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Buruli Ulcer



Diagnosis of
*Mycobacterium
ulcerans* disease

A MANUAL FOR HEALTH CARE PROVIDERS



World Health Organization

This manual was published thanks to financial support from:



Raoul Follereau

The Association Française Raoul Follereau (AFRF), France is an NGO dedicated to leprosy control in 31 countries worldwide. It also supports six research projects on leprosy, including the genome sequencing of *Mycobacterium leprae*. Long before the first International Conference on Buruli Ulcer Control and Research, Yamoussoukro, Côte d'Ivoire, 1998, AFRF had taken up the new challenge of the health and social problems caused by Buruli ulcer, working in Benin and Côte d'Ivoire since 1996. The Association also provides financial assistance to research activities on the genome sequencing of *Mycobacterium ulcerans* and on the drug treatment of the disease. It is now considering supporting other countries, starting with Ghana. AFRF is committed to mobilizing the international support needed to meet the challenges posed by Buruli ulcer. For more information, visit the AFRF website: <http://www.raoul-follereau.org>



ANESVAD, Spain is an NGO that has been working against leprosy and implementing health, social and educational projects in 28 of the poorest developing countries for over 30 years. Currently it counts on the support of over 135 000 partners and collaborators in Spain. It has recently begun work on Buruli ulcer in Côte d'Ivoire, carrying out programmes to detect the disease at an early stage and undertaking prevention, surgical treatment, training of specialized medical staff and social awareness campaigns, with the aim of limiting the impact of Buruli ulcer. For more information, visit the ANESVAD website: <http://www.anesvad.org>



Médecins Sans Frontières (MSF) is an international humanitarian aid organization that provides emergency medical assistance to populations in danger in more than 80 countries. MSF Luxembourg has been involved in Buruli ulcer control activities in Benin since 1997. MSF has upgraded the Lalo Health Centre with surgical and laboratory facilities to improve the care of patients. Apart from surgical activities, other key activities include health education in affected communities, case-finding and training of health care providers, teachers and traditional healers. In terms of Buruli ulcer research, MSF is collaborating with the Institute of Tropical Medicine, Antwerp, Belgium. For more information, visit the MSF Luxembourg's website at: <http://www.msf.lu>



The Nippon Foundation, Japan is a private grant-making foundation whose activities cover social welfare, public health, volunteer support and overseas assistance. Since 1975 it has been working through the Sasakawa Memorial Health Foundation to aid WHO in its fight to eliminate leprosy. Starting in 1998, The Nippon Foundation also began providing financial support to the WHO Global Buruli Ulcer Initiative. The Foundation, in tandem with WHO and several academic institutions, is currently exploring options for improved surgical management of the disease. Finally, it is also collaborating with WHO, AFRF and other partners to find a drug treatment for Buruli ulcer. For more information, visit The Nippon Foundation's website at: <http://www.nippon-foundation.or.jp>

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Preface

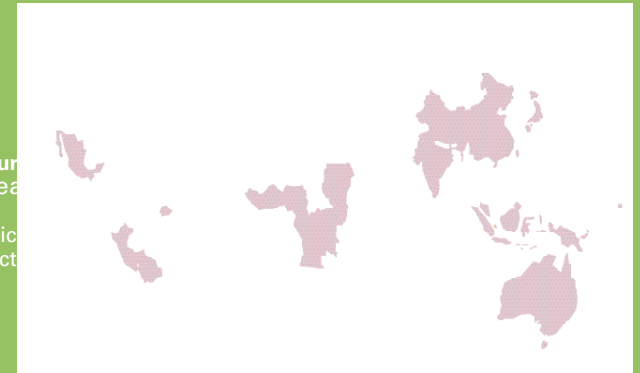
This manual is to assist health care providers and laboratory scientists to diagnose *Mycobacterium ulcerans* disease (Buruli ulcer). The manual aims to achieve a better understanding of the clinical presentation and its diagnosis. The methods described are tailored to various levels of care and available resources to improve the diagnosis and surveillance of the disease.

Please note: This manual is not intended to serve as a standard of laboratory methods. It is not a replacement for textbooks on laboratory work. Adherence to it will not ensure a successful outcome in every case, nor should it be construed as including all proper methods of laboratory diagnosis or excluding other acceptable methods aimed at the same results. Ultimate judgement regarding a particular method must be made by the health care provider or laboratory scientist in the light of the clinical findings in the patient and the available options for diagnosis.

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Figure 1. Worldwide distribution of *Mycobacterium ulcerans* disease. **Note:** Shaded areas do not represent the extent of the problem but indicate only those countries where the disease has been reported or suspected.



Introduction

In 1998, the World Health Organization (WHO) established the Global Buruli Ulcer Initiative (GBUI) in response to the growing spread and impact of Buruli ulcer, *Mycobacterium ulcerans* disease. The disease exists or has been suspected in at least 31 countries (Fig. 1). The primary objectives of the GBUI are: to raise awareness of the disease, to mobilize support for affected countries, to promote and to coordinate research activities and to coordinate the work of nongovernmental organizations (NGOs) and other partners. A summary of the achievements of the GBUI is presented in Annex 12.

In 1897, Sir Albert Cook in Uganda described skin ulcers consistent with Buruli ulcer but he did not publish these cases in the medical literature. In 1948, MacCallum et al. published the first confirmed cases of the disease. These patients were in Australia. The disease was called Bairnsdale ulcer after the main town in the original endemic region. In southeastern Australia, the disease is still often referred to as Bairnsdale ulcer but, in parts of Africa, it is called “Buruli ulcer”, the name coming from a county in Uganda where large numbers of cases were reported in the 1950s.

It is called “Buruli ulcer”, the name coming from a county in Uganda where cases were reported in the 1950s.

Pathogenesis

Once introduced into the subcutaneous tissue the organism proliferates and elaborates a toxin that has affinity for fat cells. The resulting necrosis then provides a favourable milieu for further proliferation of the organism. During the necrotic phase, there is very little or no cellular immune response and the burulin skin test is negative. By an unknown mechanism, either the toxin may be neutralized or the organism may cease to proliferate or to produce toxin. Healing seems to begin when the host develops cell-mediated immunity, at which time the burulin skin test may become positive.

The inflammatory cells then destroy the etiological agent (*M. ulcerans*) and the disease subsides with scarring. Bones may be affected by direct spread from the lesion or as a result of *M. ulcerans* bacteraemia. In contrast to other pathogenic mycobacteria, which are facultative intracellular parasites of macrophages, *M. ulcerans* occurs primarily as extracellular microcolonies.

Clinical spectrum of the disease

Clinically the disease manifests as papules, nodules, plaques, oedematous forms and ulcers. The disease may be active (ongoing infection) or inactive (previous infection with characteristic depressed stellate scars with or without other sequelae). A new case is a patient with no previous history of, or treatment for, Buruli ulcer. A recurrent case is a patient presenting within one year with a further lesion at the same or a different site. Recurrence rates vary from 16% for patients presenting early to 28% for patients presenting late. Recurrence at the same site may be due to inadequate excision. Recurrence at a different site may be due to haematogenous or lymphatic spread.

Diagnosis

Clinical: In a known endemic area, an experienced person can make the diagnosis of Buruli ulcer on clinical grounds. The following clinico-epidemiological features are important diagnostic clues:

- 1) the patient lives in or has travelled to a known endemic area;
- 2) most patients are children under 15 years of age;
- 3) about 85% of lesions are on the limbs;
- 4) lower limb lesions are twice as common as upper limb lesions.

Laboratory: Any two of the following findings are required to positively diagnose Buruli ulcers:

- 1) acid-fast bacilli in a smear stained by the Ziehl-Neelsen (ZN) technique;
- 2) positive culture of *M. ulcerans* (but this requires 6–8 weeks or longer);
- 3) histopathological study of excisional biopsy specimen (result available rapidly);
- 4) positive polymerase chain reaction (PCR) for DNA from *M. ulcerans*.

Treatment

Drug treatment: Several antimycobacterial agents have *in vitro* activity against the causative organism but no single agent has been proven to be regularly useful in the treatment of the disease. Agents used include rifampicin, rifabutin, clarithromycin, azithromycin, streptomycin and amikacin.

Combinations of agents have been used, with apparently varying success. Drug treatment alone, even with combinations of drugs, is usually ineffective when there is an established, progressing lesion. Research into drug treatment is a priority.

Surgical treatment: This is accepted as the current definitive treatment. Limiting factors include:

- 1) inadequate surgical facilities;
- 2) need for prolonged stay in hospital;
- 3) high treatment costs;
- 4) recurrence after surgical treatment (rates of 16% to 28%);
- 5) the risk of transmission of infections such as HIV.

Other adjuncts to treatment include heat and hyperbaric oxygen, which have not been definitively proven and may be impractical in developing countries.

Control and prevention

Community control strategies are currently limited by a lack of knowledge regarding the source of infection and the mode of transmission. The current standard treatment is surgery. Expert opinion is that early surgical management leads to improved results and resolution that are both cost saving. Early treatment is best promoted by an effective village-based surveillance programme. Current attitudes and beliefs may stigmatize and create fear in the affected individuals thereby delaying early and effective treatment. Educational materials should dispel such misinformation

and focus on early detection and surgery. Minor surgery (e.g., nodulectomies) may be performed at the local level.

What you should do

The current control strategy promoted by the Global Buruli Ulcer Initiative consists of:

- health education and staff training in the communities most affected;
- strengthening the health care capacity in endemic areas by upgrading surgical facilities, ensuring adequate treatment supplies and improving laboratories;
- surgical training to enable other health workers (e.g. nurses, medical assistants) to perform effective minor surgery;
- community-based surveillance to improve early detection and rapid referral for treatment in collaboration with disease control programmes such as those for leprosy and dracunculiasis;
- adoption of educational material adapted to the needs of each country;
- developing successful motivational strategies;
- rehabilitation of those already deformed by the disease.

Key points

- 1) About 70% of those infected with Buruli ulcer are children under 15 years old.
- 2) In Ghana the average cost to treat Buruli ulcer is over US\$ 780 per person.
- 3) The accepted current treatment for Buruli ulcer is usually surgery.

Clinical diagnosis



Credit: WHO

Non-ulcerative forms | Ulcerative forms | Bone involvement | Complications and sequelae | Differential diagnosis

Chapter 1

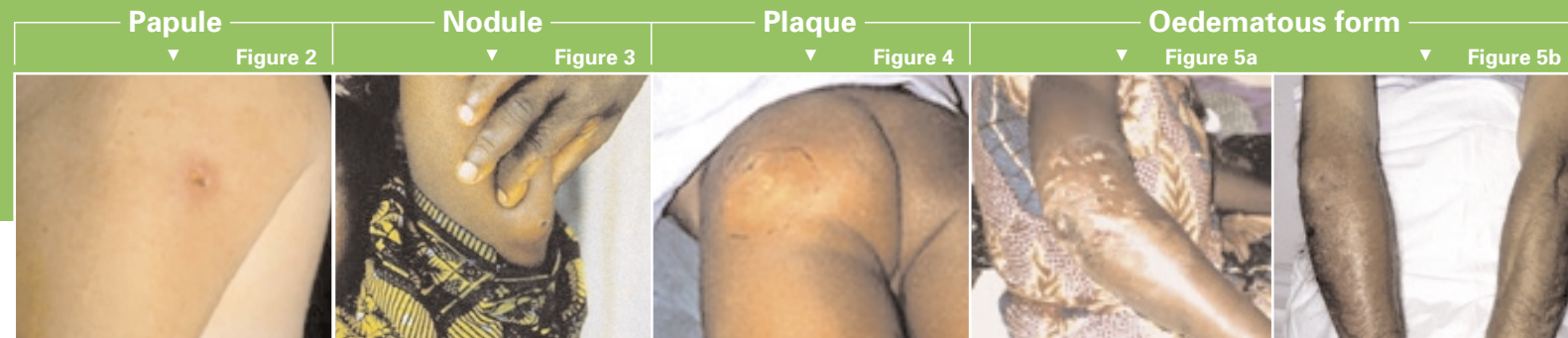
Clinical diagnosis

Objectives This chapter will assist you to recognize different forms of *Mycobacterium ulcerans* disease and to diagnose the condition irrespective of the stage at which it presents.

WHAT YOU SHOULD KNOW

Always consider the diagnosis of *Mycobacterium ulcerans* disease in patients who live in an endemic area. There are basically two presentations of *M. ulcerans* disease: non-ulcerative and ulcerative.

Non-ulcerative forms present as:



1 Non-ulcerative forms

- **Papule:** This is defined as a painless, raised skin lesion, less than 1 cm in diameter. The surrounding skin is reddened (Fig. 2). This form is commonly seen in Australia.
- **Nodule:** A nodule is a lesion that extends from the skin into the subcutaneous tissue. It is 1–2 cm in diameter. It is usually painless but may be itchy and the surrounding skin may be discoloured compared to adjacent areas (Fig. 3). This form is commonly seen in Africa.
- **Plaque:** This is a firm, painless, elevated, well-demarcated lesion more than 2 cm in diameter with irregular edges. The skin over the lesion is often reddened or otherwise discoloured (Fig. 4).
- **Oedematous form:** There is a diffuse, extensive, usually non-pitting swelling. The affected area has ill-defined margins, is firm and painless and involves part or all of a limb or other part of the body. There may be colour changes over the affected region (Fig. 5a and 5b) and the disease may be accompanied by fever.

2 Ulcerative forms

When fully developed, the ulcer has undermined edges and is indurated peripherally. The floor of the ulcer may have a white cotton wool-like appearance from the necrotic slough (Fig. 6a–d).

Figure 6a ▶
Hand



Figure 6c ▶
Back



◀ Figure 6d
Forearm



Figure 6b ▲
Leg



The ulcer is usually painless, unless there is secondary bacterial infection. When there is more than one ulcer and the ulcers are close together, they often communicate beneath intact skin.

3 Bone involvement

- **Osteomyelitis:** This is true osteomyelitis. It may be focal or multifocal. The overlying skin is often intact with no obvious lesion. Osteomyelitis may occur as a primary condition or as a metastatic condition, sometimes at a distance from a cutaneous lesion(s) or after a cutaneous lesion has healed.



Figure 7 ▶
Osteomyelitis – Leg

Mycobacterium ulcerans osteomyelitis is initially painless, but subsequently frankly painful, and well localized. There is usually an identifiable area of increased warmth. A swelling then appears and this may progress to a fistula that discharges necrotic material. Incision of the swelling reveals gelatinous tissue and, beneath this, the bone has a moth-eaten appearance. Unlike open (contiguous) osteitis, the bone is the site of necrosis to a variable extent, similar to that seen in tuberculous osteomyelitis (Fig. 7).

- **Reactive osteitis:** Reactive (contiguous) osteitis occurs as a consequence of deep destruction of overlying soft tissues. Occasionally, the bone is exposed to the point of devascularization, necrosis of cortical bone, sequestration, and osteomyelitis. The macroscopic appearance is then that of white dead bone of almost normal appearance and texture.

4 Complications and sequelae

- **Contractures**

Contractures result from scarring caused by lesions over or close to joints (Fig. 8a and 8b). Ankyloses may follow.



▲ **Figure 8a**
Contracture deformity of the upper limb



Figure 8b▶
Contracture deformity of the lower limb

- **Bleeding**

There may be continuous minor bleeding or a sudden major haemorrhage. One must be careful to avoid large blood vessels beneath a lesion.

- **Secondary infection**

Secondary bacterial infection may be caused by organisms such as staphylococci, streptococci, *Pseudomonas* sp., *Corynebacterium* sp., etc. Secondary infection may progress to cellulitis and septicaemia.

- **Extension to deep structures**

Infection may extend beneath the deep fascia to involve tendon sheaths, muscle, blood vessels, nerves, bone and joints or may destroy periorbital tissue with loss of the eye.



▲ **Figure 9**
Hypertrophic scar



Figure 10 ▶
Squamous cell carcinoma

- **Other sequelae**

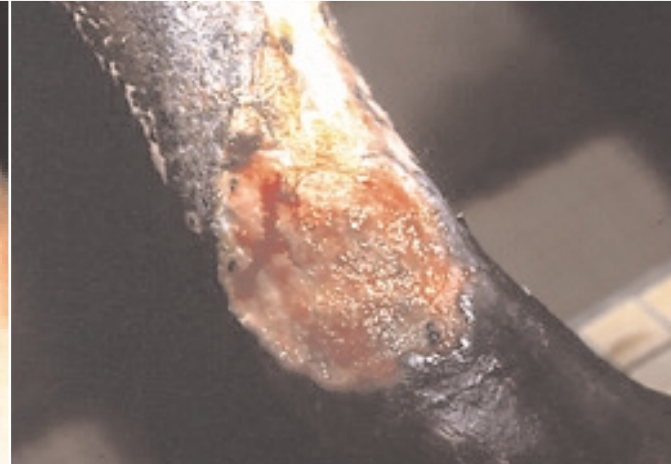
Hypertrophic scars and keloids may develop at infection and surgical sites including skin graft donor sites (Fig. 9). Squamous cell carcinoma (Marjolin's ulcer) may appear in an unstable scar or persistent ulcer many years after initial infection with *M. ulcerans*. (Fig. 10).

5 Differential diagnosis

The differential diagnosis of nodules is more difficult than that of ulcers. Some common differential diagnoses are described in Buruli Ulcer: *Mycobacterium ulcerans* infection (ref. WHO/CDS/CPE/GBUI/2000.1). Table 1 and figures 11a and 11b relate to differential diagnosis.



▲ Figure 11a
Leishmaniasis



▲ Figure 11b ▲
Tropical phagedenic ulcer

Papule	Nodule	Plaque	Oedema	Ulcer
Insect bites	Cyst	Leprosy	Cellulitis	Tropical phagedenic ulcer
Pimple	Lipoma	Cellulitis	Elephantiasis	Venous ulcer
Herpes	Onchocercoma	Mycosis	Actinomycosis	Leishmaniasis
Granuloma annulare	Boil	Psoriasis	Necrotizing fasciitis	Neurogenic ulcer
Psoriasis	Lymphadenitis	Haematoma	Osteomyelitis	Yaws
Pityriasis	Mycosis	Insect bites	Onchocercoma	Squamous cell carcinoma

Note: Infection caused by other mycobacterial organisms can be mistaken for any of the above.

