamate medium

Sucrose	75 g
Potassium dihydrogen phosphate (KH_2PO_4)	0.52 g
Disodium hydrogen phosphate (Na_2HPO_4)	1.22 g
Glutamic acid	0.72 g
Add water to	1 L

1. Adjust pH to 7.5 if necessary.
2. Aliquot into 100-ml bottles and autoclave for 20 minutes or filter-sterilize.
3. Store up to 1 year at 4 °C.

Thayer–Martin medium (nutritious selective culture medium)

GCMB (ingredients in commercial preparation) – chocolate agar

Peptone	15.0 g
Corn starch	1.0 g
Dipotassium hydrogen phosphate	4.0 g
Potassium dihydrogen phosphate	1.0 g
Sodium chloride	5.0 g
Agar	10.0 g
Haemoglobin: a dried powder of bovine haemoglobin	10.0 g
IsoVitaleX, Vitox or Kellogg's supplement enrichment	10.0 ml
Distilled water	1 L

1. Dissolve 36 g of GCMB (Difco, Becton Dickinson) in 500 ml distilled water and stir until powder dissolves completely.
2. Suspend 10 g of haemoglobin in 500 ml distilled water, make a paste before adding the water, mix thoroughly to dissolve and bring to boil.
3. Sterilize both the solutions by autoclaving at 121 °C for 15 minutes. Cool by placing the flasks in a 50 °C waterbath.
4. Remove each flask from the waterbath and aseptically add the haemoglobin solution to the GCMB flask.
5. Aseptically add 10 ml of dissolved defined supplement (e.g. Kellogg's defined supplement, IsoVitaleX or Vitox).
6. Mix the contents completely using a gentle swirling motion.
7. Immediately pour 20–25 ml of the mixture into each 9-cm Petri dish.
8. Allow the medium to solidify at room temperature.
9. Store plates inverted at 2–8 °C in sealed plastic bags for up to three weeks.

IsoVitaleX and Vitox can be purchased commercially while Kellogg's defined supplement is prepared in-house (see GCMB).

Add VCN to the chocolate agar.

Formula:

Vancomycin	300 µg/ml
Colistin	750 µg/ml
Nystatin	1250 units/ml

Tryptic soy broth (also known as trypticase soy broth and soybean-casein digest medium) ***(1)***

Acid digest of casein (tryptone)	17 g
Soya peptone	3 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Glucose	2.5 g
Distilled water	1 L

1. Suspend 30 g of the commercial powder in 1 L of distilled water, pH 7.3.
2. Mix well and dispense 2-ml aliquots into small tubes.

Transgrow medium (nutritive medium) ***(14)***

GC agar base	36 g
Haemoglobin	10 g
Glucose	1.5 g
VCNT inhibitor	10 ml
Supplement B[2]	10 ml
Distilled water	Add up to 1 L

1. Mix the ingredients to a final volume of 1 L distilled water.
2. Autoclave for 15 minutes at 121 °C.
3. Cool to 56 °C and dispense 8 ml of medium to horizontally placed glass bottles (i.e. 1-ounce prescription bottles).
4. Loosely apply a rubber-lined screwcap and allow the medium to solidify.
5. Introduce a CO_2 atmosphere into the bottle by placing the bottle (or a group of bottles) upright in a vacuum jar.
6. Exhaust the air with a vacuum pump and refill with 10% CO_2 and 90% filtered-air mixture (commercially available).
7. Allow the chamber to return to atmospheric pressure and tighten the screw-caps.
8. Store the bottle upright at 4 °C to avoid the loss of CO_2.

Preprepared media are commercially available.

Viral transport medium ***(1)***

The medium consists of Hank's balanced salt solution (HBSS, commercially available) supplemented with amphotericin B, bovine serum albumin and gentamicin.

HBSS	10.3 g
Amphotericin B	5.0 mg
Bovine serum albumin 10%	100 ml
Gentamicin	50 mg
Distilled water	Add up to 1 L

1. Dissolve 10.3 g of HBSS powder in 1 L distilled water.
2. Add amphotericin B, bovine serum albumin (dissolved in distilled water) and gentamicin.
3. Mix and adjust the pH to 7.3 with sodium bicarbonate, 7.5% solution.

Reagents and diagnostic tests

Alkaline phosphatase test ***(1)***

1. Prepare a bacterial suspension (equivalent to McFarland standard 3) in a tube containing 0.5 ml of 0.3 g/L phenol-free disodium phosphate in 0.01 mol/L Sörensen's citrate-sodium hydroxide buffer, pH 5.6.
2. Incubate for 4 hours in a 37 °C water bath.
3. Dispense four drops of 5 g/L 2,6-dibromoquinone-4-chloroimide in methanol.
4. Shake and incubate the tube at room temperature for 15 minutes.
5. Add 0.3 ml of n-butanol.
6. Shake and incubate for 5 minutes at room temperature.

API NH – ***Neisseria gonorrhoeae***

As the API NH procedures require an inoculum equivalent to a 4.0 McFarland standard, it is generally necessary to subculture the isolate before testing to obtain sufficient inoculum. The following media may be used to culture *N. gonorrhoeae* before using the API NH strip: chocolate agar with 2% IsoVitaleX or Thayer–Martin with or without antibiotic. Supplemented GCMB may also be used. Blood-based agar media (Columbia blood agar base, trypticase soy, NYC medium) may be used, although the strength of certain biochemical reactions will be modified. (This should be taken into account when reading the reaction; please see the manufacturer's instructions.) An alternative to API NH is RapID NH.

API NH system

1. Record the isolate number on the strip.
2. Place the strip in the incubation box.
3. Using a swab, pick up a few well-isolated colonies and prepare a suspension (in the NaCl provided with the kit) with turbidity equivalent to 4.0 McFarland standard, ensuring it is well mixed using a vortex mixer. Use an 18–24-hour culture. The suspension must be used immediately after preparation.
4. Distribute the prepared bacterial suspension (approximately 50 µl) in the first seven microtubes (PEN to URE, inclusive). Avoid the formation of bubbles.
5. Fill the last three microtubes with the suspension (approximately 150 µl), avoiding the formation of a convex meniscus.

[2] Supplement B contains yeast extract, L-glutamine, coenzyme 1, cocarboxylase and additional growth factors.

6. Overlay the first seven microtubes (PEN to URE, inclusive) with mineral oil (provided with the kit).
7. Incubate for 2 hours at 36 °C under aerobic conditions.
8. After the incubation period, read the reactions by referring to the reading table included in the kit (available at www.biomerieux.com, bioMérieux).

Reference strains: See the manufacturer's recommendation (bioMérieux) for quality control. Use *N. gonorrhoeae* (ATCC strain 31426) or a 2016 World Health Organization (WHO) *N. gonorrhoeae* reference strain *(15)*.

β-Lactamase tests (for the detection of plasmid-mediated resistance to penicillin)

Several tests may be used to determine whether an isolate produces β-lactamase (e.g. iodometric, phenol red) *(11)*. The easiest is the chromogenic cephalosporin (nitrocefin) test. Additionally to the nitrocefin disk and solution methods, the acidometric method and iodometric test are also used.

Acidometric method

Penicillin solution:

0.05 mol/L phosphate buffer, pH 8.0.

0.2 g/L bromocresol purple.

50 g/L buffer-free benzylpenicillin (store-frozen).

Iodometric test

Penicillin-iodine mixture (prepare fresh):

Add 1.1 ml of iodine solution (1.5 mg potassium iodine and 0.3 g iodine in 100 ml of 0.1 mol/L phosphate buffer, pH 6.4 [store in a brown bottle at 4 °C], to a vial containing 0.15 ml benzylpenicillin solution [1 million IUs per ml, stored at −20 °C]).

Starch solution:

4 g/L in distilled water, autoclave and store at 4 °C.

Bovine serum albumin (commercially available)

Formaldehyde saline fixative *(1)*

Formaldehyde 37% solution	10 ml
Sodium chloride 0.85% solution	90 ml

1. Neutralize the pH of commercial formaldehyde solutions to pH 7.0 with 5 g/L sodium carbonate solution.
2. Test with pH indicator paper.
3. Mix 10 ml of 37% formaldehyde solution with 90 ml of 0.85% sodium chloride solution.

Carbohydrate use tests *(16)*

Ingredients per 1 L of cysteine trypticase agar (CTA)

By Difco	
Tryptone	20 g
L-cysteine	0.5 g
Sodium chloride	5.0 g
Sodium sulphite	0.5 g
Agar	2.5 g
Phenol red	17 mg
By BBL	
Pancreatic digest of casein	20 g
L-cysteine	0.5 g
Sodium chloride	5.0 g
Sodium sulphite	0.5 g
Agar	2.5 g
Phenol red	17 mg

1. Suspend 28.5 g of CTA powder in 1 L distilled water. Mix thoroughly.
2. Heat with frequent agitation and boil for one minute to completely dissolve the powder.
3. Autoclave at no more than 118 °C for 15 minutes.
4. Add 5–10 g of carbohydrate, that is, glucose, maltose, sucrose or lactose before autoclaving or dissolve medium in 900 ml water, then autoclave and aseptically add 100 ml sterile 5–10% carbohydrate solution.
5. Mix the contents of the flask with a gentle swirling motion and distribute into presterilized 10-ml screw-capped tubes aseptically.
6. Cool the tubes in a slanted position to provide a slope and allow the medium to solidify at room temperature.
7. Store tubes at 4 °C.
8. Test the prepared medium for performance using stable, typical control cultures as specified by the manufacturer

Commercial kits are available.

Catalase test *(17)*

Slide (drop) method

1. Place a microscope slide inside an empty Petri dish.
2. Place a small amount of bacteria from an 18- to 24-hour-old isolated colony on the slide, using a bacteriological loop or a wooden applicator stick. Avoid carryover of agar.
3. Add a drop of 3% hydrogen peroxide (H_2O_2) to the bacteria on the slide. Do not mix.

4. Cover the Petri dish with the lid and observe for bubble formation. A microscope (40× magnification) or a magnifying glass may be required to observe weak positive reactions.

Tube method

1. Dispense 4–5 drops of 3% hydrogen peroxide (H_2O_2) to a test tube.
2. Place a small amount of bacteria from an 18- to 24-hour-old isolated colony into the tube, using a bacteriological loop or a wooden applicator stick. Avoid carryover of agar.
3. Observe for bubble formation at the end of the wooden applicator stick or bacteriological loop. Placing the tube against a dark background can enhance visibility.

Tube (slant) method

1. Obtain a 18–24 hour heavily inoculated pure culture grown on a nutrient agar slant.
2. Add 1 ml of 3% hydrogen peroxide (H_2O_2) onto the agar.
3. Observe for bubble formation against a dark background.

Ensure using microorganisms known to be catalase-positive and catalase-negative as controls.

Eosin Y Solution *(18)*

Stock solution

1. Suspend and completely dissolve 2 g eosin Y (water-soluble) in 40 ml double-distilled water.
2. Mix 160 ml of 95% ethanol.
3. Store at room temperature.

Working solution

1. Mix 200 ml of stock solution with 600 ml of 80% ethanol.
2. Add 4 ml of glacial acetic acid and mix well (in a fume hood).
3. Store at room temperature.

