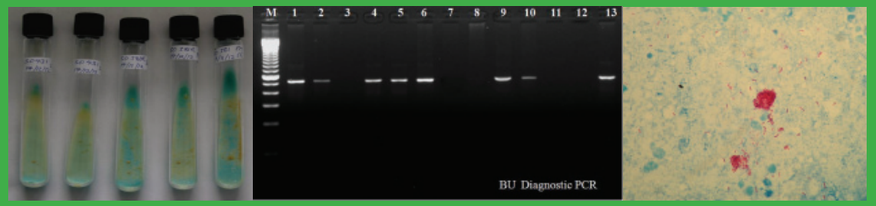

This manual provides expert guidance on the laboratory techniques and procedures used in the diagnosis of Buruli ulcer, a disease caused by *Mycobacterium ulcerans*. Aimed at laboratory technicians and scientists working on this disease, the manual details the exact procedures to follow when performing 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 accordance with the varying resources, skills and equipment typically found in countries where Buruli ulcer is endemic.

The practical value of this material is enhanced by the inclusion of more than 30 colour photographs, tables and flow charts, as well as model laboratory request forms. Subsequent chapters explain how to collect and transport clinical specimens, and the different methods available to diagnose Buruli ulcer. The core of the manual sets out diagnostic protocols and step-by-step instructions for a large number of microbiological and histopathological methods. New highlights of this manual are histopathological changes during antibiotic treatment and paradoxical reactions. The roles of national programmes, health facilities and laboratories (with special emphasis on the importance of quality assurance) in contributing to confirmation of Buruli ulcer are well described. Extensive advice on the interpretation of test results is also provided.

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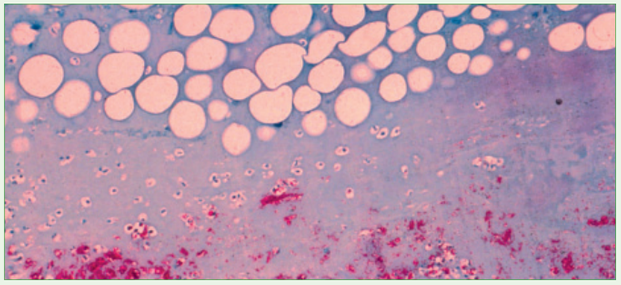
LABORATORY DIAGNOSIS OF BURULI ULCER – A MANUAL FOR HEALTH CARE PROVIDERS

LABORATORY DIAGNOSIS OF BURULI ULCER

A MANUAL FOR HEALTH CARE PROVIDERS



Edited by : Françoise Portaels

World Health Organization

LABORATORY DIAGNOSIS OF BURULI ULCER

A MANUAL FOR HEALTH-CARE PROVIDERS

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ILLUSTRATIONS AND TABLES

ILLUSTRATIONS

TABLES

ABBREVIATIONS

AFB	acid-fast bacilli
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FAM	fluorescent dye 6-carboxyfluorescein
FNA	fine needle aspiration
OADC	oleic acid, albumin, dextrose and catalase
PANTA	polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin
PCR	polymerase chain reaction
TE	trisaminomethane [Tris] hydrochloride plus ethylenediaminetetraacetic acid [EDTA] buffer
Tris	trisaminomethane
WHO	World Health Organization
ZN	Ziehl-Neelsen staining

1. INTRODUCTION AND IMPORTANCE OF LABORATORY CONFIRMATION OF BURULI ULCER

In general and in experienced hands, the clinical diagnosis of Buruli ulcer is straightforward when a patient from an area known to be endemic presents with a typical painless ulcer characterized by undermined edges. However, in areas where health workers do not see many cases of Buruli ulcer, the accuracy of clinical diagnosis may be challenging. Consequently, the number of cases may be overestimated and management of diseases other than Buruli ulcer may be poor. In 2012, the World Health Organization (WHO) published a document on the clinical diagnosis and treatment of Buruli ulcer *(1)*.

Microbiological confirmation is essential for several reasons:

1) to confirm that the disease is Buruli ulcer;
2) to determine the precise prevalence and incidence of Buruli ulcer in a given area;
3) to confirm new foci;
4) to appropriately manage the disease using antimycobacterial therapy with or without surgery;
5) to confirm the failure of treatment, or relapse or reinfection after treatment.

As more health professionals use antimycobacterial medicines to treat Buruli ulcer, the appropriate use of these medicines and the confirmation of a treatment's success or failure (points 4 and 5) become increasingly important.

There is no point-of-care rapid diagnostic test. Researchers are working to develop one. Four laboratory tests are available to confirm the clinical diagnosis (Chapter 4):

1) direct smear examination for acid-fast bacilli (AFB);
2) in vitro culture;
3) polymerase chain reaction (PCR) targeting genomic region IS*2404*;
4) histopathological examination.

However, there is some confusion in the reporting of laboratory confirmation results. In 2001, WHO recommended that at least 50% of all cases be confirmed by PCR (*2*). Some clinicians and national programmes report "50% confirmation" when specimens from 50% of their patients have been sent to the laboratory for analysis. Sending a specimen to a laboratory for microbiological confirmation does not mean that the specimen is positive for *Mycobacterium ulcerans*. The term "confirmed case" refers to a case found to be positive for *M. ulcerans* by at least one laboratory test. Given that external quality assurance results indicate that some laboratories have unsatisfactory results, two positive laboratory tests are preferred, as recommended in WHO's 2001 manual on diagnosing Buruli ulcer (*3*).

During a WHO meeting on Buruli ulcer in 2013, a new recommendation for laboratory diagnosis was proposed: national control programmes should strengthen laboratory confirmation of cases to ensure that at least 70% of all reported cases are laboratory-confirmed by positive PCR.[1]

[1] http://www.who.int/buruli/archives/en/index.html

2. COLLECTING CLINICAL SPECIMENS

Guidance on sampling techniques used for laboratory confirmation is available on WHO's website[1] and included in ***Annex 10***.

For diagnostic purposes, samples should be collected before treatment. Given the heterogeneous distribution of mycobacteria in lesions, at least two clinical specimens should be collected from each lesion. The type of clinical specimens to be collected depends on the:

- place where specimens are collected (in the field or at treatment centres);
- clinical form of the disease;
- reasons why microbiological confirmation is required.

Additional resources are available from:

- the Stop Buruli consortium;[2]
- BuruliVac;[3] and
- in ***Annex 10***.

2.1 TYPES OF CLINICAL SPECIMENS

For nonulcerative plaques and oedematous forms of the disease, the patient or the patient's relative should be asked to indicate the site at which the lesion first appeared since this is the site that is most likely to yield a positive diagnostic result.

2.1.1 FINE-NEEDLE ASPIRATION

Fine-needle aspiration (FNA) can be performed on any nonulcerative lesions, such as papules, nodules, plaques, oedemas, or ulcers that do not have undermined edges (that is, where scarred edges would prevent the collection of swabs). Samples for FNA should be taken using fine-gauge needles (such as, 21 gauge, 22 gauge or 23 gauge by 25 mm in diameter) attached to a syringe. The optimal aspiration technique is described in ***Annex 1***. Using this technique ensures that sufficient material is obtained. In the majority of cases, two FNA specimens are considered adequate.

2.1.2 SWABS

Swabs are most useful for ulcers that have undermined edges (see ***Annex 1*** for techniques to use when ulcers have scarred edges that prevent swabs from being collected). At least two swabs should be obtained from beneath the undermined edges of ulcers. The centre of the ulcer should not be swabbed because ***M. ulcerans*** is generally not present there. The technique to use for swabs is described in ***Annex*** 1.

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[1] http://www.who.int/entity/buruli/Guidance_sampling_techniques_MU_infection.pdf

[2] http://www.stopburuli.org/index.php/FNA-e-tutorial.html

[3] http://www.burulivac.eu/index.php?id=17923

2.1.3 BIOPSIES (PUNCH OR SURGICAL)

Punch biopsy is not the first choice for obtaining a sample, although it may be used in limited special circumstances, such as those indicated below. Punch biopsies (3 mm or 4 mm in diameter) must be taken after local anaesthesia has been administered; surgical biopsies may be taken under general anaesthesia during surgical excision. Biopsies should be taken from the centre of nonulcerative lesions or from the necrotic margin of ulcers that border healthy (viable) tissue. Tissue biopsy should contain the entire thickness of infected tissue, including subcutaneous adipose tissue. However, samples from the edges of nonulcerative lesions may be most suitable for histopathology.

For routine confirmation of Buruli ulcer, swabs and fine-needle aspirates are sufficient. The indications for punch or surgical biopsies are:

- to establish the differential diagnosis of Buruli ulcer;
- to investigate the cause of a paradoxical reaction (see note below and section 4.7.7 for additional information);
- to determine whether treatment has failed despite successful administration of quality-assured antibiotics;
- to establish whether cancerous changes have occurred;
- in clinical trials, to reconfirm the clinical diagnosis using at least two laboratory methods (see note below), and to evaluate the disease process and therapeutic efficacy.

Note: In its broadest sense, a paradoxical reaction is a reaction to medical treatment that is opposite to the effect that would normally be expected.

Note: If the lesion has ulcerated, a swab is preferred to a biopsy.

2.2 COLLECTING SPECIMENS IN THE FIELD

Less invasive techniques (such as swabs or FNA) can be used to collect specimens in the field to allow the decentralized diagnosis necessary for studies of prevalence or incidence, or to confirm new foci. Punch or surgical biopsies should not be collected in the field because strict sterile conditions are required and may be difficult to maintain in this setting.

2.3 COLLECTING SPECIMENS AT TREATMENT CENTRES

For routine confirmation of a case of suspected Buruli ulcer, swab specimens should be obtained from undermined ulcers; FNA specimens should be collected from papules, nodules, plaques, oedemas and non-undermined ulcers.

Swab samples or FNA samples are sufficient to establish a laboratory diagnosis in most cases. The use of punch biopsies should be reserved for exceptional circumstances as outlined in section 2.1.3. If patients require surgical treatment, surgical specimens can be sent for laboratory analysis (see also the indications for surgical biopsies outlined in section 2.1.3).

If radiological evidence of osteomyelitis is present or if osteomyelitis is discovered during surgery, bone samples should be collected to confirm *M. ulcerans* involvement.

3. STORING AND TRANSPORTING CLINICAL SPECIMENS

Before being transported to a laboratory, specimens must be labelled with the appropriate information (for example, the patient's name, the date the specimen was obtained, or the sample code), following standard practice. A permanent marker should be used to write labels. In addition, all paperwork (for example, standardized WHO data forms and other project-specific forms) must be properly filled out and forwarded with the sample to the laboratory.

Several methods of collecting, transporting and processing samples have been used in different settings; for example, samples may be divided into subsamples that undergo different laboratory tests, or separate samples may be collected for each test. Various approaches to storing and transporting samples have been validated under field conditions; for example, in some cases different types of sterile tubes, transport media or sample-collection bags have been used.

As a rule, when microbiological analyses can be performed within 24 hours, clinical specimens should be kept at 4 °C until analysis. When microbiological analyses cannot be performed within 24 hours or when refrigeration facilities are not available, a transport medium should be used for the sample. It is important to note, however, that some transport media are suitable for microbiological and molecular analysis (such as PCR) (see ***Annex 2*** for suitable transport media), but media used only for PCR analysis (trisaminomethane [Tris] plus ethylenediaminetetraacetic acid [EDTA] buffer, or a solution of alcohol and distilled water) are not suitable for culture.

3.1 FOR BACTERIOLOGICAL ANALYSIS

If samples for culture analysis are processed within 24 hours, the specimens can be placed without additives in a sterile container and kept at 4 °C. If no refrigeration facilities are available or transport to the laboratory requires several days, then transport media are essential because they protect the viability of mycobacteria.

It is recommended that FNA samples be transported in a liquid transport medium containing a Dubos broth base supplemented with oleic acid, albumin, dextrose and catalase (OADC, Becton Dickinson) and polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA, Becton Dickinson).

Swab and/or tissue specimens taken by punch or surgical biopsy (or tissue fragments taken from surgically excised skin or bone) should be transported in a liquid transport medium supplemented with 0.5% agar; this produces a semisolid transport medium (***4,5***).

All types of samples collected for diagnosis of Buruli ulcer have been successfully transported in a Dubos broth base with Dubos medium albumin supplemented with PANTA or Dubos broth with 10% OADC and PANTA (***4–8***).

The specimens in transport media should be stored at 4 °C but they can be sent at ambient temperature to national or international reference laboratories for microbiological confirmation. Transport media are described in detail in ***Annex 2***.

3.2 FOR POLYMERASE CHAIN REACTION

All bacteriological transport media described in section 3.1 allow samples (or subsamples) to be analysed using PCR (*4,5,8*).

Specimens that will be used only for PCR may also be placed in a solution of alcohol and distilled water (in a ratio of 1:1), in phosphate-buffered saline, or in TE buffer (Tris hydrochloride plus EDTA).

Note: Alcohol may interfere with certain DNA extraction procedures, such as the Gentra Puregene DNA Extraction Kit (Qiagen, Hilden, Germany), which is described in ***Annex 6***.

A cell lysis solution manufactured by Qiagen allows specimens for PCR to be stored for several months at ambient temperature and transported at ambient temperature (*6,7,9,10*). It is also possible to transport samples in liquid nitrogen (*11*).

3.3 FOR HISTOPATHOLOGICAL ANALYSIS

Biopsies for histopathological analysis should be placed in 10% formalin (*7,11,12*). Optimally, the tissue should be fixed in 10% buffered neutral formalin (pH 7.4) (see section 4.6.2).

4. METHODS OF LABORATORY CONFIRMATION AND THEIR LIMITATIONS

4.1 LABORATORY TESTS FOR DIAGNOSIS OF BURULI ULCER

Four laboratory-based tests are available to confirm the clinical diagnosis of Buruli ulcer:

1) direct smear examination for AFB;
2) in vitro culture;
3) PCR targeting genomic region IS*2404*;
4) histopathological examination.

Each of these tests has advantages and disadvantages, and the method used depends on the type of specimen collected, the purpose of the test (that is, for diagnosis or monitoring treatment outcome) and the setting in which the testing takes place (for example, in a peripheral laboratory or a reference laboratory) (***Table 1***).

Direct smear examination for AFB is available in most endemic areas as a first-line diagnostic test. WHO currently recommends that at least 70% of clinically suspected cases of Buruli ulcer be confirmed by a PCR positive result. A network of PCR laboratories has been established in endemic countries (a list of these laboratories can be found in section 6.3.4). However, only a few district-level laboratories are capable of performing PCR, so PCR is mostly restricted to well equipped, specialized reference laboratories. Culture and histopathology are not widely available in regions where Buruli ulcer is endemic.

The positivity rate of different tests depends on the quality of the samples, and may vary according to the type of sample and clinical form of the disease. For microscopy and culture, positivity rates ranging from 30% to 60% have been reported (*5–7,9,13–17*). Among true Buruli ulcer cases (that is, those confirmed by at least two positive laboratory tests), direct smear examination and culture are less frequently positive when nodules are tested (60% positivity) than when samples are collected from oedematous forms (80% positivity). ***M. ulcerans*** is particularly difficult to culture from bone (20% positivity) (*18*). Studies conducted in different African countries report PCR positivity rates from 70% to higher than 90% (*9,16,17,19–22*), and sensitivity ranging from 85% to 89% (*5,6,23,24*). The sensitivity of histopathology is about 90% (*6,7,10,12*).

To avoid a misdiagnosis caused by false-positive or false-negative results, it is recommended that two tests have positive results before a definitive diagnosis is made.

Another method of laboratory diagnosis is based on a novel DNA amplification method called loop mediated isothermal amplification (also known as LAMP) (*25–27*) . Although not currently used for routine diagnosis, this technique has the potential to become a rapid, simple and inexpensive test for ***M. ulcerans*** that could be performed at the local level without access to a sophisticated laboratory.

TABLE 1. ADVANTAGES AND DISADVANTAGES OF METHODS USED FOR LABORATORY CONFIRMATION OF BURULI ULCER[a]

Method	Advantages	Disadvantages
Direct smear examination	• Can be performed on samples collected by swab, FNA or biopsy • Easy to perform at local level • Does not require expensive materials and equipment • Rapid results	• Low sensitivity (<60%) • Needs trained personnel • Does not distinguish between viable and nonviable organisms • Requires strict quality control
In vitro culture of *Mycobacterium ulcerans*	• Can be performed on samples collected by swab, FNA or biopsy • Only method that can distinguish between viable and nonviable organisms • Can be used to monitor patients' response to antimycobacterial treatment	• Requires a sophisticated laboratory • Needs trained personnel • Slow results (at least 6–12 weeks) • Low sensitivity (20–60%) • Not useful for immediate patient care • Requires strict quality control
PCR for IS*2404*	• Can be performed on samples collected by swab, FNA or biopsy (fresh or embedded in paraffin) • Rapid results • High sensitivity and specificity (>90%)	• Requires a sophisticated laboratory • Expensive to perform • Needs highly trained personnel • Requires strict quality control • Does not distinguish between viable and nonviable organisms
Histopathology	• Fairly rapid results • High sensitivity (about 90%) • Useful in establishing differential diagnosis and monitoring response to treatment	• Requires a sophisticated laboratory • Expensive to perform • Needs highly trained personnel • Requires invasive procedure (that is, a biopsy)

FNA, fine needle aspiration; PCR, polymerase chain reaction

[a] Adapted from: ***Guidance on sampling techniques for laboratory–confirmation of*** Mycobacterium ulcerans ***infection (Buruli ulcer disease).*** Geneva, World Health Organization, 2004 (http://www.who.int/entity/buruli/Guidance_sampling_techniques_MU_infection.pdf; accessed February 2014).

4.2 SPECIMEN PREPARATION

If separate samples are collected for each test, the samples are processed as described in the annexes. If the same sample is to be used for a number of different tests, the original sample must be divided into subsamples as described in ***Annex 3.*** Aliquots of the suspension (subsamples) are then made for each test.

4.3 DIRECT SMEAR EXAMINATION

There are several staining techniques for mycobacteria: Ziehl–Neelsen, Kinyoun and auramine O. In general, the method used locally for laboratory diagnosis of tuberculosis infection can also be used to diagnose Buruli ulcer. In most cases, this will be the hot Ziehl–Neelsen method (***Annex 4***). The quantitation of smears should be done using the same method that is used locally for sputum smear examinations for tuberculosis.

4.4 IN VITRO CULTURE

4.4.1 DECONTAMINATION BEFORE CULTURE

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

Several methods have been used to decontaminate specimens before culture for mycobacteria. In general, overly strong decontamination procedures reduce the likelihood of obtaining a culture that is positive for ***M. ulcerans***. The decontamination method selected depends on which culture medium will be used. The most widely used medium is Löwenstein–Jensen. Any of the methods described in ***Annex 5*** can be used with Löwenstein–Jensen medium. However, the oxalic acid method is the method of choice because it yields low contamination rates and a higher number of positive cultures (***8***).

An overall contamination rate in the range of 2–5% is acceptable for in vitro culture of mycobacteria from non-sterile specimens. Therefore, the microbiologist has discretion in selecting the decontamination method, and the method chosen depends not only on the type of specimen but also on the degree of contamination that occurs in the laboratory (***4,8,28***).

4.4.2 CULTURE MEDIA

Löwenstein–Jensen supplemented with 0.75% glycerol is the most widely used medium (***4,8,29***), although a number of solid and liquid culture media have been used to isolate ***M. ulcerans*** (for example, Ogawa media, the BACTEC system [Becton Dickinson], Middlebrook 7H12B medium, Middlebrook 7H11 medium, and the BBL mycobacteria growth indicator tube [also known as MGIT, Becton Dickinson]). In Australia, it has been found that ***M. ulcerans*** grows best on Brown and Buckle medium, which supports the growth of most mycobacteria and differs from Löwenstein–Jensen in that it contains egg yolks and agar instead of whole eggs, and therefore does not need to be inspissated. The recipes for Löwenstein–Jensen medium, Brown and Buckle medium and Ogawa medium are provided in ***Annex 5***.

4.4.3 CULTURE CONDITIONS AND INCUBATION TIMES

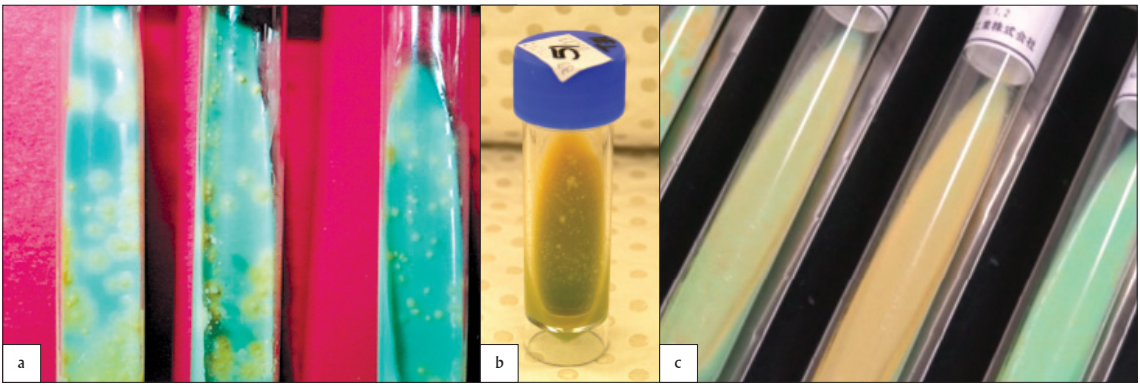
M. ulcerans grows under the same conditions as ***M. tuberculosis*** except that the optimal temperature is 29–33 °C. Primary cultures are usually positive within 6–12 weeks, depending on the bacterial load of the inoculum, but some isolates may require incubation lasting as long as 9–12 months (***4***). The duration of the incubation period should be selected according to the objective of the laboratory investigation (that is, whether short-term patient management is desired or longer-term research).

Colonies suggestive of ***M. ulcerans*** appear yellowish, rough and have well demarcated edges. African and Japanese strains are more yellowish than Australian strains (***Figure 1***), which may be only slightly pigmented. A single, typical colony should be selected for subculture on Löwenstein–Jensen medium, Brown and Buckle or Ogawa medium.