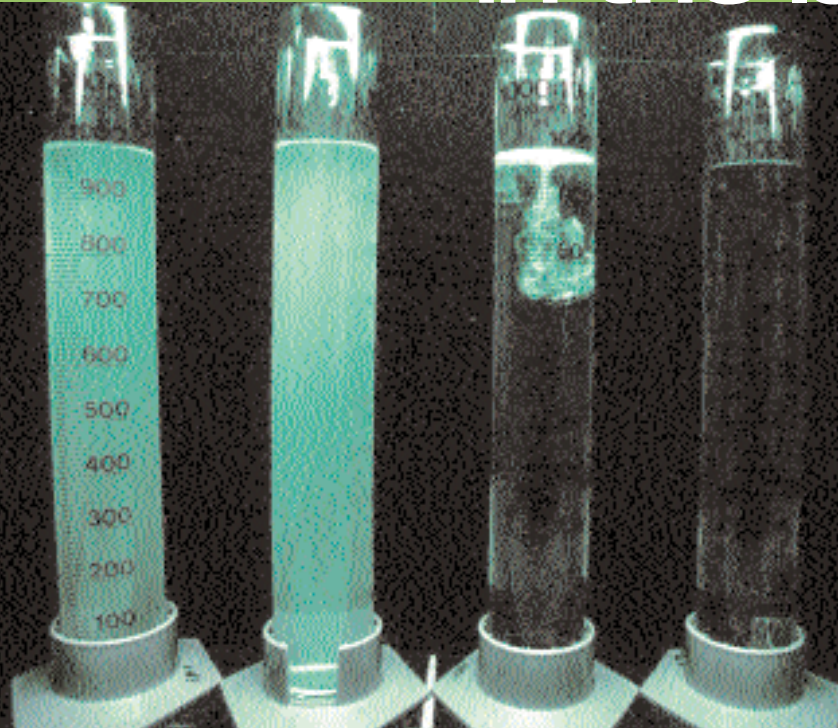
Table 1 Differential diagnoses of various forms of Buruli ulcer

Key points

- 1) Buruli ulcer disease presents as: papules, nodules, plaques, oedematous forms, ulcers and bone infections.
- 2) Contractures are easier to prevent than to correct.
- 3) Osteomyelitis may arise when an ulcer invades bone or when infection is blood-borne.

Notes

Biosafety and record-keeping in the laboratory



Credit: WHO

Handling clinical specimens | Laboratory disinfection | Record-keeping

Chapter 2

Biosafety and record-keeping in the laboratory

Objectives This chapter will assist you to understand biosafety and record-keeping when dealing with *Mycobacterium ulcerans* disease.

1 Handling clinical specimens

Mycobacterium ulcerans is an environmental pathogen, and there are very few reports of person to person transmission. Nevertheless, precautions must be taken in the laboratory. Although transmission of *M. ulcerans* to laboratory workers has not been reported, it is possible that specimens may contain other unsuspected pathogens, in particular *M. tuberculosis*, hepatitis B virus, and HIV. Basic safety standards must always be followed: gloves, gowns and use of biosafety facilities (BSL 2 or 3) whenever possible. Care must also be taken to limit the formation of aerosols.

2 Laboratory disinfection

Disinfection of nondisposable equipment and laboratory surfaces contaminated with mycobacteria requires special procedures, which differ from those used for viruses such as HIV, and other microorganisms. The use of quaternary ammonium compounds and sodium hypochlorite is discouraged: the former is ineffective and the latter is often used at suboptimal concentrations. Common antiseptics such as chlorhexidine gluconate and benzalkonium chloride exhibit no antimycobacterial activity even after treatment for 2 hours. The recommended products are indicated in Table 2.

Besides the concentration of the disinfectant, time of contact is also important and must be at least 30 minutes.

Disinfectant	Concentration
Alkaline glutaraldehyde	2%
Phenol	5%
Phenol-based disinfectants (e.g. Dettol®, Cresol®)	10%
Iodine	10%
Alcohol (for surfaces)	70%

Table 2 Disinfectants recommended for use in laboratories studying *M. ulcerans*

3 Record-keeping

Good quality laboratory records that make it possible to track each specimen through the laboratory are essential. This is best done with “bench books”. *M. ulcerans* is slow growing; therefore interim reports should be issued when available. For example, when Ziehl-Neelsen (ZN) or PCR has been performed, an interim report stating the result should be issued. When culture is first positive and a presumptive identification can be performed, it may be appropriate to issue a further preliminary report before finalizing the laboratory investigation of that specimen. *Sample laboratory request and report forms can be found in Annexes 2 and 3.*

Notes

Collection and transport of clinical specimens



Credit: IFRC/Y. Shimizu

Procedures appropriate for different care-levels | Types of clinical specimens | Storage and transport of specimens

Chapter 3

Collection and transport of clinical specimens

Objectives This chapter will assist you to collect the right clinical specimens and to transport them under appropriate conditions to the laboratory.

Tests & Procedures	Peripheral level	Intermediate level	Central level
Ziehl-Neelsen	+	+	+
Culture	–	+	+
Swabbing ulcers	+	+	+
Tissue fragments obtained from surgical specimens	–	+	+
Surgery	–	+	+
Nonsurgical therapy	–	–	+

Table 3 Specimen collection and laboratory methods for each care-level

All specimens, except swabs, must be obtained from surgically excised tissue taken in an operating theatre. Punch biopsies should never be collected in the field as they often are not diagnostic and may exacerbate the disease, promote secondary infection and delay definitive treatment. In specific situations, incisional biopsies in hospital may be performed to exclude other causes of skin lesions.

1 Procedures appropriate for different care-levels

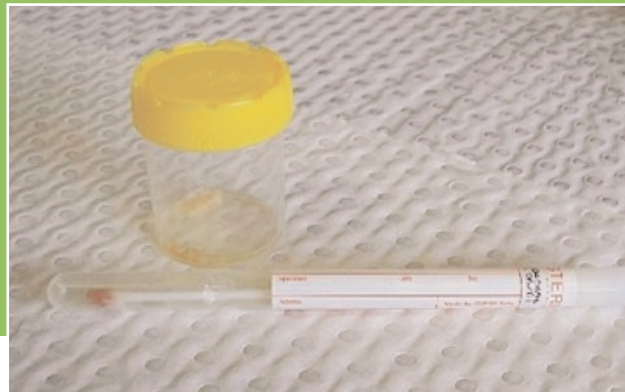
Generally, in each country there are three care-levels: peripheral (health centres and dispensaries), intermediate (general hospitals and district hospitals) and central (university teaching hospitals, regional hospitals and research centres). Specimen collection and laboratory procedures that can be performed at each level are outlined in table 3.

2 Types of clinical specimens

- **Non-ulcerative forms**

Specimens for laboratory confirmation from non-ulcerative forms (i.e., papules, nodules, plaques and oedematous forms—see Chapter 2) should be taken from the centre of the surgically excised tissue and should include the entire thickness of clinically-infected tissue.

Figure 12 ▶
Specimen collection containers



Especially for non-ulcerative plaques and oedematous forms, the patient or the patient's relative should be asked to indicate the site at which the lesion first appeared, as this is the most likely site to yield a positive diagnosis but several further biopsies should be taken from other parts of the lesion. Tissue fragments from the periphery of a lesion are not recommended for microbiological studies, because *M. ulcerans* is often not found here but such specimens may be most suitable for histopathology.

- **Ulcerative forms**

Multiple swabs should be taken from different sites, especially from beneath the undermined edges of lesions (Fig. 13). **Do not swab the slough in the centre of an ulcer.** Specimens that include all levels of the skin and subcutaneous tissue are most suitable for histopathological study.



▲ **Figure 13**
Swabbing the undermined edges
of a Buruli ulcer

- **Bone**

Diagnostic procedures to assess bone involvement should only be performed at centres providing intermediate and high-level services. For amputation specimens, the involved whole bone or curetted bone samples are required; when amputation is not necessary, curetted bone samples are appropriate.

3 Storage and transport of specimens

Sample to be stored for immediate analysis—place in a sterile container without any additives.

Sample to be transported:

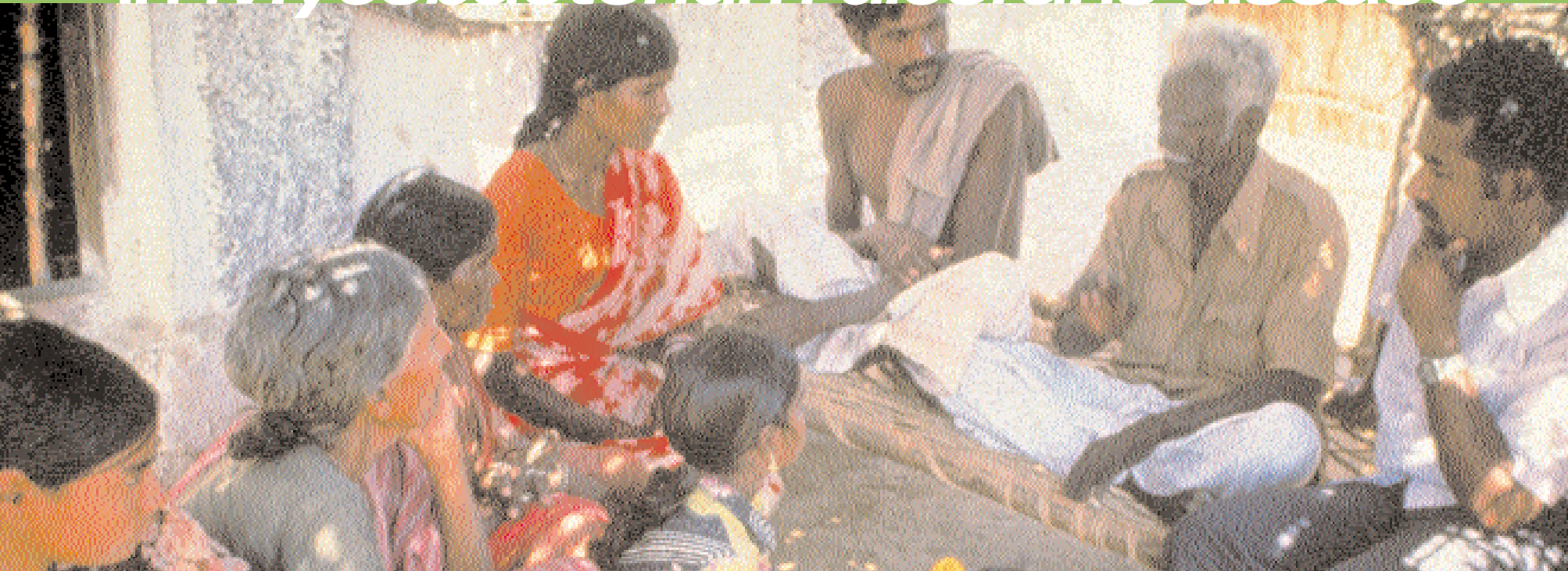
- Analysis within 24 hours—keep the sample cool (ideally at 4 °C), e.g. in an insulated container with a frozen cooling block.
- Analysis after 24 hours:
 - when refrigeration facilities are available, keep at 4 °C—do not freeze;
 - when refrigeration facilities are not available, transport medium is essential. Liquid Middlebrook 7H9 broth supplemented with polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) is recommended. Supplementation with 0.5% agar achieves a semi-solid medium. (Specimens kept in transport medium may still be culture-positive up to 21 days.)

Transport for PCR analysis

PCR is best performed directly on fresh tissue specimens prepared as described above. For ulcerative forms, dry cotton wool swabs stored in their plastic containers at ambient temperature are acceptable.

Notes

Secondary bacterial infection in *Mycobacterium ulcerans* disease



Credit: WHO

Specimens to collect | Direct examination | Culture | Antimicrobial susceptibility testing

Chapter 4

Secondary bacterial infection in *Mycobacterium ulcerans* disease

Objectives This chapter will assist you to diagnose secondary bacterial infection that may accompany *Mycobacterium ulcerans* disease.

Secondary infection of Buruli ulcers is not as common as would be expected given the extent of skin loss. The reasons for this are unclear but may include an antibiotic effect of the *M. ulcerans* toxin, mycolactone. Despite this, secondary infections with *Staphylococcus aureus* and other bacterial pathogens are well known. They should be suspected when a lesion develops cellulitis, becomes painful or the patient becomes febrile.

1 Specimens to collect

Swabs: Use sterile cotton swab to collect pus or other exudates and place into either Amies or Stuarts Transport Medium for transport to the laboratory as early as possible.

Tissue fragments: refer to Chapter 3.

2 Direct examination by microscopy

Gram's stain: Preparation of a Gram-stained smear is the method of choice for the identification of gram-positive, gram-negative bacteria and yeast cells.

Wet mount: This is to detect fungal elements and yeast cells. It is prepared by mixing the sample with 15% potassium hydroxide (KOH) on a glass slide with a cover slip. It is then heated for 15 minutes to dissolve keratin. Examine at a magnification of x400 for fungal elements and yeast cells.

3 Culture

Specimen should be processed as soon as they arrive in the laboratory. Choice of culture media depends on the result of the Gram-stain and wet mount studies.

Bacteria: In case of secondary bacterial infection, all specimens (pus or exudate) should preferably be inoculated onto a minimum of three culture media.

Plates of blood agar:

- Aerobic incubation at 35 °C for 18–20 hours for the isolation of staphylococci, streptococci, and *Candida* sp;
- Anaerobic incubation at 35 °C for 48 hours for anaerobes such as *Clostridium* sp.

MacConkey or cystine-lactose-electrolyte-deficient (CLED) medium for the isolation of gram-negative rods.

Incubation: at 35 °C for 18–20 hours for the identification of lactose and non-lactose fermenters.

Robertson's beef heart infusion broth may be inoculated as an enrichment broth and incubated 37 °C for 24 hours before sub-culturing on solid media.

Fungi: Culture on Sabouraud agar if fungal elements or yeast cells have been observed on the wet mount. Inoculated plates should be incubated at room temperature for at least 7 days.

Identification of cultured organisms: Pure subcultures of all bacteria or fungi isolated must be made and identified using standard methods. For example, coagulase test for staphylococci, bacitracin test and Lancefield grouping for streptococci species and biochemical tests for gram-negative bacilli.

4 Antimicrobial susceptibility testing

Susceptibility to antimicrobial drugs differs from place to place and region to region. It is therefore necessary for local laboratories to determine their own susceptibility patterns. This will help in selecting the most appropriate antimicrobial agent for treatment. Ideally, antimicrobial susceptibility studies should be done on all isolates.

Notes

Microbiological methods



Credit: WHO

Specimen preparation | Direct smear examination | Decontamination | *In vitro* culture | Identification

Chapter 5

Microbiological methods for diagnosis of *Mycobacterium ulcerans* disease

Objectives This chapter will assist you to understand the various microbiological methods for the diagnosis of *Mycobacterium ulcerans* disease.

A flow chart for laboratory diagnosis is presented in Annex 1.

1 Specimen preparation

Tissue specimens

- Dice tissue into small pieces in phosphate-buffered saline (PBS) or normal saline with a sterile single-use or autoclavable scalpel blade. (**Note:** If equipment is to be re-used, it must first be placed in a disinfectant, then carefully brushed before sterilization to prevent cross-contamination, especially for PCR studies. See Table 2 for recommended disinfectants).
- Mix well (e.g. with a vortex mixer).
- The specimen may also be prepared by grinding with a sterile mortar and pestle or Potter grinder. Again, care should be taken to prevent cross-contamination when cleaning these instruments.

Swabs

- Suspend swabs in a small volume of PBS or normal saline and then vortex well (e.g. 20 ml sterile tube).
- Liquid transport medium containing swab may also be shaken (vortex) directly.

2 Direct smear examination

There are several staining techniques for mycobacteria: Ziehl-Neelsen (ZN), Kinyoun and auramine-rhodamine. The method used locally for the diagnosis of tuberculosis is applicable to *M. ulcerans*. In most cases this will be the ZN stain method (see Annex 5). The quantitation of smears should be in accordance with the method used locally for microbiological diagnosis of tuberculosis.

3 Decontamination prior to culture

All specimens for primary isolation of *M. ulcerans* may contain contaminating microorganisms. Decontamination is necessary before attempting culture. The best results will be obtained from fresh specimens that are prepared and decontaminated immediately. Problems with bacterial or fungal overgrowth and loss of viable mycobacteria increase as the storage and transport time increase.

***In vitro* methods**

Several methods have been described to decontaminate specimens prior to culture for mycobacteria. Overly strong decontamination procedures will reduce the likelihood of obtaining a positive culture for *M. ulcerans*. The method of choice depends on the culture medium to be used:

- for liquid culture (BACTEC), N-acetyl-L-cysteine-sodium hydroxide or the Petroff method is recommended (see Annex 7);
- for L-J medium, any of the described methods for decontamination of mycobacteria specimens is recommended (see Annex 7).

To control for excessive decontamination in the laboratory, which will reduce the yield of positive cultures, an overall rate of contamination in the range of 2–5% of all cultures is acceptable. The method of choice is therefore at the discretion of the microbiologist and will depend on types of specimens and degree of contamination.

***In vivo* methods**

Laboratory animals (usually mice) may be used for the primary isolation of *M. ulcerans* from patient specimens to diminish problems with contamination, and may offer improved sensitivity compared to *in vitro* methods. For foot pads, a 30 µl sample—prepared as described in the section on tissue specimens above—is injected subcutaneously (100 µl if tail injection is used, see Fig. 14). At the first sign of swelling or distress, the mice are sacrificed, and foot pad or tail biopsies collected under sterile conditions are then prepared for culture as for a routine specimen, including decontamination.

Figure 14 ▶
Inoculation of *M. ulcerans*
into the tail of a mouse
Note the swelling and ulcer



4 *In vitro* culture

Culture media

For solid media, L-J is the most suitable medium for *M. ulcerans*.

For the BACTEC system, Middlebrook 7H12B medium is recommended (see Annex 8).