Germ tube test *(19,20)*

1. Suspend a yeast colony (using a wooden applicator stick) in 0.5 ml of FCS to make a light suspension.
2. Incubate the tube for 2–3 hours at 37 °C.
3. Transfer a drop of the suspension onto a microscope slide.
4. Place a coverslip.
5. Examine microscopically using 40× magnification.

The following control strains should be included when the test is performed:

- *Candida albicans* ATCC strain 14053 – Germ tube-positive;
- *Candida tropicalis* ATCC strain 66029 – Germ tube-negative.

Glucose-potassium-sodium-phosphate solution

This is HBSS without Ca^{2+} and Mg^{2+} (commercially available)

10× stock

NaCl	80.0 g
Glucose	10.0 g
KCl	4.0 g
KH_2PO_4	0.60 g
Na_2HPO_4	0.48 g
Phenol red stock (optional)	100 ml
H_2O	900 ml

1. Mix the ingredients.
2. Autoclave and store 10×stock at 4 °C until use; stock solution expires after six months.
3. Working solution is made by diluting the 10× stock 1:10 with H_2O.

Indole test *(21)*

1. Inoculate a tryptone tube with a small amount of bacteria from a pure colony.
2. Incubate for 24–48 hours at 35 °C ± 2 °C.
3. Add five drops of Kovács reagent to the tube.
4. Observe for pink/red colour formation in the reagent layer on top of the medium.

Tryptone broth

1. Dissolve 10 g tryptone and 5 g sodium chloride in 1 L distilled water.
2. Dispense 4 ml per tube.
3. Autoclave at 121 °C for 15 minutes.
4. Store at 4–10 °C.

Kovács reagent (prepare fresh; commercially available)

1. Dissolve 10 g *para*-dimethylaminobenzaldehyde (DMAB) in 150 ml reagent-grade amyl or isoamyl alcohol (butyl alcohol can be substituted).
2. If necessary, gently heat the solution to completely dissolve DMAB.
3. Slowly add 50 ml concentrated HCl.
4. Transfer the pale yellow colour solution to a brown glass bottle and store in the refrigerator.

Indole spot test

1. Inoculate the bacteria on a tryptophan-containing medium (i.e. trypticase soy agar, sheep blood agar) and incubate for 18–24 hours at the suitable temperature.
2. Place a piece of Whatman filter paper in an empty Petri dish.
3. Add 1–1.5 ml Kovács reagent onto the filter paper.
4. Smear the paper with bacteria grown for 18–24 hours.
5. Observe for pink/red colour formation.

Other methods for detecting indole production include tryptophan peptone broth, Ehrlich's reagent, motility-indole-ornithine medium and sulfide-indole-motility medium.

Latex agglutination test *(22)*

- For antibody detection – antigen bound to the surface of latex beads;
- For antigen detection – antibody bound to the surface of beads.

This protocol describes coating antigen with latex to detect antibodies.

Coating of latex

1. In a 1.5-ml tube, combine 40 µl of glycine-saline buffer with 20 µl of latex beads.
2. Add 60 µl of antigen to the latex from step 1 and incubate at 37 °C for 2 hours.
3. Centrifuge for 10 minutes at 5000 rpm.
4. Aspirate the supernatant and resuspend the pellet in 1 ml blocking buffer.
5. Centrifuge for 10 minutes at 5000 rpm.
6. Repeat the washing step once more.
7. Mix 90 µl blocking buffer with the pellet.
8. Incubate overnight at 4 °C.

For agglutination

1. Mix 200 µl of glycine-saline buffer and 4 µl of test antiserum (diluted 50 times).
2. In a 1.5-ml vial, combine 50 µl of antigen and 50 µl of diluted antiserum.
3. Incubate for 10 minutes at room temperature.
4. Add 10 µl of coated latex onto a glass slide.
5. Add 10 µl of diluted test antiserum to slide A.
6. Add 10 µl of antiserum mixed with antigen (step 1) to slide B.
7. Add 10 µl of glycine-saline buffer to slide C.
8. Using tooth picks, mix the content on each slide separately.
9. Incubate for 2 minutes at room temperature and record the results.

McFarland turbidity standards *(23)*
(commercially available)

McFarland turbidity standards are prepared by mixing the following solutions at different ratios to obtain the desired McFarland turbidity scale (e.g. 0.5, 1, 2).

McFarland turbidity scale	**0.5**	**1**	**2**	**3**	**4**
Anhydrous barium chloride 1% solution (w/v)	0.05 ml	0.1 ml	0.2 ml	0.3 ml	0.4 ml
Sulphuric acid 1% solution (v/v)	9.95 ml	9.9 ml	9.8 ml	9.7 ml	9.6 ml

Tightly seal the tubes and store at room temperature in the dark (these will remain stable for six months). Before use, invert the tubes several times to suspend the barium precipitates.

For comparison, use a background with horizontal black and white stripes.

Nitrate assimilation test *(24)*

Components of the commercial yeast carbon agar

Dextrose	10.0 g
Monopotassium phosphate	1.0 g
Magnesium sulfate	0.5 g
Calcium chloride	0.1 g
Sodium chloride	0.1 g
Inositol	2.0 mg
Methionine	2.0 mg
Tryptophan	2.0 mg
L-Histidine monohydrochloride	1.0 mg
Boric acid	0.5 mg
Calcium pantothenate	0.4 mg
Manganese sulfate	0.4 mg
Niacin	0.4 mg
Pyridoxine hydrochloride	0.4 mg
Thiamine hydrochloride	0.4 mg
Zinc sulfate	0.4 mg
Ferric chloride	0.2 mg
para-Aminobenzoic acid	0.2 mg
Riboflavin	0.2 mg
Sodium molybdate	0.2 mg
Potassium iodide	0.1 mg
Copper sulfate	40.0 µg
Biotin	2.0 µg
Folic acid	2.0 µg
Noble agar	20.0 g
Distilled water	Add up to 1 L

Auxanographic method

1. Suspend a yeast colony in sterile distilled water to yield a concentration equivalent to McFarland standard 1.
2. Melt a tube of yeast carbon agar (YCA) in boiling water and cool to 45–50 °C.
3. Add 1 ml of the yeast suspension to the cooled YCA.
4. Mix and dispense into a Petri dish.
5. Once the agar is solidified, divide the plate in half and label one side as peptone (positive control) and the other side as potassium nitrate (KNO_3).
6. Carefully place disks containing 1% peptone and 1% KNO_3 on the appropriate side of the agar surface.
7. Aerobically incubate at 25–30 °C, ensuring to keep the agar surface side up.
8. Examine the growth daily up to 4 days.

Nitrate reduction test (1)

1. Prepare a bacterial suspension equivalent to McFarland standard 3.
2. Transfer 0.04 ml of the bacterial suspension to a small tube.
3. Add 0.04 ml of 0.5 g/L sodium nitrate solution.
4. Add 0.04 ml of 0.025 mol/L phosphate buffer, pH 6.8.
5. Incubate for one hour in a 37 °C waterbath.
6. Add 0.06 ml of 8 g/L sulphanilicacid in 5 mol/L acetic acid.
7. Add 0.06 ml of 5 g/L of α-naphthylamine in 5 mol/L acetic acid.
8. Shake the tube and observe for a colour change.

Oxidase test *(25,26)*

Tetra-methyl-ρ-methylenediamine dihydrochloride	0.5 g
Distilled water	50 ml

1. Dissolve the substrate in 50 ml distilled water.
2. Place a filter paper in a Petri dish and saturate with the reagent.
3. Pick a portion of the colony to be tested using a platinum wire and rub on the filter paper.

Interpretation: A positive result is obtained when a deep purple colour appears within 10 seconds.

Oxidase reagents can be purchased commercially.

Filter paper method

1. Crush the glass ampoule containing the reagent inside the dispenser by squeezing the sides of the tube.
2. Label the filter paper with the number of the test microorganism and wet the filter paper with the reagent.
3. Pick a colony from an overnight culture with a sterile loop or applicator stick.
4. Rub the inoculum onto the reagent-saturated filter paper.
5. Examine for the appearance of a dark purple colour (positive reaction) within 10–30 seconds.

Direct agar plate method

1. Add 2–3 drops of oxidase reagent directly onto agar plates containing an overnight culture.
2. Examine for rapid colour change from pink to maroon to dark purple within 10–30 seconds.

Interpretation: Oxidase-positive microorganisms produce a purple colour within 30 seconds. Oxidase-negative microorganisms produce a light pink colour or remain colourless.

Phadebact monoclonal GC test *(27)*

In the Phadebact monoclonal GC test, two pools of murine monoclonal antibodies are separately mixed with the protein A of non-viable *Staphylococcus*, which permits the subgrouping of gonococcal isolates into WI (PorB1a [IA]) and WII/WIII (PorB1b [IB]) groups.

1. Remove freshly grown colonies of *N. gonorrhoeae* that have been presumptively identified and suspend them in 0.5 ml of 0.9% sterile phosphate-buffered saline (PBS) solution (see above) to 0.5 McFarland turbidity. Use a test tube with a cap.
2. Place the closed tube in a boiling waterbath for at least 5 minutes and then cool to room temperature.
3. Before using the gonococcal reagent, shake thoroughly. Put one drop of the WI and one drop of the WII/WIII reagents, respectively, on a slide.
4. Add one drop of the boiled gonococcal suspension to the WI reagent and one drop to the WII/WIII reagent. Be sure to include a negative control to which only PBS has been added.
5. Mix the drops thoroughly but gently with a fresh disposable loop. Use a fresh loop for each reagent.
6. Rock the slide and read the result within one minute.

Interpretation: A precipitate with either the WI or WII/WIII gonococcal reagent constitutes a positive result. A positive reaction with both reagents is an equivocal result that will need to be re-tested. If no reaction occurs with either the WI or WII/WIII gonococcal reagent, the test result is negative. (A negative result suggests that the isolates tested are not *N. gonorrhoeae*.)

Phosphate buffered saline (PBS) solution

Sodium chloride	9.0 g
Potassium chloride	0.2 g
Disodium hydrogen phosphate	1.15 g
Potassium dihydrogen phosphate	0.2 g
Distilled water	Add to make 1 L

To make 1×PBS solution:

1. Dissolve the ingredients in 800 ml distilled water.
2. Adjust pH as required (e.g. 7.4, 6.8).
3. Adjust volume to 1 L with additional distilled water.
4. Sterilize by autoclaving.

PBS is also available commercially.

Porphyrin test *(1)* (requirement for haemin [X factor])

1. Prepare a bacterial suspension equivalent to McFarland standard 3 in 0.5 ml of 2 mmol/L δ-aminolevulinic acid hydrochloride in 0.1 mol/L phosphate buffer, pH 6.9, containing 0.8 mmol/L magnesium sulfate solution.
2. Incubate for 4 hours at 37 °C in a waterbath.

3. Expose the solution to Wood's light (360-nm wavelength) in a dark room.

For bacteria other than *H. ducreyi*, haemin-impregnated discs or strips can be used to assess the haemin requirement.

Potassium hydroxide (KOH) solution 10% *(28)*

Dissolve 10 g potassium hydroxide (KOH) in 100 ml distilled water. This solution is stable for up to 2 years. Note that KOH is highly corrosive.