4.4.4 SPECIFIC IDENTIFICATION OF *MYCOBACTERIUM ULCERANS*

M. ulcerans belongs to the slow-growing group of mycobacteria. Subcultures are generally positive within 3–4 weeks of incubation at 29–33 °C, depending on the number of bacilli in the inoculum.

FIGURE 1. CULTURES OF AFRICAN, AUSTRALIAN AND JAPANESE STRAINS OF *MYCOBACTERIUM ULCERANS*



The African strains (a) and Japanese strains (c) are more yellowish than the Australian strains (b) (Courtesy of Françoise Portaels, Kazue Nakanaga and Janet Fyfe)

Identification of *M. ulcerans* is presently done by PCR. The procedures are described in *Annex 5* (section 5.4).

4.5 PCR FOR IS*2404*

The most common target sequence for PCR confirmation of Buruli ulcer is the insertion sequence IS*2404* (1274 bp long), which occurs in more than 200 copies in the *M. ulcerans* genome. Several methods for extracting and amplifying *M. ulcerans* DNA have been published (*30,31*). The sensitivity of PCR is high; however, it is relatively expensive. In laboratories that have little experience with PCR, false-positive results and false-negative results may occur; therefore, stringent quality-control measures must be implemented.

4.5.1 DNA EXTRACTION PROCEDURES

In general, DNA extraction can be performed on samples collected specifically for PCR confirmation as well as on samples or subsamples that are to be analysed using other bacteriological methods. However, it is strongly recommended that extraction procedures be performed on samples that have not been subjected to decontamination procedures (*30*).

Extraction procedures generally include physical lysis (for example, mechanical homogenization, sonication or heat treatment) or chemical lysis (for example, proteinase K digestion), or both, used in combination with subsequent precipitation and purification procedures (for example, using phenol and chloroform) to release DNA from *M. ulcerans* cells. DNA extraction methods involve both manual and automated techniques. The final volume of DNA extracted depends on the method used (*6,9,10,15,16,19,21,22,24,30–32*).

Annex 6 describes a number of commonly used extraction methods.

Due to the large number of bacilli present in some clinical specimens and in cultures, extreme care must be taken when extracting DNA from isolates (for example, for identification or genotyping) to prevent contamination of the work area, which can lead to cross-contamination of clinical specimens. Wherever possible, DNA extraction from cultures should be performed in a separate area using dedicated reagents and equipment (for example, in a separate biosafety cabinet, and using separate pipettes and centrifuge).

4.5.2 IDENTIFICATION OF *MYCOBACTERIUM ULCERANS* BY PCR

Identification of ***M. ulcerans*** by PCR may be performed on DNA extracted directly from clinical specimens (collected by swabs, FNA, or punch or surgical biopsies) (see section 2.2) or from colonies on culture media. The main advantage of PCR is that ***M. ulcerans*** disease can be definitively diagnosed within a few days to 2 weeks after receipt of a clinical specimen by the laboratory. The most commonly used methods for PCR are conventional single-step gel-based PCR and real-time PCR targeting the insertion element IS*2404*.

4.5.2.1 Gel-based PCR

The primers and PCR protocol described in the previous edition of the manual (*3*) and in ***Annex 6*** of this manual are different from those originally described by Ross and colleagues (*33*) and Stinear and colleagues (*34*), and amplify a 515 bp region of IS*2404*.

Several other gel-based PCR protocols targeting IS*2404* have been published. Guimaraes-Peres and colleagues developed a nested PCR using primers MU1 and MU2 for first-round amplification of a 568 bp fragment of IS*2404*; primers PGP3 and PGP4 were used for second-round amplification of a 217 bp product (*19*). Phillips and colleagues described a modified PCR protocol designed to reduce the risk of amplicon contamination of the PCR mix; this protocol uses primers PU4F and PU7Rbio to amplify a 154 bp product of IS*2404* (*21*).

To overcome the technical difficulties associated with using diagnostic PCR procedures in tropical countries, a dry reagent-based PCR was developed; this procedure amplifies a 492 bp sequence within IS*2404* and uses lyophilized reagents and the lyophilized primers MU5 and MU6, which are stable at ambient temperature (*10*).

Following amplification in a thermocycler, PCR products are detected by ultraviolet transillumination after electrophoresis through agarose gels stained with, for example, ethidium bromide or GelRed (Biotium). To prevent contamination, which may lead to false-positive results, care must be taken to conduct sample preparation, DNA extraction, PCR master-mix preparation, PCR amplification, and electrophoresis in separate areas of the laboratory. It is recommended that extraction controls, several negative controls, inhibition controls and positive controls be included in every PCR run as detailed in ***Annex 8***.

With experience, a laboratory worker may rely on visual comparison between the test sample and the positive control on the gel to determine the result. 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***. However, a quality assurance system must be in place and, if possible, PCR results should be compared with direct smear examination and culture results to monitor accuracy.

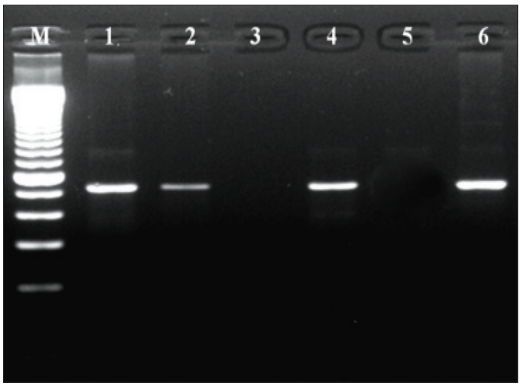
Sample results are illustrated in ***Figure 2***.

Annex 6 describes a conventional single-step gel-PCR (using primers MU1new and MU2new), the dry reagent-based-PCR assay and a real-time PCR assay.

4.5.2.2 Real-time PCR

Real-time PCR, also called quantitative real-time PCR (or qPCR), has several advantages over conventional gel-based PCR: it has increased specificity (leading to fewer false-positive results)

FIGURE 2. ELECTROPHORESIS GEL STAINED WITH ETHIDIUM BROMIDE UNDER ULTRAVIOLET LIGHT



M = 100 Kb ladder, Lanes 1, 2 and 4 = Swabs from patients with *M. ulcerans* disease; Lane 3 = Swab from patient with chronic ulcer (not caused by *M. ulcerans*), Lane 5 = Negative control and Lane 6 = Positive control (Courtesy of Dorothy Yeboah-Manu)

and sensitivity (leading to fewer false-negative results) and faster turnaround time; it is less labour intensive; DNA in the sample can be quantified; and there is a decreased risk of cross-contamination occurring owing to the closed-tube format, which avoids post-PCR handling of samples containing high numbers of amplicons.

Different real-time PCR assays targeting the insertion sequence IS*2404* have been described. The assay developed by Fyfe and colleagues has proved to be rapid and reliable, as well as highly sensitive and specific (*32*). A modified real-time PCR assay using cheaper reagents has been described by Beissner and colleagues (*35*). The detection limit of this technique is at least 100 times lower than conventional PCR. Moreover, because the assay is multiplexed with an internal positive control, it allows for the detection of PCR inhibitors that may be present in clinical samples. As a result of the decline in the costs of consumables used for real-time PCR, the cost of this analysis is comparable to that of conventional gel-based PCR. However, the availability of suitable laboratory facilities and a real-time thermocycler still remain prerequisites. The real-time PCR assay is described in ***Annex 6***.

4.6 SPECIFIC IDENTIFICATION OF OTHER MYCOBACTERIA POSITIVE FOR IS*2404*

Mycobacteria other than ***M. ulcerans*** may harbour the IS*2404* sequence (*36*), but this does not interfere with the diagnosis of Buruli ulcer by PCR since there is no evidence that these IS*2404*-positive mycobacteria cause symptoms similar to those of Buruli ulcer in humans. Moreover, IS*2404*-positive mycobacteria other than ***M. ulcerans*** can be easily differentiated from ***M. ulcerans*** by molecular techniques that can be applied directly to clinical specimens (*37,38*).

4.7 HISTOPATHOLOGICAL METHODS

A detailed history and description of the lesion that has been excised is important for an accurate evaluation and for archival purposes. The description should include the age and sex of the patient, the laboratory number or hospital number, and information on the site of lesion. Care should be taken to identify the tissue by writing with a permanent marker on the container label.

4.7.1 SELECTION OF SITE FOR BIOPSY SPECIMEN

Excisional specimens are advised. Punch biopsies (3 mm or 4 mm in diameter) are recommended for histopathological analyses.

4.7.1.1 Nonulcerative lesions

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

4.7.1.2 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.

4.7.2 FIXATION OF TISSUE

The tissue should be fixed at room temperature as soon as possible after removal in 10% buffered neutral formalin (pH 7.4). Tissue should be cut so that the thickness of the sample is not more than 10 mm. Ideally, it should be fixed in a volume of formalin at least 10 times greater than the volume of tissue. Initial fixation should be done at room temperature since the penetration of formalin is related to the temperature of the solution. Tissue should be fixed for at least 24 hours.

After fixation, samples should be transferred to a container with 70% ethanol and processed further. After fixation, samples can be shipped in smaller volumes of fixative.

Bone samples must be first fixed in formalin and then decalcified before sectioning.

Immunohistochemical staining is not imperative for diagnosis but can give deeper insight into the status of a lesion and may also be useful for the differential diagnosis. It may be done retrospectively on specimens fixed in 10% buffered neutral formalin. Tissue fixation, however, should not last longer than 24 hours since prolonged fixation may destroy antigenic sites.

Note: The 10% buffered neutral formalin fixative is sensitive to oxidation and should not be used for longer than 3 months. It should be clear and there should be no precipitate; the pH should be 6.5 or higher.

4.7.3 PREPARING HISTOPATHOLOGICAL SECTIONS

Routine processing of fixed tissue is sufficient. Paraffin sections should be cut 4–6 µm thick and stained with:

- haematoxylin and eosin;
- Ziehl–Neelsen for AFB;
- periodic acid-Schiff or Grocott's methenamine–silver for fungi; and
- tissue Gram stain for other bacteria (*Annex 7*).

Other stains may be used as indicated. The technique used for histochemistry is detailed in *Annex 7*.

4.7.4 GROSS CHANGES

Surface changes of nonulcerated lesions often show a loss of topographical markings and discolouration. Cut sections show changes in colouration, necrosis and mineralization. Lymph nodes show soft greyish-tan cut surfaces.

Cut sections of bone after decalcification show yellowish necrosis of the marrow and, often, thinning of the cortex.

4.7.5 HISTOPATHOLOGICAL CHANGES BEFORE ANTIBIOTIC TREATMENT

4.7.5.1 Skin changes

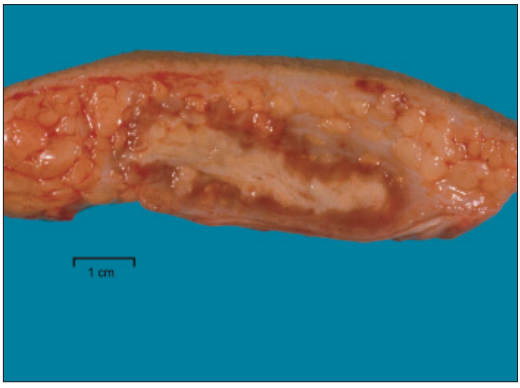
Skin changes depend on the stage of the disease.

Necrotic (active) stage: nonulcerated lesions

During this stage, the epidermis remains intact but is often hyperplastic, showing mostly psoriasiform or pseudoepitheliomatous hyperplasia.

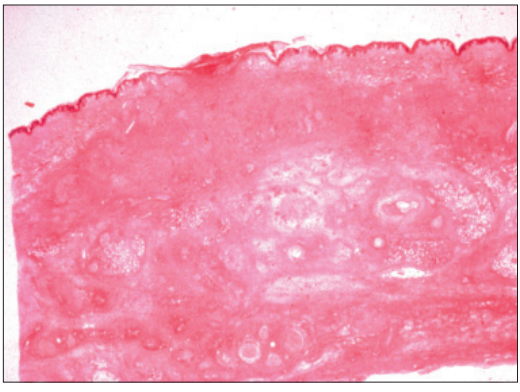
The upper dermis usually remains intact but may show various stages of degeneration, with infiltration of small numbers of inflammatory cells. There is contiguous coagulation necrosis of the lower dermis, subcutaneous tissue and underlying fascia (***Figure 3***, ***Figure 4***, ***Figure 5***).

FIGURE 3. SECTION OF A SURGICALLY RESECTED NODULE OF BURULI ULCER



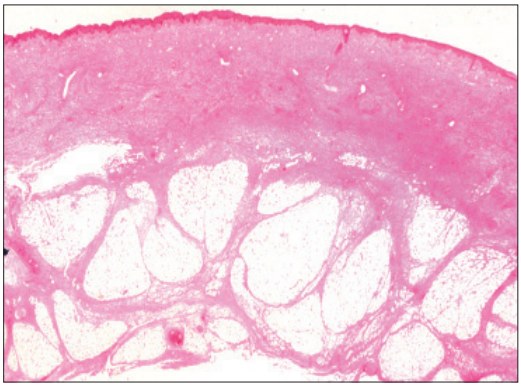
The central whitish area shows coagulation necrosis (Courtesy of John Hayman)

FIGURE 4. MICROSCOPIC SECTION OF A NODULE



Note the massive coagulation necrosis of the lower dermis and subcutaneous tissue. Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

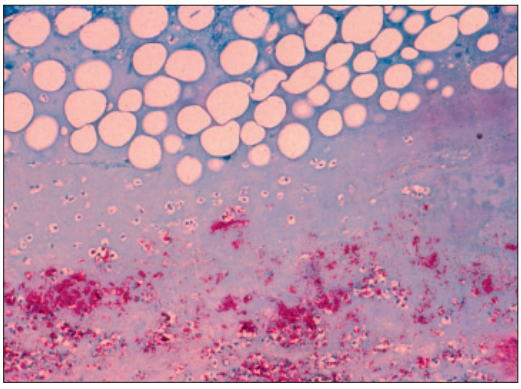
FIGURE 5. SKIN AND SUBCUTANEOUS TISSUE FROM THE CENTRE OF A NONULCERATED WIDELY DISSEMINATED LESION OF BURULI ULCER THAT COVERED 50% OF THE ABDOMEN OF A 9-YEAR-OLD BOY



The epidermis is intact. There is massive contiguous coagulation necrosis of the entire specimen. Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

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 (these are sometimes known as "fat-cell ghosts") (*Figure 6*).

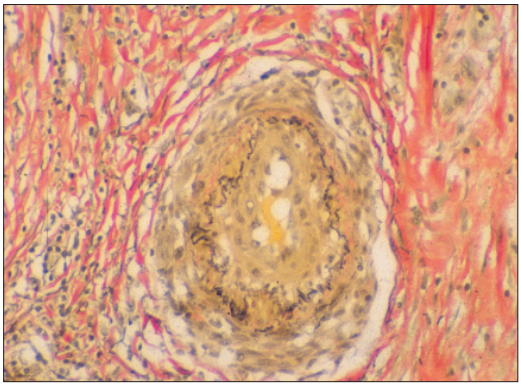
FIGURE 6. NECROTIC BASE OF BURULI ULCER SHOWING MANY FAT-CELL GHOSTS (UPPER PORTION) AND MANY ACID-FAST BACILLI (LOWER PORTION)



Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

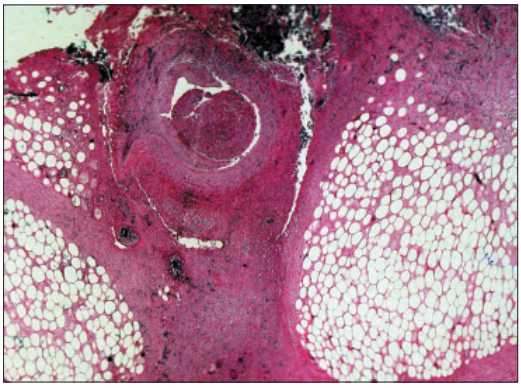
In subcutaneous tissue, occlusion of vessels by thrombi is common, and may be associated with vasculitis (*Figure 7, Figure 8*).

FIGURE 7. SEVERE VASCULITIS IN SUBCUTANEOUS TISSUE OF A BURULI ULCER LESION



Stained with Movat's (Courtesy of John Hayman)

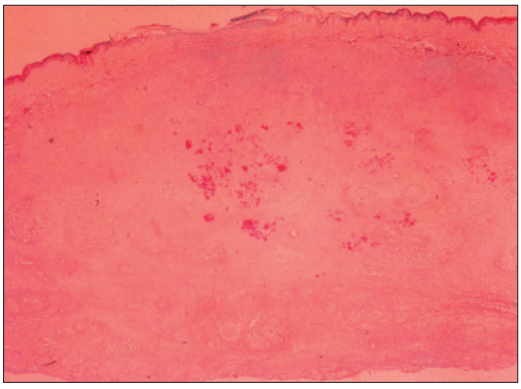
FIGURE 8. FAT-CELL GHOSTS AND VASCULITIS



Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

Varying degrees of mineralization are seen, especially in African patients. The Ziehl–Neelsen stain classically reveals large numbers of extracellular AFB, often in clusters and confined to necrotic areas (*Figure 9*).

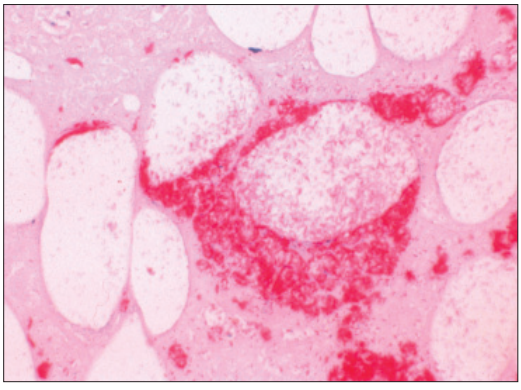
FIGURE 9. ZIEHL–NEELSEN STAIN OF A SECTION PARALLEL TO THAT IN FIGURE 4 SHOWS ACID-FAST BACILLI RESTRICTED TO THE CENTRE OF THE LESION



Necrosis extends far beyond the focus of acid-fast bacilli; (Courtesy of Wayne Meyers)

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 (*Figure 10*).

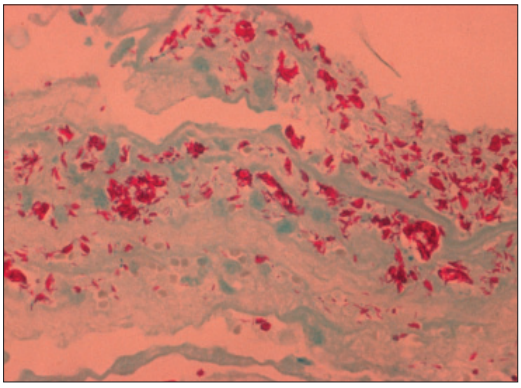
FIGURE 10. SUBCUTANEOUS TISSUE FROM THE EDGE OF A BURULI ULCER SHOWING FAT-CELL GHOSTS WITH ACID-FAST BACILLI IN THE INTERSTITIUM



Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

Continuing necrosis of the dermis usually leads to degeneration of the epidermis, and ultimately to ulceration. Necrosis, however, may spread laterally, with proliferation of AFB in the subcutaneous tissue and fascia (***Figure 11***). In such cases, ulceration of the epidermis often occurs late in the disease. When the disease progresses in this manner, patients develop the plaque form or the oedematous form.

FIGURE 11. MASSES OF ACID-FAST BACILLI INFILTRATE THE BASE OF THE EDGE OF A BURULI ULCER

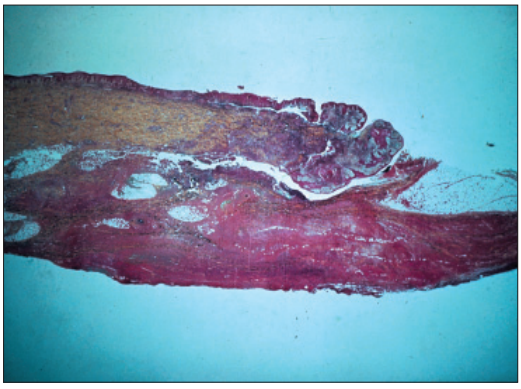


Acid-fast bacilli typically appear in clusters. Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

Necrotic (active) stage: ulcerative lesions

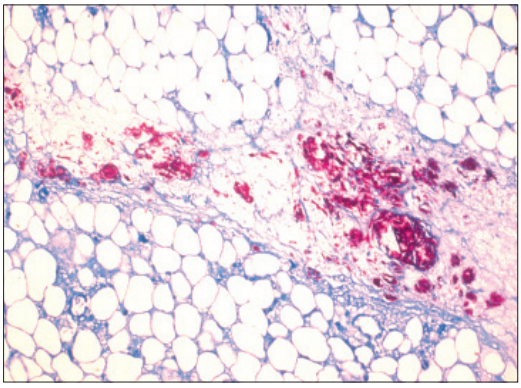
Ulcers are undermined with re-epithelialization of the edges of the lesion and undersurface of the overlying flap of the dermis (***Figure 12***). The adjacent epidermis is usually hyperplastic. The base of the pristine ulcer contains a necrotic slough of cellular debris and fibrin, sometimes with a central eschar. There is contiguous coagulation necrosis of the subcutaneous tissue and fascia, similar to that described for nonulcerated lesions (***Figure 11***, ***Figure 13***). 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 sometimes seen (***Figure 7***).

FIGURE 12. 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



(Courtesy of Wayne Meyers)

FIGURE 13. SUBCUTANEOUS TISSUE FROM THE MARGIN OF A BURULI ULCER SHOWING NECROSIS AND THICKENING OF AN INTERLOBULAR SEPTUM

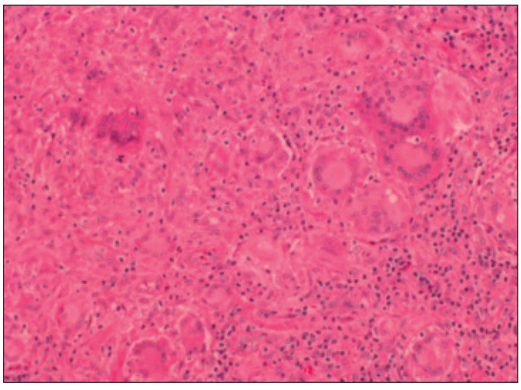


Septum contains masses of acid-fast bacilli. Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

Organizing (early granulomatous) stage

Early healing is characterized by a poorly organized granulomatous response in the dermis and subcutaneous tissue (*Figure 14*). Organizing granulomas often occur during the fourth week of antibiotic therapy.