Culture conditions

Mycobacterium ulcerans grows under the same conditions as *M. tuberculosis* except that the optimal temperature is 29–33 °C. In liquid media (e.g. in the BACTEC system) *M. ulcerans* may also show enhanced growth under micro-aerophilic conditions (2.5–5% oxygen).

Culture duration

Primary cultures are usually positive within 6–12 weeks incubation at 29–33 °C, but much longer incubation of up to 9 months may be necessary for some isolates. Duration of the incubation period should be selected according to the objective of the laboratory investigation.

Mouse inoculation**1. Specimen preparation****• Swabs**

Swirl the swab in a tube containing 1 ml sterile saline (0.85%).

Mix well.

Sample with sterile insulin syringe.

• Tissue fragments

Grind tissue fragments in a tissue grinder (Potter) with saline (2 ml).

Remove the supernatant into a sterile tube and sample with a sterile insulin syringe.

2. Animal inoculation

Foot pad inoculation: 0.03 ml in the hind foot pad.

Tail inoculation: 0.1 ml subcutaneously in the tail skin.

Animals are observed weekly for inflammation near the site of injection. The infected tissue is sampled, decontaminated and inoculated in culture media.

5 Identification

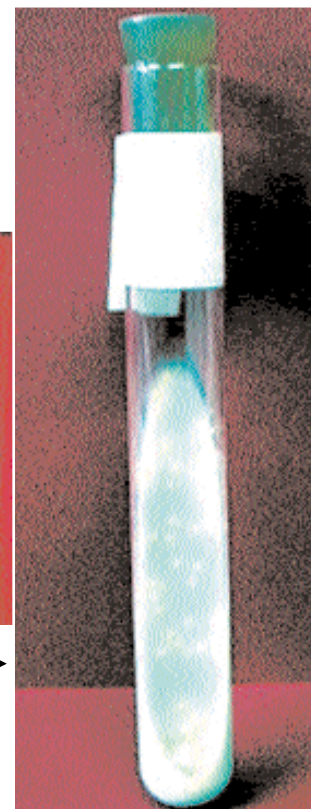
Positive primary cultures

When primary cultures are positive, colonies suggestive of *M. ulcerans* appear yellowish, rough and have well-demarcated edges. African strains are more yellowish (Fig. 15a) than Australian strains (Fig. 15b), which may be only slightly pigmented.

Figure 15a ▶
M. ulcerans isolates from Africa cultivated on L-J medium; the isolates produce a light yellow pigment



Figure 15b ▶
M. ulcerans isolate from Australia cultivated on L-J medium. The isolate does not produce pigment (nonchromogenic mycobacterium)



A single, typical colony should be selected for subculture onto L-J medium. When BACTEC is positive, L-J is inoculated from the BACTEC medium.

Identification of subculture

Growth rate

Mycobacterium sp. are classified as either slow or rapid-growers. This distinction is based on whether isolated colonies are observed before or after 7 days on a solid medium. Isolated colonies are observed after solid media are inoculated with a 10^{-4} dilution of a standard culture suspension prepared at an optical density at 580 nm of 0.25, in a tube with a diameter of 2 cm. This corresponds roughly to a suspension containing 1 mg wet weight of bacilli per ml. A further distinction is that rapidly growing, but not slowly growing species are able to develop on simple media such as nutrient agar or peptone agar.

Specific identification of *M. ulcerans***Phenotypic tests**

Mycobacterium ulcerans belongs to the slow-growing group. Tests for identification of *M. ulcerans* and related species are summarized in Table 4.

The procedures for identification of slow growing mycobacteria are indicated in Annex 9.

Specific phenotypic tests for the identification of *M. ulcerans* and related species are shown in Table 4. Differences between various subgroups according to their geographic origins are shown in Table 5. Drug susceptibility tests may be conducted for further identification. *M. ulcerans* is reliably resistant to isoniazid, para-aminosalicylic acid (PAS) and ethambutol but sensitive to rifampicin, streptomycin and several second line antituberculous drugs (see Table 6).

Table 4 Phenotypic characteristics of *M. ulcerans* and related species

Parameter	<i>M. ulcerans</i>	<i>M. marinum</i>	<i>M. shinshuense</i>
Pigmentation in the dark	+	-	+
Pigmentation in the light	+	+	+
Growth at 37 °C	-	-	-
Growth on peptone agar	+	-	-
Growth in presence of:			
Isoniazid (10 µg/ml)	M	M	-
Thiophene-2-carboxylic hydrazide (2 µg/ml)	+	+	-
Hydroxylamine (250 µg/ml)	-	+	+
p-Nitrobenzoate (500 µg/ml)	-	M	-
NaCl 5%	-	-	-
Enzymatic properties:			
Catalase, > 45 mm foam	-	-	+
Tween 80 hydrolysis (10 days)	-	+	-
Urease activity	-	+	-
Niacin production	F	-	-
Nitrate reduction	-	-	-
Acid phosphatase activity	F	+	-
+ : > 85% of strains positive; - : < 15% of strains positive; M: 50 to 85% of strains positive; F: 15 to 49% of strains positive.			

Table 5 Characteristics of the different geographical subgroups of *M. ulcerans*

	Geographic subgroups of <i>M. ulcerans</i>					
	Africa	Australia	Mexico	South America	China	Japan
Pigmentation in the dark	+ (1)	-	-	+	-	+
Pigmentation in the light	+ (1)	-	-	+	-	+
Growth at 37 °C	-	-	-	-	-	-
Growth on peptone agar	-	-	-	-	-	-
Growth in presence of:						
Isoniazid (10 µg/ml)	+	M	+	-	-	-
Thiophene-2-carboxylic hydrazide (2 µg/ml)	+	+	+	+	+	-
Hydroxylamine (250 µg/ml)	-	+	+	M	+	+
p-Nitrobenzoate (500 µg/ml)	-	-	+	-	V	-
NaCl 5%	-	-	-	-	-	-
Enzymatic properties:						
Catalase, > 45 mm of foam	-	-	-	-	-	+
Tween 80 hydrolysis (10 days)	-	-	-	-	-	-
Urease activity	-	-	-	-	-	-
Niacin production	-	-	-	-	-	-
Nitrate reduction	-	-	-	-	-	-
Acid phosphatase activity	M	-	-	-	-	-
Sequencing results (2)	Type 1	Type 2	Type 3	<i>M. marinum</i>	<i>M. shinshuense</i>	<i>M. shinshuense</i>
(1) Light yellow pigment						
(2) Data from Portaels et al., 1996						
Data from:						
Portaels et al. Chapter 11: Bacteriology of <i>M. ulcerans</i> . In: <i>Mycobacterium ulcerans</i> Infection (Buruli Ulcer) Ed: Portaels F, Meyers WM. Kluwer Academic Publishers. Resurgent and Emerging Infectious Diseases (In preparation).						
+: > 85% of strains positive; -: < 15% of strains positive; M: 50 to 85% of strains positive; V: variable results.						

Table 6 *In vitro* susceptibility of *M. ulcerans* to antimycobacterial drugs

Antimycobacterial drug	Medium	µg/ml (1)		Growth
Isoniazid	L-J	0.1	10	+
Para-aminosalicylic acid	L-J	0.5	50	+
Ethambutol	L-J	0.25	5	+
Streptomycin	L-J	2	50	-
Rifampicin	7H11	2		-
	L-J	4	40	-
Ethionamide	L-J	4	30	+
Cycloserine	L-J	5	30	-
Kanamycin	L-J	8	20	-
Clarithromycin	7H11	0.5	4	-
Ofloxacin	7H11	2		-
Ciprofloxacin	7H11	1		-
Sparfloxacin	7H11	0.5		-
Amikacin	7H11	2		-
	L-J	30		-
Capreomycin	L-J	16	20	-
Thioacetazone	L-J	2		+
Thiosemicarbazone	L-J	0.5	4	+
Viomycin	L-J	10	50	-
Thiocarbanilide	L-J	50		+
Dapsone	L-J	3		-
	L-J	1		F
	L-J	0.3		M

(1) Concentrations tested by several authors

Data from:
Portaels et al., Chapter 11: Bacteriology of *M. ulcerans*. In: *Mycobacterium ulcerans* infection (Buruli Ulcer)
Ed: Portaels F, Meyers WM. Kluwer Academic Publishers. Resurgent and Emerging Infectious Diseases
(in preparation). +: > 85% of strains positive; -: < 15% of strains positive; M: 50 to 85% of strains positive;
F: 15 to 49% of strains positive

Identification of colonies by PCR

Positive cultures may also be identified by PCR as described below and in Annex 10.

Identification of *M. ulcerans* by PCR

Identification of *M. ulcerans* by PCR may be performed directly from clinical specimens or from culture media. Although there are several published methods, currently the best method is IS2404 PCR. However, PCR is relatively expensive and is notorious for producing false-positive results in laboratories which lack experience with this technology.

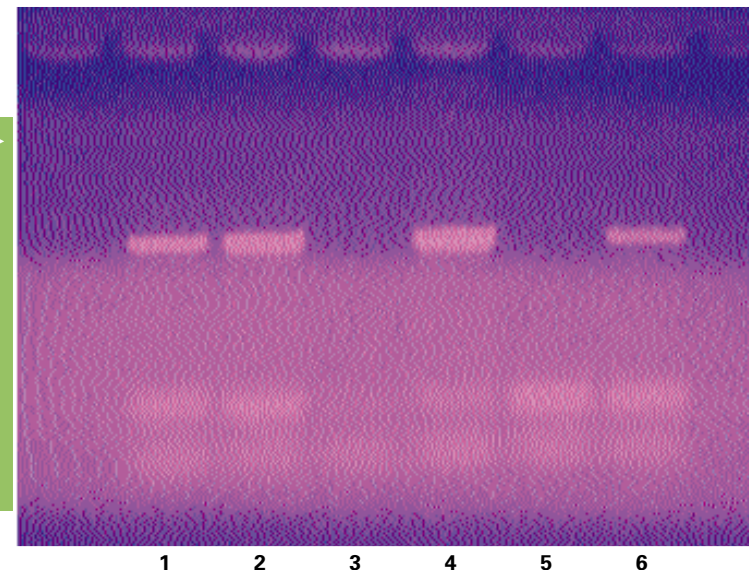
Suitable clinical specimens for PCR include dry swabs, fresh tissue or specimens kept in transport medium. Specimens should be prepared as for culture, although decontamination is not necessary. Great care must be taken to keep the sample preparation, PCR master-mix preparation and agarose gel areas of the laboratory separate to prevent cross-contamination.

It is advisable to include multiple negative controls in every PCR run. All results must be discarded if any negative control is positive. To control for inhibition, each PCR reaction is performed in duplicate. The second tube is "spiked" with approximately 100 molecules of purified *M. ulcerans* DNA. If this spiked positive control tests negative, the PCR reaction is being inhibited. Inhibition in clinical specimens can often be overcome by repeating the PCR using a 1:10 dilution of the extracted DNA sample.

PCR products are detected by ultraviolet transillumination of ethidium stained agarose gels. Presumed positive PCR results can be checked by Southern blot using an internal probe based on IS2404. With experience, it is acceptable to rely on comparison of the test sample with the positive control of the gel. If the two PCR products (positive control and test sample) align precisely, and the negative controls are negative, it can be concluded that the test sample is positive for *M. ulcerans*. Quality control measures must be in place. PCR results should be compared with culture results to monitor accuracy.

Sample results are illustrated in figure 16. It is recommended that Southern blotting or an equivalent method of verification be used to establish that the PCR product is the correct sequence when new laboratories are establishing *M. ulcerans* PCR.

Figure 16 ▶
Electrophoresis gel under UV illumination ethidium bromide stained electrophoresis gel under UV light. Lane 1-positive control; Lane 2,4,6: swab from patients with *M. ulcerans* infection; Lane 3 negative control; lane 5 swab from patient with chronic ulcer (non-*M. ulcerans*)



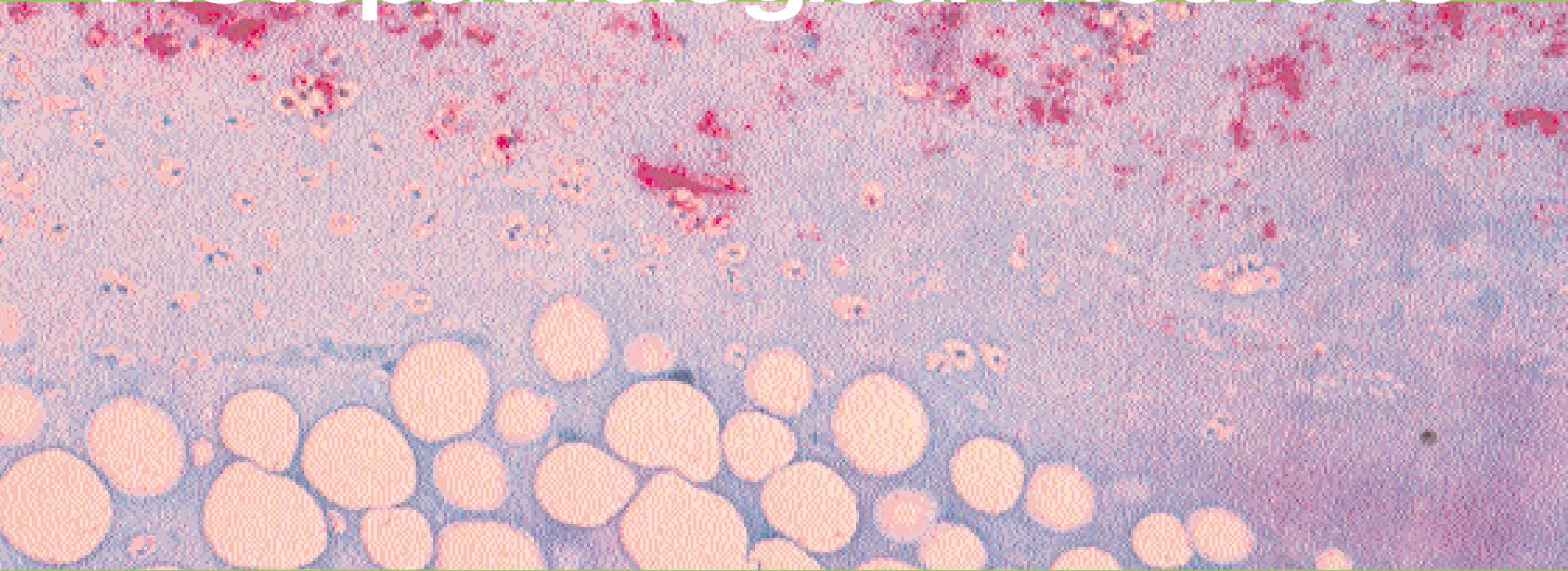
The main advantage of PCR is that *M. ulcerans* disease can be definitively diagnosed within 24 hours of receipt of a clinical specimen by the laboratory. Culture confirmation takes 6 or more weeks. It is recommended that at present PCR be used as a rapid ancillary test and not as a replacement for culture and histology.

In summary, the diagnostic PCR protocol consists of 4 phases:

- Heat and alkaline lysis (to release DNA from *M. ulcerans* cells)
- Extraction of total DNA from sample
- PCR reaction to detect *M. ulcerans*-specific DNA in extracted total DNA (primers slightly modified from Ross et al., 1997a)
- Identification of PCR product (e.g. agarose gel electrophoresis)

The full protocol is shown in Annex 10.

Histopathological methods



Credit: WHO

Selection of site for biopsy specimen | Fixation of tissue | Preparation of histopathological sections
Gross changes | Histopathological changes

Chapter 6

Histopathological methods for diagnosis of *Mycobacterium ulcerans* disease

Objectives This chapter will assist you to understand the various histopathological methods for the diagnosis of *Mycobacterium ulcerans* disease.

WHAT YOU SHOULD KNOW

A detailed history and description of the lesion that has been excised is very important for a meaningful evaluation and for archival purposes. Name, age, sex, laboratory or hospital number and site of lesion are absolutely essential.

1 Selection of site for biopsy specimen

Excisional specimens are advised. Specimens taken by punch are often unsatisfactory.

Non-ulcerative lesions

Specimens should be obtained from the presumed centre of the lesion and include all levels of the skin and subcutaneous tissue down to fascia.

Ulcerative lesions

Specimens should be taken from the edge of the ulcer and include the entire thickness of the skin and subcutis down to fascia.

2 Fixation of tissue

Optimally, the tissue should be fixed in neutral or buffered 10% formalin (pH 7.4). Ideally, the tissue should be fixed in a volume of formalin 10 times the volume of tissue for at least 24 hours before shipping. After fixation, the tissue can be shipped in smaller volumes of fixative. Care should be taken to identify the tissue with permanent markings on the container label. Bone must be decalcified before sectioning.

3 Preparation of histopathological sections

Routine processing of fixed tissue is sufficient. Sections should be cut at 4-5 microns and stained by:

1) haematoxylin and eosin; 2) Ziehl-Neelsen for AFB; 3) Grocott methenamine-silver for fungi; and 4) tissue Gram's stain for other bacteria (*see Annex 6*). Other stains are employed as indicated.

4 Gross changes

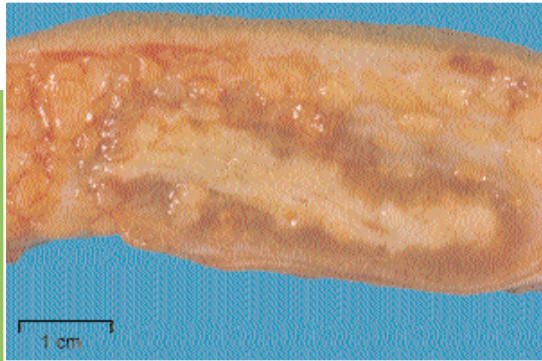
Surface changes of non-ulcerated lesions often show loss of topographic markings and discolouration.

Cut sections show changes in colouration, necrosis and mineralization. Lymph nodes show soft greyish-tan cut surfaces. After decalcification, cut sections of bone show yellowish necrosis of the marrow and often, thinning of the cortex.