Rapid carbohydrate use test *(29)*

The following reagents are commercially available:

Lysed blood agar (LBA) medium with 0.5% glucose.

Buffered balanced salt indicator solution (BSS).

Carbohydrate solutions:

10% glucose (G)

10% lactose (L)

10% sucrose (S)

10% maltose (M)

Ampicillin solution (200 mg/ml) for the β-lactamase production test.

A pure culture for the identification of suspected *N. gonorrhoeae* is obtained by subculturing a single colony on an LBA medium containing 0.5% glucose.

Emulsify two full 10-µl loops of the isolate from a pure overnight growth culture into a tube containing 1.5 ml BSS and mix well with a Pasteur pipette to obtain 10^9 microorganisms per ml.

Mark six wells of a microtitre plate as C (control), G, L, M, S and P'ase (penicillinase).

Add 25 µl of 10% sterile carbohydrate solution to the G, L, M, S wells and 25 µl of ampicillin solution to the P'ase well. The first well without any sugar will serve as the control.

Add 100 µl (four drops) of bacterial suspension to each of the six wells.

Read after *2–4* hours of incubation at 3*5–3*7 °C in air (not in CO_2). It is recommended that the β-lactamase reaction is examined again after 24 hours because slow β-lactamase reactions occur with occasional strains.

Note: If *N. gonorrhoeae* is suspected and a doubtful result is obtained with the rapid carbohydrate use test, check the purity of the culture and confirm the identity by using other tests, such as serological tests.

RapID NH system *(30)*

Bacteria should be grown as pure culture on non-selective (nutrient agar, chocolate agar, tryptic soy agar ± 5% sheep blood) or selective (Thayer–Martin agar, NYC agar, Martin-Lewis agar) media and confirmed with Gram stain and oxidase test before the test. When using the 1-hour procedure, only use selective agar.

1. Using a bacteriological loop or a cotton swab, suspend bacteria equivalent to McFarland standard 3 in 1 ml RapID inoculation fluid. Use 18–24-hour cultures. The suspension must be used within 15 minutes after preparation.
2. Inoculate a purity plate using this bacterial suspension. Incubate the plate at 35–37 °C for 18–24 hours.
3. Peel back the lid of the RapID NH system over the inoculation port.
4. Transfer 1 ml of inoculation fluid with bacteria onto the upper right-hand corner of the system. Re-seal the lid.
5. Place the system on a flat surface and tilt back (away from the test cavities) at a 45-degree angle.
6. To evenly distribute the inoculum, rock the system from side to side.
7. Slowly tilt the system forward until all the inoculum flows into the reaction cavities.
8. Place the system on a flat surface.
9. Incubate in a non-CO_2 incubator for 4 hours at 35–37 °C. The system can be placed on the chipboard incubation trays provided with the kit.
10. After incubation, add the reagents provided and score the panels according to the manufacturer's instructions, which are provided with the kit.

Sodium azide *(31)*

To prepare a 10% stock solution, dissolve 10 g sodium azide in 100 ml distilled water. The solution can be stored at room temperature.

Sörensen's citrate-sodium hydroxide buffer (0.01 mol/L)

1. Dissolve 2.1 g citric acid monohydrate in 20 ml of 1 mol/L NaOH.
2. Add distilled water to 1 L (=0.01 mol/L disodium citrate).
3. Adjust to pH 5.6.

To prepare 100 ml of working solution, add 69.3 ml of the above solution to 30.7 ml of 0.01 mol/L HCl.

SP4 medium *(32)*

Ingredients in commercial preparations

Pancreatic digest of casein	10.0 g
Pancreatic digest of gelatin	5.0 g
PPLO broth without crystal violet	3.5 g
Polymyxin B	50.0 mg
Amphotericin B	5.0 g
FCS	170.0 ml
Connaught Medical Research laboratories 1066 medium 10X	50.0 ml
Yeast extract	35.0 ml
Yeastolate 10%	20.0 ml
Penicillin	1 000 000 U
Phenol red	18.0 mg/L
Deionized water	690 ml

SP4 broth/agar with glucose: Glucose 5.0 g/L, pH 7.4 ± 0.2 at 25 °C

SP4 broth with arginine: Arginine 5.0 g/L, pH 7.4 ± 0.2 at 25 °C

SP4 broth with urea: Urea 1.0 g/L, pH 6.0 ± 0.2 at 25 °C

SP4 agar: Agar 9.0 g/L

Thiazine stain *(33)*

Ingredients in commercial preparations: Methylene blue, azure b, potassium phosphate monobasic, sodium phosphate monobasic, sodium azide, Triton X-100, polydimethylsiloxane and methyl cellulose. Solution pH 6.8.

Urease test *(34)*

Christensen's urea agar method

1. Using a pure culture grown for 18–24 hours, heavily streak an entire Christensen's urea agar slant. Make sure not to stab the butt.
2. Incubate the tubes (with caps loosened) at 35 °C.
3. Observe for colour change at 6 hours, 24 hours and daily up to 6 days. Avoid over-incubation because this can lead to false-positive results due to protein hydrolysis.

Recipe for Christensen's urea agar

Peptone	1 g
Dextrose	1 g
Sodium chloride	5 g
Potassium phosphate, monobasic	2 g
Urea	20 g
Phenol red	0.012 g
Agar	15–20 g

1. Dissolve the ingredients, except agar, in 100 ml distilled water and filter-sterilize using 0.45-mm filters.
2. Dissolve the agar in 900 ml distilled water. Boil to dissolve completely and autoclave at 121 °C for 15 minutes, and cool to 50–55 °C.
3. Add the filter-sterilized urea base (step 1) to the cooled agar and mix well.
4. Add 4–5 ml per sterile tube (13 × 100 mm) and slant the tubes. Let agar solidify.
5. Store at 4–8 °C.
6. Warm at room temperature. Do not re-heat because this can decompose urea.

Stuart's urea broth method

1. Using a pure culture grown for 18–14 hours, heavily inoculate a tube of Stuart's urea broth.
2. Shake the tube gently and incubate (with caps loosened) at 35 °C.
3. Observe for colour change at 8, 12, 24 and 48 hours.

Recipe for Stuart's urea broth

Yeast extract	0.1 g
Potassium phosphate, monobasic	9.1 g
Potassium phosphate, dibasic	9.5 g
Urea	20 g
Phenol red	0.01 g

1. Dissolve the ingredients in 1 L distilled water and filter-sterilize (0.45-mm filter).
2. Add 3 ml per sterile tube (13 × 100 mm).
3. Store at 4–8 °C.
4. Warm at room temperature. Do not reheat because this can decompose urea.

Rapid urease test kits are commercially available.

Venereal Disease Research Laboratory (VDRL) test *(35)*

Prepare fresh:

1. Dispense 0.4 ml of VDRL-buffered saline to the bottom of a round glass-stoppered bottle (30 ml) with a flat inner bottom.
2. Slowly dispense 0.5 ml VDRL antigen (drop by drop) onto the saline in the bottle; during this process gently, but continuously rotate the bottle on a flat surface.
3. Pipette 4.1 ml of buffered saline.
4. Cap and shake the bottle using a bottom-to-top-to-bottom motion (30 times in 10 seconds).
5. Use the suspension within 8 hours, making sure to gently swirl the bottle every time the antigen is used.

VDRL antigen

- This is a colourless alcoholic solution containing 0.03% cardiolipin, 0.9% cholesterol and 0.21% ± 0.01% lecithin.
- The antigen should be stored either in the dark at room temperature or refrigerated at 2–8 °C, but not frozen.
- Bottles or vials that contain precipitate should be discarded.

VDRL-buffered saline, pH 6.0 ± 0.1 (1.0% NaCl) (commercially available)

- This contains 0.5 ml formaldehyde neutral (ACS), 0.037 g Na_2HPO_4 anhydrous, 0.170 g KH_2PO_4, 10 g NaCl and 1 L distilled water.
- The solution should be adjusted to pH 6.0 ± 0.1 and stored in screw-capped bottles.

0.9% Saline

- Add 0.9 g dry sodium chloride to 100 ml distilled water.

10.0% Saline

- Add 10 g dry sodium chloride to 100 ml distilled water.

Vitox *(36)*

Ingredients in commercial preparations

Vitamin B_{12}	0.1 mg
Adenine	10.0 mg
L-Glutamine	100.0 mg
Guanine	0.3 mg
para-Aminobenzoic acid	0.13 mg
L-Cystine	11.0 mg
Nicotinamide adenine dinucleotide (coenzyme 1)	2.5 mg
Cocarboxylase	1.0 mg
Iron (III) nitrate	0.2 mg
Thiamine hydrochloride	0.03 mg
Cysteine hydrochloride	259.0 mg

Vitox rehydration fluid

Distilled water	10.0 ml
Glucose	1.0 g

1. Combine one vial of Vitox rehydration fluid with one vial of Vitox.
2. Mix well.
3. Cool the desired growth medium to 50 °C.
4. Add the supplement to 500 ml (2% v/v) or 1 L (1% v/v) of the desired medium.

Stains

Giemsa stain (10%) *(26)*

1. Prepare the smear on the slide.
2. Fix with methanol for 2–3 minutes.
3. Cover with diluted stain for 10 minutes (Leishman's stain) or 30 minutes (Giemsa stain).
4. Rinse in flowing buffered water or PBS, pH 7.0–7.2.
5. Air-dry.

Preprepared stains are commercially available.

Stock solution

Giemsa powder	0.5 g
Glycerol	33 ml
Methanol absolute, acetone free	33 ml

1. Dissolve the Giemsa powder in glycerol by placing the mixture in a 55 °C waterbath for 90 minutes.
2. When crystals are dissolved, add absolute methanol.
3. Store at room temperature.

Working solution (prepare immediately before use)

Stock solution	1 ml
Phosphate buffer	23 ml

Phosphate buffer

Solution 1 (0.1 mol/L):

$Na_2HPO_4 \cdot 2H_2O$	17.84 g

Make up to 1 L with distilled water.

Solution 2 (0.1 mol/L):

KH_2PO_4	13.62 g

Make up to 1 L with distilled water.

1. Mix 56.1 ml of solution 1 with 43.9 ml of solution 2.
2. Add 900 ml of distilled water.
3. Adjust to 6.9 pH.

Rapid 1-minute Giemsa method

1. Prepare the smear on the slide.
2. Dip five times in a fixative.
3. Dip six times in eosin solution (see recipe above).
4. Dip six times in a thiazine dye mixture (see recipe below).
5. Rinse with phosphate buffer, pH 6.8.

Gram stain (1)

The Gram stain procedure is described in Chapter 2 and is also reported in this annex as this stain procedure is also used for other pathogens. The reagents can be purchased commercially or prepared in-house *(1)*. Ready-to-use kits are also commercially available.