FIGURE 14. EARLY HEALING OF A BURULI ULCER IN THE ORGANIZING PHASE: LYMPHOCYTES, EPITHELIOID CELLS AND GIANT CELLS



Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

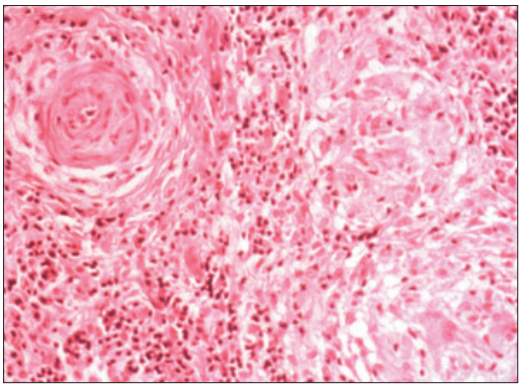
The granulomatous infiltration comprises swollen macrophages (epithelioid cells), Langhans giant cells and lymphocytes. These eventually form organized tuberculoid granulomas. Foamy macrophages, lymphocytes and plasma cells are sometimes seen at the margin of necrotic fat. AFB are scarce or absent.

Healing stage

As healing advances, granulation tissue is followed by fibrosis and a depressed scar (*Figure 15, Figure 16*). AFB are seldom seen.

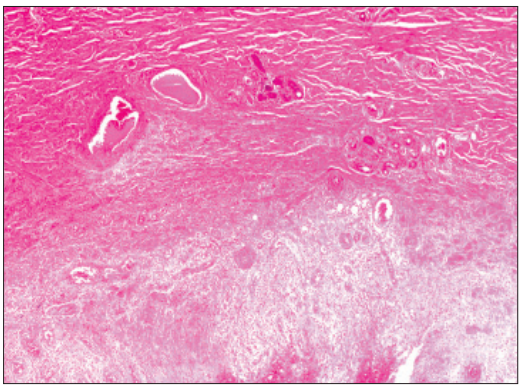
A few patients with old, untreated lesions may also develop granulomas during spontaneous healing.

FIGURE 15. WELL-FORMED, DELAYED HYPERSENSITIVITY GRANULOMA IN A HEALING BURULI ULCER



Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

FIGURE 16. ADVANCED STAGE OF A HEALING BURULI ULCER SHOWING SCARRING OVER MOST OF THE SECTION

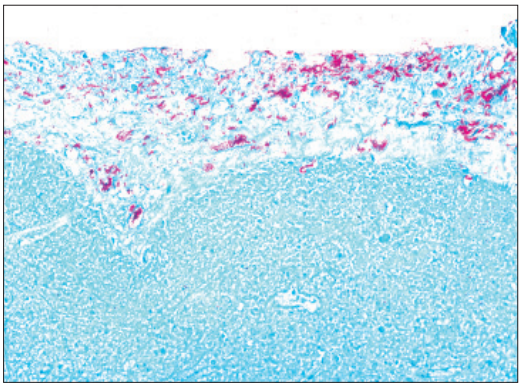


Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

4.7.5.2 Lymph nodes

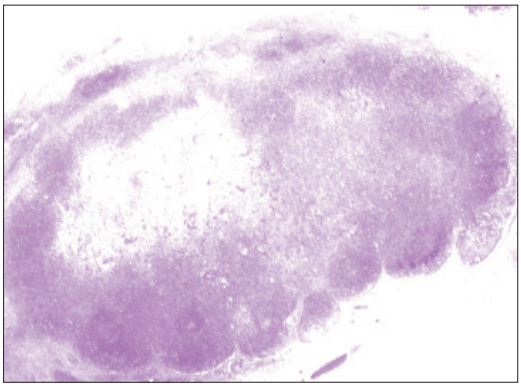
Although clinical lymphadenopathy is rarely seen, significant lymphadenitis is often seen histopathologically, both in lymph nodes adjacent to lesions and in regional nodes. Lymph nodes adjacent to lesions may show marked invasion of the capsule by AFB (*Figure 17*). The parenchyma is often markedly necrotic, with destruction of cortical lymphoid tissue (*Figure 18*). In such cases, the entire node may be invaded by AFB. Regional lymph nodes, however, may show only sinus histiocytosis. Granulomatous changes and AFB are usually not seen.

FIGURE 17. LYMPHADENOPATHY IN BURULI ULCER



The parenchyma of the node is necrotic, and the capsule is heavily infiltrated by acid-fast bacilli. Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

FIGURE 18. NECROTIC LYMPHADENITIS IN A LYMPH NODE PROXIMAL TO A BURULI ULCER

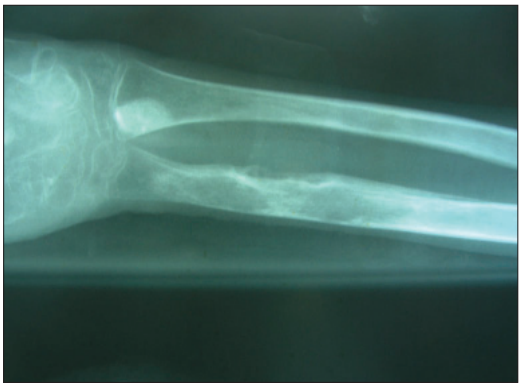


The medulla is destroyed and only remnants of the cortical lymphoid tissue remain. Stained with haematoxylin and eosin (Parallel sections stained with Ziehl–Neelsen show large numbers of acid-fast bacilli in Figure 17) (Courtesy of Wayne Meyers)

4.7.5.3 Bone changes

Bone may be affected by direct extension from an overlying lesion or at a site distant from recognized lesions, presumably by haematogenous spread of *M. ulcerans* (*Figure 19*).

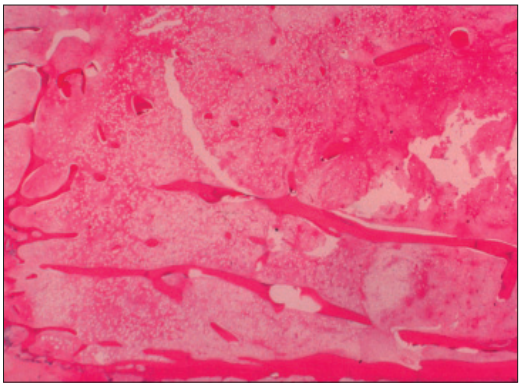
FIGURE 19. X-RAY OF THE LEG SHOWING DESTRUCTION OF THE BONE



The patient had a Buruli ulcer over the affected area (Courtesy of Giovanni Battista Priuli)

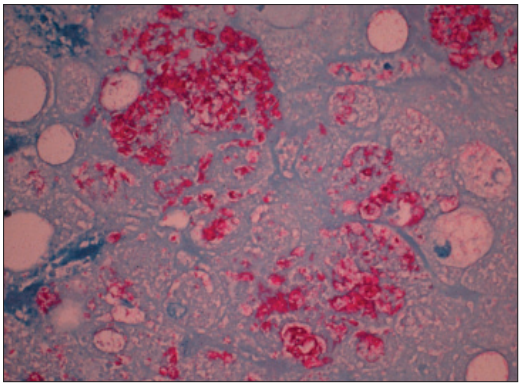
Histopathologically, the marrow is extensively necrotic, and bone trabeculae are eroded (***Figure 20***). AFB are present in varying numbers, most often in the necrotic marrow (***Figure 21***, ***Figure 22***).

FIGURE 20. OSTEOMYELITIS OF THE TIBIA SHOWING NECROSIS OF THE MARROW AND EROSION OF TRABECULAE



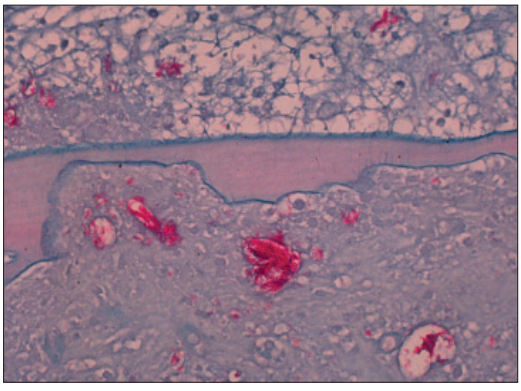
Stained with haematoxylin and eosin (Courtesy of Wayne Meyers)

FIGURE 21. OSTEOMYELITIS OF THE TIBIA WITH MASSES OF ACID-FAST BACILLI IN NECROTIC MARROW



Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

FIGURE 22. OSTEOMYELITIS OF THE TIBIA SHOWING NECROSIS OF MARROW AND A TRABECULA OF BONE UNDERGOING DISSOLUTION IN AN AREA OF ACID-FAST BACILLI



Stained with Ziehl–Neelsen (Courtesy of Wayne Meyers)

Although some lesions in bone seem to be purely an effect of ***M. ulcerans***, approximately 50% of osteomyelitic lesions are coinfected by pyogenic organisms, such as streptococci, staphylococci and ***Corynebacterium*** spp. 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***.

4.7.5.4 Patients with extensive disease

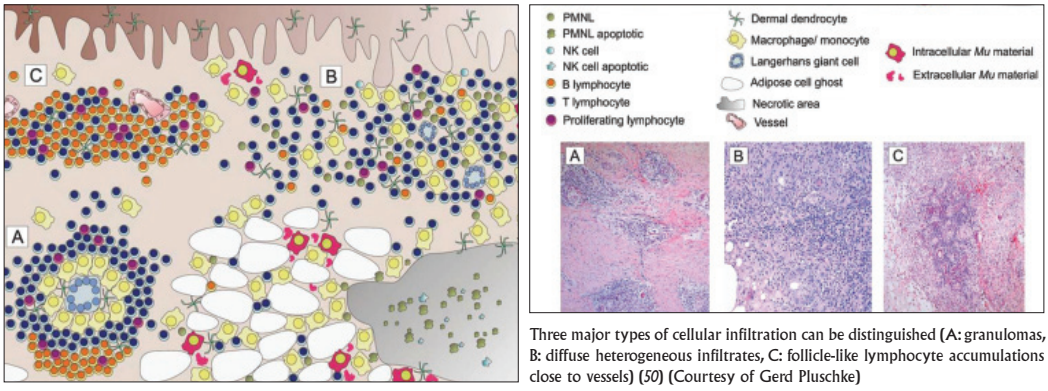
Patients with aggressive oedematous lesions involving large areas of the body often have widespread oedema and impaired renal function or other evidence suggesting that visceral organs are involved. These patients sometimes die early in the course of the disease. Although some authorities suspect that these events are attributable to a systemic effect of the toxin, the only way to be certain is to increase efforts to study the pathophysiology of these patients and to study autopsy specimens.

4.7.6 IMMUNOCHEMISTRY AND ANALYSIS OF CHEMOTHERAPY-ASSOCIATED LOCAL IMMUNE RESPONSES

Histopathological hallmarks of untreated Buruli ulcer lesions include the presence of focally distributed extracellular clusters of AFB, massive tissue necrosis, fat-cell ghosts and a remarkably low cellular infiltration in the centre of the lesions (***39–41***). Signs of chronic inflammation, granulomatous reactions and intracellular AFB may, however, be seen at the periphery of untreated lesions, particularly in patients with a tendency to self-heal (***42***).

During chemotherapy, chronic leukocyte infiltration, most likely triggered by immunostimulating products and antigens released from killed mycobacteria, gradually begins; this leads to the development of diffuse infiltrates in all areas of the affected dermal connective tissue and adipose tissue. This process culminates in the development of ectopic lymphoid structures (***Figure 23***). It is commonly assumed that mycolactone levels decline during chemotherapy, allowing infiltrating leukocytes to reach extracellular mycobacteria, leading to phagocytosis and their destruction. AFB start to be internalized by phagocytes as early as 2 weeks after treatment starts, when ***M. ulcerans*** can still be cultured from tissue homogenates (***41***).

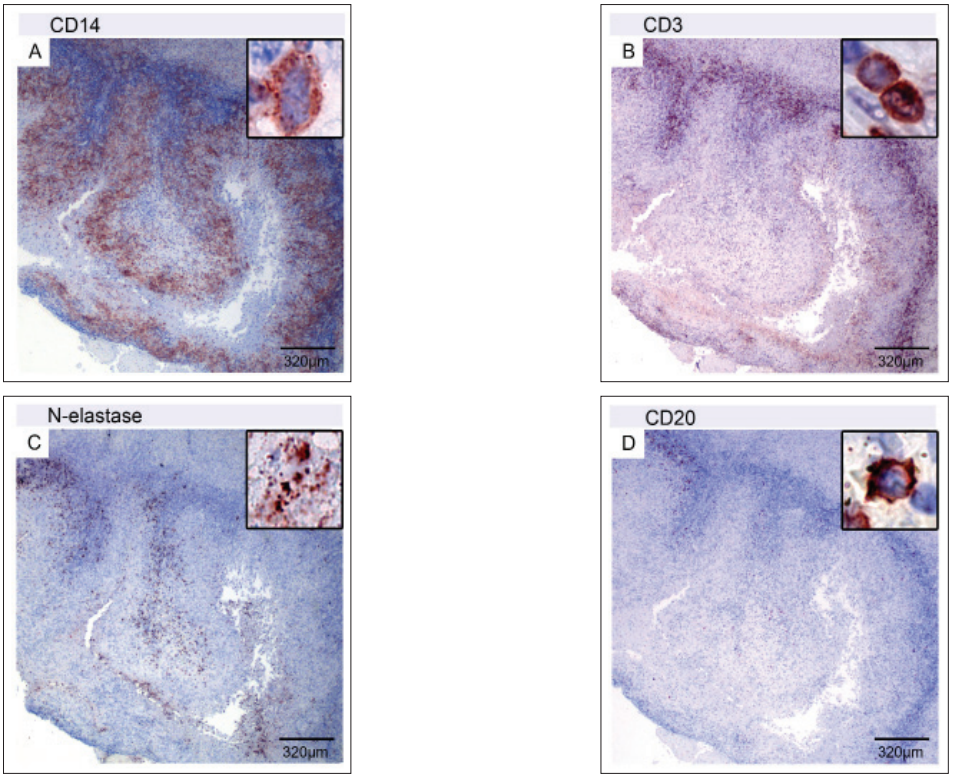
FIGURE 23. SCHEMATIC OVERVIEW OF CELLULAR INFILTRATION PATTERNS AND DISTRIBUTION OF MYCOBACTERIAL MATERIAL IN BURULI ULCER LESIONS TREATED WITH ANTIBIOTICS



Three major types of cellular infiltration can be distinguished (A: granulomas, B: diffuse heterogeneous infiltrates, C: follicle-like lymphocyte accumulations close to vessels) (***50***) (Courtesy of Gerd Pluschke)

Immunohistochemical studies give insight into the cellular composition of the chronic leukocyte infiltrates. Remainders of early neutrophilic infiltrates may be immunohistochemically detectable inside the central necrotic areas (***Figure 24***). In treated lesions, macrophages and monocytes positive for CD14 cells typically become abundant in adipose tissue and around necrotic areas (***Figure 24***), but concurrent neutrophilic infiltrates are often a consequence of secondary infection. The gathering of antigen-presenting cells around necrotic areas, the development of clusters of B cells (***Figure 25***) and highly organized epithelioid granulomas (***Figure 26***) indicate that antigen recognition and processing is stimulating active and specific local immune responses to ***M. ulcerans.***

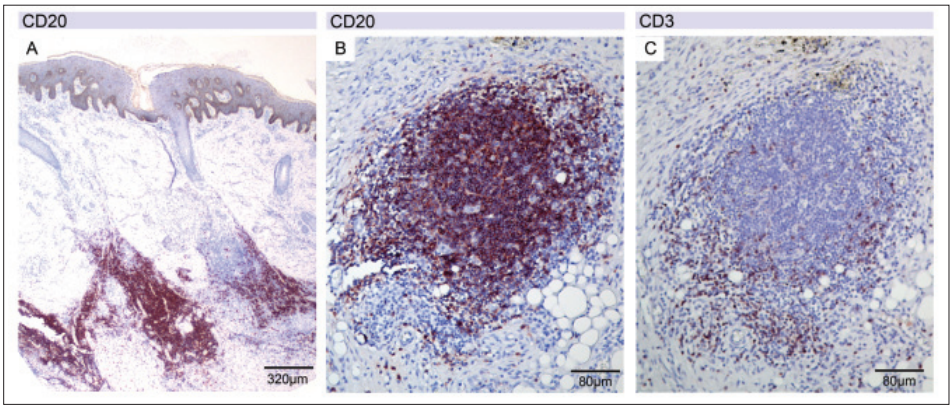
FIGURE 24. BANDS OF LEUKOCYTES SURROUNDING THE UNINFILTRATED NECROTIC CENTRE OF A BURULI ULCER LESION



A belt of CD14+ monocytes and macrophages (A) overlaps a more external second belt of CD3+ T cells (B). In the necrotic core, N-elastase positive neutrophilic debris (C) was found, but no intact neutrophils (D insert). Clusters of CD20+ B cells were located away from the necrotic core (D) (*48*) (Courtesy of Gerd Pluschke)

The outer layer of granulomas is mainly composed of T lymphocytes with $CD4^+$ T cells usually outnumbering $CD8^+$ T cells. These lymphocyte belts are interspersed with dermal dendrocytes. Focal clusters of B cells appear at the outer margins of the layer of T lymphocytes. Antigen-presenting cells, in particular Langhans giant cells and epithelioid macrophages, form the centre of granulomas. In human tuberculosis granulomas, the central core is usually necrotic; however, this is not observed in the lesions of Buruli ulcer patients treated with antibiotics.

FIGURE 25. ACCUMULATION OF B CELLS IN BURULI ULCER LESIONS



(A) Band of CD20+ B cells; **(B)** cluster of CD20+ B cells with a few interspersed CD3+ T cells; **(C)** (*48*) (Courtesy of Gerd Pluschke)

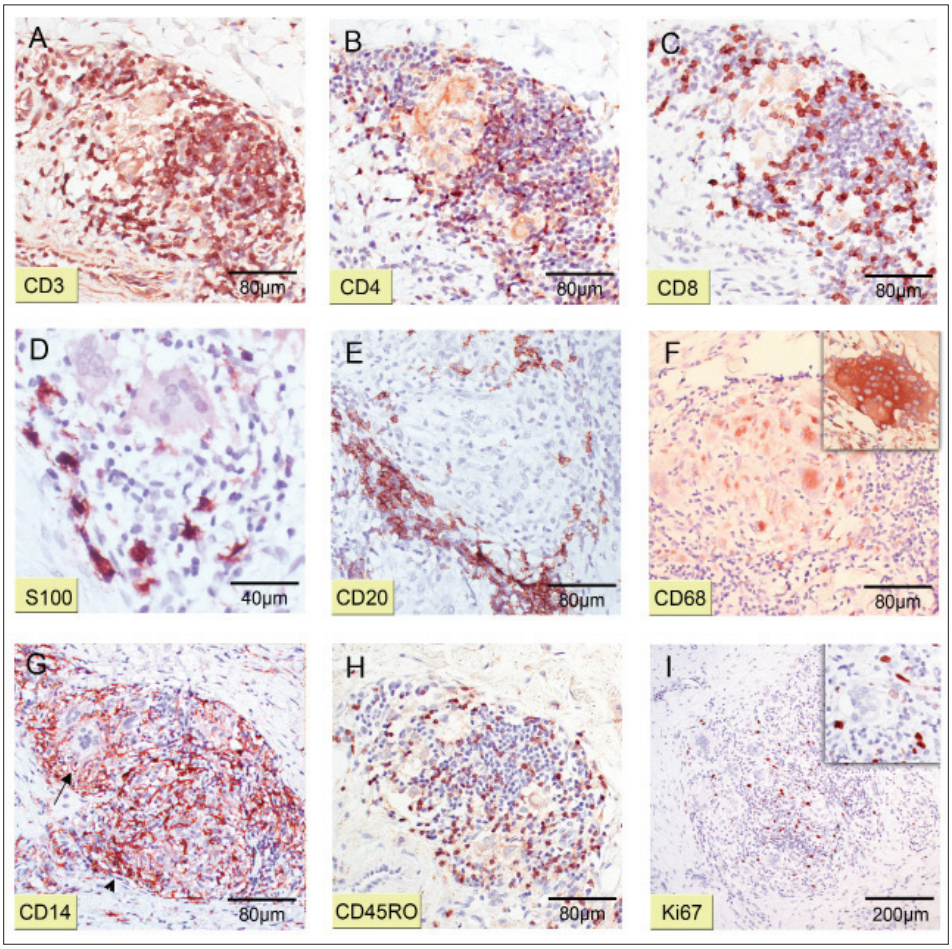
Because the distribution of different types of leukocytes in Buruli ulcer lesions may be heterogeneous, the analysis of a single biopsy may not be representative of the entire lesion. Furthermore, there is great variation in individuals' responses in terms of histopathological findings and clinical outcome. Although ectopic lymphoid tissue is thought to develop in infectious diseases primarily to sequester pathogens, this process is often accompanied by tissue damage. In Buruli ulcer, the development of paradoxical reactions during treatment (*43,44*), including an enlargement of ulcers and an accelerated progression of nonulcerated plaques and oedemas to ulcerative lesions, may represent in some patients a local immune reconstitution inflammatory syndrome. Such a transient worsening of recognized lesions or the appearance of new disease-specific lesions despite appropriate treatment, or both, has long been described in cases of leprosy and tuberculosis.

In Buruli ulcer, deterioration of lesions after initiation of antimycobacterial therapy may be related to factors such as:

- the burden of mycobacterial debris and necrotic tissue remaining in the lesion (*45*);
- superinfection; and
- the composition and organizational status of cellular infiltrates (*46*).

Reactivation phenomena, such as the development of new ulcerations or painful nodules weeks to months after treatment has been completed, may suggest the priming of the adaptive immune system by successful treatment of primary lesions, not a failure of antimycobacterial chemotherapy (*47,48*).