5 Histopathological changes

Skin changes

Necrotic (active) stage: non-ulcerated lesions



◀ **Figure 17**
Section of surgically resected nodule of *M. ulcerans* disease. The central whitish area represents coagulation necrosis

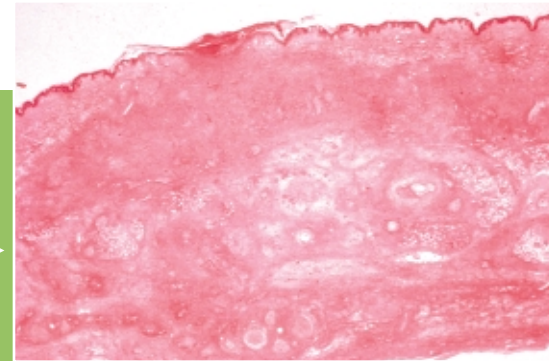
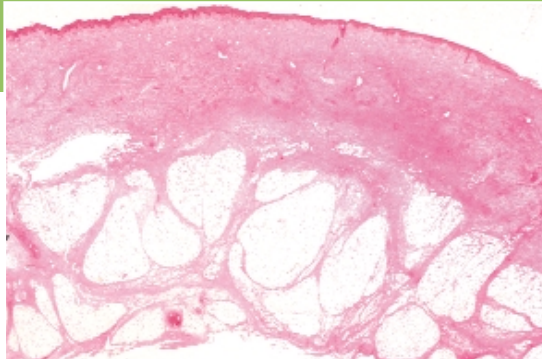


Figure 18 ▶
Microscopic section of a nodule. Note the massive coagulation necrosis of the lower dermis and subcutaneous tissue. H & E x2



◀ **Figure 19**
Skin and subcutaneous tissue from centre of a non-ulcerated widely disseminated lesion of *M. ulcerans* infection that covered 50 percent of the abdomen of a nine year old boy. Epidermis is intact. There is massive contiguous coagulation necrosis of the entire specimen. H & E x2

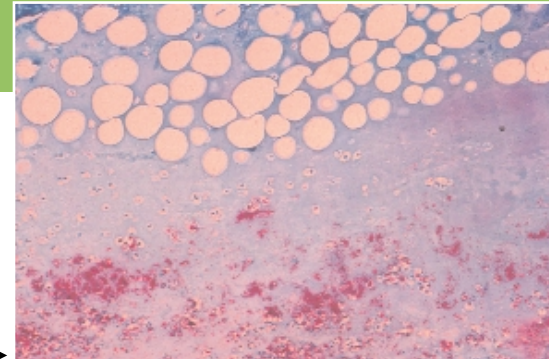
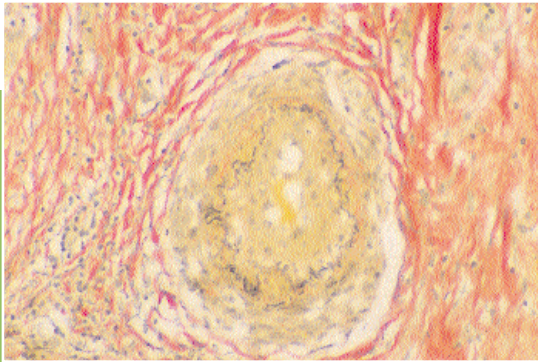


Figure 20 ▶
Necrotic base of Buruli ulcer showing many fat cell ghosts (upper portion) and many AFB (lower portion). ZN stain x50

The epidermis is intact, but is often hyperplastic. The upper dermis is usually intact but may show various stages of degeneration with infiltration of small numbers of inflammatory cells. There is contiguous coagulation necrosis of the lower dermis, subcutaneous tissue and underlying fascia (Fig. 17–19). There is oedema with remarkably few inflammatory cells, unless the lesion is infected secondarily by pyogenic bacteria. Adipose cells swell, but may lose their nuclei and retain their cell wall (fat cell ghosts—Fig. 20).

Vasculitis is common in the subcutaneous tissue, often with occlusion of vessels by thrombi (Fig. 21 and Fig. 22). Varying degrees of mineralization are seen, especially in African patients. The ZN stain classically reveals large numbers of extracellular acid-fast bacilli (AFB); often in clusters and confined to the necrotic areas (Fig. 23).



◀ **Figure 21**
Severe vasculitis in subcutaneous tissue of lesion of Buruli ulcer. Movat x80

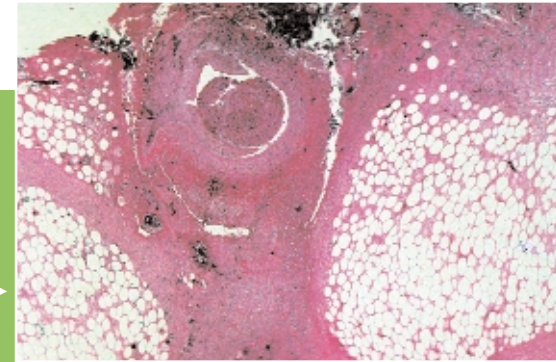
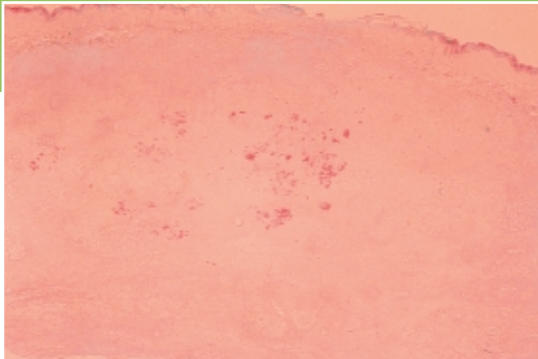


Figure 22 ▶
Fat cell ghosts and vasculitis. H & E stain x50



◀ **Figure 23**
ZN stain of a section parallel to that of Figure 18 shows AFB restricted to the centre of the lesion. Necrosis extends far beyond the focus of AFB x2

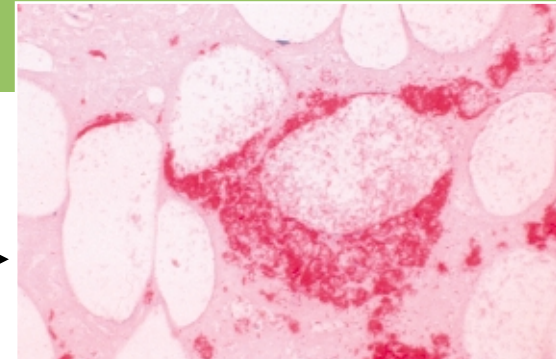
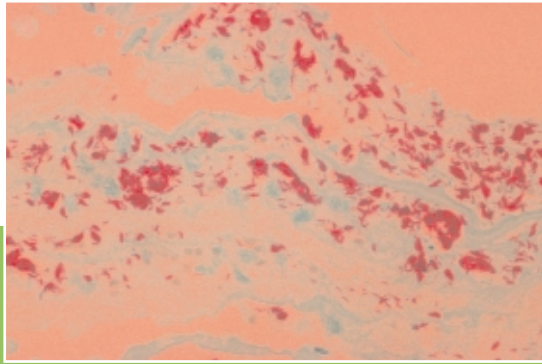


Figure 24 ▶
Subcutaneous tissue from the edge of a Buruli ulcer showing fat cell ghosts with AFB in the interstitium. ZN stain x100

Most bacilli are in the deeper areas of the specimen but may invade the interstitium of the adipose tissue and lobular septa of the subcutaneous tissue (Fig. 24).

Continuing necrosis of the dermis usually leads to degeneration of the epidermis and ultimate ulceration. Necrosis, however, may spread laterally with proliferation of AFB in the subcutaneous tissue and fascia (Fig. 25). Ulceration of the epidermis in such cases is often a very late event. The spread of disease in this manner leads to the plaque and oedematous forms of the disease.



◀ **Figure 25**
Masses of AFB infiltrate the base
of the edge of a Buruli ulcer.
AFB typically in clusters. ZN x100

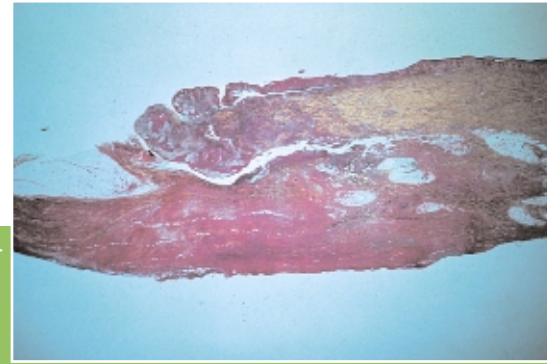
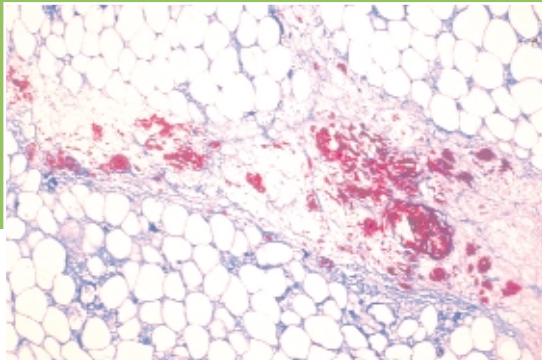


Figure 26 ▶
Biopsy specimen from the edge of a
Buruli ulcer showing undermining of
the dermis and massive necrosis of the
skin, dermis, subcutis and the fascia



◀ **Figure 27**
Subcutaneous tissue from margin of
a Buruli ulcer showing necrosis and
thickening of an interlobular septum.
Septum contains masses of AFB.
ZN stain x50

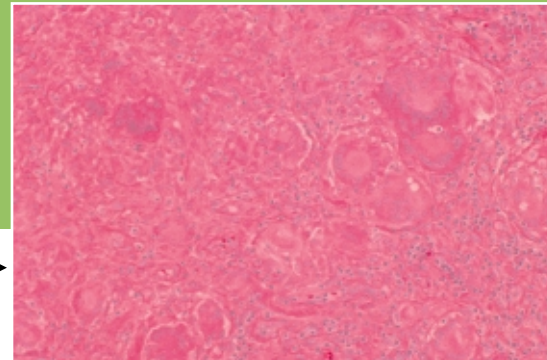
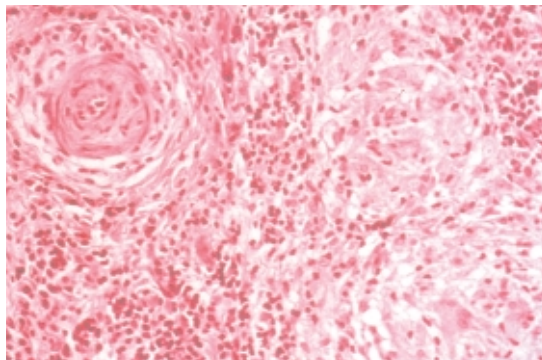


Figure 28 ▶
Early healing of a Buruli ulcer in the
organizing phase: lymphocytes,
epithelioid and giant cells. H & E x50



◀ **Figure 29**
Well formed delayed hypersensitivity
granuloma in healing Buruli ulcer.
H & E x50

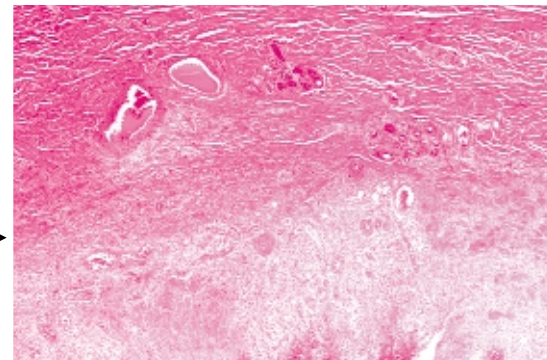


Figure 30 ▶
Advanced stage of healing
Buruli ulcer showing scarring
over most of the section.
H & E x25

Necrotic (active) stage: ulcerative lesions

Ulcers are undermined with reepithelialization of the edges of the lesion and undersurface of the overlying flap of the dermis (Fig. 26). Adjacent epidermis is usually hyperplastic. The base of the pristine ulcer contains a necrotic slough of cellular debris and fibrin, sometimes with a central eschar. There is contiguous coagulation necrosis of the subcutaneous tissue and fascia similar to that described for non-ulcerated lesions (Fig. 25 and Fig. 27). AFB are located in the base of the central slough and necrotic subcutaneous tissue. The disease rarely extends into underlying muscle. Vasculitis and mineralization are seen often (Fig. 21).

Organizing (early granulomatous stage)

Early healing is characterized by a poorly organized granulomatous response in the dermis and subcutaneous tissue (Fig. 28).

The granulomatous infiltration comprises swollen macrophages (epithelioid cells), Langhans' giant cells and lymphocytes. These eventually form organized tuberculoid granulomas. Foamy macrophages, lymphocytes and plasma cells are sometimes seen at the margin of necrotic fat. AFB are scarce or absent.

Healing stage

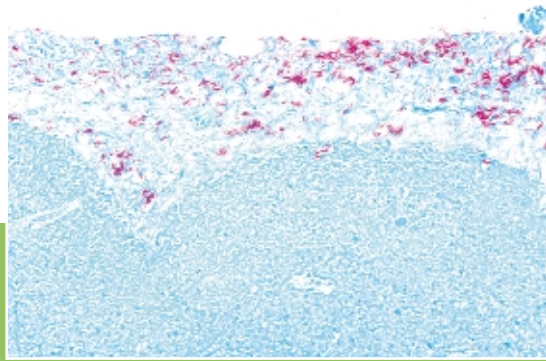
As healing advances, granulation tissue forms followed by fibrosis and a depressed scar (Fig. 29 and Fig. 30). AFB are seldom seen.

Lymph nodes

Although clinical lymphadenopathy is rarely appreciated, significant lymphadenitis is often seen histopathologically, both in lymph nodes adjacent to lesions and in regional nodes. Those adjacent to lesions may show marked invasion of the capsule by AFB (Fig. 31).

The parenchyma is often markedly necrotic with destruction of cortical lymphoid tissue (Fig. 32).

In such cases the entire node may be invaded by AFB. Regional lymph nodes, however, may show only sinus histiocytosis. Granulomatous changes are usually not seen, and AFB are rarely seen in regional nodes.



◀ **Figure 31**
Lymphadenopathy in Buruli ulcer. The parenchyma of the node is necrotic and the capsule is heavily infiltrated by AFB. ZN stain x100

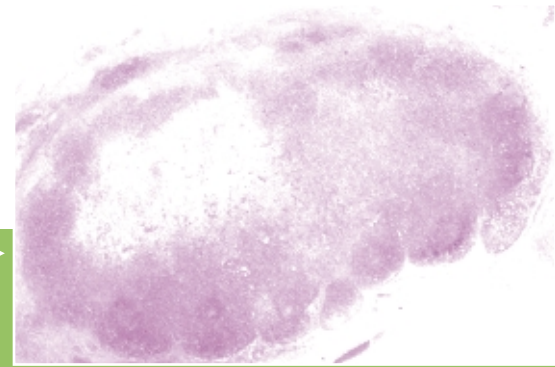


Figure 32 ▶
Necrotic lymphadenitis in a lymph node proximal to Buruli ulcer. The medulla is destroyed and only remnants of the cortical lymphoid tissue remain. ZN stained parallel sections showed large numbers of AFB. H & E stain x5



◀ **Figure 33**
X-ray of the leg showing destruction of the bone. **Note:** the patient had a Buruli ulcer over the affected area

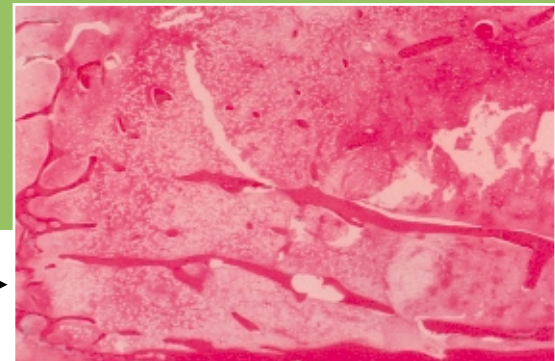
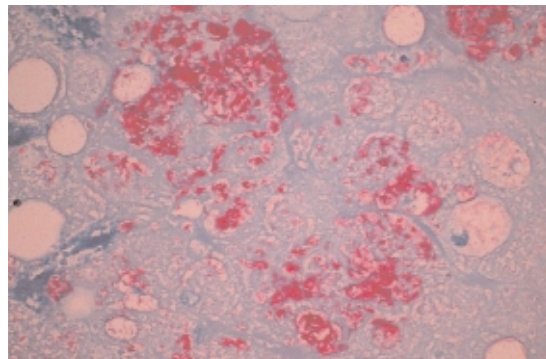


Figure 34 ▶
Osteomyelitis of tibia showing necrosis of the marrow and erosion of trabeculae. H & E stain x2.5



◀ **Figure 35**
Osteomyelitis of tibia with masses of AFB in necrotic marrow. ZN stain x50

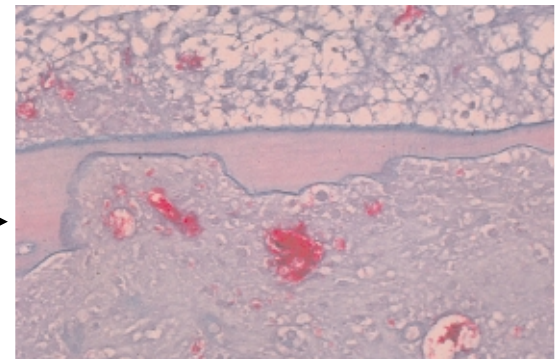


Figure 36 ▶
Osteomyelitis of tibia showing necrosis of marrow and a trabecula of bone undergoing dissolution in area of AFB. ZN stain x100

Bone changes

Bone may be affected by direct extension from an overlying lesion of Buruli ulcer, or at a site distant from recognized lesions, presumably by haematogenous spread of *M. ulcerans* (Fig. 33).

Histopathologically, the marrow is extensively necrotic and the bone trabeculae are eroded (Fig. 34). AFB are present in varying numbers, most often in the necrotic marrow (Fig. 35 and Fig. 36).