Crystal violet solution (primary stain)

Iodine solution (mordant stain)

Acetone-ethanol (decolorizing agent)

Safranin solution (counterstain)

1. Cover the fixed smear with crystal violet for 30 seconds. Gently rinse with cold tap water.
2. Flood the slide with iodine solution for 30 seconds. Gently rinse with cold tap water.
3. Decolourize with acetone (fast and harsh), acetone-ethanol or 95% ethanol (slow and gentle) alone until the purple colour stops flooding out of the smear. It is best to hold the slide in a gloved hand near running water. The time of discolouration will depend on which agent is used and the thickness of the smear and will be shortest (typically a few seconds) for acetone and require longer (up to a minute) for ethanol. Excessive discolouration must be avoided because Gram-positive bacteria may appear as Gram-negative. Disregard the thick portions of an uneven smear, which may stain blue.
4. Rinse quickly under running water to stop the discolouration and drain off excess water.
5. Counterstain with safranin, neutral red or fuchsin for one minute.
6. Rinse with running water and gently blot the slide with absorbent paper.

Crystal violet solution

Solution A (10%)		**Solution B (10%)**	
Crystal violet powder	2 g	Ammonium oxalate	0.8 g
Ethanol 95%	20 ml	Distilled water	80 ml

1. Mix solutions A and B to produce crystal violet staining reagent. Store for 24 hours and filter through paper.

Acetone-ethanol (1:1 ratio)

Acetone	50 ml
Ethanol 95%	50 ml

Iodine solution

Potassium iodide	2 g
Iodine crystals	1 g
Distilled water	300 ml

1. Most references suggest grinding iodine and potassium iodide in a mortar.
2. Add water slowly as the chemicals are being ground until the iodine is dissolved.

Safranin solution

Stock solution		**Working solution**	
Safranin	5 g	Stock solution	10 ml
Ethanol 95%	100 ml	Distilled water	90 ml

Leishman stain

The Leishman stain can be used as an alternative to the Giemsa stain for *Klebsiella granulomatis* (donovanosis) after fixation of the material on the slide with methanol.

Stock solution

1. Add 1.5 g Leishman powder to 1 L methanol and mix well, using a few glass beads.
2. Allow the stock solution to stand at room temperature for 24 hours.

Working solution

1. Prepare a fresh daily working solution by diluting one part of stock solution in two parts of distilled water (buffered water with 3.76 g/L disodium hydrogen phosphate and 2.10 g/L potassium dihydrogen phosphate, pH 7.0–7.2).
2. Store in a tightly closed bottle to prevent moisture entering the stock solution.

Method:

1. Prepare a smear from the ulcer edges (where the Donovan bodies are most likely to be found) on a glass slide.
2. Cover the slide with the working solution Leishman stain for 10 minutes or up to 30 minutes with Giemsa stain.
3. Rinse the slide in buffered water or PBS solution (pH 7.0–7.2).
4. Air-dry the slide and examine under a light microscope using oil immersion (1000× magnification).

MB stain *(37, 38)*

MB	0.3 g
Ethanol	30 ml
Distilled water	100 ml

1. Dissolve dye in ethanol.
2. Add distilled water.
3. Prepare and fix a smear on a glass slide.
4. Flood the slide with the MB stain for one minute.
5. Wash the stain off the slide using running tap water.
6. Rinse, gently blot with absorbent paper and examine microscopically.

MB/gentian violet (GV) stain *(39)*

The MB/GV stain is prepared by adding four parts MB (Loeffler formula) with one part GV (Huker formula).

1. Prepare a thin smear from a clinical gonococcal swab, and the appropriate control culture, on separate labelled glass slides.
2. Heat-fix by passing the slide over a flame.
3. Add MB/GV stain for 30–60 seconds.
4. Rinse, air-dry and examine microscopically.
5. *N. gonorrhoeae* will appear as dark purple cocci or diplococci.

GV dye

1. Dissolve 1 g GV in 20 ml of 95% ethanol.
2. Add 60 ml 5% formalin and dissolve completely.
3. Filter the solution to remove any precipitate.

MB dye

Dissolve 0.5 g MB powder in 100 ml distilled water

Warthin–Starry silver impregnation stain *(40,41)*

1. Cut (i.e. 5 µm) and deparaffinize tissue sections and hydrate to triple-distilled water.
2. Submerge slides containing deparaffinized tissue in 1% silver nitrate solution and microwave until the solution boils, approximately for 45–60 seconds.
3. Submerge the slides in developer solution and microwave for 45–60 seconds. As the developer boils, brown marks will appear in solution.
4. Quickly rinse in hot running water.
5. Rinse twice in distilled water.
6. Dehydrate in graded alcohols.
    d. Submerge in 95% alcohol.
    e. Submerge in absolute alcohol.
7. Clear two to three times in xylene.
8. Mount with synthetic resin (i.e.Permount).

1% Citric acid

Citric acid	1 g
Distilled or deionized water	100 ml

Acidulated water

Distilled or deionized water	500 ml

Add enough of the 1% citric acid to bring the pH to 4.0.

1% Silver nitrate solution for impregnation

2% Silver nitrate solution	25 ml
Acidulated water, pH 4.0	25 ml

2% Silver nitrate solution for developing solution

Silver nitrate	5 g
Acidulated water, pH 4.0	250 ml

5% Gelatin solution

Gelatin, high-grade	2.5 g
Acidulated water, pH 4.0	50 ml

0.15% Hydroquinone solution

Hydroquinone crystals	0.075 g
Acidulated water, pH 4.0	50 ml

Developer solution *(prepare immediately before use)*

2% Silver nitrate solution	12 ml
5% Gelatin solution	30 ml
0.15% Hydroquinone solution	16 ml

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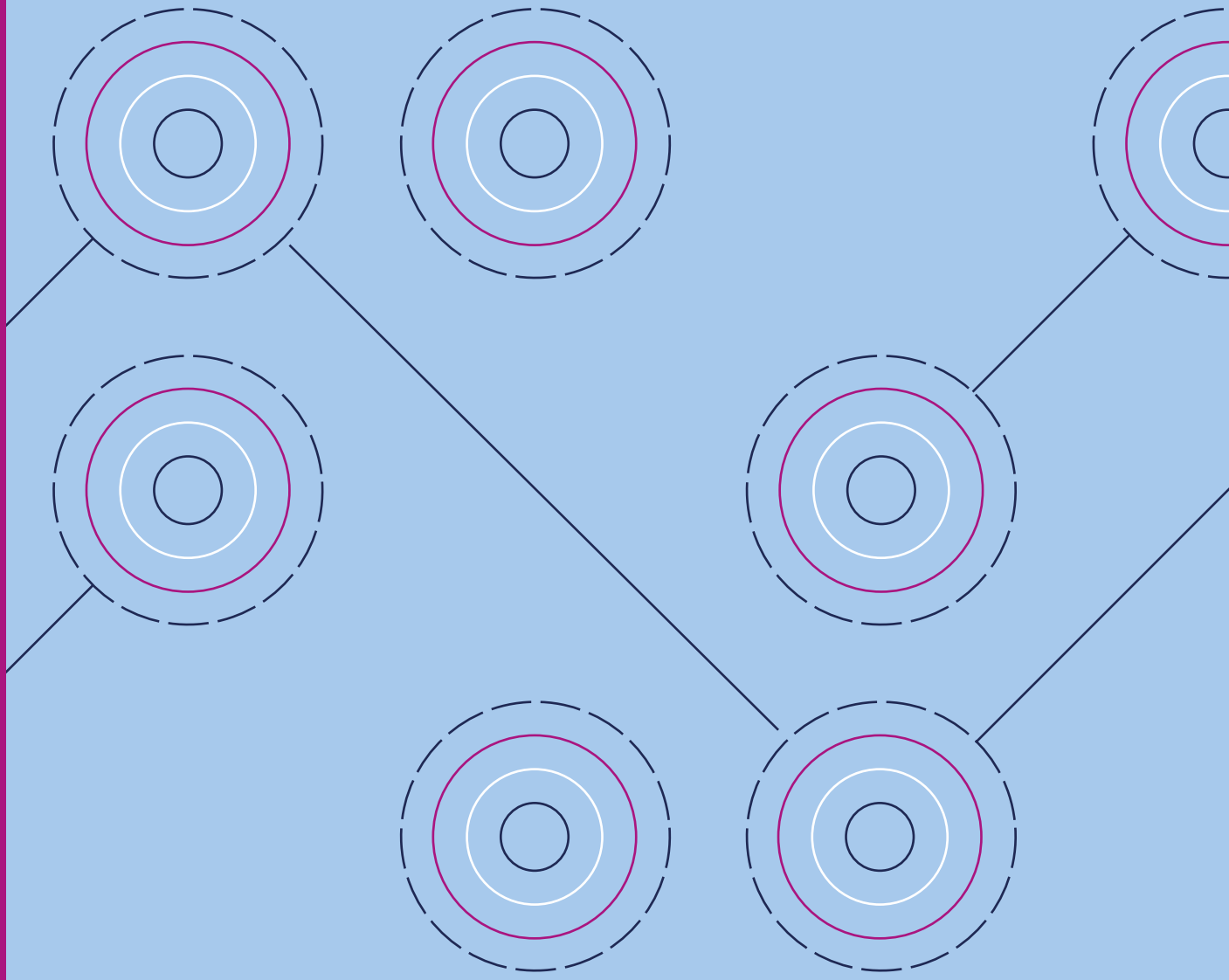
Annex 2. Laboratory supplies



Annex 2. Laboratory supplies

Sumudu Perera and Jo-Anne Dillon

Note: Laboratory supplies are listed in the order they are mentioned in each chapter. Only the product or reagent manufacturer's name has been indicated. This list is not intended as a comprehensive list of every manufacturer. Local distributors are not identified.

Chapter 2. Microscopy and principles of staining

- ***Equipment***
  - Brightfield transmitted light microscope
  - Darkfield or dark-ground microscope (and/or attachments)
  - Dual or triple head or a video facility (to interpret microscopic images)
  - Phase-contrast microscope
  - Gas burner
  - Fluorescence microscope (and/or attachments to a transmitted light microscope)
  - Room that can be darkened
  - Sink
- ***Reagents***
  - Saline
  - Gram stain (crystal violet, Lugol's iodine, decolourizers [alcohol, acetone], counterstain [safranin, neutral red, carbol fuchsin])
  - Alcohol (for fixing slides)
  - Immersion oil
  - Appropriate antibody attached to a fluorochrome
  - Water
  - Methylene blue
  - Giemsa stain
- ***Consumables***
  - Swabs or loops
  - Slides
  - Coverslips
  - Dropper or Pasteur pipettes
  - Bottles (to store Gram stain chemicals)

Chapter 3. Point-of-care (POC) tests and test principles

- ***Equipment***
  - Refrigerator (+4 °C)
  - Centrifuge instrument
  - Real-time polymerase chain reaction (PCR) instrument
- ***Reagents***
  - For specific reagents required for each kit/test refer to the manufacturer's instructions
- ***Consumables***
  - Blood collection needles, syringes, devices
  - Oral fluid collection devices
  - Sterile urine cup
  - Swabs
  - Syringes
  - Plungers
- ***Diagnostic tests***
  - WHO-prequalified rapid diagnostic tests (RDTs) for HIV screening. Professional use only. Specimen types: fingerstick capillary blood, oral fluid, plasma, serum, venous blood.
    - ABON HIV 1/2/O Tri-Line HIV Rapid Test (ABON Biopharm, China)
    - Bioline HIV-1/2 3.0 (Abbott Diagnostics, USA)
    - Determine HIV Early Detect (Abbott Diagnostics, USA)
    - Determine HIV-1/2 (Abbott Diagnostics, USA) – approved by the U.S. Food and Drug Administration (FDA)
    - INSTI HIV-1/HIV-2 Antibody Test (bioLytical Laboratories, Canada) – FDA-approved
    - MERISCREEN HIV 1-2 WB (Meril Diagnostics, India)
    - One Step Anti-HIV (1&2) Test (InTec Products, China)