FIGURE 26. DETAILED ORGANIZATION OF A GRANULOMA IN A BURULI ULCER LESION TREATED WITH ANTIBIOTICS



Histological serial sections were stained with antibodies against different cellular surface markers or cytoplasmic markers (the counterstain was haematoxylin). (A, B, C) Staining for CD3, CD4 and CD8 cells, respectively, reveals a belt of helper lymphocytes as well as cytotoxic T lymphocytes surrounding the centre of the granuloma. (D) S100+ dermal dendrocytes spread among T lymphocytes in the outer layer. (E) Focus on CD20+ B lymphocytes at the border of the granuloma. (F) CD68+ antigen-presenting cells in the centre; the insert shows large Langhans giant cell. (G) Large amounts of membrane-bound (arrow) and soluble (arrowhead) CD14 can be observed. (H) Distribution of activated (CD45RO+) lymphocytes. (I) Proliferating (Ki67+) cells indicate the active status of granulomas (*50*) (Courtesy of Gerd Pluschke)

4.7.7 PARADOXICAL REACTIONS

In tuberculosis and other mycobacterial infections, the term paradoxical reaction describes the clinical worsening of pre-existing lesions or the development of new lesions in patients who had initially improved while receiving antimycobacterial treatment (***44,45,47–49***). In the treatment of Buruli ulcer, the timing of paradoxical reactions is unpredictable (they may occur from a few days to many months after the start of chemotherapy); the duration and severity of the reactions are unpredictable as well. Paradoxical reactions that occur during antimycobacterial treatment seem to be more common and severe in HIV-positive individuals who are also on highly active antiretroviral therapy. In these cases, the underlying mechanism of the paradoxical reaction may be an immune reconstitution inflammatory syndrome (also known as IRIS). However, paradoxical reactions also occur in patients who are not immunocompromised; in these cases, the exact mechanisms are not well understood.

The definition of paradoxical reaction in Buruli ulcer includes the enlargement of ulcers, accelerated progression of nonulcerated plaques and oedemas to ulcerative lesions, and the emergence of new lesions during chemotherapy. There may be different underlying mechanisms; therefore it is not correct to regard as IRIS all paradoxical reactions occurring during treatment for Buruli ulcer.

Because mycolactone causes local (and some systemic) immune suppression in Buruli ulcer lesions, practically all patients with Buruli ulcer being treated with antimycobacterial chemotherapy develop vigorous local immune responses. In many patients, massive infiltration of lesions and the development of atopic lymphoid tissue do not seem to interfere with wound healing. Therefore, strong local immune responses are a good marker of the success of antimycobacterial treatment (with the killing of ***M. ulcerans*** and the corresponding decline in mycolactone levels), and strong immune responses are not necessarily an indication of complications related to the immune responses. In patients being treated for Buruli ulcer, it is difficult to differentiate between deterioration resulting from paradoxical reaction and deterioration resulting from treatment failure.

5. QUALITY ASSURANCE

If laboratory testing for Buruli ulcer is unreliable, patients with the disease may not be diagnosed, causing them to develop more severe disease, and also causing the burden of disease in an area to be underestimated. Alternatively, patients without the disease may be treated unnecessarily and the disease burden may be overestimated. Therefore, it is essential to ensure that all laboratory tests are accurate and reliable; this can be achieved through a process known as quality assurance.

The accuracy and reliability of laboratory tests are critical to the success of programmes to control Buruli ulcer. All parts of the testing system must be monitored to ensure the quality of the overall process, to detect and reduce errors, and to improve consistency among testing sites. Quality assurance should be done by each laboratory (as internal quality control) and also by a reference laboratory (as an external quality assessment).

- **Internal quality control**: This refers to the systematic internal monitoring of work practices, technical procedures, equipment and materials; it includes monitoring the quality of reagents (such as stains).
- **External quality assessment**: This is a process that assesses a laboratory's performance. It may include the onsite evaluation of laboratories, the use of panel tests, and blinded rechecking of test results.

Internal quality control and external quality assessment systems are detailed in ***Annex 8*** (based on the acid-fast direct smear microscopy training package, Current Laboratory Practice Series) (***51***).

6. ROLES OF NATIONAL PROGRAMMES, HEALTH FACILITIES AND LABORATORIES

In order to ensure the quality of laboratory confirmation of Buruli ulcer, national programmes, health facilities and laboratories all have an important role to fulfill.

6.1 NATIONAL BURULI ULCER CONTROL PROGRAMMES

- ensure that local laboratories and national reference laboratories have appropriate resources to process diagnostic samples;
- monitor the activities of local laboratories and national reference laboratories;
- collate results from all laboratories and forward them to national and international authorities (such as ministries of health and WHO);
- identify training needs at the laboratories, and coordinate training with laboratories for staff at health facilities when appropriate.

6.2 HEALTH FACILITIES

- screen patients suspected of having Buruli ulcer for nonulcerative or ulcerative lesions;
- obtain appropriate specimens for diagnostic confirmation;
- label specimens and complete BU 03 form (*Annex 9*);
- transport specimens to a local laboratory or national reference laboratory;
- collect results from the laboratory;
- transfer results onto the BU 01 and BU 03 forms (*Annex 9*);
- transfer results to the national programme.

6.3 LABORATORIES

6.3.1 PERIPHERAL LABORATORIES

- receive and process swabs and FNA specimens using the direct smear examination;
- record the smear results within 24 hours and transfer them to the national programme within the time specified by reporting guidelines;
- send 20% of all processed specimens to the national reference laboratory to be monitored for external quality assurance purposes.

6.3.2 NATIONAL REFERENCE LABORATORY

- receives and processes swabs, FNA specimens and, if needed, specimens from punch or excision biopsies using direct smear examination, PCR, culture and histopathology (in some laboratories);
- sends results of smear stainings to the health facility within 24-48 hours, 2 weeks for PCR,

4 weeks for histopathology and 6 months for cultures. The health facility should report results to the national programme within the time specified by the country's reporting guidelines;

- receives about 20% of all processed specimens from peripheral laboratories to conduct external quality assurance of microscopy results;
- regularly organizes microscopy proficiency testing for peripheral laboratories using sample panels; ideally, this occurs once a year. Blinded retesting may be done occasionally for laboratories that consistently have problems during proficiency testing;
- organizes microscopy trainings:
  - for technicians who are new to the microscopy network;
  - for some technicians if specific problems are found during the external quality assurance process;
  - for all technicians if there is an important change in the procedures used within the network.
- regularly supervises the work of peripheral laboratories; ideally, this occurs quarterly;
- regularly organizes blinded rechecking of slides from peripheral laboratories; ideally, this occurs quarterly. Panel testing may be done for laboratories that consistently have problems during the blinded rechecking process; it may be done annually for all laboratories.

6.3.3 INTERNATIONAL (SUPRANATIONAL) REFERENCE LABORATORY

- receives and processes swabs, FNA specimens and, if needed, specimens from punch or excision biopsies using direct smear examination, PCR and occasionally culture and histopathology;
- sends results to the health facility within 24-48 hours for smears, 2 weeks for PCR, 4 weeks for histopathology and 6 months for cultures. The health facility should report results to the national programme within the time specified by the country's reporting guidelines;
- regularly organizes PCR proficiency testing for national reference laboratories using sample panels; ideally, this occurs once a year. Blinded retesting may be done occasionally for laboratories that consistently have problems during proficiency testing. In this case, 10% of all processed specimens from the national reference laboratory are retested for external quality assurance purposes;
- validates and implements new diagnostic technologies;
- regularly supervises the work of peripheral laboratories; ideally, this occurs quarterly;
- regularly organizes blinded rechecking of slides from peripheral laboratories; ideally, this occurs quarterly. Panel testing may be done for laboratories that consistently have problems during the blinded rechecking process; it may be done annually for all laboratories.

6.3.4 GLOBAL NETWORK OF LABORATORIES CONFIRMING *MYCOBACTERIUM ULCERANS* DISEASE (BURULI ULCER)

A network of PCR laboratories has been established in endemic and non-endemic countries. These laboratories are shown in ***Table 2***.

The external quality assessment results of PCR, performed by most of these laboratories, has been published (***52***).

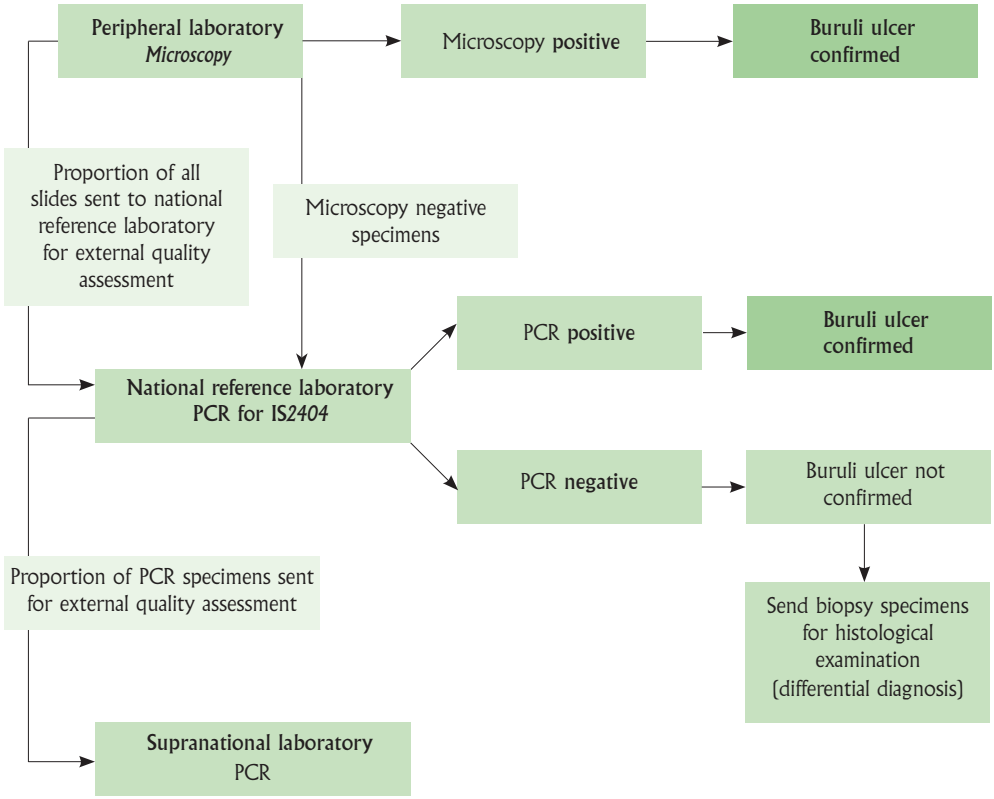
TABLE 2. GLOBAL NETWORK OF LABORATORIES FOR CONFIRMING *MYCOBACTERIUM ULCERANS* DISEASE (BURULI ULCER)

Country	Name of laboratory	Contact person
1. Australia	Victorian Infectious Diseases Reference Laboratory, Melbourne* Mycobacterium Reference Laboratory, Queensland	Dr Janet Fyfe *Janet.Fyfe@mh.org.au* Ms Caroline Lavender *Caroline.Lavender@mh.org.au* Dr Sushil Pandey *sushil_pandey@health.qld.gov.au*
2. Belgium	Institute of Tropical Medicine, Antwerp, Belgium*	Professor Françoise Portaels *fportaels@itg.be* Professor Bouke de Jong *bdejong@itg.be* Dr Miriam Eddyani *MEddyani@itg.be*
3. Benin	Mycobacterial Reference Laboratory, Cotonou Buruli ulcer case-finding and treatment center "Raoul et Madeleine Follereau" de Pobè	Dr Dissou Affolabi *affolabi_dissou@yahoo.fr* Professor Séverin Anagonou *anagonou_severin@yahoo.fr* Dr Estelle Marion *stel.marion@yahoo.fr*
4. Cameroon	Pasteur Centre of Cameroon, Yaoundé	Dr Sara Eyangoh *eyangoh@pasteur-yaounde.org*
5. Central African Republic	Pasteur Institute, Bangui	Dr Fanny-Elodie Minime-Lingoupou *flingoupou@yahoo.fr*
6. Côte d'Ivoire	Pasteur Institute, Abidjan	Professor Mireille Dosso *mireilledosso@yahoo.fr* Dr Solange Kakou Ngazoa *ngazoa_solange@yahoo.fr* Dr N'Guetta Aka *aka_nguetta@yahoo.fr*
7. Democratic Republic of the Congo	National Institute of Biomedical Research (INRB), Kinshasa	Professor Anatole Kibadi Kapay *akibadi@yahoo.fr* Mrs Bibiche Mundabi *mbd852000@yahoo.fr*
8. France	University and University Hospital of Angers - Institute of Biological Sciences in Health - IRIS, Angers	Dr Laurent Marsollier *laurent.marsollier@inserm.fr*
9. French Guiana	Pasteur Institute, Cayenne	Dr Anne-Sophie Drogoul *anneso.drogoul@gmail.com*
10. Germany	Department of Infectious Diseases and Tropical Medicine, University Hospital, Ludwig-Maximilians University, Munich	Dr Gisela Bretzel *bretzel@lrz.uni-muenchen.de* Dr Marcus Beissner *beissner@lrz.uni-muenchen.de*
11. Ghana	Noguchi Memorial Institute for Medical Research, Accra Kumasi Center for Collaborative Research, Kumasi	Professor Dorothy Yeboah-Manu *DYeboah-Manu@noguchi.mimcom.org* Dr Anthony Ablordey *AAblordey@noguchi.mimcom.org* Dr Richard Phillips *rodamephillips@gmail.com* Mr Michael Frimpong *mfrimpong28@gmail.com*
12. Japan	Leprosy Research Center, National Institute of Infectious Diseases, Tokyo	Dr Kazue Nakanaga *nakanaga@nih.go.jp*
13. Switzerland	Swiss Tropical and Public Health Institute, Basel	Professor Gerd Pluschke *Gerd.Pluschke@unibas.ch*
14. Togo	National Institute of Hygiene, Lomé	Dr Abiba Banla-Kere *kerebanla@hotmail.com*

*Institute of Tropical Medicine, Antwerp, Belgium is a WHO Collaborating Center for the Diagnosis and Surveillance of *Mycobacterium Ulcerans* Infection; Victorian Infectious Diseases Reference Laboratory, Melbourne, Australia is a WHO Collaborating Center for *Mycobacterium ulcerans* infection

7. PROPOSED ALGORITHM FOR DIAGNOSING BURULI ULCER

FIGURE 27. ALGORITHM FOR DIAGNOSING BURULI ULCER AT THE PERIPHERAL, NATIONAL REFERENCE AND SUPRANATIONAL LEVELS (*53*)



PCR, polymerase chain reaction

Proposed algorithm for laboratories diagnosing Buruli ulcer at the peripheral, national reference and supranational levels (*53*) (Courtesy of Dissou Affolabi)

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ANNEXES

ANNEXES

ANNEX 1:
COLLECTING CLINICAL SPECIMENS

For all clinical specimens, the relevant clinical information should be obtained for every patient using the BU 03 form designed by WHO (see http://www.who.int/buruli/control/ENG_UB_03.pdf or ***Annex 9***). This section covers only specimen collection for routine diagnosis of cases or patients. These days, surgical and punch biopsies are reserved for specific indications (see ***Annex 10***).

1.1 PROCEDURE FOR OBTAINING A FINE-NEEDLE ASPIRATION

Using the appropriate aspiration technique ensures that sufficient tissue will be obtained. The optimal technique is explained and illustrated with photos and videos in the step-by-step tutorial on fine-needle aspiration (FNA) and collecting swabs on the website of the Stop Buruli consortium.[1]

1.1.1 MATERIALS FOR FNA

The following materials are needed:

1. a 10 ml syringe and 21 gauge, 22 gauge or 23 gauge needles;
2. microtubes with liquid transport medium; the transport medium used depends on the individual laboratory and on the type of analysis being performed – that is, whether the specimen will be cultured or tested only by Polymerase Chain Reaction (PCR). Specimens that will be used only for PCR may be placed in a solution of alcohol and distilled water (in a ratio of 1:1), in phosphate-buffered saline or in TE buffer (10mM trisaminomethane [Tris] hydrochloride plus 1mM ethylenediaminetetraacetic acid [EDTA];
3. glass slides;
4. a pencil and a permanent marker;
5. disposable sterile latex gloves;
6. cotton-wool pads;
7. alcohol for disinfecting;
8. wound-dressing material.

Make sure that everything is ready before you proceed:

- label glass slides in pencil (with the patient's number and date);
- label microtubes in permanent marker (with the patient's number and date).

1.1.2 METHODS

Your working environment should be as clean as possible.

Take the patient's clinical history and perform a physical examination, then explain to the patient what you intend to do. Tell the patient that you need to perform a test called a fine-needle aspiration to find out what is causing the lesion (nodule, plaque, oedema or ulcer) so that the

[1] http://www.stopburuli.org/index.php/FNA-e-tutorial.html

correct treatment can be given. Explain that you will insert a small needle into the lesion. Explain that the procedure will take less than 1 minute and may be a little painful. Let the patient know that you will collect two samples to improve the chances of finding out what is causing the patient's symptoms.

Explain that if the test results are negative, the patient may need to have another test after 1 or 2 weeks because sometimes more than one test is needed to obtain accurate results. Ask the patient whether she or he has any questions.

The assistant should prepare a tray with gauze or cotton-wool pads soaked in alcohol, a capped 21 gauge, 22 gauge or 23 gauge needle with a syringe inserted, a dry piece of gauze, an adhesive plaster and a sample bottle that was obtained from the laboratory.

Before you perform the aspiration, put on clean sterile gloves to protect the patient and yourself. Make sure that you do not touch anything that could contaminate your gloved hands.

Carefully clean the lesion with the gauze or cotton-wool pads soaked in alcohol. Make sure that you disinfect the area well, by wiping the skin several times up and down. Gently palpate the lesion. Locate the site where skin tissue is softest, or estimate the centre of the lesion. This will be the site where you will insert the needle. You may use the marker to outline the lesion to serve as a guide for placing the needle.

Take the syringe, and carefully remove the cap from the needle. Insert the needle into the skin, and gently advance the needle into the subcutaneous tissue. With the needle inside the tissue, apply full suction to create negative pressure. Slowly move the needle back and forth while maintaining suction pressure. Change the angle of the needle three times, and then gently release the suction. Be careful not to draw blood from a blood vessel during this process. The patient may feel some discomfort, so you need to watch her or him and offer reassurance if necessary.

Withdraw the needle, and apply a piece of dry gauze to stop any bleeding. Re-examine the puncture site. Apply a second piece of dry gauze and an adhesive plaster if there is further bleeding at the site.

Gently flush the contents of the needle into the bottle provided by the laboratory, and then label the microtube in permanent marker with the patient's number and date.

Note: To aspirate the bacteria, the needle must be advanced through the subcutaneous tissue to the fat tissue. This is where the bacteria are located in nonulcerative lesions.

Make sure that only the tip of the needle (approximately 1 cm) goes into the subcutaneous tissue. If the needle is inserted too deeply, it will go past the subcutis and fascia. Use a new needle and syringe if you need to obtain a second sample.

Note: Only experienced physicians or experienced health workers should perform FNA; continuing training and regular supervision should be provided to health workers to improve their skills.

1.1.3 PROCESSING AN FNA SAMPLE

If microscopy can be done locally, a drop of aspirate is ejected onto a slide (***Annex 4***). The remainder is expelled into the liquid transport medium (***Annex 2***).

Gently draw some of the liquid transport medium into the needle and then expel it into the microtube. To ensure that all of the sample is transferred, repeat this three times. Then close the microtube.

Be careful that you do not injure yourself with the needle. Discard the syringe and needle in a safety box. The sample (accompanied by a correctly completed BU 03 form) should be sent as soon as possible to the nearest laboratory.

Note: For some types of lesions, especially plaques, the aspiration may appear to be a "dry tap" (that is, it may look like there is no aspirate visible in the syringe). Despite this, some cells will be collected by the aspiration.

1.2 PROCEDURE FOR OBTAINING A SWAB SPECIMEN

Obtaining a swab is a simple procedure, but it first has to be explained to the patient.

1.2.1 MATERIALS FOR SWAB

The following materials are needed:

1. alcohol for cleaning the skin;
2. an alcohol swab (a piece of cotton-wool or gauze dipped in alcohol);
3. a sterile swab on a wooden or plastic applicator;
4. disposable gloves;
5. a permanent marker;
6. transport medium; the transport medium used depends on the individual laboratory and on the type of analysis being performed – that is, whether the specimen will be cultured or tested only by PCR.

1.2.2 METHODS

Your working environment should be as clean as possible.

Take the patient's clinical history and perform a physical examination, then explain to the patient what you will do. Tell the patient that you need to collect a small amount of tissue from the ulcer to find out what is causing the illness so that the correct treatment can be given. Explain that you will use a piece of dry cotton-wool on an applicator to gently wipe under the edges of the ulcer to collect some of the tissue.. Let the patient know that you will collect two samples to improve the chances of finding out what is causing the patient's symptoms. Explain that the procedure will take less than 1 minute and may be a little uncomfortable.

Explain that if the test results are negative, the patient may need to have another test after 1 or 2 weeks because sometimes more than one test is needed to obtain accurate results. Ask the patient whether she or he has any questions.

Ask your assistant to prepare a simple tray with a permanent marker, a cotton swab stick, a microtube with semi-solid transport medium, clean disposable gloves, and a swab or piece of cotton-wool soaked in alcohol or disinfectant. Before you perform the procedure, put on clean gloves to protect the patient and yourself. Make sure that you do not touch anything that could contaminate your gloved hands.

Using the cotton-wool soaked in alcohol or disinfectant, carefully clean the skin surrounding the ulcer twice, avoiding the base of the ulcer.

Gently insert the cotton-wool on the applicator underneath the edges of the ulcer. Rotate the swab and wipe the tissues beneath the edges of the ulcer in a downward and clockwise manner. Gently insert the swab into the sterile tube, and label the tube with the patient's name, age, sex and the date the sample was obtained.

1.3 ADDITIONAL RESOURCES

More detailed documentation can be obtained on the websites of the Stop Buruli consortium[1] and BuruliVac.[2]

[1] http://www.stopburuli.org/index.php/FNA-e-tutorial.html

[2] http://www.burulivac.eu/index.php?id=17923

ANNEX 2:
TRANSPORT MEDIA FOR BACTERIOLOGICAL ANALYSES

Several transport media are recommended for bacteriological analyses (that is, direct smear examination, culture and polymerase chain reaction [PCR]): a liquid transport medium for FNA, a semisolid transport medium for swabs and biopsies, and polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA) transport medium, which can be used for all types of samples.