Although some lesions in bone seem to be purely an effect of the *M. ulcerans* in the bone, approximately 50% of the osteomyelitic lesions are coinfecting by pyogenic organisms such as streptococci, staphylococci and *Corynebacterium* sp. In such instances, there is suppuration and the organisms may be visible in Gram's stained sections. Well formed granulomas may develop producing a chronic osteomyelitis that is probably caused by *M. ulcerans*.

Patients with extensive disease (comment)

Patients with aggressive oedematous lesions involving large body areas often have widespread oedema and impaired renal function, or other evidence suggesting visceral organ involvement. Such patients sometimes die early in the course of the disease. While some authorities suspect that these events are attributable to a systemic effect of the toxin, this question can only be resolved by increased efforts to study the pathophysiology of such patients and by the study of autopsy specimens.

NOTES ON INTERPRETATION OF LABORATORY TESTS

As with all laboratory tests, the quality of the results produced depends on the quality and prompt delivery of the samples. It is not advisable to conclude that a patient does not have Buruli ulcer even if all tests are negative. Such situations may arise if biopsies or swabs are taken from areas where no organisms are present, or transport times are prolonged.

For example, in one series, 500 patients were considered to have proven Buruli ulcer using a strict definition of having at least two of the following tests positive: culture, PCR, histology or ZN. When each diagnostic method was considered alone, the following sensitivities were obtained: ZN 40–80%, culture 20–60%, histology > 90%, PCR > 90%. ZN and culture in particular are dependent on the type of clinical lesion. For example ZN sensitivity for nodules was 40%, for ulcers 60% and plaques 80%. For culture, bone specimens were positive in only 20% of cases, 50% for ulcers and 60% for plaques. Sensitivity of culture may be further improved by initial passage in mice (up to 75%). Clearly it is not advisable to exclude the diagnosis or to conclude that the patient has Buruli ulcer based on any one laboratory test.

Although uncommon, false positive ZN or PCR results or even false positive culture results have occurred. If the result from the laboratory does not fit with the clinical presentation, or is questioned by the clinician, laboratory tests should be repeated on freshly collected specimens.

Ideally, results obtained using several modalities and multiple samples should be considered together. However, in practice, in endemic areas, experienced clinicians commonly make accurate presumptive diagnosis on clinical grounds alone, or by using a combination of clinical appearances and a ZN stained smear.

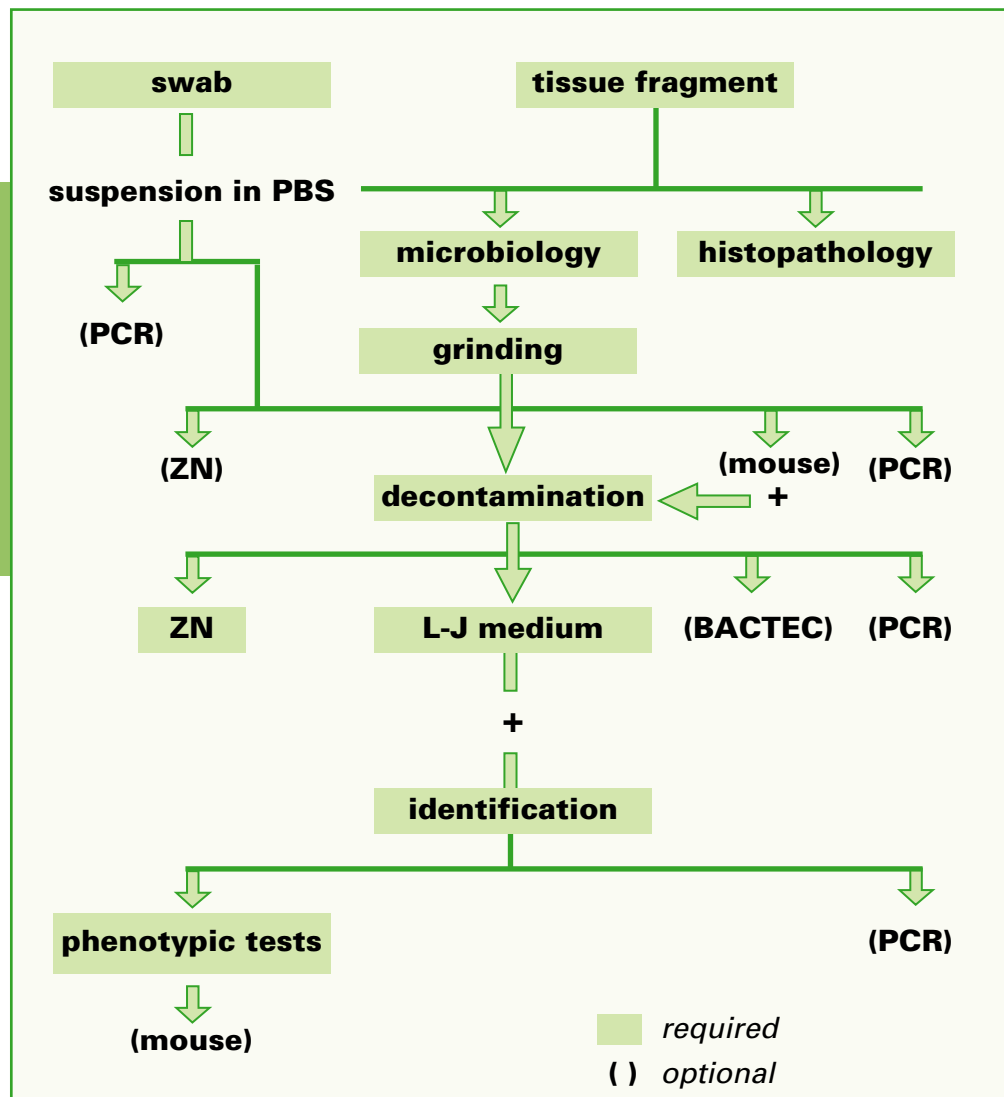
Annexes



Credit: WHO

Flow-chart for the laboratory diagnosis | Laboratory request form | Laboratory report form | Preparation of culture media
Microbiological staining techniques | Histopathological staining techniques | Decontamination methods | *Mycobacterium
ulcerans* culture with BACTEC 460 TB instrument | Biochemical and culture tests used for identification of slow-growing
mycobacteria | PCR protocol | Manufacturers' addresses | Work of WHO on Buruli ulcer | Some research institutions
involved in Buruli ulcer activities | Some NGOs and others involved in Buruli ulcer activities | Members of the WHO
Advisory Group on Buruli ulcer | Suggested reading

1 Flow-chart for the laboratory diagnosis of *Mycobacterium ulcerans* disease



2 Laboratory request form

A. Institutional information

1. Name of institution, address
2. Subdistrict District Region Country
3. Name of officer completing the form (last/first) 4. Title Speciality

B. Patient information

5. Health facility ID number Patient identification number
6. Name (last/first) 7. Age (months/years) 8. Sex M F
9. Address
10. Occupation of patient
11. Brief description of the lesion 12. Site of first lesion
13. List family contacts
14. Patient classification New case Recurrent case Same site
Different site

C. Location of lesion(s)

15. *Upper limb:* Right Left Abdomen Back Buttocks, perineum
- Lower limb:* Right Left Thorax Head and neck

D. Clinical form

16. Unifocal forms

- Active:* Nodule Papule Plaque Oedema Ulcer Osteomyelitis
- Inactive:* Scar Amputation Others, specify

17. Multifocal forms (please indicate the location of each clinical form)

Location of lesion(s)	Clinical form
a.
b.
c.
d.
e.
f.

E. Specimen for laboratory diagnosis**Institutional information**

- a. Name of institution, address
- b. Subdistrict District Region Country
- c. Name of officer completing the form (last/first)
- d. Title Speciality

Specimen laboratory number

18. Site of collection
19. Nature of specimen
20. Transport medium
21. Conditions for transportation
22. Date and time of collection

F. Tests requestedZN Culture PCR Histopathology

Received in laboratory Date (dd/mm/yy)..... Time

Name of officer Signature

3 Laboratory report form

Tests summary	ZN	Culture	PCR on specimen	Histopathology
Positive	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Negative	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Tests details	ZN	
Positive	<input type="checkbox"/>	Name of scale
Negative	<input type="checkbox"/>	Results
	Culture	
Positive	<input type="checkbox"/>	Time from inoculation to first positive primary culture (in weeks)
Negative	<input type="checkbox"/>	Species isolated
	PCR on specimen	
Positive	<input type="checkbox"/>	Comments
Negative	<input type="checkbox"/>	
	Histopathology	
Positive	<input type="checkbox"/>	Please attach report
Negative	<input type="checkbox"/>	Comments

Signature of microbiologist/technologist Date (dd/mm/yy)

4 Preparation of culture media

Löwenstein-Jensen (L-J) medium

There are three groups of components which are prepared separately and then added to make the medium:

1. Mineral solution
2. Malachite green solution
3. Homogenized whole eggs

• Mineral solution

– potassium dihydrogen phosphate anhydrous (KH ₂ PO ₄)	2.40 g
– magnesium sulphate (MgSO ₄ ·7H ₂ O)	0.24 g
– magnesium citrate	0.60 g
– asparagine	3.60 g
– glycerol (reagent grade)	12 ml
– distilled water	600 ml

Dissolve the ingredients following the order above, in distilled water, by heating. Autoclave at 121 °C for 30 minutes to sterilize. Cool to room temperature. This solution may be kept indefinitely and may be stored in suitable amounts in the refrigerator.

• Malachite green solution 2%

– malachite green dye	2 g
– sterile distilled water	100 ml

Using aseptic techniques, dissolve the dye in sterile distilled water by placing the solution in an incubator for 1 to 2 hours. This solution cannot be stored indefinitely and may precipitate or change to less-deeply coloured solution. In either case, discard and prepare a fresh solution.

• Homogenized whole eggs

Fresh hen's eggs, not more than 7 days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in soap solution. Rinse eggs thoroughly and soak them for 15 minutes in 70% ethanol. Remember to wash your hands before handling the clean, dry eggs. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of the complete medium

– mineral solution	600 ml
– malachite green solution	20 ml
– homogenized eggs (20–25 eggs depending on the size)	1000 ml

The complete egg medium is distributed in 6–8 ml volumes in sterile 14 or 28 ml McCartney bottles or in 20 ml volumes in 20x 150 mm screw-capped test tubes and the tops tightly closed. Inspissate the medium (*see below*) within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Coagulation of medium (inspissation)

Before loading, heat the inspissator to 80 °C to hasten the build up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium at 80–85 °C for 45 minutes. Do not reheat the medium.

The quality of egg media deteriorates when coagulation is done at a too high temperature or for too long. Discolouring of the coagulated medium may be due to excessive temperature. The appearance of holes or bubbles on the surface of the medium also indicates faulty coagulation procedures.

Discard poor quality media.

Sterility check

After inspissation, the whole batch of media or a representative sample of culture bottles should be incubated at 35–37 °C for 24 hours as a sterility check.

Storage

Eggs may sometimes contain antibiotics which inhibit the growth of mycobacteria. The origin of the eggs must be known to control their quality. The media should be dated, stored and may be kept in the refrigerator for several weeks with caps tightly closed to prevent the medium from drying out. For optimal isolation of mycobacteria, L-J media should not be older than 4 weeks.

Middlebrook 7H10 and 7H11 agar medium

Middlebrook 7H10 may be made from basic ingredients or prepared from commercially available 7H10 agar-powdered base and Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment. 7H11 is 7H10 agar enriched by the addition of an enzymatic digest of casein. It is best to prepare 7H10 and 7H11 medium in small quantities of 200–400 ml to minimize the heating needed to melt the agar. Boiling the basal medium before autoclaving (either to solubilize the agar or to provide stocks of prepared base that may be stored and boiled for later use) should be avoided because repeated heating compromises the quality of the medium.

When Middlebrook 7H10 or 7H11 medium is used, it must be incubated under micro-aerophilic conditions (2.5–5.0% oxygen). The exposure of Middlebrook 7H10 or 7H11 agar to either daylight or heat may result in the release of formaldehyde in sufficient concentration to inhibit the growth of mycobacteria.

5 Microbiological staining techniques

Ziehl-Neelsen (ZN) staining

The reagents described here are strictly for use with the hot Ziehl-Neelsen method only. The hot method is superior to cold methods, such as the Kinyoun. Concentrations recommended are slightly different from what is often found in handbooks. The fuchsin concentration is slightly higher and the methylene blue lower, providing the best possible contrast (strong red bacilli with a light blue background). Other concentrations and cold methods may give satisfactory results under otherwise optimal conditions. However, when other conditions (microscope, light, technician training) are less well controlled, it is strongly recommended to use the concentrations given below and the hot method for a better colour contrast.

REAGENTS

• Fuchsin

- | | |
|-------------------------------------|-------------------|
| – basic fuchsin | 10 g |
| – 95% ethanol (technical grade) | 100 ml |
| – dissolve basic fuchsin in ethanol | Solution 1 |

• Phenol

- | | |
|-------------------------------------|-------------------|
| – phenol crystals | 5 g |
| – distilled water | 85 ml |
| – dissolve phenol crystals in water | Solution 2 |

Mix 10 ml of **solution 1** with 90 ml of **solution 2** and store in a tightly stoppered amber-coloured bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for at least 12 months. Filter before or at the time of use.

• Decolourizing solution

- | | |
|----------------------------------|-------|
| – concentrated hydrochloric acid | 3 ml |
| – 70% ethanol (technical grade) | 97 ml |

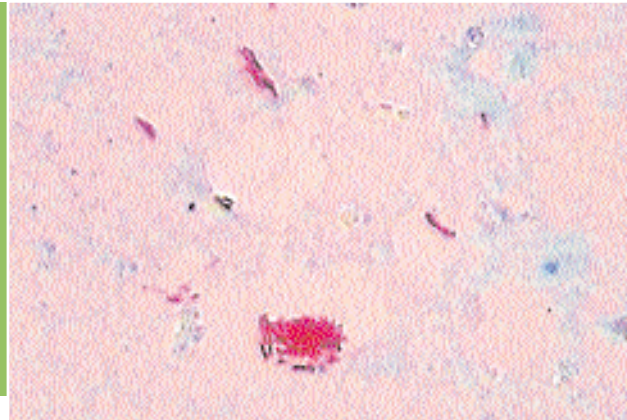
Carefully add concentrated hydrochloric acid to 70% ethanol. Always add acid slowly to alcohol, not vice versa. Store in an amber-coloured bottle. Label bottle with name of reagent carbolfuchsin and date of preparation. Can be stored at room temperature indefinitely.

Counterstain

- methylene blue chloride 0.1 g
- distilled water 100 ml

Dissolve methylene blue chloride in distilled water in a tightly stoppered amber-coloured bottle. Label bottle with name of reagent and dates of preparation and expiry. Can be stored at room temperature for at least 12 months.

Figure 37 ▶
ZN stained smear from a Buruli ulcer
showing red extracellular AFB against
a blue background

**Procedure**

1. Place the numbered slides on a staining rack in batches (maximum 12). Ensure that the slides do not touch each other.
2. Flood entire smear with ZN carbol-fuchsin which has been filtered prior to use; the most practical way is to pour stain over the slide through a funnel equipped with filter-paper.
3. Heat the slide slowly until it is steaming for 3–5 minutes. Do not let the stain boil dry.
4. Rinse with gentle stream of running water until free stain is washed away.
5. Flood the slide with the decolourizing solution for 3 minutes.
6. Rinse the slide thoroughly with water. Drain excess water from the slide.
7. Repeat steps 5 and 6 if the smear is still too red.
8. Flood the slide with counterstain.
9. Allow the smear to counterstain, usually for a maximum of 60 seconds. If after repeated exposure to acid-alcohol, the smear cannot be sufficiently discoloured, counterstain a bit longer.
10. Rinse the slide thoroughly with water. Drain excess water from the slide.
11. Allow smears to air-dry. Do not blot. Keep slide out of direct sunlight and read as soon as possible.

Quantitation scale

No. of AFB seen on average	No. of fields to screen	Report
No. of AFB / 100 immersion fields	100	No AFB observed
1–9 AFB / 100 immersion fields*	100	Record exact figure
10–99 AFB / 100 immersion fields	100	+
1–10 AFB / 1 immersion field	50	++
> 10 AFB / 1 immersion field	20	+++

*A finding of three or fewer bacilli in 100 fields does not correlate well with culture positivity, but should be reported.

Fluorochrome staining**REAGENTS****Auramine O**

- auramine powder 0.1 g
- 95% ethanol (technical grade) 10 ml
- dissolve auramine in ethanol **Solution 1**

Note: Auramine is carcinogenic, direct contact with skin should be avoided.

Phenol

- phenol crystals 3.0 g
- distilled water 87 ml
- dissolve phenol crystals in water **Solution 2**

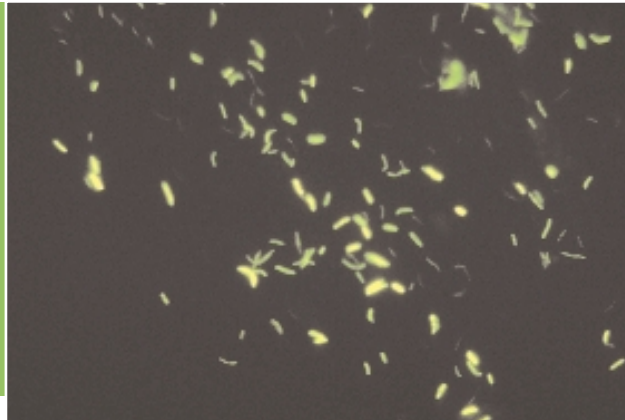
Mix **solutions 1** and **2** and store in a tightly stoppered amber-coloured bottle away from heat and light. Do not use after 3 months. A precipitate usually forms but does not indicate deterioration; however, the solution should be filtered during the staining procedure.

Decolourizing solution

- concentrated hydrochloric acid 0.5 ml
- 70% ethanol (technical grade) 100 ml

Carefully add concentrated hydrochloric acid to the ethanol. Always add acid slowly to alcohol, not vice versa. Store in amber-coloured bottle. Label bottle with name of reagent and date of preparation. Keeps indefinitely.