- One Step HIV 1/2 Whole Blood/Serum/ Plasma Test (Guangzhou Wondfo Biotech, China)
- Diagnostic Kit for HIV (1+2) Antibody (Colloidal Gold) V2 (Shanghai Kehua Bio-Engineering, China)
- DPP HIV-1/2 Assay (Chembio Diagnostic System, USA) – FDA-approved
- First Response HIV 1-2.O Card Test (Version 2.0) (Premier Medical, India)
- Geenius HIV 1/2 Confirmatory Assay (Bio-Rad Laboratories, France)
- HIV 1/2 STAT-PAK (Chembio Diagnostics, USA) – FDA-approved
- HIV 1/2 STAT-PAK Dipstick Assay (Chembio Diagnostics, USA)
- OraQuick HIV-1/2 Rapid Antibody Test (OraSure Technologies, USA)
- Rapid Test for Antibody to HIV (Colloidal Gold Device) (Beijing Wantai Biological Pharmacy Enterprise, China)
- STANDARD Q HIV 1/2 Ab 3-Line Test (SD Biosensor, USA)
- SURE CHECK HIV 1/2 Assay (Chembio Diagnostics, USA)
- TrinScreen HIV (Trinity Biotech Manufacturing, Ireland)
- Uni-Gold HIV Test (Trinity Biotech Manufacturing, Ireland)

WHO-prequalified RDTs for HIV screening – self-testing:

- CheckNow HIV (Abbott Diagnostics, USA) – fingerstick capillary blood
- INSTI HIV Self Test (bioLytical Laboratories, Canada) – fingerstick capillary blood
- Mylan HIV Self Test (Atomo Diagnostics, Australia) – fingerstick capillary blood
- OraQuick HIV Self-Test (OraSure Technologies, USA) – FDA-approved, oral fluid
- SURE CHECK (Chembio Diagnostics, USA) – fingerstick capillary blood
- Wondfo HIV Self-Test (Guangzhou Wondfo Biotech, China) – fingerstick capillary blood

RDTs for syphilis screening:

- Alere Determine Syphilis TP (Abbott Laboratories, USA) – whole blood (fingerstick), plasma, serum
- Reveal TP (Syphilis) (MedMira Laboratories, Canada) – whole blood (venous, fingerstick), plasma, serum
- SD Syphilis 3.0 (SD Bioline/Abbott Laboratories, Republic of Korea) – whole blood (venous, fingerstick), plasma, serum
- Syphicheck-WB (Tulip Diagnostics/Qualpro, India) – whole blood (venous, fingerstick), plasma, serum
- VISITECT SYPHILIS (Omega Diagnostics, United Kingdom) – whole blood (venous, fingerstick)

Multiplex RDTs for syphilis and HIV/syphilis

- Non-*Treponema pallidum/T. pallidum* Syphilis Test (Chembio Diagnostics, USA) – CE-marked
- DPP Syphilis Screen and Confirm Assay (Chembio Diagnostics, USA)– whole blood (fingerstick, venepuncture)

WHO-prequalified combined HIV/syphilis screening assays:

- First Response HIV 1+2 syphilis (Premier Medical Corporation, India)
- SD BIOLINE HIV/Syphilis Duo (Abbott Laboratories, USA) –serum, plasma
- STANDARD Q HIV/Syphilis Combo Test (SD Biosensor, Republic of Korea)

Additional combo HIV/syphilis screening assays:

- DPP HIV-Syphilis (Chembio Diagnostics, USA) – serum, plasma
- INSTI Multiplex HIV-1/HIV-2/Syphilis Antibody Test (bioLytical Laboratories, Canada) – serum, plasma
- Multiplo Rapid TP/HIV Antibody Test (MedMira Laboratories, Canada) – serum, plasma
- *OnSite* HIV/Syphilis (CTK Biotech, USA) – serum, plasma, whole blood

Antibody-based RDTs for screening of *Chlamydia trachomatis*. Specimen types: cervical swab, vaginal swab, penile urine.

- ACON Chlamydia (ACON Laboratories, USA)
- aQcare Chlamydia TRF kit (Medisensor, Republic of Korea)
- BioRapid CHLAMYDIA Ag Test (Biokit, Spain)
- Chlamydia Rapid Test SAS (Diagnostics for the Real World, United Kingdom)
- Clearview Chlamydia (Abbott Laboratories, USA)
- Chlamydia Test Card (Ultimed Products, Germany)
- HandiLab-C (HandiLab, USA)
- QuickVue (QuidelOrtho, USA)

Point-of-care (POC) immunoassays for screening of *Neisseria gonorrhoeae*

- ACON CT/NG Duo (ACON Laboratories, USA) – endocervical swab

- ACON NG (ACON Laboratories, USA) – endocervical swab
- BioStar Optical ImmunoAssay-Gonorrhea (Thermo Fisher Scientific, USA) – urine
- OneStep Gonorrhea RapiCard InstaTest (Cortez Diagnostics, USA) – endocervical swab, male urethral swab

Antigen detection-based RDT for *Trichomonas vaginalis*

- OSOM Trichomonas Test (Sekisui Diagnostics, USA) –clinical laboratory improvement amendments waived by the U.S. FDA

Commercially available integrated nucleic acid amplification test (NAAT)-based platforms

- ARIES Systems (Luminex, USA)
- EasyNAT System (Ustar Biotechnologies, China)
- GeneXpert system (Cepheid, USA)
- HG Swift (HiberGene Diagnostics, Ireland)
- binx io Diagnostic System (binx health, USA)
- m-PIMA (Abbott Laboratories, USA)
- SAMBA II (Diagnostics for the Real World, USA)
- Sexual Health Test (Visby Medical, USA)
- SOLANA (QuidelOrtho Corporation, USA)
- Truelab Real Time micro PCR System (Molbio, India)

Chapter 4. Principles of molecular tests for the diagnosis of STIs

- ***Equipment***

Separate rooms for specimen nucleic acid extraction, PCR master mix preparation and testing

Chapter 6. Urogenital mycoplasmas

- ***Equipment***

Centrifuge

Freezer (–70 °C)

Fridge (+4 °C)

Panther system (Hologic, USA)

Cobas 6800/8000 systems (Roche Diagnostics, Switzerland)

Alinity m system (Abbott Molecular, USA)

GeneXpert System (Cepheid, USA)

PCR instrument

- ***Reagents***

SP4 medium or Vero cells for minimum inhibitory concentration testing

PCR reagents

- ***Consumables***

Swabs for collecting samples

Sterile urine cups

Cryovials

- ***Diagnostic tests***

U.S. FDA-approved NAATs:

- Aptima *Mycoplasma genitalium* (AMG) Assay (Hologic, USA) – CE-IVD-marked, urogenital specimens
- cobas TV/MG Test (Roche Diagnostics, Switzerland) – vaginal swabs, male urine
- Alinity m STI Assay (Abbott Molecular, USA) – vaginal swabs, male urine, endocervical swabs

CE-IVD-marked NAATs:

- ResistancePlus MG (SpeeDx, Australia)
- S-DiaMGRes Assay (Diagenode Diagnostics, Belgium)
- RealAccurate TVMGres Assay (PathoFinder, Netherlands)
- Allplex MG & AziR Assay (Seegene, Republic of Korea)

Near-patient NAAT:

- ResistancePlus MG FleXible (SpeeDx, Australia)

Assays detecting *par*C mutations:

- Allplex MG & MoxiR Assay (Seegene, Republic of Korea)
- LightMix Modular *par*C Kit (TIBMolbiol, Germany)
- MGMO qPCR (NYtor, Netherlands)

Chapter 7. Gonorrhoea

- ***Equipment***

Balance

Freezer (–80 °C)

Freezer (–20 °C)

Fridge (+4 °C)

Gas burner

Hot plate

Incubator (36 ± 1 °C, approximately 70–80% humid atmosphere, 5 ± 1% CO_2) – candle extinction jar with moistened cotton wool ball or towels; jar with CO_2-generating envelopes; CO_2 incubator with water bowl; other equipment for enhanced humidity can be used

0.5 McFarland nephelometric standard

37 °C storage for Bijou bottle

Microscope (light microscope)

pH meter

Plastic ruler (mm) or vernier callipers

Steer's replicator (multipoint inoculators or calibrated loop) (CMI-Promex, USA)

Vortex

Water bath

Speculum

Matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) mass spectrometry (MS)

Glass bottle

Polycarbonate screw-capped Bijou bottle (5 ml, plastic)

Autoclave or oven

Air-tight container with desiccant

ETEST applicator tool or forceps

Small vial

Lyophilizer

- ***Reagents***

Immersion oil

Antimicrobial discs (Oxoid)

Antimicrobial powders (Sigma-Aldrich or the pharmaceutical manufacturer, if available)

Brain-heart infusion broth (Oxoid)

Trypticase soy broth

Nutrient broth

Chocolate agar slopes

Liquid paraffin

Cysteine trypticase agar (containing glucose, maltose and sucrose at a final concentration of 1–2%)

ETEST strips (bioMérieux, France) or other commercially available gradient strip tests

GC Medium Base (Difco)

Non-selective GC agar medium

GCVIT medium

Distilled water

0.1 mol/L phosphate buffer, pH 7.0

0.1 mol/L HCL

95% ethanol or glacial acetic acid

Glycerol (15–20%) (Sigma-Aldrich)

Cryoprotective nutritive broth

Penicillin solution

Penicillin-iodine mixture

Starch solution

70% ethanol

Liquid nitrogen

Microbank fluid with cryobeads (commercially available)

Skimmed milk

Gram stain (crystal violet, Lugol's iodine, decolourizers [acetone, acetone-ethanol, ethanol], counterstain [safranin, fuchsin])

Media – these media may be commercially obtained from local suppliers or prepared in-house

- Non-nutritive transport medium (containing charcoal)
- Non-nutritive transport media – Amies, Stuart, Copan ESwab, VCM swabs (stored at +4 °C before transport)
- Nutritive transport media – Jembec, Transgrow, Gono-Pak, InTray GC system (stored at ±36 °C before transport)
- Selective culture agar media – Thayer–Martin, modified Thayer–Martin media, (NYC) agar
- Non-selective culture agar media

Selective culture media with 3–4 mg/L vancomycin

Selective culture media with 2 mg/L vancomycin or 1 mg/L lincomycin

Nutritive transport system with an atmosphere with enhanced concentration of CO_2 – when transit time exceeds 48 hours

IsoVitaleX (BD)/Vitox (Oxoid)

Methylene blue

Absorbent paper

Mueller-Hinton broth (Oxoid), sterile saline or equivalent solution

Nitrocefin discs, nitrocefin solution or lyophilized nitrocefin powder

Oxidase reagent – 1% aqueous solution of tetramethyl-*para*-phenylenediamine dihydrochloride (commercially available BactiDrop oxidase)