If only PCR analysis is to be performed on the sample, the following solutions may also be used: phosphate-buffered saline (see ***Annex 5*** for preparation), TE buffer (10mM Tris hydrochloride plus 1mM EDTA, with pH 8.0), a cell lysis solution (for example, manufactured by Qiagen), or a solution of alcohol and distilled water in a ratio of 1:1.

2.1 LIQUID TRANSPORT MEDIUM

The steps to prepare 200 ml liquid transport medium are given below.

Dubos broth base (Becton Dickinson product number: 238510)

1. Add 1.3 g Dubos broth base to 180 ml distilled water.
2. Dissolve by heating.
3. Sterilize at 121–124 °C for 15 minutes with a loosened screw cap.
4. Cool to below 50 °C.
5. Add aseptically 20 ml oleic acid, albumin, dextrose and catalase (OADC) supplement (see below for preparation).
6. Add aseptically 5 ml PANTA (see below for preparation).
7. Incubate the complete medium at 37 °C for 24 hours to check its sterility.
8. Distribute 1 ml in sterile 2 ml vials.

The steps to prepare 1 litre of OADC supplement are given below.

OADC supplement

1. Add 0.6 ml oleic acid to 50 ml sodium hydroxide (NaOH) 0.05 N.
2. Stir for about 30 minutes.
3. Add 50 g bovine serum albumin fraction V (Acros Organics product number: 94349-60-7).
4. Add 920 ml distilled water.
5. Add 15 ml glucose.
6. Dissolve by stirring for about 2 hours.
7. Adjust pH to 6–7.
8. Filter.
9. Incubate for 24 hours at 37 °C to test its sterility.

Note: It is also possible to use the Becton Dickinson BBL mycobacteria growth indicator tube (also known as MGIT) supplemented with OADC enrichment and PANTA antibiotic mixture (product number: 245116). Lyophilized PANTA is ready to use after reconstitution.

The steps to prepare 5 ml of PANTA antimicrobial supplement are given below.

PANTA (Becton Dickinson, product number: 442188)

1. Add 5 ml distilled water to 1 vial of lyophilized PANTA.
2. Add aseptically to the Dubos medium.

Note: The liquid transport medium can be stored at 2–8 °C for a maximum of 6 months.

2.2 SEMISOLID TRANSPORT MEDIUM

The steps to prepare 200 ml semisolid transport medium are given below.

Dubos broth base (Becton Dickinson product number: 238510)

1. Add 1.3 g Dubos broth base to 180 ml distilled water.
2. Add 1.0 g agar (final concentration, 0.5% agar).
3. Dissolve by heating.
4. Sterilize at 121–124 °C for 15 minutes with a loosened screw cap.
5. Cool to below 50 °C.
6. Add aseptically 20 ml OADC supplement (see 2.1 for preparation).
7. Add aseptically 5 ml PANTA (see 2.1 for preparation).
8. Incubate the complete medium at 37 °C for 24 hours to check its sterility.
9. Distribute 1 ml in sterile 2 ml tubes.

Note: The semisolid transport medium can be stored at 2–8 °C for a maximum of 6 months.

2.3 PANTA TRANSPORT MEDIUM

The steps to prepare 246 ml PANTA transport medium are given below.

Dubos broth base (Becton Dickinson product number: 238510)

1. Dissolve 1.5 g Dubos broth base in 204 ml distilled water. Heating is not necessary.
2. Sterilize at 121–124 °C for 15 minutes with a loosened screw cap.
3. Cool to below 50 °C.
4. Filter glycerol (1,2,3-propanetriol) through a sterile bottle-top filter (this will take a while because of the viscosity of glycerol).
5. Add aseptically 12 ml of the sterile filtered glycerol to the broth.

Dubos medium albumin (Becton Dickinson product number: 230910)

1. Add aseptically 24 ml Dubos medium albumin to the sterile broth.

PANTA (Becton Dickinson product number: 245114)

1. Dissolve two lyophilized PANTA vials by adding 3 ml distilled water to each.
2. Add aseptically the 6 ml of the dissolved PANTA to the Dubos–glycerol mixture.
3. Incubate the PANTA transport medium for 24 hours at 37 °C to test its sterility.
4. Add aseptically 500 µl of PANTA transport medium into sterile 2 ml tubes, thus preparing transport containers that are ready to use.

Note: The PANTA transport medium can be stored at 2–8 °C for a maximum of 6 months.

ANNEX 3:
PREPARING SPECIMENS FOR BACTERIOLOGICAL ANALYSES

The following approaches are recommended.

3.1 FINE-NEEDLE ASPIRATION

1. Inoculate the FNA into appropriate transport medium (*Annex 2*) as described in *Annex 1.*
2. Vortex well.

3.2 SWABS

1. Break off the end of a swab and place it in a 5 ml tube or bottle containing glass beads, or in a tube containing a transport medium.
2. Suspend swabs in a small volume of phosphate-buffered saline or normal saline; the volume needed depends on the number of tests to be performed. **Note:** Swabs kept in semisolid transport medium should first be removed from the medium and then placed in phosphate-buffered saline or normal saline.
3. Vortex well.

3.3 TISSUE SPECIMENS FROM PUNCH BIOPSY OR SURGICAL BIOPSY

1. Grind tissue (fresh or in transport medium) into small pieces (for example, in a Petri dish) with a sterile single-use or autoclavable scalpel or scissors. **Note:** If equipment is to be re-used, it must first be placed in an appropriate disinfectant, and then carefully brushed before sterilization to prevent cross-contamination, especially for PCR studies. Recommended disinfectants are 2% alkaline glutaraldehyde, 5% phenol, 10% phenol-based disinfectants (for example, Dettol), 10% iodine or 70% alcohol.
2. Place diced tissue in a 5 ml tube or bottle containing glass beads.
3. Add phosphate-buffered saline or normal saline; the volume needed depends on the number of tests to be performed.
4. Mix well (for example with a vortex mixer).

Note: The specimen may also be prepared by grinding with a sterile mortar and pestle or Potter grinder.

Note: Care should be taken to prevent cross-contamination when cleaning the instruments.

ANNEX 4:
DIRECT SMEAR EXAMINATION

4.1 PREPARING SPECIMENS

4.1.1 FNA (SEE *ANNEX 1* FOR INFORMATION ON OBTAINING A SAMPLE BY FNA)

1. Use a pencil to label a glass slide with the serial number from the laboratory registry.
2. Carefully detach the needle from the syringe.
3. Gently draw some air into the syringe then reattach the needle.
4. Advance the syringe plunger gently to eject a drop of the aspirate onto a glass slide.
5. Prepare a smear of the aspirate on the labelled slide, air dry and heat fix by passing the slide through a flame three times.

4.1.2 SWABS

1. Use a pencil to label a glass slide with the serial number from the laboratory registry.
2. Place the swab in a 5 ml tube or bottle containing glass beads.
3. Add 2 ml of phosphate-buffered saline.
4. Vortex well.
5. Prepare a smear of the suspension on the labelled slide, air dry and heat fix by passing the slide through a flame three times.

Note: If glass beads and vortexes are not available, a smear may also be made by:

1. labelling a glass slide;
2. placing a drop of sterile saline on it;
3. wiping the swab in the saline;
4. air drying and heat fixing by passing the slide through a flame three times.

4.1.3 TISSUE

1. Use a pencil to label a glass slide with the serial number from the laboratory registry.
2. Prepare a smear of the suspension on the slide, air dry and heat fix by passing the slide through a flame three times.

Note: Smears may also be prepared directly from tissue without grinding (*13*).

4.2 ZIEHL–NEELSEN STAINING

In general, the method used locally for laboratory diagnosis of tuberculosis can also be used to diagnose *Mycobacterium ulcerans* disease. In most cases, this will be the hot Ziehl–Neelsen staining method. The number of acid-fast bacilli found in a smear should be reported using the same grading scales as those used locally for sputum smear examination for tuberculosis.

The reagents described here are used only with the hot Ziehl–Neelsen method. The hot method is superior to cold methods, such as the Kinyoun. The concentrations recommended here are slightly different from those often found elsewhere. The fuchsin concentration is slightly higher, and the methylene blue is lower, providing the best possible contrast (that is, strong red bacilli against a light blue background). Other concentrations and cold methods may give satisfactory

results under optimal conditions. However, when other conditions (such as the microscope, light or technician training) are less well controlled, it is strongly recommended that the concentrations given below and the hot method are used to provide better colour contrast.

4.2.1 REAGENTS

The steps for preparing the reagents are given below.

4.2.1.1 Carbol fuchsin 1% staining solution

Phenol crystals	50 g
95% ethanol or methanol	100 ml
Distilled water	50 ml
Basic fuchsin	10 g
Distilled water	800 ml

The best way to prepare carbol fuchsin (1 litre) is as follows:

1. Mix 100 ml alcohol (95% ethanol or methanol) with 50 g of phenol in a conical flask of minimum 1 litre.
2. Add about 50 ml distilled water, mix again.
3. Add 10 g of basic fuchsin powder, mix until complete dissolution.
4. Add 800 ml distilled water, mix well (e.g. by transferring a few times to a second 1 litre conical flask if no larger volume is available). A magnetic stirrer is useful. Heating is not needed.
5. Store in a tightly stoppered amber-coloured bottle.
6. Label the bottle with the name of reagent (carbol fuchsin) and the dates of preparation and expiry.
7. Filter before using, or at the time of use.

Note: The reagent can be stored at room temperature out of direct sunlight for 12 months. Other ways of preparing this solution do not always give a good result and are not recommended.

4.2.1.2 Decolourizing solution

70% ethanol (technical grade)	97 ml
Concentrated hydrochloric acid (HCl)	3 ml

1. Carefully add concentrated hydrochloric acid (HCl) to 70% ethanol.
2. Always add acid slowly to alcohol, not vice versa.
3. Store in an amber-coloured bottle.
4. Label the bottle with name of reagent (decolourizing solution or acid–alcohol) and the date of preparation.

Warning: HCl is an irritant and should be handled with care.
Note: The decolourizing solution can be stored at room temperature long-term.

4.2.1.3 Counterstain

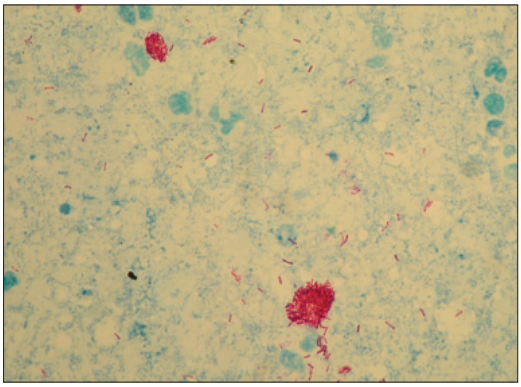
Methylene blue chloride	0.1 g
Distilled water	100 ml

1. Dissolve methylene blue chloride in distilled water in a tightly stoppered amber-coloured bottle.
2. Label the bottle with name of reagent (methylene blue solution), and the dates of preparation and expiry.

Note: The counterstain can be stored at room temperature for 12 months.

Figure 28 shows a Ziehl–Neelsen stained smear acid-fast bacilli as red rods against a blue background.

FIGURE 28. ZIEHL–NEELSEN STAINED SMEAR ACID-FAST BACILLI



Ziehl–Neelsen stained smear of a Buruli ulcer lesion showing red extracellular acid-fast bacilli against a blue background (Courtesy of Kristina Lydia Huber)

4.2.2 PROCEDURE

Prepare a smear (not too thick nor too thin) from the suspension obtained after preparing the specimen as indicated in ***Annex 3.*** A suitable smear means that one should be able to read a printed text through the unstained smear placed at some distance from the text.

1. Place the labelled slides on a staining rack in batches with a maximum of 12 slides per batch. Ensure that the slides do not touch each other.
2. Flood each entire smear with the filtered carbol fuchsin; the most practical way to do this is to pour the stain over the slide through a funnel lined with filter paper.
3. Heat each slide slowly until it is steaming; do this for 15 minutes. Do not let the stain boil or dry.
4. Rinse with a gentle stream of running water until all free stain has been washed away.
5. Flood the slide with the decolourizing solution for 3 minutes.
6. Rinse the slide thoroughly with water. Drain the excess water from the slide.
7. Repeat steps 5 and 6 if the smear is still too red.
8. Flood the slide with the counterstain.
9. Allow the smear to counterstain; this usually takes a maximum of 60 seconds.
10. Rinse the slides thoroughly with water. Drain the excess water from the slides.

11. Allow smears to air dry. Do not blot them. Keep the slides out of direct sunlight. Read as soon as possible using a 8x or 10x eyepiece and the 100x objective of a light microscope and immersion oil (800x or 1000x magnification).
12. After reading the slides, remove the immersion oil by letting it soak into tissue paper or toilet paper. Store the slides in boxes.

4.2.3 GRADING SCALE FOR ZIEHL–NEELSEN MICROSCOPY

No. of AFB seen on average	No. of fields to screen	Report
0/100 immersion fields	100	No AFB observed
1–9/100 immersion fields[a]	100	Record exact figure
10–99/100 immersion fields	100	+
1–10/1 immersion field	50	++
>10/1 immersion field[b]	20	+++

AFB, acid-fast bacilli

[a] A finding of ≤3 bacilli in 100 fields does not correlate well with culture positivity, but should be reported.

[b] ***Mycobacterium ulcerans*** often forms clumps of densely packed AFB that are too numerous to be counted accurately. When large numbers of AFB are observed, the report should be "+++".

4.3 FLUOROCHROME STAINING

Some laboratories use fluorescence microscopy with auramine O to diagnose tuberculosis. Fluorescence microscopy is indicated when the number of daily specimens exceeds 30 specimens per laboratory worker. It has been shown that equivalent results are obtained for detecting ***M. ulcerans*** with either Ziehl–Neelsen or auramine O stains (*13*).

4.3.1 REAGENTS

The steps for preparing the reagents are given below.

4.3.1.1 Staining solution

Auramine O (solution 1)

Auramine powder	0.1 g
95% ethanol (technical grade)	10 ml

Dissolve auramine in ethanol.

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

Phenol (solution 2)

Phenol crystals	3.0 g
Distilled water	87 ml

Dissolve phenol crystals in water.

1. Mix **solution 1** and **solution 2** together.
2. Store in a tightly stoppered amber-coloured bottle away from heat and light.
3. Label the bottle with the name of the reagent (auramine O) and the dates of preparation and expiry.

Note: The solution can be stored at room temperature in a closed cupboard for a maximum of 3 months.

Note: A precipitate often forms but this does not indicate deterioration; however, the solution should be filtered before being used for staining.

4.3.1.2 Decolourizing solution

70% ethanol (technical grade)	100 ml
Concentrated HCl	0.5 ml

1. Carefully add concentrated HCl to 70% ethanol.
2. Always add acid slowly to alcohol, not vice versa.
3. Store in an amber-coloured bottle.
4. Label the bottle with the name of the reagent (decolourizing solution or acid–alcohol) and the date of preparation.

Note: The decolourizing solution keeps indefinitely.

Potassium permanganate ($KMnO_4$)	0.5 g
Distilled water	100 ml

4.3.1.3 Counterstain

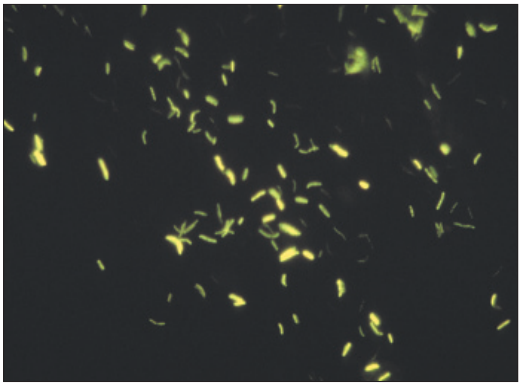
1. Dissolve potassium permanganate ($KMnO_4$) in distilled water in a tightly stoppered amber-coloured bottle.
2. Label the bottle with the name of the reagent (potassium permanganate) and the dates of preparation and expiry.

Note: The solution can be stored at room temperature for a maximum of 3 months.

Note: The 0.5% $KMnO_4$ solution gives a dark background. This makes it difficult to keep the smear in focus. At lower concentrations, it is not as dark; however, weaker solutions are unstable and are thus not preferred by many laboratories.

Figure 29 is a smear stained with fluorochrome (auramine O); it shows acid-fast bacilli as bright yellow rods against a dark background.

FIGURE 29. SMEAR STAINED WITH FLUOROCHROME (AURAMINE O)



Smear stained with fluorochrome (auramine O) showing acid-fast bacilli as bright yellow rods against a dark background (Courtesy of Alfred Michael Emmerson)

4.3.2 PROCEDURE

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

1. Place labelled smears on a staining rack in batches (with a maximum of 12 per batch). Ensure that the slides do not touch each other.
2. Use a funnel equipped with Whatman number 1 filter paper to pour the stain on the slides. Flood each entire smear with auramine O.
3. Allow to stand for 15 minutes, ensuring that the staining solution remains on the smears. **Note:** Do not heat the slides.
4. Rinse with water and drain. Distilled water is recommended, but this is often not available in field laboratories. Experience from some laboratories has shown that tap water is satisfactory. A suitable alternative to tap water would be to use dechlorinated water (that is, water that has been exposed to air for 24 hours).
5. Decolourize with 0.5% acid–ethanol for 2 minutes.
6. Rinse with water and drain.
7. Flood smears with counterstain for 2 minutes. Time is critical because counterstaining for longer periods may quench the fluorescence of acid-fast bacilli (AFB).
8. Rinse with water and drain.
9. Allow smears to air dry. Do not blot them. Read the slides as soon as possible using a magnification of 200–250x (20x or 25x eyepiece and 10x objective).
10. After they have been read, store the slides in the dark (that is, in a closed slide-box).

4.3.3 GRADING SCALE FOR FLUORESCENCE MICROSCOPY

The number of AFB indicates how infectious the patient is. It is important to record exactly what you see.

What you see (200x)	What you see (400x)	What to report
No AFB in one length	No AFB in one length	No AFB observed
1–4 AFB in one length	1–2 AFB in one length	Confirmation required*
5–49 AFB in one length	3–24 AFB in one length	Scanty
3–24 AFB in one field	1–6 AFB in one field	+
25–250 AFB in one field	7–60 AFB in one field	++
>250 AFB in one field	>60 AFB in one field	+++

*Confirmation required by another technician or preparation of another smear, to be stained and read.

ANNEX 5:
IN VITRO CULTURE OF *MYCOBACTERIUM ULCERANS*

In vitro culture of ***M. ulcerans*** consists of four steps:

1. preparing the specimen;
2. decontaminating the specimen (to remove contaminating microorganisms);
3. inoculating and incubating on Löwenstein–Jensen, Brown and Buckle or Ogawa medium;
4. identifying ***M. ulcerans***.

5.1 PREPARING SPECIMENS

Use the suspensions prepared as described in ***Annex 3.***

5.2 DECONTAMINATING SPECIMENS

Several decontamination methods exist, and the method chosen depends on the level of culture contamination experienced by the laboratory and is at the laboratory's discretion.

5.2.1 OXALIC ACID METHOD

5.2.1.1 Reagents

Digester (4% NaOH solution)

NaOH pellets (analytical grade)	20 g
Distilled water	500 ml

1. Weigh 20 g of NaOH pellets.
2. Dissolve in 500 ml distilled water in a 500 ml bottle.
3. Autoclave at 121 °C for 15 minutes with a loosened screw cap.
4. After the solution has cooled, fasten the cap.

Note: The digester can be stored at 2–8 °C for a maximum of 6 months.

Phosphate–buffered saline

Phosphate-buffered saline	1 tablet
Distilled water	100 ml

1. Dissolve 1 tablet of phosphate-buffered saline in 100 ml distilled water.
2. Autoclave at 121 °C for 15 minutes with a loosened screw cap.
3. After the solution has cooled, fasten the cap.

Note: The phosphate-buffered saline can be stored at 2–8 °C for a maximum of 12 months.

0.2% malachite green

Malachite green	200 mg
Distilled water	100 ml

1. Dissolve 200 mg malachite green in 100 ml distilled water.
2. Autoclave at 121 °C for 15 minutes with a loosened screw cap.
3. After the solution has cooled, fasten the cap.

Note: The solution can be stored at 2–8 °C for a maximum of 12 months.

5% oxalic acid

Oxalic acid	5 g
Distilled water	100 ml

1. Dissolve 5 g oxalic acid in 100 ml distilled water.
2. Autoclave at 121 °C for 15 minutes with a loosened screw cap.
3. After the solution has cooled, fasten the cap.

Note: The solution can be stored at 2–8 °C for a maximum of 12 months.

0.8% cycloheximide (actidione)

Cycloheximide	0.4 g
Distilled water	50 ml

1. Weigh 0.4 g cycloheximide.
2. Dissolve cycloheximide in 50 ml distilled water.
3. Autoclave with a loosened screw cap.
4. After the solution has cooled, fasten the cap.

Note: The solution can be stored at 2–8 °C for a maximum of 12 months.

5.2.1.2 Procedure

Note: Ensure that all manipulations are performed in a sterile manner to avoid contaminating the reagents and samples.

Note: Use gloves, and work in a biological safety cabinet.

1. Place absorbent paper on the work area of the safety cabinet and soak it with an appropriate disinfectant, such as a 10% phenol-based disinfectant (for example, Dettol).
   Note: All manipulations of contaminated material should be performed over this paper to avoid the spattering of falling drops, and so that any spills are immediately disinfected.

2. Transfer the sample to a 50 ml container, such as a Falcon tube (for example, tubes from Greiner bio-one or Becton Dickinson), and add the following reagents, taking care not to touch the tube if the same pipette will be used for more than one tube:
   a. 5 ml of 0.2% malachite green
   b. 5 ml of 1 N NaOH digester
   c. 1 ml of 0.8% cycloheximide.
3. Vortex, then leave for 30 minutes at room temperature.
4. Centrifuge for 15 minutes at room temperature at 3500 x g.
5. Decant the supernatant into a container for liquid waste.
6. Add 10 ml of 5% oxalic acid to the sediment.
7. Leave for 20 minutes at room temperature.
8. Centrifuge for 15 minutes at room temperature at 3500 x g.
9. Decant the supernatant into a container for liquid waste.
10. Resuspend the pellet obtained after decontamination by transferring a few drops of sterile distilled water, and pipetting manually or using a vortex if needed.
11. Inoculate the culture medium immediately.