Figure 38 ▶
Fluorochrome stained smear showing
AFB as bright yellow rods against
a dark background

**Counterstain**

- potassium permanganate (KMnO_4) 0.5 g
- distilled water 100 ml

Dissolve potassium permanganate in distilled water in a tightly stoppered amber-coloured bottle. Label bottle with name of reagent and dates of preparation and expiration. Store at room temperature for up to 3 months.

The potassium permanganate (0.5%) as described above tends to give a very dark background. This makes it difficult to keep the smear in focus. At lower concentrations this effect is less, however, such weak solutions of KMnO_4 are unstable and not preferred by many laboratories.

Procedure

Prepare fairly thick smears from homogenized biopsy material. Such smears are easier to examine because of the more visible background.

1. Place numbered smears on a staining rack in batches (maximum 12). Ensure that the slides do not touch each other.
2. Flood entire smear with auramine O. Use a funnel equipped with a Whatman #1 filter-paper to pour the stain on the slides. Allow to stand for 15 minutes, making sure that the staining solution remains on the smears. *Do not heat!*
3. Rinse with water and drain. Distilled water is usually recommended but this is often not available in field laboratories. Experience from some laboratories has shown that use of tap water is always satisfactory. A suitable alternative would be dechlorinated water (i.e. water that has been exposed to air for 24 hours).
4. Decolourize with 0.5% acid-ethanol for 2 minutes.
5. Rinse with water and drain.
6. Flood smears with counterstain for 2 minutes. Time is critical because counterstaining for longer periods may quench the fluorescence of AFB.
7. Rinse with water and drain.
8. Allow smears to air-dry. Do not blot. Read as soon as possible, keep slides in the dark (i.e. in a closed slide-box).

Quantification scale (see that for ZN staining)

Fluorescent microscopy magnification	200 or 250x	400x	630x
Number of AFB count	Divide observed count by 10	Divide observed count by 4	Divide observed count by 2

To adjust for altered magnification of the fluorescent microscope, divide the number of organisms seen by the factor provided and refer to the quantification table for ZN smear for the appropriate value to report.

6 Histopathological staining techniques

Adapted from technical SOP 5.47, Armed Forces Institute of Pathology (AFIP), Washington DC, USA

1. Harris' haematoxylin and eosin (H & E) procedure (without mercury)

Note: This procedure stains tissue elements and bacteria more intensely than many other H & E procedures. Use of potassium permanganate in place of mercury salts is less hazardous to the environment and individuals.

The procedure is intended for specimens fixed in 10% buffered neutral formalin and tissue sections cut at 4-6 μm thick. Control tissue should contain nuclei, cytoplasmic structures, connective tissue and if possible bacteria.

SOLUTIONS

• Harris' hematoxylin

- potassium or ammonium alum 100 g
- distilled water 500 ml

Dissolve with the aid of heat.

In a separate container combine the following:

- hematoxylin crystals 5 g
- absolute ethanol 50 ml
- distilled water 250 ml

Dissolve (may be warmed) and add:

- 0.25% potassium permanganate 250 ml

Allow to stand 3 minutes stirring and combine this solution with the above alum solution.

Cool in running water, and add 20 ml of glacial (100%) acetic acid. Filter before use.

• 1% acid-alcohol

- 95% ethanol 736 ml
- deionized water 263.2 ml
- concentrated hydrochloric acid 10 ml

• Ammonia water

- deionized water 1000 ml
- 28% ammonium hydroxide 4 ml

- **1% Eosin stock solution**

- eosin Y, water-soluble
- deionized water

1 g
100 ml

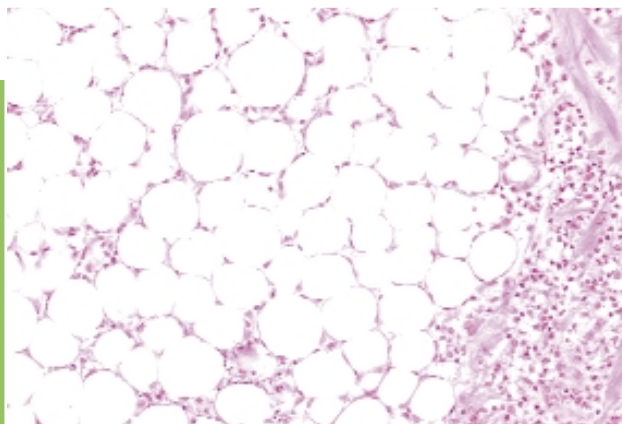


Figure 39 ▶

This section showing panniculitis is from a Buruli ulcer patient stained by the H & E method

Note: the nuclei of cells are blue and the connective tissue is pink

- **1% Phloxine stock solution**

- phloxine B
- deionized water

1 g
100 ml

- **Eosin—phloxine solution**

Combine the following:

- eosin stock solution
- phloxine stock solution
- 95% ethanol
- glacial acetic acid

100 ml
10 ml
780 ml
4 ml

This solution is good for approximately one week.

Staining procedure

1. Deparaffinize slides and hydrate to water.
2. Stain in freshly filtered Harris haematoxylin for 10 minutes.

3. Wash in warm running tap water for 5 minutes.
4. Dip twice in 1% acid-alcohol to differentiate.
5. Stop the differentiation by dipping in warm tap water and then dipping in weak ammonia water or saturated lithium carbonate until section begins to turn bright blue.
6. Wash in warm running tap water for 10 minutes.
Note: if nuclear staining is weak, return to step 2. If the background is not clear return to step 4 but use only 1 quick dip in the acid alcohol.
7. Counterstain in eosin-phloxine for 2 minutes
8. Dehydrate and clear through 2 changes successively of 95% ethanol, absolute ethanol and xylene. Slides should remain in each for 2 minutes.
9. Mount in a resinous mounting medium.

2. Ziehl-Neelsen (ZN) method for acid-fast organisms

Adapted from technical SOP 5.23, Armed Forces Institute of Pathology (AFIP), Washington, DC, USA

Note: This technique is used to demonstrate acid-fast organisms other than *Nocardia* sp. and leprosy bacilli. The procedure is intended for specimens fixed in 10% buffered neutral formalin and sections cut at 4–6 micrometers thick. Control sections should contain known *M. tuberculosis* or *M. ulcerans*.

SOLUTIONS

• ZN carbolfuchsin solution

- | | |
|----------------------------------|--------|
| – phenol (fused crystal, melted) | 25 ml |
| – absolute ethanol | 50 ml |
| – basic fuchsin | 5 g |
| – deionized water | 500 ml |

Store in a warm but open place to maintain the solution in liquid form.

• Acid-alcohol

- | | |
|----------------------------------|--------|
| – 70% ethanol | 100 ml |
| – concentrated hydrochloric acid | 1 ml |

• Methylene blue solution (working)

- | | |
|---------------------------|--------|
| – methylene blue crystals | 3 g |
| – deionized water | 600 ml |

Staining procedures

1. Deparaffinize and hydrate to deionized water.
2. Stain in ZN carbofuchsin for 30 minutes.

Note: if organisms fail to stain, prepare new carbofuchsin solution.

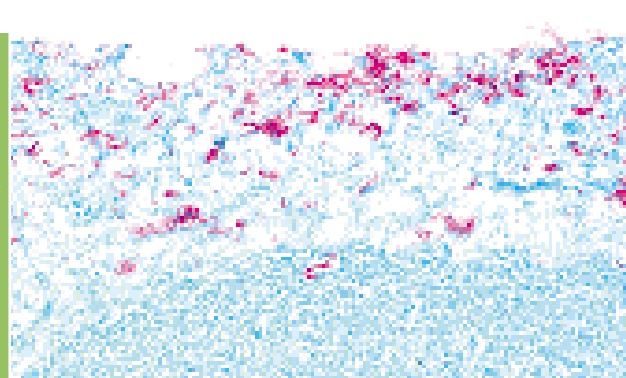


Figure 40 ▶
This section of a lymph node from a Buruli ulcer patient is stained by the ZN method. The AFB are red and the background tissue is blue

3. Wash in cool tap water for 10 minutes.

Note: if tap water is chlorinated, wash for a shorter time.

4. Differentiate slides individually with acid-alcohol.
5. Wash in running water for 3 minutes.
6. Counterstain by dipping slide individually in working methylene blue solution then rinsing them in tap water.
7. Dehydrate and clear in 2 successive changes in 95% ethanol, 100% ethanol and xylene.
8. Mount in a resinous mounting medium:
 - AFB:red
 - Background:blue

3. Grocott's method for fungi (GMS)

Adapted from technical SOP 5.10, Armed Forces Institute of Pathology (AFIP), Washington, DC, USA

Note: This technique demonstrates all forms of fungi, however, *Histoplasma capsulatum* and *Nocardia asteroides* may require extended time in the methenamine-silver solution. The procedure is intended for specimens fixed in 10% buffered neutral formalin and tissue sections cut at 4-6 µm thick. Control tissues must be from a known fungal infection containing fungal elements—histoplasmosis and nocardiosis tissues should not be used.

SOLUTIONS

- **4% Chromic acid**

- chromic trioxide (warning: irritant) 4 g
- distilled water 100 ml

- **5% Silver nitrate**

- silver nitrate 5 g
- distilled water 100 ml

- **3% methenamine***

- methenamine 30 g
- distilled water 1000 ml

- **1% Sodium metabisulfite**

- sodium metabisulfite 1 g
- distilled water 100 ml

- **1.0% Gold chloride**

- yellow gold chloride 5 g
- sterile water 500 ml

- **0.1% Gold chloride**

- 1.0% gold chloride 10 g
- sterile water 100 ml

- **2% Sodium thiosulfate**

- sodium thiosulfate 2 g
- sterile water 100 ml

* (synonyms: hexamethylenetetramine, hexamine, hexamethylenamine)

• **Stock methenamine-silver nitrate solution**

- 5% silver nitrate solution 50 ml
- 3% methenamine solution 1000 ml

Note: A white precipitate forms but immediately dissolves on shaking. The clear solution remains usable for months. Store in the refrigerator.

• **5% Sodium borate**

- sodium borate (decahydrate) 5 g
- distilled water 1000 ml

• **Working methenamine-silver nitrate solution**

Note: Make fresh for each batch of tissues.

- stock methenamine-silver nitrate solution 25 ml
- distilled water 25 ml
- 5% sodium borate solution 2 ml

• **Stock 0.2% light green**

- light green, SF yellowish (C.I. # 42095) 0.2 g
- distilled water 100 ml
- glacial acetic acid 0.2 ml

• **Working light green solution**

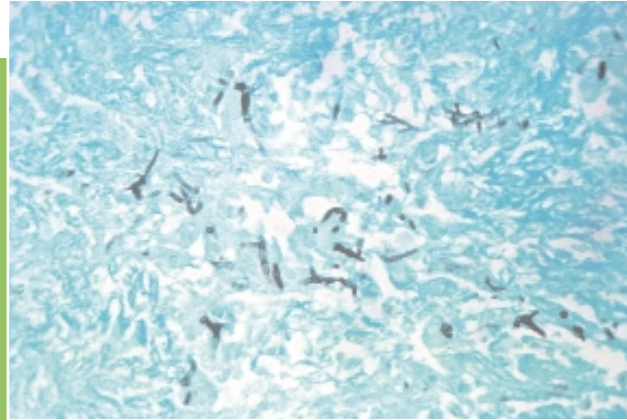
- stock light green solution 10 ml
- distilled water 50 ml

Staining procedure:

1. Deparaffinize and hydrate to distilled water.
2. Oxidize in 4% chromic acid solution for 1 hour.
3. Wash in tap water for at least 20 minutes; slides must be colourless.
4. Reduce in 1% sodium metabisulfite for 1 minute to remove any residual chromates.
5. Wash in tap water at least 5 minutes.
6. Rinse with 6 changes of distilled water.
7. Place in freshly made working methenamine-silver nitrate solution in a 60 °C oven for 60 to 70 minutes or until sections turn toasty brown. Check microscopically.

8. Rinse in 6 changes of sterile water.
9. Tone in 1% gold chloride for 1 to 5 minutes—check microscopically; fungi will be black and the background should be pink to grey.
10. Rinse in distilled water.

Figure 41 ▶
Histopathologic section of a phaeomycotic cyst in skin. This GMS stained section shows black-stained hyphae of the causative fungus, *Philalophora repens*



11. Remove unreduced silver by placing in 2% sodium thiosulfate solution for 2 to 5 minutes.
12. Wash thoroughly in tap water.
13. Counterstain in stock light green solution for 4 minutes.
14. Rinse in distilled water.
15. Dehydrate, clear and mount in a resinous mounting medium.

4. Brown-Hopps methods for gram-positive and gram-negative bacteria

Adapted from technical SOP 5.4, Armed Forces Institute of Pathology (AFIP), Washington DC, USA

Note: This procedure demonstrates many gram-positive and most gram-negative bacteria and is intended for specimens fixed in 10% buffered neutral formalin and tissue sections cut at 4–6 µm thick. Controls must contain gram-positive and gram-negative organisms. Inflamed appendix is often a useful source of control tissue.

SOLUTIONS

• Crystal violet

- crystal violet 1 g
- deionized water 100 ml

• Gram's iodine

- iodine crystals 1 g
- potassium iodide 2 g
- deionized water 5 ml

When iodine and potassium iodide are completely dissolved, add an additional 295 ml of deionized water.

• 1% Basic fuchsin

- basic fuchsin 1 g
- deionized water 100 ml

• Gallego's solution

- deionized water 100 ml
- concentrated formalin (37–40%) 2 ml
- glacial acetic acid 1 ml

• Picric acid-acetone

- picric acid (dried, see below) 0.05 g
- acetone (water-free) 500 ml

Note: Drying picric acid—Place a layer of wet picric acid (more than you actually need) less than 2 mm thick between four large sheets of coarse filter-paper (two sheets above and two below). Squeeze as much water out as possible by rolling a bottle or other round object over the filter-paper. The picric acid can now be weighed. Put the excess back with the wet picric acid. Use the weighed picric acid immediately. Rinse the filter-paper in running water until all the yellow colour is removed before discarding. Always store picric acid crystals under water to avoid an explosion.

• Acetone-xylene

- acetone 100 ml
- xylene 100 ml

Staining procedures:

1. Deparaffinize and hydrate to 95% ethanol.

Note: A horizontal staining rack is used for steps 2 through 8. Staining dishes are used for the remaining steps.

2. Place 15 to 20 drops of 1% crystal violet solution on each slide. Leave on the slide for 1 to 2 minutes. Agitate gently.
3. Rinse in tap water.

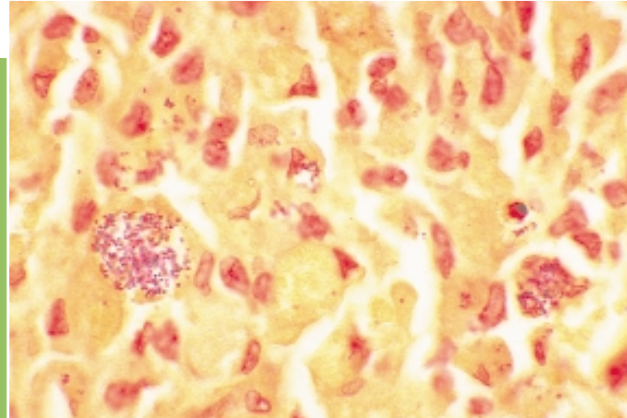


Figure 42 ▶

Brown-Hopps (B & H) gram-stain of tissue infected by *Rhodococcus* sp.

Note: several clusters of blue-stained (gram-positive) bacteria

4. Place slides in Gram's iodine for 1 minute.
5. Rinse in tap water.
6. Decolourize slides with acetone until the last of the crystal violet colour starts to stream away.
7. Wash slides well in water immediately.
8. Pour 1% basic fuchsin on the slide for 5 minutes.
9. Rinse slides in tap water.
10. Place slides in Gallego's solution, 2 changes, 60 seconds in each with vigorous agitation.
11. Rinse slides in tap water.
12. Place slides in acetone for 30 seconds.
13. Place slides in picric acid-acetone for 2–3 minutes.
14. Place slides in acetone-xylene solution, 2 changes, 30 seconds in each.
15. Clear slides in xylene, 2 changes.
16. Mount in a resinous mounting medium.

7 Decontamination methods

1. Sodium hydroxide (modified Petroff)

PREPARATION

• 4% sodium hydroxide (NaOH) solution

- sodium hydroxide pellets (analytical grade) 4 g
- distilled water 100 ml

Dissolve NaOH in distilled water by heating. Autoclave at 121 °C for 15 minutes. This solution may be kept indefinitely and may be stored in the refrigerator.

• Sterile saline

- sodium chloride (NaCl) pellets (analytical grade) 0.85 g
- sterile distilled water 100 ml

Autoclave at 121 °C for 15 minutes.

METHOD

1. For each 2 ml of specimen (swab, pus or homogenized tissue) add approximately 2 ml of 4% NaOH or more.
2. Tighten the cap of the container and shake to digest.
3. Let stand for 15 minutes at room temperature with occasional shaking.
4. Centrifuge at 3000x g for 15 minutes.
5. Pour off supernatant.
6. Add 15 ml sterile saline or distilled water and resuspend sediment.
7. Centrifuge at 3000x g for 15 minutes.
8. Decant supernatant and inoculate into culture medium immediately.

2. Digestion decontamination N-acetyl-L-cysteine-sodium hydroxide method

PREPARATION

• Solution A: NaOH 4%

- NaOH 4 g
- distilled water 100 ml

- **Solution B: sodium citrate 2.9%**

- sodium citrate 2 H₂O 2.9 g
- distilled water 100 ml

Mix equal parts of solution A and solution B.

Distribute in screw-cap flasks.

Autoclave at 121 °C for 20 minutes.

On the day of use, add N-acetyl-L-cysteine at 1% concentration in the flask.

Mix well.

As N-acetyl-L-cysteine loses its mucolytic activity on standing, the final reagent must be used within 24 hours.

METHOD

- **Swabs**

Swirl the swab into 2 ml sterile distilled water in a centrifuge tube, then remove the swab.