0.9% phosphate-buffered saline (PBS)

GC agar base supplemented with 1% defined growth supplement or 1% IsoVitaleX/Vitox or other media, such as Diagnostic Sensitivity Test Agar with 5% lysed horse blood and 1% IsoVitaleX/Vitox

Growth supplement – 1.1 g L-cysteine, 0.03 g guanine HCl, 3 mg thiamine HCL, 13 mg *para*-aminobenzoic acid, 0.01 g B12, 0.1 g cocarboxylase, 0.25 g nicotinamide adenine dinucleotide, 1 g adenine, 10 g L-glutamine, 100 g glucose and 0.02 g ferric nitrate (in 1 L H2O)

External quality assessment specimen panels – United Kingdom National External Quality Assessment Services, Quality Control of Molecular Diagnostics or by informal exchange of samples between laboratories

2016 WHO *Neisseria gonorrhoeae* reference strains (WHO Collaborating Centre for Gonorrhoea and other STIs, Sweden; WHO Collaborating Centre for STI and AMR, Australia; and the National Collection of Type Cultures, United Kingdom)

ATCC 49226 *N. gonorrhoeae* reference strain

- ***Consumables***

Cryovials

Glass slides

Racks for tubes (15 ml or 50 ml) and for cryovials (2 ml)

Charcoal-coated Dacron or Rayon swabs

Swabs (Dacron or Rayon) on plastic shafts – endocervix, vagina, rectum, oropharynx

Swabs (Dacron or Rayon) on aluminium shafts – urethra, conjunctiva

Sterile urine cups

Genelock tubes

Endocervical brushes

Assay-specific collection kits for NAATs

Absorbent paper

Filter paper

Dropper or Pasteur pipettes

Membrane filters (0.2 µm)

90-mm Petri dishes

140- to 150-mm Petri dishes
Plastic bags to store agar plates
Bacteriological loop
Foil

- ***Tests (commercially available kits)***

Chromogenic cephalosporin test
Oxidase test (BactiDrop oxidase)
Carbohydrate use test
Carbohydrate use and detection of pre-formed enzymes

- API NH (bioMérieux, France)
- RapID NH (Remel, Thermo Fisher Scientific, USA)
- Gonochek-II (EY Laboratories, USA)

Antibody detection tests

- GonoGen II (New Horizons Diagnostics Corporation, USA)
- Phadebact monoclonal GC test (Boule Diagnostics, Sweden)

Nucleic acid probe assays

- Gen-Probe PACE 2 (Hologic, USA)
- Digene Hybrid Capture 2 (HC2) CT/NG (Qiagen, Germany)

U.S. FDA-approved NAATs

- RealTime CT/NG (Abbott Molecular, USA) – endocervical samples, vaginal and urethral swabs, urine, conjunctival samples
- Alinity m STI (Abbott Molecular, USA) – endocervical samples, vaginal, anorectal and oropharyngeal
- BD CTQx/GCQx (Becton Dickinson, USA) – endocervical samples, vaginal swabs, urine
- BD CTGCTV2 on MAX (Becton Dickinson, USA) – endocervical samples, vaginal swabs, urine
- Xpert CT/NG (Cepheid, USA) – endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine, conjunctival samples
- Aptima Combo 2 (Hologic, USA) – endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine
- Aptima *N. gonorrhoeae* (Hologic, USA) – endocervical samples, vaginal swabs, urine
- cobas CT/NG (4800) (Roche, Switzerland) – endocervical samples, vaginal and urethral swabs, urine
- cobas CT/NG (6800/8800) (Roche, Switzerland) – endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine
- cobas CT/NG (5800) (Roche, Switzerland) – endocervical samples, vaginal, anorectal and oropharyngeal swabs, urine

NAATs not approved by the U.S. FDA

- Laboratory-developed and commercial NAATs targeting the *cpp*B gene, cytosine DNA methyltransferase gene, *opa* genes and *por*A pseudogene

POC-NAAT approved by the U.S. FDA

- Binx io CT/NG (binx health, USA) – vaginal swabs, penile urine
- Visby Medical Sexual Health (CT/NG/TV) (Visby Medical, USA) – vaginal swabs

Chapter 8. Chlamydial infection

- ***Equipment***

Freezer (−80 °C)
Fridge (+4 °C)
m2000 RealTime System (m2000sp automated sample preparation instrument, m2000rt real-time thermal cycler) (Abbott Molecular, USA)
Alinity m instrument (Abbott Laboratories, USA)
Viper System with XTR Technology (Becton Dickinson, USA)
BD MAX System (Becton Dickinson, USA)
GeneXpert/GeneXpert Infinity (Cepheid, USA)
Panther system (Hologic, USA)
Cobas 4800 systems (Roche Diagnostics, Switzerland)
Cobas 6800/8800 systems (Roche Diagnostics, Switzerland)
Cobas 5800 system (Roche Diagnostics, Switzerland) – for smaller throughput laboratories
AC power adaptor
Access to electricity (110V or 220V power sources)
binx *io* system (binx health, USA)
Enzyme-linked immunosorbent assay (ELISA) microplate reader
Balance
Biohazard containment hood
Centrifuge
Cryovials
Light microscope
Fluorescence microscope
Haemocytometer
Incubator (+37 °C)
Multi-channel pipette
Sonicator
Vacuum flask
Vortex
Heatblock
Hearing and eye protection

- ***Reagents***

Liquid cytology medium (LCM)
Methanol
Avidin/biotin
Horseradish peroxidase

Fluorescently labelled polyclonal antibodies against *C. trachomatis* lipopolysaccharide (Thermo Fisher Scientific, USA)

Trypsin

Microscope mounting fluid

Glucose-potassium-sodium-phosphate solution

Iscove's modified Dulbecco's medium (IMDM-VG) (Sigma-Aldrich, Merck, Germany)

Sucrose-phosphate-glutamate (SPG) storage medium

SPG containing fetal calf serum (FCS) and antibiotics (i.e. vancomycin, gentamicin, nystatin)

Modified Eagle's Medium (MEM-VG)

PBS

McCoy cell lines

- ***Consumables***

Dacron or Rayon swabs on plastic shafts – vaginal, rectum, oropharynx

Dacron or Rayon swabs on plastic shafts, liquid cytology brush or broom – endocervix

Dacron or Rayon swabs on aluminium shafts – urethra, conjunctiva, nasopharynx

Sterile urine cups

Sterile pipettes for culture medium aspiration

Cryovials

Coverslips 22 × 50 mm

Glass slides

Dropper or Pasteur pipettes

Sealing film

Tissue culture flasks

Tissue culture plates (96-well)

Tissue culture vials (5 ml) containing 13-mm glass coverslip

- ***Diagnostic tests***

U.S. FDA-approved commercially available NAATs

- Abbott RealTime CT/NG (Abbott Molecular, USA)– endocervical swabs, vaginal swabs, urethral swabs, urine, conjunctival samples (conjunctival samples only validated not FDA-approved)
- Abbott RealTime CT (Abbott Molecular, USA) – endocervical swabs, vaginal swabs, urethral swabs, urine, conjunctival samples (conjunctival samples only validated not FDA-approved)
- Alinity m STI Assay (Abbott Laboratories, USA) – endocervical swabs, vaginal swabs, anorectal swabs, oropharyngeal swabs, penile urine
- ProbeTec ET CTQx/GCQx (Becton Dickinson, USA) – endocervical swabs, vaginal swabs, urethral swabs, urine
- CTGCTV2 (Becton Dickinson, USA) – endocervical specimen (swab or LCM), vaginal swabs, urine
- Xpert CT/NG (Cepheid, USA) – endocervical swabs, vaginal swabs, anorectal swabs, oropharyngeal swabs, urine, conjunctival samples (conjunctival samples only validated not FDA-approved)
- Aptima Combo 2 Assay for CT/NG (Hologic, USA) – endocervical samples (swabs or LCM), vaginal swabs, anorectal swabs, oropharyngeal swabs, urethral swabs, urine
- Aptima CT Assay (Hologic, USA) – endocervical swabs, vaginal swabs, urine (not available in the USA)
- cobas CT/NG v.2 to use on the cobas 4800 system (Roche Diagnostics, Switzerland) – endocervical specimens (swabs and LCM), vaginal swabs, urethral swabs, first-catch urine
- cobas CT/NG assay for use on the cobas 6800/8800 and 5800 systems (Roche Diagnostics, Switzerland) – endocervical specimens (swabs and LCM), vaginal swabs, anorectal swabs, oropharyngeal swabs, urine

U.S. FDA-approved POC-NAATs

- binx *io* CT/NG assay (binx health, USA) – vaginal swabs, penile urine
- Sexual Health Test (Visby Medical, USA) (CT/NG/TV) – vaginal swabs

U.S. FDA-approved antigen POC tests (should only be used when adequate laboratory facilities are lacking)

- Clearview (Alere, USA)
- QuickVue (Quidel, USA)

Chapter 9. Trichomoniasis

- ***Equipment***

Fridge (+4 °C)

Incubator (+37 °C)

Light microscope

Centrifuge

AC power adaptor

Water bath

Heat block

Alinity m instrument (Abbott Laboratories, USA)

Viper System with XTR Technology (Becton Dickinson, USA)

BD MAX System (Becton Dickinson, USA)

Panther system (Hologic, USA)

Cobas 5800/6800/8800 Systems (Roche Diagnostics, Switzerland)

GeneXpert System (Cepheid, USA)

- ***Reagents***

  LCM

  Modified Diamond's medium

  Kupferberg medium

  InPouch TV Culture System (BioMed Diagnostics, USA)

  Saline

  Amies medium

- ***Consumables***

  Glass slides

  Coverslips

  Dropper or Pasteur pipettes

  Dacron or Rayon swabs on aluminium shafts

  Dacron or Rayon swabs on plastic shafts – for culture and microscopy

  Cotton swabs on wooden shafts – not recommended for POC tests or NAATs

  Sterile urine cups

  Culture tubes

  Anaerobic jar

  Pouch holders for microscopy

- ***Diagnostic tests***

  Antigen-based POC test

  - OSOM Trichomonas Rapid Test (Genzyme Diagnostic, USA) – vaginal swabs

  Culture kit

  - InPouch TV culture system (BioMed Diagnostics, USA)

  POC-NAAT (U.S. FDA-approved)

  - Xpert TV (Cepheid, USA) – endocervical swabs, vaginal swabs, urine
  - Sexual Health Test (Visby Medical, USA) – vaginal swab

  NAAT

  Many of the U.S. FDA-approved laboratory-based chlamydia/gonorrhoea NAATs have claimed to detect *Trichomonas vaginalis.*

  Antibody tests – should not be used for routine diagnosis of trichomoniasis.