5.2.2 SODIUM HYDROXIDE METHOD (MODIFIED PETROFF METHOD)

5.2.2.1 Preparation

4% NaOH solution

NaOH pellets (analytical grade)	4 g
Distilled water	50 ml

1. Dissolve NaOH in distilled water by heating.
2. Autoclave at 121 °C for 15 minutes.

Note: This solution can be kept long-term and may be stored in the refrigerator, although this is not necessary.

Sterile saline

Sodium chloride (NaCl) pellets (analytical grade)	0.85 g
ESterile distilled water	100 ml

1. Dissolve NaCl in distilled water.
2. Autoclave at 121 °C for 15 minutes.
3. Store the solution in a refrigerator.

Note: This solution can be kept long-term.

5.2.2.2 Procedure

1. For each 2 ml of suspension prepared from the specimen (taken from a swab, FNA or homogenized tissue) add 2 ml of 4% NaOH.
2. Tighten the cap of the container, and shake to digest.
3. Let stand for 15 minutes at room temperature; shake occasionally.
4. Centrifuge at 3000 x g for 15 minutes.
5. Pour off the supernatant.
6. Add 15 ml sterile saline or distilled water, and resuspend the sediment.
7. Centrifuge at 3000 x g for 15 minutes.
8. Decant the supernatant and resuspend the pellet obtained after decontamination by transferring a few drops of sterile distilled water, and pipetting manually or using a vortex if needed.
9. Inoculate the culture medium.

5.2.3 N-ACETYL-L-CYSTEINE METHOD

5.2.3.1 Preparation

For 100 ml N–acetyl–L–cysteine–NaOH solution

4% sterile NaOH (see section 5.2.2)	50 ml
2.9% sterile sodium citrate dihydrate	50 ml
N-acetyl-L-cysteine	0.5 g

1. Add sodium citrate solution to NaOH solution.
2. Distribute into screw-cap flasks.
3. Autoclave at 121 °C for 20 minutes.
4. On the day of use, add N-acetyl-L-cysteine at 1% concentration to the flask, and mix well.

Note: The final reagent must be used within 24 hours because N-acetyl-L-cysteine loses its mucolytic activity.

For 1 litre phosphate buffer

Disodium hydrogen phosphate (Na_2HPO_4) (water free)	4.74 g
Potassium dihydrogen phosphate (KH_2PO_4) (water free)	4.54 g
Distilled water	1000 ml

1. Dissolve 4.74 g Na_2HPO_4 and 4.54 g KH_2PO_4 in 1000 ml distilled water.
2. Autoclave at 121 °C for 15 minutes.

Note: The phosphate buffer can be stored at room temperature or in a refrigerator; it is stable for 6 months. The phosphate buffer may also be prepared using a ready-to-use tablet of phosphate-buffered saline as described in 5.2.1.1.

Tween 80 5% solution

Tween 80 is available as a ready-to-use preparation.

5.2.3.2 Procedure

1. For each 1 ml of suspension prepared (up to 10 ml) from the specimen (taken from a swab, FNA or homogenized tissue samples in a 50 ml container) add the same amount of N-acetyl-L-cysteine–NaOH solution.
2. Incubate by shaking at moderate speed for 15 minutes at room temperature.
3. Add 10 ml sterile phosphate-buffered saline.
4. Add 2 drops 5% Tween 80.
5. Centrifuge at 3000 x g for 20 minutes.
6. Pour off the supernatant.
7. Add 1 ml sterile phosphate-buffered saline and resuspend the sediment.
8. Inoculate the culture medium.

5.3 INOCULATING CULTURE MEDIA

5.3.1 PREPARING LÖWENSTEIN–JENSEN MEDIUM

5.3.1.1 Components

There are three components of Löwenstein–Jensen medium; they are prepared separately and then combined to make the medium:

1. mineral solution;
2. malachite green solution;
3. homogenized whole eggs.

Mineral solution

(KH_2PO_4)	2.40 g
Magnesium sulfate ($MgSO_4.7H_2O$)	0.24 g
Magnesium citrate	0.60 g
Asparagine	3.60 g
Potato flour	30.00 g
Glycerol (reagent grade)	12 ml
Distilled water	600 ml

1. Dissolve the ingredients by heating in distilled water in the order listed in the table above.
2. Autoclave at 121 °C for 30 minutes.
3. Cool to room temperature.

Note: This solution may be kept long-term and should be stored in the refrigerator.

2% Malachite green solution

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 hour to 2 hours.

Note: This solution can be stored for a maximum of 12 months, and may precipitate or change to a less deeply coloured solution. If either of these occurs, discard the solution and prepare fresh solution.

Homogenized whole eggs

1. Clean fresh hen's eggs that are ≤7 days old by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap.
2. Let the eggs soak for 30 minutes in the soap solution.
3. Rinse eggs thoroughly and soak them for 15 minutes in 70% ethanol.
   Note: Remember to wash your hands before handling the clean, dry eggs.
4. Crack the eggs with a sterile knife into a sterile flask, and beat them with a sterile egg whisk or whip them in a sterile blender.

Note: Eggs sometimes contain antibiotics that inhibit the growth of mycobacteria. The origin of the eggs must be known to ensure their quality.

5.3.1.2 Preparing the complete medium

Mineral solution	600 ml
Malachite green solution	20 ml
Homogenized eggs (20–25 eggs)	1000 ml

Note: The use of a sterile magnetic stirrer is helpful to mix the components.

1. Distribute 6–8 ml of the complete egg medium into sterile 14 ml or 28 ml McCartney bottles; alternatively, distribute 20 ml volumes into 20 x 150 mm screw-cap test tubes and close the tops tightly.
2. Inspissate the medium (see instructions below) within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Inspissation (coagulation of the medium)

1. Heat the inspissator to 80 °C to hasten the build-up of the temperature before loading with the bottles.
2. 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.
3. 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 the result of excessive temperature. The appearance of holes or bubbles on the surface of the medium also indicates faulty coagulation procedures.
4. Discard any 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 2 weeks to check their sterility. A reference strain may also be inoculated on the medium to verify good growth.

Storage

The media should be dated, and may be stored in the refrigerator for several weeks as long as the caps remain tightly closed to prevent the medium from drying out.

Note: For optimal isolation of mycobacteria, the media should be kept at 2–8 °C for a maximum of 6 months.

5.3.2 PREPARING BROWN AND BUCKLE MEDIUM

Depending on the volume of media prepared, the method makes approximately 600 slopes (6.5 litres) or 150 slopes (1.625 litres).

5.3.2.1 Materials

The materials needed are:

- 2 sterile 1 litre measuring cylinders;
- 3 boxes of 30 ml sterile V-bottomed screw-cap plastic bottles;
- 6 racks that hold 100 bottles;
- A sterile lead for the peristaltic pump.

5.3.2.2 Reagents

Reagents	For 6,5 litres (l)	For 1,625 litres (l)
Dipotassium hydrogen orthophosphate anhydrous (K_2HPO_4)	36 g	9 g
Potassium dihydrogen orthophosphate (KH_2PO_4)	12 g	3 g
Agar number 3 (Oxoid LP0013)	68 g	17 g
50% glycerol	100 ml	25 ml
Distilled water	4.8 l	1.2 l
Additives		
Sterile egg yolks (from 90–95 eggs)[a]	1.6 l	400 ml
2% malachite green (filtered and sterilized)[a]	64 ml	16 ml

[a] See below for preparation instructions.

Sterile egg yolks

1. Place eggs into a stainless steel container with a lid, and add 70% alcohol to cover the eggs. **Note:** 70% alcohol can be reused for 1 month unless eggs have been broken and it becomes cloudy.
2. Leave for 30 minutes.
3. Remove eggs with sterile tongs, and place on a sterile towel; leave them to air dry in a laminar flow cabinet.
4. Wearing sterile surgical gloves, crack each egg aseptically on the edge of a sterile beaker; remove the egg yolk by straining through a gloved hand or using a sterile egg separator.
5. Drop the egg yolk into a 1 litre sterile measuring cylinder and discard the albumin.
6. When the cylinder has been filled to the required mark (that is, either 400 ml or 800 ml depending on the volume of medium being prepared), stir gently with a sterile masher to homogenize the yolks.

Note: This procedure can be done the day before the yolks are needed, and the yolks can be stored at 4 °C overnight. The yolks should be brought to room temperature before they are used.

2% malachite green

1. Weigh 10 g of malachite green.
2. Dissolve in 500 ml of distilled water.
3. Place on a stirrer for 1 hour.
4. Sterilize by autoclaving at 121 °C for 15 minutes.
5. Cool to 50 °C.
6. Filter through a Sartorius P20 plus filter (catalogue number: 18056 D) or equivalent using a peristaltic pump and a sterile lead.
7. Store in a sterile bottle.

Note: Malachite green will retain insoluble particles if it is not autoclaved and then filtered.

5.3.2.3 Procedure (media preparation)

Using an autopreparator:

1. Measure out 4.8 litres of distilled water and 100 ml of 50% glycerol and place in the vat of an autopreparator (such as an AES Chemunex S8000 Automatic Media Preparator, or equivalent);
2. Weigh the salts and the agar; add to the vat;
3. Mix and let soak for 10 minutes;
4. Measure the pH and record it;
5. Sterilize at 121 °C for 15 minutes;
6. Cool to 56 °C for ≥45 minutes to cool down the top of the autopreparator;
7. Aseptically add the sterile egg yolks and malachite green to the autopreparator and let mix;
8. Change the pouring temperature to 60 °C, and aseptically dispense 9 ml of media in tubes using a peristaltic pump and sterile lead;
9. Slope in racks that hold 100 bottles, and leave to set;
10. Rinse the lead in hot water immediately after use to prevent the agar from setting;
11. Measure and record the pH. The pH should be within the range 6.8–7.0. If the pH is not correct, discard the slopes;
12. Label and date the bottles;
13. Remove the required number of slopes for quality-control testing.

Note: The medium should be a pale olive green colour.

Note: The shelf-life of Brown and Buckle medium is 6 months at room temperature.

5.3.2.4 Quality control

Before using the medium, a representative sample of culture bottles should be incubated at 35–37 °C for 2 weeks to check their sterility.

5.3.3 PREPARING OGAWA MEDIUM

Ogawa medium is named "1%", "2%" or "3%" Ogawa medium according to the concentration of potassium dihydrogen phosphate (KH_2PO_4) in the mineral solution. For the laboratory diagnosis of Buruli ulcer or other non-tuberculous mycobacterial diseases, 2% Ogawa medium is used.

5.3.3.1 Materials

The materials needed are:

- 2 sterile 200 ml measuring cylinders and 2 sterile 500 ml conical flasks;
- 2 sterile funnels and 3 pieces (30 x 30 cm) of sterile cotton gauze;
- a sterile magnetic stirrer, a sterile beaker and 2 sterile stainless spoons;
- 40–45 sterile screw-cap test tubes (18 x 180 mm).

5.3.3.2 Components

There are three components of Ogawa medium; they are prepared separately and then combined to make the medium:

1. mineral solution;
2. malachite green solution;
3. homogenized whole eggs.

Note: Mineral solution and malachite green solution are prepared separately and may be kept long-term in the refrigerator. Whole egg solution is prepared when needed.

Mineral solution

Potassium dihydrogen phosphate (KH_2PO_4)	2.0 g
Monosodium glutamate	0.5 g
Magnesium citrate	0.1 g
Soluble starch (reagent grade)	3.0 g
Sodium pyruvate	0.2 g
Distilled water	100 ml

1. Dissolve all the components listed in the table above.
2. Autoclave at 121 °C for 30 minutes.
3. Cool to room temperature.

2% malachite green solution

Malachite green oxalate	1 g
Distilled water	50 ml

Prepare the malachite green solution as described above (see 5.3.2.2.).

Note: This solution does not need to be completely dissolved; add it to the whole egg solution with a wide-orifice-pipette to transfer insoluble powder.

Homogenized whole eggs

1. Soak eggs 10 minutes in 70% ethanol, then leave them to air dry in a laminar flow cabinet.
2. Crack the eggs with a sterile stainless spoon into a sterile beaker and mix them using another sterile stainless spoon.
3. Filter whole egg solution through a piece of sterile cotton gauze supported by a sterile funnel placed on a 200 ml sterile cylinder.

5.3.3.3 Procedure (media preparation)

Mineral solution	100 ml
Glycerol (reagent grade)	4 ml
Malachite green solution	4 ml
Homogenized eggs (5 to 6 eggs)	200 ml

1. Mix all the reagents in a sterile 500 ml conical flask with a sterile magnetic stirrer and filtrate through two-ply sterile cotton gauze supported by a sterile funnel.
2. Distribute 7 ml of the complete egg medium into sterile screw-cap test tubes (18 x 180 mm) and close the cap (not too tightly).
3. Check media before inspissation. Defoam the media by popping bubbles with a heated needle or by taking up the bubbles with a sterile pipette. Overnight storage in this condition is allowed.
4. Heat the inspissator to 70 °C.
5. Place the tubes in a slanted position in the inspissator, and coagulate the medium at 90 °C for 60 minutes.
6. Cool media in a water bath immediately after coagulation.
7. Discard any 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 2 weeks to check their sterility. A reference strain may also be inoculated on the medium to verify good growth.

Storage

The media should be dated, and may be stored in the refrigerator for several weeks as long as the caps remain tightly closed to prevent the medium from drying out.

5.4 INOCULATING AND INCUBATING THE CULTURE MEDIA

1. Inoculate about 0.2 ml of the specimen on each tube of Löwenstein–Jensen, Brown and Buckle or Ogawa medium.
2. Disperse the inoculated suspension over the medium by gently moving the tubes.
3. Leave the tubes inclined overnight in a place where they are protected from light.
4. The tubes can be left inclined on the work bench at room temperature or in an incubator.
5. Incubate the inoculated media in an incubator at 33 °C (30–35 °C) for 6 months (9 months may be necessary for some specimens).

6. After 1 week of incubation, inspect the tubes for growth; using a lamp, check for possible contamination or early growth. Suspicious growth can be checked for purity by microscopy. Contaminated tubes should be discarded (after registration).
7. Mycobacterial colonies can be subcultured to obtain abundant growth or directly identified.
8. Tubes that have no growth can be incubated for longer.
9. During the next 6 months, read cultures every week or every 2 weeks as described above. The maximum incubation time for primary isolation of ***M. ulcerans*** is 12 months. After this period, all tubes with no growth should be considered negative.

5.5 IDENTIFYING *M. ULCERANS*

M. ulcerans belongs to the slow-growing group of mycobacteria. Primary cultures are usually positive within 6–12 weeks, and subcultures are generally positive within 3–4 weeks of incubation at 29–33 °C on Löwenstein–Jensen medium, Brown and Buckle or Ogawa medium. Colonies suggestive of ***M. ulcerans*** appear yellowish and rough, and have well demarcated edges. African and Japanese strains are more yellowish than Australian strains (***Figure 1***), which are cream in colour.

Identification of ***M. ulcerans*** is done by PCR. The most common target sequence for PCR confirmation of Buruli ulcer is the insertion sequence IS*2404*. DNA from primary cultures or subcultures is extracted by suspending a few colonies in TE buffer (10mM Tris hydrochloride plus 1mM EDTA with pH 8.0), and using heat to inactivate them at 100 °C for 8–10 minutes.

Procedures for extracting DNA from cultures and detecting ***M. ulcerans*** DNA by PCR are described in ***Annex 6*** below.

ANNEX 6:
POLYMERASE CHAIN REACTION (PCR) PROTOCOLS

6.1 PREVENTING FALSE-POSITIVES: THE THREE-ROOM PRINCIPLE

The risk of false-positive results occurring during PCR reactions is real. When performing PCR, it is vital to guard against false-positives caused by contamination. In order to minimize the risk of false-positive results, strict rules regarding clothing, specimens, reagents, writing materials, and preparing samples must be respected. It is important that DNA extraction from cultures and clinical specimens is performed using dedicated reagents and equipment.

The three-room PCR principle should be followed.

Room 1:

- strictly no DNA extracts, templates or amplicons;
- preparation of PCR reaction mix only.

Room 2:

- low-level DNA room (no PCR amplicons);
- preparation of tissue samples and swabs for PCR;
- work performed in a class II biological safety cabinet or a PCR series cabinet.

Room 3:

- high-level DNA room (PCR amplicons are present in high copy numbers);
- PCR amplification and post-PCR manipulation room;
- used for running agarose gels.

Note: Equipment, clothing, reagents, specimens and writing materials should never be moved from room 3 to room 2 or to room 1, or from room 2 to room 1.

General procedures

- Laboratory coats and gloves must be worn in each room; and lab coats should remain associated only with particular rooms – that is, laboratory coats for room 1 must be worn only in room 1.
- Both bleach and ultraviolet radiation degrade DNA and RNA. Bleaching and treating work surfaces with ultraviolet radiation can lower the risk of contaminating samples with DNA or RNA.
  - After work, benches in the DNA extraction area should be cleaned with bleach and treated with ultraviolet light.
  - DNA extraction rooms must be cleaned weekly with a bleach solution of 0.5–1%; desks must be cleaned with a 50% ethanol solution.

Note: The distilled water should be changed monthly.

6.2 OVERVIEW OF PROTOCOL FOR DIAGNOSTIC PCR

There are four steps in the protocol for using PCR to diagnose *M. ulcerans* disease:

1. preparing the specimen;
2. extracting the DNA;
3. PCR amplification (by conventional gel-based PCR or real-time PCR);
4. quality assurance.

6.3 PREPARING SPECIMENS

For PCR analysis, suspensions prepared for bacteriological analysis, as described in *Annex 3*, or specimens placed in alcohol, phosphate buffered saline or TE buffer (10mM Tris hydrochloride plus 1mM EDTA with pH 8.0), or cell lysis solution may be used.

Note: If a cell lysis solution (for example, manufactured by Qiagen) is used as a transport buffer, samples may be subjected to direct heat inactivation, and this can be followed by DNA extraction using Qiagen's Gentra Puregene procedure (see 6.4.2).

Note: Always include a negative control in phosphate-buffered saline (for Qiagen extraction in cell lysis solution respectively), and include the control in the whole procedure.

The procedure for specimens prepared according to *Annex 3* is as follows:

FNA

1. Transfer 0.5 ml specimen to 1.5 ml microfuge tube.
2. Wash (see below).

Swabs and tissue samples

1. Transfer 1 ml specimen to 1.5 ml microfuge tube.
2. Wash (see below).

Wash

1. Spin 1.5 ml tube for 10 minutes at high speed in a microfuge.
2. Carefully remove and discard supernatant.
3. Wash pellet with 1 ml phosphate-buffered saline, then spin for 10 minutes.
4. Carefully remove phosphate-buffered saline and resuspend pellet in 180 µl distilled water.

Note: The distilled water should be stored in a Teflon bottle and changed monthly.

Paraffin blocks

1. Transfer at least 6 x 20 µm-thick paraffin-embedded tissue sections (cut with a sterile microtome, or scraped carefully from the block using a sterile scalpel blade) to a 1.5 ml screw-capped tube.
2. Add 1 ml Histolene or xylene and incubate at room temperature until paraffin has dissolved (1–5 minutes). **Note:** Include a reagent control tube containing no paraffin sections.
3. Centrifuge tubes at maximum speed in a microfuge for 5 minutes to pellet tissue.

4. Remove supernatant using a fine tip transfer pipette, being careful to avoid the pellet.
5. Discard supernatant into a container suitable for volatile solvents.
6. Resuspend pellet in 1 ml absolute ethanol and incubate for 5 minutes at room temperature.
7. Centrifuge at maximum speed for 5 minutes.
8. Remove supernatant using a fine tip transfer pipette, being careful to avoid the pellet, and briefly air dry.
9. Extract DNA from tissue using either the Maxwell method or the Gentra Puregene procedure as described below.

6.4 DNA EXTRACTION PROCEDURES

The goal of DNA extraction is to:

- inactivate infectious agents;
- purify and concentrate mycobacterial DNA in clinical specimens for PCR analysis.

Note: DNA from cultures is extracted by suspending a few colonies in TE buffer (10mM Tris hydrochloride plus 1mM EDTA with pH 8.0) and using heat to inactivate them at 100 °C for 8–10 minutes.

6.4.1 MAXWELL METHOD

6.4.1.1 Preparation

Lysis buffer

Trisaminomethane hydrochloride (Tris HCl) 1M, pH 7.5	0.5 ml
NaCl (5 M)	100 µl
EDTA (0.5 M)	1 ml
10% sodium dodecyl sulfate	2.5 ml
Distilled water	46.9 ml

Add the above solutions together in the order they are mentioned.

Note: The lysis buffer can be stored at 4 °C for 6 months.

Proteinase K (20mg/ml)

Proteinase K	1000 mg
Distilled water	50 ml

1. Dissolve the proteinase K in water.
2. Aliquot 1 ml per tube.

Note: The proteinase K can be stored at –20 °C for a maximum of 2 years.

6.4.1.2 Method

1. Add 50 µl of proteinase K to 200 µl of lysis buffer.
2. Add 250 µl of this mixture (lysis buffer plus proteinase K) to 200 µl of sample suspension.
3. Heat at 62 °C and shake regularly for at least 3 hours (preferably overnight).
4. Use 400 µl of the mix with the Maxwell 16 Tissue DNA Purification Kit (manufactured by Promega, Leiden, the Netherlands) and the Maxwell 16 Instrument (also manufactured by Promega) following the manufacturer's instructions.

Note: The final volume of DNA extract is 300 µl. Store the DNA extract at ≤18 °C or at 2–8 °C if further analysis will be completed within 2 days.

6.4.2 GENTRA PUREGENE PROCEDURE

The Gentra Puregene Cell Kit (Qiagen) extraction procedure can be used for all types of clinical samples and culture material.

6.4.2.1 Reagents

- The Puregene DNA extraction kit contains:
  - cell lysis solution, which can also be used as transport buffer;
  - DNA hydration solution;
  - protein precipitation solution;
  - lysozyme (10 mg/ml);
  - proteinase K (20 mg/ml).

- The following alcohols are also required:
  - ethanol 70%;
  - isopropanol (2-propanol).