- **Tissue**

Grind tissue fragments in a sterile mortar with pestle or in a sterile tissue grinder (Potter) with sterile saline (2 ml).

Remove supernatant into a sterile centrifuge tube.

Add 2 ml of N-acetyl-L-cysteine-NaOH mixture; tighten the tubes and mix on vortex for 20 seconds.

For decontamination, keep the tubes for 20 minutes at room temperature.

Dilute the digested and decontaminated specimen with 30 ml of sterile distilled water.

Mix by inversion and centrifuge at 3000x g for 20 minutes.

Discard the supernatant fluid in a jar containing disinfectant.

Resuspend sediment in 1 ml of sterile distilled water; mix well.

With a syringe, inoculate 0.5 ml of the sample in a 7H12B bottle and 0.2 ml on L-J medium.

Spread 0.1 ml of the sample on a slide and stain.

Incubate the media at 30 °C.

Note: The mucolytic agent NALC (used for rapid digestion of sputum) enables the decontaminating agent (NaOH) to be used at a lower final concentration (1%). Sodium citrate is included in the digestion mixture to bind the heavy metal ions which may be present in the specimen and may inactivate the NALC.

- **NALC method, if properly performed, provides more positive cultures than other methods (resulting in the killing of approximately 30% of the bacilli).**
- **Time needed to process a single specimen is about 40 minutes; 20 specimens would take approximately 60 minutes.**
- **NALC loses activity rapidly in solution so fresh digestion mixture should be made daily.**
- **The indicated specimen exposure time should be strictly adhered to and a 1:10 dilution of resuspended sediment must be made to decrease the concentration of any toxic components that may inhibit growth from the specimen.**

8 *Mycobacterium ulcerans* culture with BACTEC 460 TB instrument

Culture medium system

One vial of Middlebrook 7H12B medium can be inoculated and incubated at 30 °C. 7H12B medium may be used as a stand-alone medium, however, for maximum recovery of *M. ulcerans*, one tube of L-J medium (or modification) or 7H10 or 7H11 Middlebrook media may also be used.

Addition of antimicrobial supplement (PANTA)

Contaminations may be reduced by supplementing the medium with a mixture of antimicrobials prior to inoculation. PANTA supplement contains polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin, and is available in a lyophilized form. (BACTEC 12B medium becomes selective after the addition of PANTA.)

Reconstitute the lyophilized PANTA with 5 ml of reconstituting fluid and add 0.1 ml of this mixture to each vial of 12B medium (4 ml) before inoculation of the specimen.

To establish CO₂ atmosphere

The BACTEC 460 TB should be connected to a supply of 5%–10% CO₂ in air.

Prior to inoculation, all 12B vials on BACTEC must be tested to eliminate vials with high background readings and to establish a CO₂ enriched atmosphere. Any vial showing an initial Growth Index (GI) of 20 or more is eliminated prior to inoculation.

Inoculation of culture media

Use a tuberculin syringe with permanently attached needle for all inoculations.

Using a new syringe for each specimen, inoculate 0.5 ml of the specimen into the medium vial.

After every inoculation of a 12B vial, clean the rubber stopper of each vial with an appropriate disinfectant (e.g. 70% ethanol).

All inoculated vials should be incubated at 30 °C. Growth of the culture is checked weekly.

M. ulcerans is a slow growing bacterium. Vials should be kept for at least two months, or longer if possible.

GI readings of 10 or less are considered negative and a GI of more than 10 is positive. Positive vials are tested twice a week. The generation time of *M. ulcerans* in mice is approximately 3.5 days.

9 Biochemical and culture tests used for the identification of slow-growing mycobacteria

Most of these tests are based on studies carried out by the International Working Group on Mycobacterial Taxonomy which described highly reproducible tests for use in systematics of the genus *Mycobacterium* (Wayne et al., 1974, 1976). The tests recommended as minimal standard tests for the identification of slow growing mycobacteria were described in 1992 by Vincent Lévy-Frébault and Portaels.

Figure 43 ▶

The first two tubes from the left were inoculated with a photochromogenic strain (no pigmentation in the dark but pigmentation in the light).

The next two tubes were inoculated with a scotochromogenic strain (pigmentation produced in the dark and in the light)

The last two tubes were inoculated with an African *M. ulcerans* strain (pigmentation produced in the dark and in the light)



Pigmentation and photoreactivity. Mycobacteria may synthesize carotenoid pigments which confer a yellow-to-red pigmentation to colonies. Carotenogenesis is achieved in the absence of light by scotochromogenic mycobacteria. For these mycobacteria, the influence of light is essential, and prolonged incubation in light can only intensify the pigmentation. Photochromogenic mycobacteria require exposure to light and oxygen for carotenogenesis. Other mycobacteria do not develop pigmentation under any light conditions. However, a few nonphotochromogenic strains may develop some pigmentation as they age (weak to bright yellow or pink to coral). Because of these variable characteristics of pigment formation, pigmentation must be determined as follows: a cell suspension, properly diluted to obtain isolated colonies, is inoculated into two tubes containing the appropriate solid medium. It is important to obtain isolated colonies to ensure the maximal oxygenation necessary for carotenogenesis. One of the tubes is wrapped or placed in any device that ensures total darkness. The tube protected in this way and the unshielded tube are incubated at the optimal temperature until growth is plainly visible in the unshielded tube.

The test has to be performed as soon as well-developed colonies are observed because photoinduction of carotenogenesis may not occur in old cultures and, as mentioned above, pigmentation can develop slowly while the control remains non-pigmented. The tube incubated in the dark is placed at a distance of 20 cm from a 60 W fluorescent lamp (or equivalent tungsten bulb) for 1 to 2 hours. Good aeration of the culture, which is properly ensured by loosening the cap, is critical as carotenogenesis is oxygen dependent. The cultures are then incubated again and examined for pigmentation 24 hours after illumination.

A comparison of the pigmentation of the shielded tube that was exposed once to light with the unshielded tube determines whether the mycobacterium is scotochromogenic, photochromogenic, or nonphotochromogenic on the basis of the criteria as described in Figure 43.

Resistance to isoniazid, thiophene-2-carboxylic hydrazide, hydroxylamine, p-nitrobenzoic acid and NaCl

Resistance to inhibitory agents is assessed by incorporating each inhibitory agent into separate tubes of L-J medium. Most compounds are added into the uncoagulated solution prepared as shown in Table 7.

Table 7 Preparation of inhibitory agents*

Drug	Final concentration (µg/ml)	Solvent	Concentration in stock solution (mg/ml)	Amount used for 100 ml of medium (ml)
Isoniazid	1	water	0.1	1
Isoniazid	10	water	1	1
Thiophene-2-carboxylic hydrazide	2	50% aqueous ethanol	0.1	2
Hydroxylamine	500	water	25	2
p-Nitrobenzoic acid	500	propylene glycol	50	1

**In addition, a 5% (final concentration) NaCl solution was prepared by adding 5 g of NaCl to 100 ml of water.*

From the standard culture suspension, 10⁻² and 10⁻⁴ dilutions are prepared, and 0.2 ml portions of the 10⁻² dilution are inoculated into tubes of medium containing inhibitory agents. In addition, control tubes are inoculated with 0.2 ml of the 10⁻² and 10⁻⁴ dilutions. The colonies are counted after no fewer than 3 weeks incubation or when growth is visible in the control tubes. If growth on a drug-containing medium is less than the growth of the 10⁻⁴ dilution control (i.e. less than 1% resistance), the culture is reported as susceptible; growth on drug-containing medium equal to the growth of the 10⁻² dilution control (i.e. more than 1% resistance) is reported as resistant. Colonies in the 10⁻² dilution control tubes must be numerous (more than several hundred colonies or confluent growth); otherwise, interpretation of the test may be inaccurate.

Catalase activity. The test used to detect catalase activity is semiquantitative and reflects differences in enzyme kinetics (Wayne et al, 1976). For this test, butts (not slants) of L-J medium are used (Fig. 44).

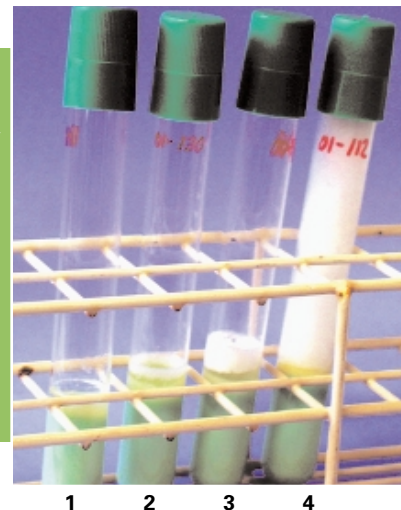
The flat circular surface of the medium is inoculated with 3 drops of an undiluted suspension of mycobacteria, prepared as described above for growth rate determinations.

Figure 44 ►

Tube 1 was inoculated with a strain without catalase activity (no foam)

Tubes 2 and 3 were inoculated with two strains producing less than 45 mm foam

Tube 4 was inoculated with a catalase positive strain producing more than 45 mm foam



The tubes are incubated at the optimal temperature for growth. After the medium is examined to ensure that there is growth, 1 ml of a freshly prepared assay reagent is added. The assay reagent consists of a mixture of 0.5 ml of 30% H₂O₂ and 0.5 ml of 10% Tween 80 in H₂O. The tube is placed in an upright position, and the height of the foam column generated after exactly 5 minutes is measured. A column of foam that rises more than 45 mm above the surface of the medium is recorded as positive. If the column is less than 31 mm, the result is recorded as negative. If the foam column is between 31 and 45 mm, the test should be repeated. Agar media may not be substituted for L-J medium, as they yield erratic results.

Tween 80 hydrolysis. The Tween 80 hydrolysis test (Wayne et al., 1974) is based on the fact that neutral red is orangish-yellow, even at a pH corresponding to the pH of the red form, when it is complexed with Tween 80 (a polyethylene derivative of sorbitan monooleate). Hydrolysis of Tween 80 leads to a change in the colour of the substrate to pink. This colour change is not due to a pH shift caused by the production of oleic acid but to the destruction of Tween 80.

The substrate solution consists of 0.5 ml of Tween 80 in 100 ml of 0.067 M phosphate buffer (pH 7.0) to which 2 ml of a 1% aqueous solution of neutral red is added. The solution is dispensed into screw-cap tubes (16 by 125 mm) in 4 ml amounts, and the preparations are sterilized by autoclaving. After cooling, a loopful of bacteria is suspended in 4 ml of substrate, and each tube incubated at 30–33°C for up to 10 days. A change in colour from amber to pink or red is recorded after 24 h and 5 and 10 days of incubation as a positive reaction. The pink colour must develop in the fluid itself, so the suspension should not be shaken until after each reading is made. Otherwise, the fact that the cells themselves may be neutral red-positive and take up the pink dye may lead to a false-positive reading (Wayne, 1985).

Urease activity. The method of Steadham (1979) is used. A loopful of actively growing culture on L-J medium is emulsified in 1 ml of urea broth prepared as shown in Table 8.

Table 8 Urease activity (the Steadham method)

Component	Amount
Peptone	1 g
Dextrose	1 g
Sodium chloride	5 g
Potassium phosphate (monobasic)	0.4 g
Urea	20 g
Phenol red sodium (1% solution)	1 ml
Tween 80	0.1 ml
Distilled water	1 000 ml

The tubes are incubated at 30–33 °C and are read after 1 and 7 days.

A change in the colour of the broth from bright yellow to dark pink or red is an indication of the breakdown of urea and is read as a positive reaction.

Niacin detection. Some mycobacteria have a block in the nicotinamide-adenine dinucleotide (NAD) scavenging pathway and accumulate extracellular niacin. The standard method used for the niacin assay requires 3 to 6 weeks incubation on L-J medium. A culture is covered with 1 ml of sterile distilled water, and the surface of the culture is broken with a spatula to improve extraction of the niacin, which is *excreted and accumulates* in the medium. The tube is placed horizontally to ensure maximal contact of the water with the culture.

After a 20-minute extraction period, 0.5 ml of the liquid is transferred to a screw-cap tube; then 0.5 ml of a solution containing 4% aniline in 95% ethanol and 0.5 ml of a 10% cyanogen bromide solution are added. The tube is tightly closed immediately, for safety reasons. If niacin is present, a yellow colour develops within 5 minutes.

To avoid any misinterpretation because of yellow pigments extracted from chromogenic mycobacteria, aniline can be replaced by a solution containing 3% benzidine in absolute ethanol. This method produces a red colour for positive reactions.

Note: Benzidine is carcinogenic and its use may be forbidden in some countries. This compound, as well as cyanogen bromide, must be handled with special care; a worker should wear a mask and gloves to weigh the powder and should handle the solution in a safety cabinet.

Paper strips impregnated with reagents are commercially available, and the instructions of the manufacturer must be followed. The colour developed in the liquid, and not the colour on the paper strip, denotes the reaction.

Nitrate reductase activity. The nitrate reductase test usually employs simple buffered substrate solution containing 0.01 M NaNO_3 in M/45 phosphate buffer (pH 7.0) prepared with 0.085 g of NaNO_3 , 0.117 g of KH_2PO_4 , and 0.485 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 100 ml of distilled water. A loopful of cells is suspended in 2 ml of the substrate solution, and the preparation is incubated at 37 °C for 2 hours. Nitrite formation is detected by adding 1 drop of an aqueous hydrochloric acid solution (1/1, vol/vol) and then 2 drops of a 0.2% sulfanilamide solution and 2 drops of a 0.1% naphthylethylenediamine solution. Colour intensity, which may range from pale pink (+/-) to deep red (5+), is determined by comparison with the colour standards described below. On this scale a positive reaction must be at least a 2+.

The substrate solution and all test reagents must be stored in the dark at 4 °C. If a precipitate forms or if a reagent changes colour, the solution should be discarded, and a fresh one prepared. Colour standards for interpretation are prepared as described below. A working buffer (**solution 1**) is prepared by combining 35 ml of 0.067 M disodium phosphate (9.47 g of anhydrous Na_2HPO_4 per litre), 5 ml of 0.067 M monopotassium phosphate (9.07 g of KH_2PO_4 per litre), and 100 ml of 0.067 M trisodium phosphate (25.47 g of $\text{Na}_3\text{PO}_4 - 12 \text{H}_2\text{O}$ per litre). To 10 ml of **solution 1**, 0.1 ml of 1% ethanolic phenolphthalein and 0.2 ml of 0.01% bromthymol blue (1 ml of 1% ethanolic bromthymol blue in 100 ml of distilled water) are added to make **solution 2**. A 2 ml portion of **solution 2** in an appropriate tube (tube 1) represents the 5+ colour standard. Then 2 ml of **solution 1** is placed into seven additional tubes (tubes 2 to 8), and 2 ml of **solution 2** is added to tube 2. A 2 ml portion is transferred to the next tube, and serial dilutions of 2 ml are made in the remaining tubes using distilled water as diluent (2 ml is discarded from tube 8). Tube 2, the tube containing equal amounts of **solutions 1 and 2**, corresponds to the 4+ colour standard; tubes 3, 5, 6 and 8 correspond to the 3+, 2+, 1+ and +/- colour standards, respectively.

Acid phosphatase activity. The substrate solution for the acid phosphatase activity test (Wayne, 1985) consists of 100 mg of the pyridine salt of phenolphthalein phosphate dissolved in 100 ml of 0.2 M acetic acid-sodium acetate buffer (pH 5.2). The buffer should be steamed at 100 °C for 30 minutes and cooled to room temperature before the phosphate substrate is added. A tube containing 1 ml of substrate is inoculated with a loopful of cells and the preparation is incubated at 37 °C for 4 hours. Then 1 ml of 10% Na_2CO_3 in H_2O is added to stop the reaction and develop the colour.

Suitable dilutions of a colour standard stock (1 mg of free phenolphthalein per ml of 95% ethanol) are made in H_2O to yield phenolphthalein concentrations of 2.5, 5, and 10 $\mu\text{g}/\text{ml}$. Aliquots are treated with sodium carbonate solution in the same manner as the test solutions. A test preparation that is colourless or a test preparation that is pink and has an intensity less than that of the 2.5 $\mu\text{g}/\text{ml}$ standard is scored as negative. Reaction mixtures that have intensities corresponding to the intensity of the 5 or 10 $\mu\text{g}/\text{ml}$ standard are recorded as positive.

10 Polymerase chain reaction (PCR) protocol

Preventing false positives—The three-room principle (“3-room”)

When performing PCR, it is vital to guard against false-positives due to contamination.

The “3-room” PCR principle is recommended:

- **Room 1:**
 - strictly no DNA
 - preparation of PCR reaction mix only
- **Room 2:**
 - low level DNA room
 - preparation of tissue samples and swabs for PCR
 - work is performed in a Class II biological safety cabinet or a PCR series cabinet
- **Room 3:**
 - high level DNA room
 - PCR amplification and post PCR manipulation room
 - running of agarose gels
 - southern blot hybridization

Equipment, clothing, specimens, writing materials, etc. should never be moved from room 3 to rooms 1 and 2, or from room 2 to room 1.

DIAGNOSTIC PCR PROTOCOL

The diagnostic PCR for *M. ulcerans* infection consists of 4 major steps:

1. Specimen preparation
2. Heat and alkaline lysis (to release DNA from *M. ulcerans* cells)
3. Extraction and purification of total DNA from sample
4. PCR amplification and visualisation of PCR product (agarose gel electrophoresis)

1. Specimen preparation

Always include a phosphate buffered saline (PBS) negative control which is taken through the whole procedure.