  Laboratory-based nucleic acid amplification tests (FDA-approved)

  - Alinity m STI Assay (Abbott Laboratories, USA) – vaginal swabs, endocervical swabs, urine
  - AmpliVue Trichomonas Assay (Quidel, USA) – vaginal swabs
  - ProbeTec ET CTQx/GCQx (Becton Dickinson, USA) – endocervical swabs, vaginal swabs
  - CTGCTV2 (Becton Dickinson, USA) – endocervical swabs, vaginal swabs, urine
  - Aptima TV (Hologic, USA) – endocervical swabs, vaginal swabs
  - cobas TV/MG assay (Roche Diagnostics, Switzerland) – endocervical swab, vaginal swab, urine, meatal swabs
  - SOLANA Trichomonas Assay (Quidel, USA) – vaginal swabs, urine

Chapter 10. Bacterial vaginosis

- ***Equipment***

  Light microscope

  Speculum

  Panther system (Hologic, USA)

- ***Reagents***

  10% potassium hydroxide (KOH)

  Gram stain (crystal violet, Lugol's iodine, decolourizers [alcohol, acetone], counterstain [safranin, neutral red, carbol fuchsin])

  Saline

- ***Consumables***

  Cotton swabs

  Glass slides

  Coverslips

  pH indicator paper strips, that is, Whatman narrow-range pH paper (pH range 3.8–6.0 or 2.0–9.0)

  Dropper or Pasteur pipettes

- ***Diagnostic tests***

  POC tests

  - OSOM BVBlue Test (Genzyme Diagnostics, MA)
  - FemExam (Cooper Surgical, USA)
  - BD Affirm VP (Becton Dickinson, USA)
  - VGTest spectrometry (3QBD, Israel)

  Commercially available NAATs or direct-probe assays

  - Aptima BV Assay (Hologic, USA)
  - Allplex Vaginitis Screening Assay (Seegene, Republic of Korea)
  - Vaginitis Plus (VG+), NuSwab (Laboratory Corporation of America Holdings, USA)
  - SureSwab Advanced Bacterial Vaginosis (BV), transcription-mediated amplification (Quest Diagnostics, USA)
  - BD MAX Vaginal Panel (Becton Dickinson, USA)
  - OneSwab BV Panel (Medical Diagnostic Lab, USA)
  - Bacterial vaginosis PCR kit (ATRiDA, the Netherlands)
  - AmpliSens Florocenosis/Bacterial vaginosis-FRT (InterLab Service, Russia)

Chapter 11. Candidiasis

- ***Equipment***
  - Incubator at 36 °C
  - Light microscope
  - MALDI–TOF MS
- ***Reagents***
  - 10% potassium hydroxide (KOH) solution
  - Bovine or horse serum
  - Gram stain (crystal violet, Lugol's iodine, decolourizers [alcohol, acetone], counterstain [safranin, neutral red, carbol fuchsin])
  - Saline
  - Sabouraud dextrose agar with chloramphenicol or chromogenic agar
  - Transport medium (Amies)
- ***Consumables***
  - Cotton swabs
  - Coverslips
  - Glass slides
  - Narrow-range pH paper strips
  - Dropper or Pasteur pipettes
  - Inoculation loop
  - Test tubes
- ***Diagnostic tests***
  - BD MAX Vaginal Panel (Becton Dickinson, USA)
  - Allplex Vaginitis Screening Assay (Seegene, Republic of Korea)
  - BD Affirm VPIII (Becton Dickinson, USA)

Chapter 12. Herpes simplex virus (HSV) infections

- ***Equipment***
  - Fridge (+4 °C)
  - Freezer (−80 °C) or liquid nitrogen
  - Incubator (+36 °C) with 5% CO_2 atmosphere
  - Cooler box
  - Centrifuge
  - Light microscope
  - Stereoscopic microscope
  - Fluorescence microscope
  - Homogenizer
  - Vortex
  - Moist chamber
  - Mechanical stirrer
- ***Reagents***
  - Universal transport medium
  - Viral transport medium
  - Culture growth medium
  - Ice
  - Primers from HSV DNA sequences common to both HSV-1 and HSV-2 (HSV DNA polymerase [*pol F*], HSV thymidine kinase, glycoprotein B domain)
  - Primes and probes for HSV DNA sequences specific for HSV-1 or HSV-2 (*gG, gD, gI*)
  - Intra-laboratory and inter-laboratory quality controls – certified and registered reference panels comprising coded control specimens
  - Primary human diploid fibroblasts and cell lines (i.e. MRC-5 cells [human fibroblasts]), Vero cells [monkey kidney], Hep-2 cells [laryngeal squamous cell carcinoma], baby hamster kidney cells, rabbit kidney cells)
  - Fluorescein isothiocyanate (FITC)-labelled or IP-labelled type-specific monoclonal antibodies
  - Fluorescein-labelled HSV-1-specific monoclonal antibody
  - Fluorescein-labelled HSV-2-specific monoclonal antibody
  - 5% FCS
  - PBS
  - Glycerol
  - 2SP medium
  - Herpes cell maintenance medium
  - Rabbit or mouse HSV-specific polyclonal or HSV-1-specific or HSV-2-specific monoclonal antibodies
  - Anti-rabbit or mouse horseradish peroxidase-labelled antibody
- ***Consumables***
  - Dacron swabs on wire/plastic shafts
  - Tissue culture roller tubes
  - Shell vials or multi-well plates
  - Tissue culture tubes
  - Glass slides
  - Coverslips
  - Dropper or Pasteur pipettes
  - Polytetrafluoroethylene-coated glass slides
- ***Diagnostic tests***
  - Methods to detect HSV in lesions
    - PCR – skin lesions, vesicular fluid/exudate from vesicle base, mucosal surfaces, aqueous or vitreous humour, cerebrospinal fluid (CSF), blood
    - Loop-mediated isothermal amplification – skin lesions, vesicular fluid/exudate from vesicle base, mucosal surfaces, aqueous or vitreous humour, CSF, blood
    - Helicase-dependent amplification – skin lesions, vesicular fluid/exudate from vesicle base, mucosal surfaces, aqueous or vitreous humour, CSF, blood
    - Immunofluorescence – lesional smear, vesicular fluid/exudate from vesicle base
    - Latex agglutination – skin lesions, vesicular fluid/exudate from vesicle base
    - Viral culture – skin lesions, vesicular fluid/exudate from vesicular base, biopsy material, conjunctival or corneal smear

- Tzanck smear – scraping skin/mucosal lesional base, biopsy material, conjunctival or corneal smear

Methods to detect anti-HSV antibodies in blood

- Haemagglutination – serum
- ELISA – serum
- Western blot – serum
- Multiplex flow immunoassays – serum
- Luciferase immunoprecipitation assay – serum
- Microfluidic-based POC device – whole blood

Diagnostic tests for HSV-2

- Type-specific enzyme-linked immunoassay (EIAs) – HerpeSelect (Focus Technologies, USA)
- Microfluidic-based POC devices for HSV-2-specific antibodies – capillary blood or serum

Chapter 13. Syphilis

- ***Equipment***

Binocular light microscope with 10× eyepieces, 10× objective

Darkfield or dark-ground microscope (and/or attachments)

Fluorescence microscope with 10× eyepieces, 10× and 40× objectives

Darkened room

ELISA reader

Vortex

Centrifuge

Fridge (+4 °C)

Freezer (−20 °C)

Freezer (≤ −70 °C)

Shaker

Rotator for rapid plasma reagin (RPR) test

Waterbath

Incubator

Chemiluminescence device/photomultiplier system

Screw-cap bottles

pH meter

Lyophilizer

30-ml bottles with flat inner-bottom surface or 25-ml stoppered flask

Mechanical rotator adjustable to 180 ± 2 rpm, circumscribing a circle 19 mm in diameter on a horizontal plane

- ***Reagents***

Saline

Acetone or methanol

95% ethanol

Biotinylated goat anti-human immunoglobulin G (IgG)-labelled with streptavidin-peroxidase

Fluorescein-labelled anti-*T. pallidum* immunoglobulin

Biotinylated anti-rabbit immunoglobulin antibodies

Rabbit anti-*T. pallidum* immunoglobulin antibodies

FITC-labelled antihuman immunoglobulin

Immersion oil

Low fluorescence, non-drying immersion oil

Formalin

Paraffin

PBS

PBS pH 7.2 with 2% Tween-80

PBS with 0.5% bovine serum albumin and 0.1% sodium azide

Treponema pallidum passive particle agglutination (TPPA) sample diluent

Peroxidase-conjugated avidin-biotin complex

Horseradish peroxidase

Streptavidin horseradish peroxidase

Enzyme substrate for EIA

Stop solution for EIA

Phycoerythrin conjugated to goat anti-human IgG or isoluminol-antigen conjugate

Biotinylated human or rabbit anti-*T. pallidum* antiserum

Venereal Disease Research Laboratory (VDRL) antigen (0.03% cardiolipin, 0.9% cholesterol, 0.21% ± 0.01% lecithin)

VDRL-buffered saline, pH 6.0 ± 0.1 (1.0% NaCl) – commercially available

0.9% saline

10.0% saline

- ***Consumables***

Blood tubes

Needles and syringes

Syringes 2 ml or 5 ml

Tubes to collect CSF

Tubes to collect saliva

Cotton swabs

Gauze swabs

Gauze

Thin stainless steel or platinum spatula, or bacteriological loop

Paraffin

Formalin

Microscope slides

- Standard
- 25.4 × 76.2-mm, frosted, 1-mm thick, 2 circles, 1 cm inside diameter
- 5 × 7.5 cm, 12 paraffin or ceramic rings, 14 mm in diameter

Coverslips
- Standard
- no. 1, 22-mm square
- Cover for slides while on rotator
- Sponge for RPR test

T. pallidum antigen slides, commercial antigen suspension or *T. pallidum* Nichols strain

Bacteriological loop (2-mm diameter, 26-gauge, platinum)

Absorbent paper

Slide board with moist chamber and paper towels

Staining dishes (glass/plastic, removable slide carriers)

Test tubes (12 × 75 mm) and holders

U-shaped microtitre plates and plate covers

Slide holder for 5 × 7.5 cm slides

Ringmaker to make paraffin rings

- ***Diagnostic tests***

Direct detection methods
- Darkfield microscopy
- Direct fluorescent antibody test
- Immunohistochemistry – avidin-biotin peroxidase complex technique

Commercial NAATs are not approved by the U.S. FDA and are not available in the USA; however, in-house NAATs are available (specimens: any lesion exudate, body fluids and fresh, frozen or fixed and paraffin-embedded tissues)
- SpeeDx PlexPCR VHS (SpeeDx, Australia) – commercially available in Europe and Australia

Serological tests (non-treponemal – detects lipoidal antigen)
- RPR test (Becton Dickinson, USA) – serum, plasma
- Toluidine red unheated serum test (New Horizon Diagnostics, USA) – serum, plasma
- VDRL test – serum, plasma, CSF
- Wassermann reaction test – serum, CSF

Serological tests (treponemal)
- Fluorescent treponemal antibody absorption (FTA-Abs) – serum, plasma, CSF
- *T. pallidum* haemagglutination assay (indirect agglutination assay) – serum, plasma
- Microhaemagglutination assay for *T. pallidum* (indirect agglutination assay) – serum, plasma
- TPPA (Fujirebio Holdings, Japan; indirect agglutination assay) – serum, plasma
- EIA – serum
- Chemiluminescence immunoassay – serum

Commercially available POC or rapid tests

Quantitative non-treponemal serological tests
- Rapid tests (pre-qualified by WHO)
- Treponemal test – whole blood, plasma, serum
- Dual treponemal and non-treponemal (lipoidal antigen) tests – whole blood, plasma, serum (Chembio Diagnostic Systems, USA)
- Dual syphilis and HIV tests – whole blood, plasma, serum

Chapter 14. Lymphogranuloma venereum (LGV)

- ***Tests (commercially available kits)***

A commercial *C. trachomatis* NAAT (Chapter 8) should be used first for specific detection of LGV. If *C. trachomatis*-positive, a subsequent NAAT targeting the unique LGV-specific 36-bp deletion of the *phpH* gene should be used as a secondary confirmation test. When these NAATs are unavailable, *C. trachomatis*-specific serological assays could be used.