6.4.2.2 Preparation

1. Prepare a reaction tube as a negative extraction control by adding 700 µl cell lysis solution buffer to a 2.0 ml microfuge tube. This control will be treated the same way as clinical samples but must not contain any diagnostic material.
2. To extract ***M. ulcerans*** DNA from clinical samples, place swabs and 3 mm punch biopsies in 700 µl cell lysis solution and FNA samples (the content of the syringe) in 300 µl cell lysis solution.
3. To extract ***M. ulcerans*** DNA from cultures, add one inoculation loop of culture material to 700 µl cell lysis solution.
4. Use heat to inactivate clinical samples by incubating at 95 °C for 20 minutes.

6.4.2.3 Procedure

The steps should be performed in the order described below.

Step A. Cell lysis

For FNA, punch biopsies and tissue samples

1. Add 10 µl proteinase K (that is, 20 mg/ml) to each sample and incubate in the thermomixer at 55 °C at moderate speed overnight until complete lysis of the tissue.
2. Inactivate proteinase K by incubating in the thermomixer at 80 °C for 20 minutes.
3. Cool to room temperature.
4. Add 15 µl lysozyme (that is, 10 mg/ml) and incubate in the thermomixer at 37 °C at moderate speed for 1 hour.

For swab samples and culture material

1. Add 15 µl lysozyme (that is, 10 mg/ml) to samples, and incubate in the thermomixer at 37 °C at moderate speed for 1 hour.
2. Add 10 µl proteinase K (that is, 20 mg/ml) to samples and incubate in the thermomixer at 55 °C at moderate speed for 4 hours (or overnight) until complete lysis.
3. Proteinase K is inactivated by incubating at 80 °C for 20 minutes.

Step B. Protein precipitation

1. Place samples in an ice bath for 5 minutes.
2. Add 230 µl protein precipitation solution.
3. Vortex vigorously at high speed for 20 seconds to mix uniformly.
4. Place samples in an ice bath for 5 minutes.
5. Centrifuge at 13000 x g for 5 minutes. The precipitated proteins will form a tight pellet. If the pellet is not tight, repeat steps 3–5.
6. Prepare the appropriate number of new 2 ml microfuge tubes containing 700 µl isopropanol.

Step C. DNA precipitation

1. Pour the supernatant containing DNA into a clean 2 ml microfuge tube containing 700 µl isopropanol; leave behind the precipitated protein pellet.
2. Mix by inverting gently 10 times.
3. Centrifuge at 13000 x g for 5 minutes.
4. Pour off the supernatant.
5. Add 700 µl 70% ethanol, and invert the tubes to wash DNA pellets.
6. Centrifuge at 13000 x g for 5 minutes. Carefully pour off the ethanol. The pellet may be loose, so pour slowly and watch the pellet.
7. Invert and drain the tube on a clean absorbent paper towel (place each tube at a different spot), and allow to air dry, or dry in the thermomixer at 65 °C until the tube is completely dry (about 20 minutes). **Note:** The ethanol must be completely evaporated or it may inhibit the PCR.

Step D. DNA hydration

1. Add 200 µl DNA hydration solution (or 50 µl for FNA samples) to each sample.
2. Rehydrate the DNA by carefully pipetting up and down about 20 times.
3. Incubate in the thermomixer at 65 °C for 30 minutes.

6.5 PCR AMPLIFICATION

PCR exponentially amplifies the target DNA. This is accomplished using a cyclic reaction among different steps conducted at different temperatures. The temperatures are specific to each step and for each PCR reaction, and they mainly depend on the nucleotide sequence of the primers:

- denaturation – makes the DNA single stranded (mostly done at 94 °C);
- annealing – anneals the primers (mostly done at 50–70 °C);
- prolongation – extends the complementary DNA chain (mostly done at 72 °C).

The PCR reaction also demands the use of correct buffer conditions, the presence of the target DNA, deoxynucleotide triphosphates (dNTPs) and an enzyme, most often the *Taq* polymerase, a thermostable enzyme originating from *Thermus aquaticus*.

The PCR reaction is considered successful only if the positive PCR control and the negative PCR control give the desired result.

The DNA can be amplified by conventional gel-based PCR or by real-time PCR.

6.5.1 CONVENTIONAL GEL-BASED PCR

A simple visual detection of the amplicons is done by electrophoresis on an agarose gel followed by an ethidium bromide staining.

Note: Ethidium bromide is highly mutagenic and should be handled carefully.

6.5.1.1 Single-round PCR

Single-round PCR has four steps:

1. preparing the master-mix;
2. adding template DNA (as prepared in section 2 of this annex);
3. performing amplification;
4. visualizing the PCR product.

Step A. Preparing the master-mix (for example, using reagents 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 (µl)
Distilled water	9
Thermo buffer (10 x)	2
Magnesium chloride ($MgCl_2$) (25 mM)	1.2
dNTPs (2 mM)	2
M. ulcerans 1 primer[a] (20 µM)	1
M. ulcerans 2 primer[b] (20 µM)	1
Taq polymerase (5 units/µl)	0.2

dNTPs, deoxynucleotide triphosphates

[a] *M. ulcerans* 1 GAT CAA GCG TTC ACG AGT GA.

[b] *M. ulcerans* 2 G GC AGT TAC TTC ACT GCA CA.

These quantities will make 16.4 µl master-mix, which is usually produced in volumes of ≥164 µl and then aliquoted into multiple tubes.

Step B. Adding template DNA to tubes containing the master-mix

1. Add 15 µl of master-mix to each PCR tube.
2. Add 5 µl of test DNA sample; the total volume for each PCR tube becomes 20 µl.
3. Always include PCR negative controls and positive controls in each run.
4. Check for the presence of PCR inhibitors in every sample.

Step C. Performing amplification

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

94 °C for 4 minutes
94 °C for 40 seconds
60 °C for 40 seconds
72 °C for 40 seconds } 35 cycles[a]
72 °C for 5 minutes
4 °C – hold the sample at this temperature until ready to analyse on agarose gel.

[a] One cycle consists of the three phases (or temperatures) indicated by the bracket.

Step D. Visualizing the PCR product

1. To visualize the product of PCR, 9 µl reaction products plus 1 µl sample buffer are electrophoresed through 2% agarose gels containing ethidium bromide (0.5 µg/ml) (see notes below).
2. Gels are run in Tris acetate plus EDTA buffer (40 mM Tris acetate plus 1 mM EDTA) – for example, at 100 volts for 20 minutes (depending on the apparatus).
3. Visualize PCR products stained with ethidium bromide using an ultraviolet transilluminator.
4. Samples are considered positive if they yield a 515 bp product that lines up exactly with the positive control.
5. All negative controls must be negative.
6. A negative result in the sample to test for inhibition (see above) indicates that the PCR is inhibited. Repeat the PCR using a 1:10 dilution of the test DNA. If it is still inhibited, no result can be given for that sample.

Note: To prepare the sample buffer:

1. Dissolve 250 mg bromophenol blue in 33 ml 150 mM Tris (pH 7.6);
2. Add 60 ml glycerol and 7 ml of distilled water;
3. Store at room temperature.

Note: Ethidium bromide is highly mutagenic and should be handled carefully.

6.5.1.2 Dry reagent-based PCR

Reagents

The following reagents will be needed:

- PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences);
- lyophilized primers MU5 and MU6 (see note below);
- purified distilled water, molecular grade;
- low electroendoosmosis agarose (standard agarose);
- 10 x buffer made with 1M Tris borate plus 10 mM EDTA, pH 8.3 (also known as TBE buffer);
- GelRed (Biotium);
- 10 x gel loading buffer dye (such as BlueJuice, manufactured by Invitrogen);
- 100 bp DNA ladder (such as those manufactured by Invitrogen).

Note: Following the manufacturer's recommendation for the vacuum concentrator used, 2.5 µl of primer (10 µM) MU5 (5` AGC GAC CCC AGT GGA TTG GT `3) and MU6 (5` CGG TGA TCA AGC GTT CAC GA ´3) are lyophilized batchwise in 0.2 ml PCR reaction tubes.

Preparing the PCR reaction tubes

1. Add one PuReTaq Ready-To-Go-PCR Bead (containing PCR buffer, $MgCl_2$ and dNTPs) to each PCR reaction tube containing the lyophilized primers MU5 and MU6.
2. Add 22.5 µl distilled water to dissolve beads completely.

Adding template DNA

- Diagnostic samples
  - Every diagnostic sample (DNA extract) is tested undiluted and in a 10^{-1} dilution.
  - Add 2.5 µl original DNA extract or diluted extract to PCR reaction tubes.

Performing amplification

Reaction tubes are transferred to the thermocycler. Amplification is carried out for 1 hour and 30 minutes as shown.

Steps	Temperature	Duration	Number of cycles
Initial denaturation	95 °C	10 minutes	1
Denaturation	95 °C	10 seconds	} 35[a]
Annealing of primers	58 °C	10 seconds	
Extension	72 °C	30 seconds	
Final extension	72 °C	10 minutes	1
Hold	15 °C	As long as necessary	

[a] For denaturation, annealing of primers and extension, one cycle consists of the three phases (or temperatures) indicated by the bracket.

6.5.1.3 Internal quality control

- Inhibition controls
  - Inhibition controls (diluted and undiluted) are processed in parallel for all diagnostic samples to prevent false-negative results caused by inhibition processes.
  - For the inhibition-control reaction, 1.25 µl of diagnostic (unknown) DNA or dilution plus 1.25 µl positive-control DNA is added to the respective tube.

- Positive and negative controls

In addition to diagnostic samples and inhibition controls, the following controls are also processed.

Type of control	Purpose	Content of reaction
Negative extraction control	Negative control of the extraction process used to exclude contamination occurring during extraction	2.5 µl of the negative extraction control
No template control	Negative control of PCR used to exclude contamination of reagents	2.5 µl distilled water without any DNA
Positive PCR control	Positive control (run control of PCR to determine specific amplification	2.5 µl positive DNA[a]

PCR, polymerase chain reaction

[a] For a positive control, either DNA extract from a patient who has already tested positive can be used or extracted culture material.

Note: To prepare positive sample of ***M. ulcerans*** DNA (for inhibition testing and positive control):

1. extract ***M. ulcerans*** DNA by suspending a few colonies in TE buffer (10mM Tris hydrochloride plus 1mM EDTA with pH 8.0);
2. use heat to inactivate (100 °C for 8–10 minutes);
3. use 1 µl in PCR reaction.

6.5.1.4 Analysing PCR amplicons and interpreting results

Performing agarose gel electrophoresis

To prepare the working concentration of the buffer made with 1M Tris borate plus 10 mM EDTA at pH 8.3 (also known as TBE buffer), dilute 50 ml of 10-fold concentrated TBE buffer in 950 ml deionized water (5% dilution).

1. To prepare a 1.5% agarose gel, heat 1.5 g agarose and 100 ml 0.5 x buffer in a plastic Erlenmeyer flask for 3 minutes at 600 watts in a microwave oven. **Note:** Bubbles indicate that the temperature is correct.
2. After heating, cool the fluid to approximately 50 °C. This can be done by holding the flask under running water. Shake the flask to ensure that the liquid cools uniformly.
3. After cooling, add 10 µl GelRed and shake the flask gently.
4. Seal the electrophoresis tray and pour the fluid into the agarose gel electrophoresis tray. Carefully remove bubbles using a pipette tip. Then place the spacer comb or combs at the correct location in the tray. Allow the agarose gel to cool to room temperature; then remove the spacer comb carefully.

5. Put the congealed gel into the agarose gel electrophoresis chamber, which must be filled with 0.5 x buffer (up to the fill line).
6. To load the gel, mix 15 µl of dry reagent-based PCR amplicon with 3 µl of commercially available gel loading dye (e.g. 10 x BlueJuice); or alternatively, 1 ml dye may be prepared by mixing 500µl glycerol with 497.5 µl distilled water and 2.5 µl Bromophenol Blue.
7. Load 10 µl of the 100 bp DNA ladder (be sure to use the working solution) into one sample slot per line.
8. Apply the correct voltage, and electrophoresis is carried out as described below.

Size of gel	Voltage	No. of spacer combs	Time for gel electrophoresis
12 x 12 cm	100 volts	2	55 minutes

Analysing and interpreting agarose gel electrophoresis

- Following agarose gel electrophoresis, amplicons are visualized using ultraviolet light (302 nm) in the gel-documentation unit.
- A positive reaction produces a band of 492 bp in length with primers MU5 + MU6 (dry reagent-based PCR) and of 515 bp with primers MU1 + MU2 (single round PCR); a negative reaction produces no band.
- Results of diagnostic samples are interpreted only according to the results of the inhibition controls.

The possible results are shown below.

Result of sample	Result of inhibition control	Interpretation of results
Negative	Positive	Sample negative
Positive	Positive	Sample positive
Negative	Negative	Sample inhibited[a]
Positive	Negative	Sample positive

[a] If this result occurs, then samples should be tested at a higher dilution.

The dry reagent-based PCR reaction is considered successful only if the positive control yields a positive result and the negative control yields a negative result.

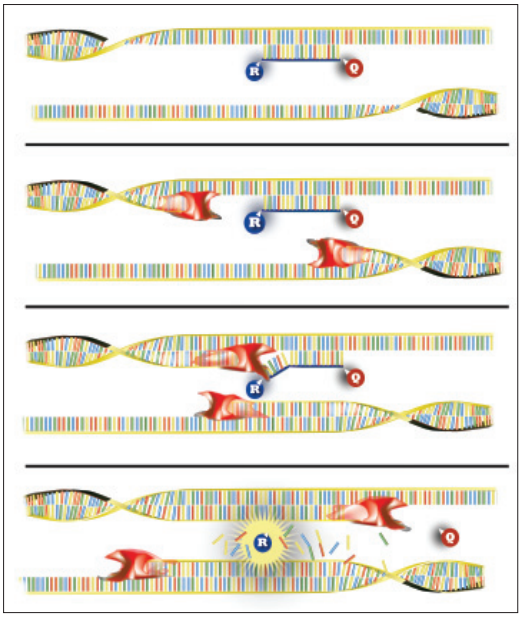
6.5.2 REAL-TIME QUANTITATIVE PCR PROTOCOL

6.5.2.1 Background

The real-time PCR assay described below was developed by Fyfe and colleagues[1] and described in detail in Lavender and Fyfe,[2] and uses a minor groove-binding TaqMan probe. TaqMan probes are hydrolysis probes designed to increase the specificity of real-time PCR. The hydrolysis probe or TaqMan chemistry relies on the 5′–3′ exonuclease activity of the *Taq* polymerase, which degrades a hybridized (non-extendible) DNA probe during the extension step of the PCR reaction.

This probe is designed to hybridize to a region within the amplicon, and is dual labelled (with a reporter fluorophore and a quencher molecule). The close proximity of the quencher suppresses the fluorescence of the reporter fluorophore. Once the exonuclease activity of the *Taq* polymerase degrades the probe, the fluorescence of the reporter increases at a rate that is proportional to the amount of template present, allowing the DNA in the starting material to be quantified (*Figure 30*). However, in a diagnostic context, quantitation is not generally required.

FIGURE 30. TAQMAN REAL-TIME POLYMERASE CHAIN REACTION



(Courtesy of Koen Vandelannoote)

Hydrolysis probes allow several target sequences to be detected in the same reaction by using specific probes with different coloured labels (this is known as multiplexing). In this assay, the probe that is designed to detect a region within IS*2404* (IS*2404* TP) is labelled with the fluorescent dye 6-carboxyfluorescein (FAM) at the 5′ end, and with a minor groove-binding nonfluorescent quencher (known as MGBNFQ) at the 3′ end.

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[1] Fyfe J et al. Development and application of two multiplex real-time PCR assays for the detection of *Mycobacterium ulcerans* in clinical and environmental samples. *Applied and Environmental Microbiology*, 2007, 73:4733–4740.

[2] Lavender CJ, Fyfe JA. Direct detection of *Mycobacterium ulcerans* in clinical specimens and environmental samples. *Methods in Molecular Biology*, 2013, 943:201–216.

A commercially available TaqMan exogenous internal positive control is incorporated into each reaction to ensure that a negative result for a particular sample is a true IS*2404* negative and not caused by the presence of PCR inhibitors. The internal positive control probe has a VIC fluorophore attached to its 5' end and a major groove-binding nonfluorescent quencher attached to its 3' end.

Amplification and sequence detection are performed using a real-time PCR instrument, such as the ABI Prism 7000 Sequence Detector, Eppendorf Mastercycler Realplex, or equivalent.

6.5.2.2 Materials

The following materials are needed to perform real-time quantitative PCR:

- TaqMan PCR master-mix (for example, from Applied Biosystems or equivalent);
- primers IS*2404* TF and IS*2404* TR (see below);
- probe IS*2404* TP (see below);
- Exogenous Internal Positive Control Reagents (manufactured by Applied Biosystems);
- nuclease-free water;
- 96-well optical reaction plate (for example, from Applied Biosystems or equivalent);
- optical adhesive film (for example, from Applied Biosystems or equivalent).

6.5.2.3 Procedure

The steps should be performed in the order described below.

Step A. Preparing the master–mix

Mixtures for real-time PCR for IS*2404* with an internal positive control contain 0.9 µM concentrations of each primer, a 0.25 µM concentration of the probe, the TaqMan PCR master-mix (1x), and TaqMan internal positive control reagents (1x), in a total volume of 24 µl.

Primer or probe	Sequence (5'–3')
IS*2404* TF	AAAGCACCACGCAGCATCT
IS*2404* TR	AGCGACCCCAGTGGATTG
IS*2404* TP	6 FAM-CGTCCAACGCGATC-MGBNFQ

The master-mix is made in a 1.5 ml or 2.0 ml tube. As a general rule, for every twelfth reaction one additional reaction is made. The 24 µl of the mix is then distributed into each well of the reaction plate.

The reagents needed and the amounts needed for each reaction are shown below.

Reagent (starting concentration)	Amount to add (µl) for 1 reaction
TaqMan PCR master-mix (2x)	12.5
IS*2404* TF (18 µM)	1.25
IS*2404* TR (18 µM)	1.25
IS*2404* TP (5 µM)	1.25
Exogenous DNA for internal positive control (50x)	0.5
Exogenous mix for internal positive control (10x)	2.5
Nuclease-free water	4.75
Total	**24**

Step B. Add template DNA to tubes containing master–mix

Add to the wells 1 µl of template DNA. Three control wells are included in each run: a no-template control (1µl of nuclease-free water instead of template DNA), a positive control (1µl of a dilute solution of ***M. ulcerans*** genomic DNA) and a negative control (1µl of an extract prepared with only the reagents used for extracting DNA from the samples). Seal the reaction plates with optical adhesive covers using an applicator; then place them in the real-time PCR instrument with or without a compression pad (depending on the model of real-time PCR instrument).

Note: It is important to make it clear that a normal thermocycler cannot be used for real-time PCR. An instrument that both amplifies and detects the fluorescence is essential.

Note: The dilution of the genomic DNA will depend on the initial concentration of the preparation. The appropriate dilution needs to be determined empirically. A 10^{-6} dilution of the preparation may be used, which gives a Ct of 25–30 in the assay. The more dilute the preparation the less the chances of introducing contamination, but if it is too dilute then there is a greater chance that it will fail.

Step C. Amplifying and detecting

Amplification and detection are performed in a real-time PCR instrument using the following programme:

- 1 cycle at 95 °C for 10 minutes (to activate the *Taq* enzyme);
- 40 cycles at 95 °C for 15 seconds (melting); and
- 1 minute at 60 °C (for annealing and extending).

Note: Discard the reaction plates immediately after each run without opening the thermal seal to reduce the chances of amplicon contamination.

Step D. Analysing and interpreting the results

After completing the run, first analyse the results for the IS*2404* FAM detector. If required, manually move the threshold line so that it is above the background-noise level.

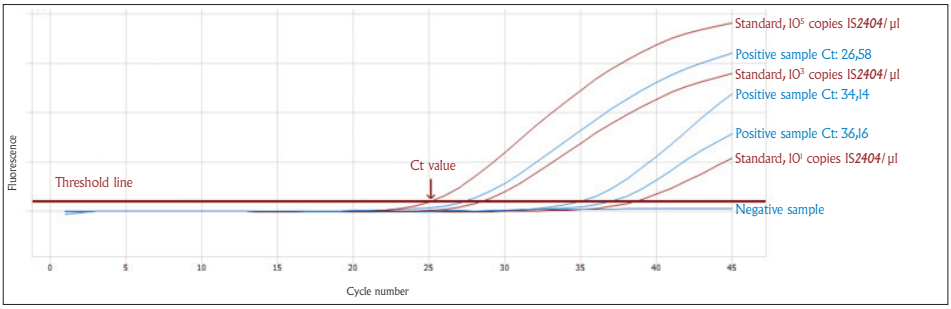
Repeat the process for the internal positive control VIC detector. In the presence of a strong FAM signal for the IS*2404* assay, a negative assignment or signal, or both, can be obtained for the internal positive control VIC detector. This is a result of the limiting primer concentrations used in the internal control assay.

Thus, the results are interpreted for each sample as shown below.

If the IS*2404* FAM detector is	And the internal positive control VIC detector is	Then the IS*2404* result is
+	+/–	+
–	+	–
–	–	Possible inhibition

For positive IS*2404* samples, the cycle threshold value for the FAM detector represents the cycle at which the amplification curve crosses the threshold line; this provides an indication of the concentration of ***M. ulcerans*** DNA in the sample. The cycle threshold value is inversely related to the concentration of DNA in the sample (that is, a high cycle threshold value indicates a low DNA concentration; a low cycle threshold value indicates a high DNA concentration). ***Figure 31*** illustrates the quantitative real-time PCR amplification curves.

FIGURE 31. AMPLIFICATION CURVES FOR QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION



(Courtesy of Pieter Bomans, Miriam Eddyani and Koen Vandelannoote)

6.6 QUALITY ASSURANCE

It is crucial that all of the quality assurance practices described in ***Annex 8*** are adhered to in order to prevent contamination and false-positive results. Diagnosis of Buruli ulcer by PCR should not be performed if these quality assurance measures cannot be undertaken in the laboratory.

ANNEX 7:
HISTOPATHOLOGICAL STAINING TECHNIQUES

7.1 THE FIXATIVE

Details on fixation procedures are given in section 4.6.2 of the manual.