• Fresh tissue

- dice tissue into small pieces in small volume of PBS (e.g. 2 to 5 ml) with disposable scalpel
- vortex well, transfer 1 ml to 1.5 ml microfuge tube
- wash (*see below*)

• Swabs

- break off end of swab, place in 5 ml tube; add PBS (e.g. 2 ml; vortex well)
- transfer 1 ml to 1.5 ml microfuge tube
- wash (*see below*)

• Paraffin blocks

- scrape 4 to 5 thin sections using a disposable scalpel and place in 1.5 ml microfuge tube
- add 1 ml xylene, mix, stand for 10 minutes at room temperature
- spin for 5 minutes at high speed in a microfuge
- decant xylene, and repeat xylene deparaffination 2 or 3 times
- repeat twice more using 100% ethanol
- air dry pellet
- resuspend in PBS, cut into small pieces with disposable scalpel
- vortex well, and transfer 1 ml to 1.5 ml microfuge tube
- wash (*see below*)

Washes

- spin 1.5 ml microfuge tube for 2 minutes at high speed in a microfuge
- decant PBS, and wash pellet with 1 ml PBS, spin for 2 minutes
- decant PBS and resuspend pellet in 180 μ l distilled water (stored in Teflon bottle; change monthly)

2. Heat and alkaline lysis (to release DNA from *M. ulcerans* cells)

- add 20 μ l 0.5 M NaOH to tube containing sample in 180 μ l distilled water
- add 4 μ l 10% sodium dodecyl sulfate (SDS)
- mix, place on heating block at 95 °C for 15 minutes

3. Extraction of total DNA from sample

- **Perform 2 phenol¹/chloroform (1:1) extractions:**

- add 200 µl phenol/chloroform to sample
- vortex well, spin at high speed in a microfuge for 5 minutes
- transfer top layer (aqueous phase) to new tube containing 200 µl phenol/chloroform and repeat

- **Perform 1 chloroform extraction:**

- add 200 µl chloroform to sample
- vortex well, spin at high speed in a microfuge for 5 minutes
- transfer top layer (aqueous phase) to new tube

- **Ethanol precipitation:**

- add 200 µl distilled water to purified sample
- add 1 µl glycogen²
- add 16 µl 5M NaCl
- add 800 µl 100% ethanol
- spin at high speed in a microfuge for 15 minutes
- decant supernatant and wash pellet with 500 µl 70% ethanol, spin for 1 minute
- air dry pellet
- resuspend pellet in 50 µl of distilled water (test DNA sample ready for PCR)

¹ Phenol: equilibrated with Tris buffer pH 8 nuclease free (Amersham)

² Glycogen: 20 mg/ml (Boehringer Mannheim)

4. PCR amplification and visualization of PCR product (5 steps)

- A. Make Mastermix
- B. Add test DNA (prepared in section 3)
- C. Perform amplification
- D. Visualize PCR product
- E. Perform southern hybridisation to check sequence of PCR product (optional)

A. Make mastermix (all reagents supplied by Promega)

The following is sufficient for one reaction (in practice, quantities are scaled up to allow sufficient master-mix for 10 or more PCR reactions).

Reagent (starting concentration)	Quantity to add in μl
distilled water	9
thermo buffer (10x)	2
MgCl ₂ (25 mM)	1.2
dNTPs (2 mM)	2
** <i>M. ulcerans</i> 1 primer (20 μ M)	1
** <i>M. ulcerans</i> 2 primer (20 μ M)	1
Taq polymerase (5 units/ μ l)	0.2
	<u> </u>
	= 16.4 μ l "mastermix"

** *M. ulcerans* 1–GAT CAA GCG TTC ACG AGT GA

** *M. ulcerans* 2–GGC AGT TAC TTC ACT GCA CA

(usually produced in volumes of 164 μ l or more and then aliquoted into multiple tubes)

B. Add test DNA to tubes containing mastermix:

- add 15 μ l of mastermix into each PCR tube
- add 5 μ l of test DNA sample; total volume for each PCR tube is then 20 μ l
- always include PCR negative and positive controls with each run
- to check for the presence of PCR inhibitors; instead of 5 μ l of DNA sample add 4 μ l of DNA sample and 1 μ l of positive control *M. ulcerans* DNA equivalent to 100 molecules* (PCR result from this tube must be positive to exclude presence of inhibition)

C. Perform amplification

Amplification cycles are performed in an automated thermal cycler using the following protocol:

94 °C	—	4 minutes	
94 °C	—	40 seconds	} 35 cycles
60 °C	—	40 seconds	
72 °C	—	40 seconds	
72 °C	—	5 minutes	
4 °C	—	hold sample until ready to analyse on agarose gel	

D. Visualization of PCR product:

- 9 µl reaction products plus 1 µl sample buffer** are electrophoresed through 2% agarose gels containing ethidium bromide (0.5 µg/ml)
- gels are run in TAE buffer (40 mM Tris acetate, 1 mM EDTA), e.g. at 100 v for 20 minutes (not crucial depends on your apparatus)
- visualise ethidium bromide stained PCR products using a UV trans-illuminator
- samples are considered positive if they yield a 515 bp product that lines up exactly with positive control
- all negative controls must be negative
- a negative result in the sample to test for “inhibition” (see above) indicates that the PCR is being inhibited. Repeat the PCR using a 1:10 dilution of the test DNA. If still inhibited, no result can be given for that sample

E. Checking sequence of PCR product by Southern blot hybridization (optional for quality control – Boehringer Mannheim supply both DIG-RNA and DIG-DNA labelling kits. Refer to Ross et al. *Journal of Clinical Microbiology*, 1997; **35**:1696–1700)**• Southern transfer:**

- transfer DNA fragments to positively charged nylon membranes (Boehringer Mannheim)
- use vacuum transfer apparatus (Hybaid, UK) with a vacuum of 40 cm water in alkaline transfer buffer (0.5 M NaOH, 1.5 M NaCl)
- allow membrane to air dry, 37 °C for 1 hour

• Prehybridization and hybridization:

- place blot in plastic bag containing 20 ml standard prehybridization solution per 100 cm² of membrane surface area
- seal bag and prehybridize in 68 °C water bath, shaking for at least 1 hour
- discard the prehybridization solution from the bag. Add the standard hybridization solution containing the DIG-labelled probe; allow probe to hybridize overnight at 68 °C
- next day, pour hybridization solution with probe from bag into a polypropylene tube (freeze at – 20 °C as it can be re-used many times)
- wash membrane twice in 2x wash solution for 5 minutes per wash at room temperature
- wash membrane twice in 0.1x wash solution for 15 minutes per wash at 78 °C in a shaking water bath. DIG-labelled probe is the 200 bp product obtained from *M. ulcerans*-NEST1/2 PCR amplification described by Ross et al. Probes may be DNA or RNA. RNA probes may be more convenient for high-volume laboratories; however, you will need to use a DIG-RNA labelling mix

• **DIG-Detection of Southern blot:**

(refer to non-radioactive nucleic acid detection kit [Boehringer Mannheim] for additional information)

- following post-hybridization washes, equilibrate membrane in DIG buffer I
- transfer to fresh dish and block membrane in DIG buffer II for at least 30 minutes, at room temperature with gentle agitation
- dilute anti-DIG alkaline phosphatase 1:5000 in DIG buffer II, add to blocked membrane, incubate at room temperature for 30 minutes with gentle agitation
- transfer membrane to fresh dish and wash 2x 15 minutes per wash in DIG buffer I, at room temperature
- equilibrate membrane in DIG buffer III for 2 minutes
- detect membrane using CSPD chemiluminescent substrate (Boehringer Mannheim) dilute CSPD (25 mM) 1:100 in DIG buffer III
- add to membrane, incubate 15 minutes at 37 °C to enhance luminescent reaction
- expose for 5 to 30 minutes at room temperature to X-ray film (Kodak X-omat scientific imaging film)
- process film in automated developer

NOTES

* **Preparation of positive control *M. ulcerans* DNA (inhibition testing and use as positive control)**

- extract *M. ulcerans* DNA (Boddinghaus et al. *Journal of Clinical Microbiology*, 1990; 28:1751–1759)
- digest 0.5 µg DNA (10^8 mol) with enzyme Alu I (Promega) for 2 hours, 37 °C, in 20 µl volume
- perform 1 phenol/chloroform extraction
- ethanol precipitate DNA
- resuspend in 100 µl distilled water (10^8 mol/100 µl)
- dilute to 10^2 mol/µl
- use 1 µl in PCR reaction

** **Sample buffer preparation**

- dissolve 250 mg bromophenol blue in 33 ml 150 mM Tris pH 7.6
- add 60 ml glycerol and 7 ml of distilled water
- store at room temperature

11 Manufacturers' addresses

The following list for manufacturers of commonly used media and reagents does not indicate special endorsement of these products and/or manufacturers by WHO. Please follow closely the manufacturer's instructions when using these media and reagents.

Becton-Dickinson

250 Schilling Circle
Cockeysville, MD 21030, USA
Phone: (1) 800 638 8663
Fax: (1) 410 584 8129

BBL Microbiology Systems

(orders through Fisher Scientific Co.)
PO Box 4829
Norcross, GA 30091, USA
Phone: (1) 800 766 7000
Fax: (1) 800 926 1166

bioMérieux SA

69280 Marcy-l'Étoile, France
Phone: (33) 478 87 2000
Fax: (33) 478 87 2090

BACTEC System

Becton-Dickinson and Company
7 Loveton Circle, Sparks
MD 21152, USA

Wellcome Diagnostics

Laboratories Wellcome SA
Division Diagnostics
159, rue Nationale
75640 Paris Cedex 13, France

12 Work of WHO on Buruli ulcer

On the advice of world experts, WHO has taken the leadership in coordinating Buruli ulcer control and research efforts worldwide. This is essential in order to maintain effective function and focus. WHO/GBUI activities since early 1998—when the Initiative was established—include the following:

- 1) A preliminary meeting of an ad hoc Task Force was held in February 1998. Later, an Advisory Committee of 18 experts was established. This Committee includes world authorities on Buruli ulcer and representatives from endemic countries. Some group members contributed to the recently published monograph and this manual.
- 2) The first International Conference on Buruli Ulcer Control and Research was held in Yamoussoukro, Côte d'Ivoire, 6–8 July 1998. This resulted in an increased awareness of the disease. At this Conference, the *Yamoussoukro Declaration on Buruli ulcer* was signed by three heads of state and the Director-General of WHO. The report of this Conference, in English and in French, is available for distribution.
- 3) Assessments in Benin, Côte d'Ivoire, Ghana, and Togo were conducted between March and July 1998 with the aim of understanding the problem of Buruli ulcer and discussing the importance of the disease with various government authorities. As a result, focused programmes have been established in Benin, Côte d'Ivoire, Ghana, Guinea, and Togo. More countries are establishing programmes.
- 4) Progress has been achieved in raising the awareness of the significance of Buruli ulcer. However, as the disease is still unknown to many, more work needs to be done. A newly established website (www.who.int/gtb-buruli) will assist to make available much needed information. The first WHO educational leaflets, in English and in French, targeting community workers at district and village levels, have been printed and distributed in endemic countries.
- 5) Standard case definitions and forms for the surveillance and clinical management of patients, as well as standard guidelines for treatment and referral of patients, have been developed by the WHO Advisory Committee in consultation with other experts worldwide.
- 6) A WHO scientific working group, consisting of some 40 world experts in the disease, known as the International *Mycobacterium ulcerans* Study Team (IMuST), has been established in collaboration with A/Prof. John Hayman, from the Monash University, Melbourne, Australia. The IMuST seeks to develop control and research activities, and to help coordinate the world's efforts against Buruli ulcer.

7. Collaborating centres will be established in some international research institutions to support research by facilitating exchange of materials and assisting in training activities.
8. Research in the following priority areas with potential impact on control of the disease has been identified by the WHO Advisory Group and IMuST:
 - operational steps in the implementation of adequate control measures;
 - mode(s) of transmission;
 - environmental changes that favour emergence of the disease;
 - surveys to determine the burden of the disease;
 - chemical structure of the toxin;
 - rapid methods of diagnosis; and
 - action of known antimicrobial drugs on *M. ulcerans*, starting with animal models and continuing to clinical trials.

13 Some research institutions involved in Buruli ulcer activities

- Armed Forces Institute of Pathology (AFIP), Washington DC, USA
- Bactériologie et Hygiène, Faculté de Médecine Pitié-Salpêtrière, Paris, France
- Centers for Disease Control and Prevention (CDC), Atlanta, GA, USA
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- Nippon Medical School, Tokyo, Japan
- Noguchi Memorial Institute for Medical Research, Accra, Ghana
- Pasteur Institute of Guiana, Cayenne, French Guiana
- Plastic Surgery & Burns Center, Korle-Bu Teaching Hospital, Accra, Ghana
- St George's Hospital Medical School, London, England
- Swiss Tropical Institute, Basel, Switzerland
- Unité Génétique Moléculaire Bactérienne, Pasteur Institute, Paris, France

14 Some nongovernmental organizations and others involved in Buruli ulcer activities

- Acción Sanitaria y Desarrollo Social (ANESVAD), Spain
- Aide aux Lépreux Emmaüs-Suisse (ALES), Switzerland
- American Leprosy Missions (ALM), USA
- Association Française Raoul Follereau (AFRF), France
- Associazione Italiana Amici di Raoul Follereau (AIFO), Italy
- Catriona Hargreaves Charitable Trust (CHCT), England
- Damien Foundation, Belgium
- Directorate General for International Cooperation, Belgium
- Fondation Luxembourgeoise Raoul Follereau (FFL), Luxembourg
- Government of Japan
- Humanitarian Aid Relief Team (HART), USA
- Japan Tissue Engineering Co., Ltd, Japan
- Kobe International University, Japan
- Médecins Sans Frontières (MSF), Luxembourg & Switzerland
- MAP International, West Africa, Côte d'Ivoire
- Pharmaciens Sans Frontières, France
- Projet Humanitaire Afrique Nord Sud (PHANS), France
- Pfizer Pharmaceuticals Inc., USA
- Rotary Club of Milan, Italy
- Sasakawa Memorial Health Foundation, Japan
- The Nippon Foundation, Japan

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16 Suggested reading

1. WHO–Laboratory services in tuberculosis control. Part III: Culture. WHO, 1998, 95 p.
2. CDC Laboratory manual. Isolation and identification of *Mycobacterium tuberculosis*. A guide for the level II laboratory. 1981, 143 p.
3. Vincent Lévy-Frébault, Portaels F. Proposed minimal standards for the genus *Mycobacterium* and for description of new slowly growing *Mycobacterium* species. *International Journal of Systematic Bacteriology*, 1992; **42**:315–323.
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7. Ross BC et al. Development of a PCR assay for rapid diagnosis of *Mycobacterium ulcerans* infection. *Journal of Clinical Microbiology*, 1997; **35**:1696–1700.
8. Portaels F et al. Variability in 3' end of 16S rRNA sequence of *Mycobacterium ulcerans* is related to geographic origin of isolates. *Journal of Clinical Microbiology*, 1996; **34**:962–965.
9. Palomino JC, Portaels F. Effects of decontamination methods and culture conditions on viability of *Mycobacterium ulcerans* in the BACTEC system. *Journal of Clinical Microbiology*, 1998; **36**:402–408.
10. Steadham JE. Reliable urease test for identification of *mycobacteria*. *Journal of Clinical Microbiology*, 1979; **10**:134–137.
11. Wayne LG. The “atypical” *mycobacteria*: recognition and disease association. *Critical Reviews of Microbiology*, 1985; **12**:185–222.
12. Wayne LG et al. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for pigment, urease, resistance to sodium chloride, hydrolysis of Tween 80, and b-galactosidase. *International Journal of Systematic Bacteriology*, 1974; **24**:412–419.
13. Wayne LG et al. Highly reproducible techniques for use in systematic bacteriology in the genus *Mycobacterium*: tests for niacin and catalase for resistance to isoniazid, thiophene-2-carboxylic acid hydrazide, hydroxylamine, and p-nitrobenzoate. *International Journal of Systematic Bacteriology*, 1976; **26**:311–318.
14. Rikimaru T et al. Efficacy of common antiseptics against *mycobacteria*. *International Journal of Tuberculosis and Lung Diseases*, 2000; **4**:570–576.

Available further materials

- Buruli ulcer (*Mycobacterium ulcerans* infection). WHO/CDS/CPE/GBUI/2000.1 (*English, French, Spanish*)
- Report: 3rd Advisory Buruli ulcer (*Mycobacterium ulcerans* infection). WHO/CDS/CPE/GBUI/2000.2 (*English, French*)
- Management of *Mycobacterium ulcerans* disease (Buruli ulcer). WHO/CDS/CPE/GBUI/2001.3 (*English, French, Spanish*)
- Buruli ulcer comic. WHO/CDS/CPE/GBUI/2001.5 (*English, French*)
- Brochure on Buruli ulcer. WHO/CDS/CPE/SMT/2001.6 (*English, French, Spanish*)
- Report: 4th Advisory Buruli ulcer (*Mycobacterium ulcerans* infection). WHO/CDS/CPE/GBUI/2001.6 (*English, French*)
- Posters and leaflets on Buruli ulcer (*English, French*)
- 9 minutes 25 seconds video on Buruli ulcer (*English, French, Spanish*)
- Epi info software for data management (*English, French*)
- Training video—diagnosis and management of Buruli ulcer (*English, French*)

For more information, contact:

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This manual provides an expert guide to laboratory techniques and procedures used in the diagnosis of Buruli ulcer, a complex disease caused by *Mycobacterium ulcerans*. The disease, which remains incompletely understood, affects impoverished rural populations in a growing number of tropical countries, where it imposes a huge social and economic burden.

Addressed to health care workers and laboratory scientists in endemic countries, the manual aims to facilitate a better understanding of both the clinical behaviour of the disease and the exact procedures to follow when performing a range of diagnostic tests. Recommended procedures, intended for use throughout the health system, are presented at levels appropriate for peripheral, district, and central services and in line with the varying resources, skills, and equipment typically found in endemic countries. The practical value of this material is enhanced through the inclusion of over 50 colour photographs, tables, flow charts, and model laboratory request and reporting forms.

The opening chapter, on clinical diagnosis, describes the different nonulcerative and ulcerative forms of Buruli ulcer, the features of bone involvement, and common complications and sequelae. Subsequent chapters cover biosafety precautions and record keeping, the collection and transport of clinical specimens, and the diagnosis of secondary bacterial infections. Against this background, the core of the manual sets out diagnostic protocols and step-by-step instructions for a large number of microbiological and histopathological methods. Extensive advice on the interpretation of test results is also provided.

Guidelines for clinical management are set out in a companion volume,
Management of *Mycobacterium ulcerans* disease: A manual for health care providers