Chapter 15. Chancroid

- ***Equipment***

Centrifuge

Freezer (–70 °C) or lyophilizer for long-term storage

Fridge (+4 °C)

Candle jar or incubator (not higher than 35 °C, humidified 5% CO_2-enriched atmosphere or microaerophilic conditions)

Light microscope

McFarland standards (10^8 colony-forming units (CFU)/ml and 10^9 CFU/ml)

Waterbath

Wood's light (wavelength 360 nm)

Dark room

Hot plate

Vortex

Steers replicator

- ***Reagents***

Transport media (Amies or Stuart's medium). A thioglycolate-haemin-based medium containing L-glutamine and bovine albumin maintains the viability of *H. ducreyi* for several days at 4 °C.

Semisolid chocolate agar with and without vancomycin (3 µg/ml)

GC agar base with 1–2% haemoglobin, 5% FCS, 10% cofactors-vitamins-amino acids (CVA) enrichment and vancomycin (3 µg/ml)

Mueller-Hinton agar with 5% chocolate horse blood, 1% CVA enrichment and vancomycin (3 µg/ml)

Charcoal-based medium

Gram stain (crystal violet, Lugol's iodine, decolourizers [alcohol, acetone], counterstain [safranin, neutral red, carbol fuchsin])

Oxidase test (tetramethyl-*p*-phenylenediamine hydrochloride)

Nitrate reduction test (0.5 g/L sodium nitrate solution, 0.025 mol/L phosphate buffer, pH 6.8, 8 g/L sulfanilic acid in 5 mol/L acetic acid, 5 g/L α-naphthylamine in 5 mol/L acetic acid)

Porphyrin test (2 mmol/L δ-aminolevulinic acid hydrochloride in 0.1 mol/L phosphate buffer, pH 6.9, containing 0.8 mmol/L magnesium sulfate)

Alkaline phosphatase (ALP) test (0.3 g/L phenol-free sodium phosphate in 0.01 mol/L Sörensen's citrate-sodium hydroxide buffer, pH 5.6, 5 g/L 2,6-dibromoquinone-4-chlorimide in methanol, 0.3 ml *n*-butanol)

Mueller-Hinton agar or GC agar base enriched with 1% haemoglobin, 5% FCS and 1% IsoVitaleX enrichment supplement

Mueller-Hinton agar or GC agar (without vancomycin)

Antimicrobials for susceptibility testing (sulfamethoxazole, trimethoprim, tetracycline, chloramphenicol, erythromycin, kanamycin (or streptomycin), ciprofloxacin (or fleroxacin), ceftriaxone (or cefotaxime))

Mueller-Hinton agar base

Haemoglobin

Distilled water

1% *H. ducreyi* supplement

Tryptic soy broth

Chocolate agar stabs

Media for long-term storage (skimmed milk, FCS + 10% dimethylsulfoxide, skimmed milk + 20% glycerol)

- ***Consumables***

  Bacteriological loops

  Cotton swabs or dry gauze

  Ice packs to transport specimens at 4 °C.

  Dropper or Pasteur pipette

  Microcentrifuge tubes

  Glass culture tubes

  Strips of filterpaper

  Containers for autoclaving agar

  Petri dishes (internal diameter 9 cm)

  Orange 25 G needle and syringe

- ***Diagnostic tests***

  Phenotypic tests

  - Oxidase test
  - Nitrate reduction test
  - Porphyrin test
  - ALP test
  - Catalase test
  - Indole test
  - Urease test
  - Oxidative fermentation test
  - Aminopeptidase activity
  - Activity with β-naphthylamide derivatives

  No U.S. FDA-approved commercial NAATs are available for the detection of *H. ducreyi*, although several laboratory-developed tests are described in literature.

Chapter 16. Donovanosis (granuloma inguinale)

- ***Equipment***

  Fridge (+4 °C)

  Light microscope

  Punch biopsy forceps

  Incubator (+43 °C)

- ***Reagents***

  95% ethanol

  Giemsa staining reagents (fixative, eosin solution, thiazine dye mixture, phosphate buffer, pH 6.8, methanol, buffered water or PBS, pH 7.0–7.2)

  Leishman's stain reagents

  Warthin–Starry silver impregnation reagent

  Paraffin

  Monocyte/Hep-2 cell culture

  Transport medium including nucleic acid preservatives

  Immersion oil

  Formaldehyde-saline fixative

  Distilled water

  Hot water

  Glycerol

  Acid silver nitrate solution

  Xylene

  Five-day old embryonated chicken eggs

- ***Consumables***

  Glass slides

  Cotton swabs

  Container to put formaldehyde-saline fixative in

- ***Diagnostic tests***

  No commercial NAATs are available. Research-based PCR tests targeting the *phoE* gene and genital ulcer disease have been developed.

Chapter 17. Human papillomavirus (HPV) infections

- ***Equipment***

  Centrifuge

  Vortex

  Light microscope

  Freezer (–70 °C)

  Fridge (+4 °C)

- ***Reagents***

  Acetic acid

HPV-specific DNA probes

Immersion oil

Vial transport medium

Positive and negative cell line controls

Global HPV LabNet TYPING proficiency panels (The International HPV Reference Center) for verification of genotyping accuracy of HPV tests

Global HPV LabNet Screening proficiency panels (The International HPV Reference Center) for screening needs

- ***Consumables***

Glass slides

Swabs

Brushes

Lavage devices

Urine cups

Formalin

Paraffin

- ***Tests (see also Chapter 17)***

Historical methods

Direct-probe hybridization assays (i.e. dot blot, Southern blot)

HPV messenger RNA assay

Aptima HPV test (Hologic, USA) – FDA-approved for cervical cancer screening in combination with cytology. Clinician-collected cervical samples have higher sensitivity than self-collected samples.

HPV NAATs – validated clinically for cervical cancer screening

- HC2 HPV DNA Test (QIAGEN, USA) – FDA-approved. Used as a standard comparator test for clinical validation of other HR HPV DNA tests
  - *care*HPV test (QIAGEN, USA) – low-cost, clinical use. Same technology as HC2 test.
- GP5+/6+ PCR-EIA (Diassay, the Netherlands). Used as a standard comparator test for clinical validation of other HR HPV DNA tests
- Abbott RealTime High Risk HPV Test (Abbott Molecular, USA) – WHO-prequalified for in vitro diagnostics
- Anyplex II HPV HR Detection (Seegene, Republic of Korea)
- BD Onclarity HPV Assay (BD Diagnostics, USA) – FDA-approved for HPV alone primary screening
- Cobas 4800 HPV Test (Roche Molecular Diagnostics, USA) – FDA-approved for HPV alone primary screening
- HPV-Risk Assay (Self-Screen, the Netherlands)
- PapilloCheck HPV-Screening Test (Greiner Bio-One, Germany)
- Xpert HPV (Cepheid, USA) – WHO-prequalified for in vitro diagnostics
- Alinity m HR HPV Assay (Abbott Molecular, USA)
- Cobas 6800/8800 HPV Test (Roche Molecular Diagnostics, USA) – FDA-approved for HPV alone primary screening
- Aptima HPV Assay (Hologic, USA) – FDA-approved only with co-testing with cytology
- The NB care HPV test is prequalified but not formally validated according to Meijer's criteria or VALGENT – FDA-approved only with co-testing with cytology
- CLART HPV 4S (GENOMICA, Saudi Arabia)
- NeuMoDx (NeuMoDx Molecular, a QIAGEN company, USA)
- OncoPredict SCR (Hiantis, Italy)
- Aptima mRNA (Hologic, MA, USA)

Host/viral molecular progression markers (being evaluated)

- Host factor p16INK (p16)
- Ki67
- Methylation markers

In-house, laboratory-developed assays can be used in routine diagnostics.

Chapter 18. Human immunodeficiency virus (HIV) infections

- ***Equipment***

Flow cytometer (single-platform or dual-platform)

Haematology analyser

Fluorescence microscope

Freezer (–20 °C) for storing antibodies

Fridge (+4 °C)

- ***Reagents***

Fluorescein-tagged secondary antibodies

Secondary antibodies conjugated with an enzyme and corresponding substrate

Labelled monoclonal antibodies for flow cytometry (anti-CD4, anti-CD3, anti-CD8 and anti-CD45)

- ***Consumables***

  Blood tubes

  Needles and syringes

  Colour reagent (colloidal gold)

  Cotton-tipped swabs

  Membrane strips (with separated HIV-specific proteins or recombinant proteins or peptides)

  Slide (for HIV-infected fixed cells)

  Filter paper (for spotting blood)

- ***Diagnostic tests***

  Serological tests

  - Enzyme immunoassays (EIAs: first-, second-, third- and fourth-generation). Detect viral antigen p24 and HIV antibodies

  Rapid tests. More than 50 test kits are available worldwide (specimen types: serum, plasma, whole blood, oral fluid).

  - Immunoconcentration devices
  - Lateral flow cassettes or strips

  Confirmatory assays

  - Immunofluorescence assays
  - Western blot (WB)
  - Dot or line immunoassays

  Detection of HIV RNA, DNA or p24

  - Commercial kits for HIV diagnosis of infants
  - POC PCR tests – recommended for resource-constrained settings
  - Viral load testing – the WHO prequalification programme has been assessing commercially available viral load testing platforms; both laboratory-based and POC testing platforms (specimen types: plasma preferred, if not dried-blood spots)
  - Clinical laboratory-based CD4 testing (POC or near-care settings)– HIV viral load testing is the preferred approach for diagnosis and confirming treatment failure. When HIV viral load testing cannot be performed adequately, CD4 testing is recommended.

HIV drug resistance (HIVDR) testing (specimen: plasma, dried-blood spots)

- Genotypic testing
- Sanger sequencing to detect nucleotide sequence changes in the *pol* gene leading to alterations in protease, reverse transcriptase and integrase enzymes
- Commercially available kits for HIV-1 genotyping:
  - ViroSeq HIV-1 Genotyping System (Celera Diagnostics, USA)
  - HIV-1 Genotyping Kit with Integrase (Thermo Fisher Scientific, USA)
- Many next-generation sequencing technologies have been adopted for HIVDR genotyping in research and clinical settings in resource-rich countries, including 454 pyrosequencing technique.
  - Phenotypic testing – Due to costs and lack of a standardized approach for drug resistance testing, phenotypic testing is not recommended.

World Health Organization

Department of Global HIV, Hepatitis and Sexually Transmitted Infections Programmes

Email:
hiv-aids@who.int

Website:
https://www.who.int/teams/global-hiv-hepatitis-and-stis-programmes/about

20, avenue Appia
1211 Geneva 27
Switzerland

www.who.int

9789240077089