The fixative known as 10% buffered formalin is a 4% buffered formaldehyde solution: it is **NOT** a 10% formaldehyde solution. Historically, it was prepared as a 10% solution made from a stock bottle of 37–40% formaldehyde. It can be purchased ready for use as "10% buffered neutral formalin", or made up fresh from crystalline paraformaldehyde.

Note: Paraformaldehyde is hazardous. A protective mask, eyeglasses, gloves and a fume-hood must be used when handling it. Therefore, purchasing 10% buffered neutral formalin is recommended.

7.2 HARRIS' HAEMATOXYLIN AND EOSIN PROCEDURE (WITHOUT MERCURY)

Note: This procedure stains tissue elements and bacteria more intensely than many other haematoxylin and eosin procedures. Using potassium permanganate ($KMnO_4$) in place of mercury salts is less hazardous to people and the environment.

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

7.2.1 SOLUTIONS

7.2.1.1 Harris' haematoxylin

Potassium alum or ammonium alum	100 g
Distilled water	500 ml

Dissolve with the aid of heat.

In a separate container combine the following:

Haematoxylin crystals	5 g
Absolute ethanol	50 ml
Distilled water	250 ml

Dissolve (may be warmed to dissolve).

Then add the following:

0.25% $KMnO_4$	250 ml

1. Stir for at least 3 minutes (until completely dissolved).
2. Combine this solution with the alum solution.
3. Cool in running water.
4. Add 20 ml of glacial (100%) acetic acid.
5. Filter before use.

7.2.1.2 Eosin–phloxine solution

1% acid–alcohol

95% ethanol	736 ml
Deionized water	263.2 ml
Concentrated HCl	10 ml

Warning: HCl is an irritant and should be handled with care.

1. Add deionized water to 95% ethanol.
2. Carefully add concentrated HCl to the water-ethanol solution, not vice-versa.

Note: This solution can be stored at room temperature long-term.

Ammonia water

Deionized water	1000 ml
28% ammonium hydroxide	4 ml

Note: This solution can be stored at room temperature long-term.

1% eosin stock solution

Eosin Y (water soluble)	1 g
Deionized water	100 ml

Note: This solution can be stored at room temperature long-term.

1% phloxine stock solution

Phloxine B	1 g
Deionized water	100 ml

Note: This solution can be stored at room temperature long-term.

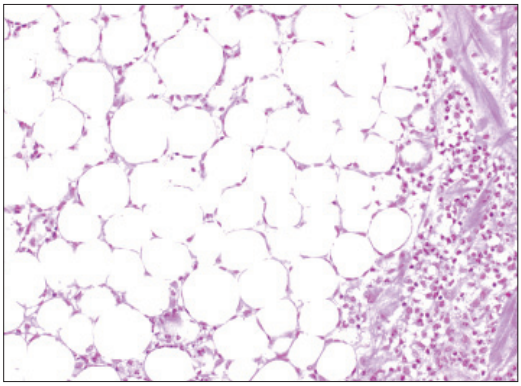
To make the eosin–phloxine solution, combine the following:

Eosin stock solution	100 ml
Phloxine stock solution	10 ml
95% ethanol	780 ml
Glacial (100%) acetic acid	4 ml

Note: This solution can be stored at room temperature and lasts for approximately 1 week.

Figure 32 shows panniculitis from a Buruli ulcer patient stained by the Harris' haematoxylin and eosin method.

FIGURE 32. SECTION STAINED WITH HARRIS' HAEMATOXYLIN AND EOSIN SHOWING PANNICULITIS IN A PATIENT WITH BURULI ULCER



The nuclei of cells are blue and the connective tissue is pink (Courtesy of Wayne Meyers)

7.2.2 STAINING PROCEDURE

1. Deparaffinize the slides and hydrate with water.
2. Stain in freshly filtered Harris' haematoxylin for 10 minutes.
3. Wash in warm running water for 5 minutes.
4. Dip twice in 1% acid–alcohol to differentiate.
5. Stop the differentiation by dipping in warm water and then dipping in weak ammonia water or saturated lithium carbonate until the section begins to turn bright blue.
6. Wash in warm running water for 10 minutes. **Note:** If nuclear staining is weak, repeat steps 2–6. If the background is not clear, repeat step 4 but use only one quick dip in the acid–alcohol. Then repeat steps 5 and 6.
7. Counterstain in eosin–phloxine for 2 minutes.
8. Dehydrate the slide.
9. Clear slides by immersion into 95% ethanol (twice for 2 minutes each time), 100% ethanol (twice for 2 minutes each time) and xylene (twice for 2 minutes each time).
10. Mount in a resinous mounting medium.

7.3 ZIEHL-NEELSEN METHOD FOR ACID-FAST ORGANISMS

Note: This technique is used to demonstrate acid-fast organisms other than *Nocardia* spp. and leprosy bacilli.

The procedure is used for specimens fixed in 10% buffered neutral formalin, and for tissue sections cut 4–6 µm thick. Control sections should contain known ***Mycobacterium tuberculosis*** or ***M. ulcerans***.

7.3.1 SOLUTIONS

7.3.1.1 Ziehl–Neelsen carbol fuchsin solution

Phenol (fused crystal, melted)	25 ml
Absolute ethanol	50 ml
Basic fuchsin	5 g
Deionized water	500 ml

Note: Store in a warm place in a room or on a shelf to maintain the solution in liquid form. Well prepared reagents can be stored for at least 6 months.

7.3.1.2 Acid–alcohol

70% ethanol	100 ml
Concentrated HCl	1 ml

Warning: HCl is an irritant and should be handled with care.

1. Carefully add concentrated HCl to 70% ethanol.
2. Always add acid slowly to alcohol, not vice versa.
3. Store in an amber-coloured bottle.

Note: This solution can be stored at room temperature long-term.

7.3.1.3 Working methylene blue solution

Methylene blue crystals	3 g
Deionized water	600 ml

1. Dissolve methylene blue chloride in deionized water in a tightly stoppered amber-coloured bottle.
2. Label the bottle with the name of the reagent (methylene blue solution), and the dates of preparation and expiry.

Note: The methylene blue solution can be stored at room temperature for 12 months.

7.3.2 STAINING PROCEDURE

1. Deparaffinize the slides and hydrate with deionized water.
2. Stain in Ziehl–Neelsen carbol fuchsin for 30 minutes. **Note:** If organisms fail to stain, prepare new carbol fuchsin solution.
3. Wash in cool water for 10 minutes. **Note:** If the water is chlorinated, wash for a shorter time. Wash until the water is clear

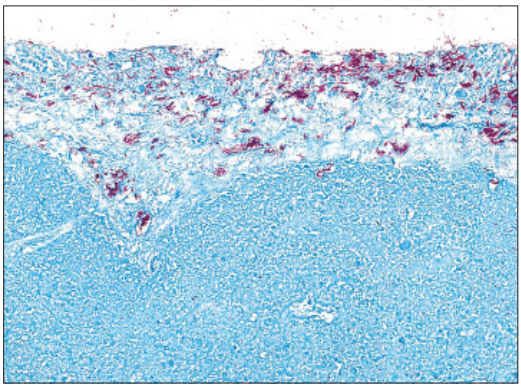
4. Differentiate slides individually with acid–alcohol.
5. Wash the slides in running water for 3 minutes.
6. Counterstain by dipping each slide individually into the working methylene blue solution and then rinsing in water.
7. Dehydrate the slide.
8. Clear slides by immersion into 95% ethanol (twice for 2 minutes each time), 100% ethanol (twice for 2 minutes each time) and xylene (twice for 2 minutes each time).
9. Mount in a resinous mounting medium.

7.3.2.1 Staining results

Acid-fast bacilli	Red
Background	Blue

Figure 33 shows a section of a lymph node from a Buruli ulcer patient stained by the Ziehl-Neelsen method.

FIGURE 33. SECTION OF A LYMPH NODE STAINED USING THE ZIEHL–NEELSEN METHOD FROM A PATIENT WITH BURULI ULCER



The acid-fast bacilli are red and the background tissue is blue (Courtesy of Wayne Meyers)

7.4 GROCOTT'S METHENAMINE–SILVER METHOD FOR FUNGI

Note: This technique demonstrates all forms of fungi; however, ***Histoplasma capsulatum*** and ***Nocardia asteroides*** may require extended time in the methenamine–silver solution.

This procedure is used for specimens fixed in 10% buffered neutral formalin, and for tissue sections cut 4–6 µm thick. Control tissue must be from a known fungal infection containing fungal elements: histoplasmosis and nocardiosis tissue should not be used.

7.4.1 SOLUTIONS

4% chromic acid

Chromic trioxide	4 g
Distilled water	100 ml

Warning: Chromic trioxide is an irritant.
Note: This solution can be stored at room temperature long-term.

5% silver nitrate

Silver nitrate	5 g
Distilled water	100 ml

Note: This solution can be stored at room temperature long-term.

3% methenamine (also known as hexamethylenetetramine, hexamine, hexamethylenamine)

Methenamine	30 g
Distilled water	1000 ml

1% sodium metabisulfite

Sodium metabisulfite	1 g
Distilled water	100 ml

1% gold chloride

Yellow gold chloride	5 g
Sterile water	500 ml

0.1% gold chloride

1.0% gold chloride	10 ml
Sterile water	90 ml

2% sodium thiosulfate

Sodium thiosulfate	2 g
Sterile water	100 ml

Stock methenamine–silver nitrate solution

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

Notes: A white precipitate forms after mixing the two solutions but dissolves after shaking. The clear solution is stable for several months if stored at 4 °C. The solution should be stored in the refrigerator.

5% sodium borate

Sodium borate (decahydrate)	50 g
Distilled water	1000 ml

Working methenamine–silver nitrate solution

Stock methenamine–silver nitrate solution	25 ml
Distilled water	25 ml
5% sodium borate solution	2 ml

Note: This solution should be made fresh for each batch of tissue samples.

Stock 0.2% light green solution

Light green, SF yellowish (also known as CI 42095)	0.2 g
Distilled water	100 ml
Glacial (100%) acetic acid	0.2 ml

Working light green solution

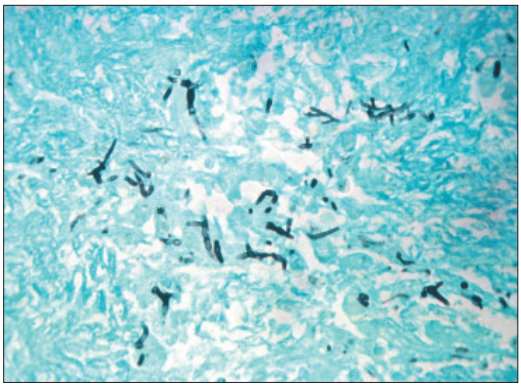
Stock light green solution	10 ml
Distilled water	50 ml

7.4.2 STAINING PROCEDURE

1. Deparaffinize the slides and hydrate with distilled water.
2. Oxidize in 4% chromic acid solution for 1 hour.
3. Wash in 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 water for at least 5 minutes.
6. Rinse with six changes of distilled water.
7. Place in freshly made methenamine–silver nitrate working solution in a 60 °C oven for 60–70 minutes or until sections turn light brown (a toasty colour). Check microscopically in order to stop the reaction before overstaining occurs.
8. Rinse in six changes of sterile water.
9. Tone in 0.1% gold chloride for 1–5 minutes and then check microscopically; fungi will be black and the background should be pink to grey.
10. Rinse in distilled water.
11. Remove unreduced silver by placing in 2% sodium thiosulfate solution for 2–5 minutes.
12. Wash thoroughly in water.
13. Counterstain in stock 0.2% light green solution for 4 minutes.
14. Rinse in distilled water.
15. Dehydrate.
16. Clear slides by immersion into 95% ethanol (twice for 2 minutes each time), 100% ethanol (twice for 2 minutes each time) and xylene (twice for 2 minutes each time).
17. Mount in a resinous mounting medium.

Figure 34 shows a histopathological section of a phaeomycotic cyst in skin stained with Grocott's methenamine-silver nitrate.

FIGURE 34. HISTOPATHOLOGICAL SECTION OF A PHAEOMYCOTIC CYST IN SKIN STAINED WITH GROCOTT'S METHENAMINE-SILVER NITRATE



Section shows black-stained hyphae of the causative fungus, *Phialophora repens* (Courtesy of Wayne Meyers)

7.5 BROWN–HOPPS METHODS FOR GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA

Note: This procedure shows many Gram-positive bacteria and most Gram-negative bacteria.

This procedure is used for specimens fixed in 10% buffered neutral formalin, and for tissue sections cut 4–6 µm thick. Controls must contain Gram-positive and Gram-negative organisms. An inflamed appendix is often a useful source of control tissue.

7.5.1 SOLUTIONS

Crystal violet

Crystal violet	1 g
Deionized water	100 ml

Note: This solution can be stored at room temperature long-term.

Gram's iodine

Iodine crystals	1 g
Potassium iodide	2 g
Deionized water	5 ml + 295 ml

When iodine and potassium iodide are completely dissolved in 5 ml deionized water, 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 (100%) acetic acid	1 ml

Picric acid–acetone

Picric acid (dried, see below)	0.05 g
Acetone (water-free)	500 ml

To dry picric acid:

1. place a layer of wet picric acid (more than you need) that is less than 2 mm thick between four large sheets of coarse filter paper (two sheets above the picric acid and two below);
2. squeeze out as much water as possible by rolling a bottle or other round object over the filter paper;
3. after squeezing and rolling, weigh the picric acid;
4. put any excess back with the wet picric acid;
5. use the weighed picric acid immediately;
6. rinse the filter paper in running water until all the yellow colour is removed, then discard it.

Note: Always store picric acid crystals underwater to avoid an explosion.

Acetone–xylene

Acetone	100 ml
Xylene	100 ml

7.5.2 STAINING PROCEDURE

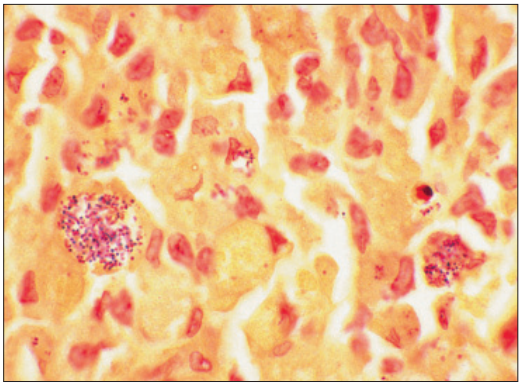
Note: Use a horizontal staining rack for steps 2–8. Use staining dishes for all other steps.

1. Deparaffinize the slides and hydrate with 95% ethanol.
2. Place 15–20 drops of 1% crystal violet solution on each slide. Leave on the slide for 1–2 minutes, and agitate gently.
3. Rinse in water.
4. Place slides in Gram's iodine for 1 minute.
5. Rinse in water.
6. Decolourize slides with acetone until the last of the crystal violet colour starts to stream away.
7. Immediately wash slides well in water.
8. Pour 1% basic fuchsin on the slide and let stand for 5 minutes.
9. Rinse the slides in water.
10. Place the slides in Gallego's solution for 60 seconds and agitate vigorously. Repeat once.
11. Rinse the slides in water.
12. Place the slides in acetone for 30 seconds.

13. Place the slides in the picric acid–acetone solution for 2–3 minutes.
14. Place the slides in the acetone–xylene solution for 30 seconds. Repeat once.
15. Clear slides by immersion into xylene (twice for 2 minutes each time).
16. Mount in a resinous mounting medium.

Figure 35 shows a tissue infected by ***Rhodococcus*** spp. after Brown–Hopps Gram stain.

FIGURE 35. BROWN–HOPPS GRAM STAIN OF TISSUE INFECTED BY *RHODOCOCCUS* SPP.



Note the several clusters of blue-stained (Gram-positive) bacteria (Courtesy of Wayne Meyers)

7.6 PERIODIC ACID-SCHIFF (PAS) STAINING FOR FUNGI

Note: This procedure is used to demonstrate glycogen and fungi; it is also useful for the differential diagnosis of cutaneous ulceration.

7.6.1 SOLUTION

0.5% periodic acid

Periodic acid	0.5 g
Distilled water	100 ml

Sulfurized water

Distilled water	180 ml
1N HCl	10 ml
10% sodium metabisulfite hydrogenosulfite	10 ml

10% Sodium bisulfite

Sodium metabisulfite	1 g
Distilled water	10 ml

Schiff's reagent

Basic fuchsin	1 g
Distilled water (boiling)	200 ml

1N HCl 20 ml

Sodium metabisulfite	1 g
Activated dehydrated charcoal	2 g

1. Shake the first two items together vigorously.
2. Cool to 50 °C.
3. Filter.
4. Add 20 ml of 1N HCl to the Schiff's reagent.
5. Cool to 25 °C.
6. Add 1 g of sodium metabisulfite to the solution.
7. Allow to stand in a dark place in a clean, tightly closed flask for 24 hours.
8. Add 2 g of activated dehydrated charcoal to the solution.
9. Shake for 3 minutes.
10. Filter.
11. The solution should be clear.
12. Store at 4 °C in a foil-covered bottle.

Ammonia water

Deionized water	1000 ml
28% ammonium hydroxide	5 ml

Ethanol–formalin

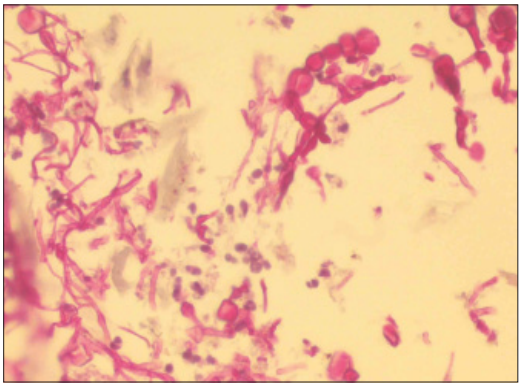
100% ethanol	40 ml
4% formalin	160 ml

Figure 36 shows fungal elements in a cutaneous ulceration stained with the periodic acid–Schiff method.

7.6.2 STAINING PROCEDURE

1. Deparaffinize the slides and hydrate with:
   1.a. toluene for 5 minutes; do this three times;
   1.b. alcohol 80% for 5 minutes;
   1.c. ethanol–formalin for 5 minutes;
   1.d. running water.
2. Oxidize in 0.5% periodic acid for 15 minutes.
3. Rinse in running water.
4. Stain with Schiff's reagent for 15 minutes, then rinse in warm water (about 60 °C) for about 10 minutes to develop the pink colour.

FIGURE 36. FUNGAL ELEMENTS IN A CUTANEOUS ULCERATION STAINED WITH PERIODIC ACID–SCHIFF AND SHOWN WITH HIGH MAGNIFICATION



(Courtesy of Jean-Jacques Roux)

5. Dip the slide in a solution of sulfurized water for 10 seconds; do this three times.
6. Wash in running water for 5 minutes.
7. Counterstain in Mayer's haematoxylin for 5 minutes (Mayer's haematoxylin can be bought as a ready-to-use reagent).
8. Wash in running water.
9. Dip in diluted 0.5% ammonia water for 1 minute.
10. Wash in running water.
11. Dehydrate in 100% ethanol for 1 minute; do this three times.
12. Clear in toluene (twice for 1 minute each time).
13. Mount in a resinous mounting medium.

7.6.3 STAINING RESULTS

Nuclei	Blue
Fungi	Red
Background	Pink or purple

7.7 IMMUNOHISTOCHEMISTRY

Note: This technique is used to stain specific antigens through a specific antigen–antibody interaction.

The procedure is used for specimens that have been fixed for 24 hours in 10% buffered neutral formalin, and for tissue sections cut 4–6 µm thick.

7.7.1 SOLUTIONS

Phosphate–buffered saline (PBS)

Sodium chloride (NaCl)	8 g
Potassium chloride (KCl)	0.2 g
Potassium phosphate (KH_2PO_4)	0.2 g
di-natriumhydrogen phosphate dihydrate (Na_2HPO_4 x 2 H_2O)	1.42 g
Distilled water	1000 ml

Adjust the pH to 7.2.

Note: Prepare a new batch of solution every time.

Pretreatment buffer: EDTA

EDTA working solution

Ethylenediaminetetraacetic acid disodium salt solution 0.5 M	1.4 ml
Distilled water	700 ml

Adjust pH to 8.

Note: Prepare a new batch of solution every time.

Pretreatment buffer: citrate

Stock citric acid 0.1 M solution

Citric acid	19.2 g
Distilled water	1000 ml

Note: The solution can be stored at 4 °C for 6 months.

Stock sodium citrate 0.1 M solution

Sodium citrate dihydrate	29.4 g
Distilled water	1000 ml

Note: The solution can be stored at 4 °C for 6 months.

Citric acid working solution

Stock citric acid 0.1 M solution	14 ml
Stock sodium citrate 0.1 M solution	56 ml
Distilled water	630 ml

Adjust the pH to 6.

Note: Prepare a new batch of working solution every time.

Pretreatment buffer: trypsin

Stock trypsin buffer solution

NaCl	0.44 g
Trisaminomethane hydrochloride	0.4 g
Calcium chloride	0.05 g
Distilled water	50 ml

Adjust pH to 7.8.

Note: The solution can be stored at 4 °C for 6 months.

Stock 2% trypsin solution

Trypsin bovine	0.02 g
Stock trypsin buffer solution	1 ml

Note: Prepare the solution, and quickly split it into 50 µl aliquots for storage at –20 °C to avoid self-digestion.

Trypsin working solution

Stock 2% trypsin solution (at -20 °C)	50 µl
Stock trypsin buffer solution	950 µl

Note: Prepare immediately before use.

0.3% hydrogen peroxide (H_2O_2)

30% hydrogen peroxide (H_2O_2)	2 ml
Distilled water	200 ml

Note: Prepare a new batch every time, and use immediately.

Blocking solution

Animal serum	15 µl
Phosphate-buffered saline	1 ml

Use serum from the animal in which the secondary antibody was produced.

Note: Prepare a new batch of solution every time.

Stock 1° antibody dilution buffer

Tween-20	100 µl
Phosphate-buffered saline	1 ml

Note: The solution can be stored at 4 °C for 6 months.

Working solution

1° antibody dilution buffer

Stock 1° antibody dilution buffer	10 µl
Phosphate-buffered saline	1 ml

Note: Prepare a new batch of solution every time.

2° antibody dilution buffer

Animal serum	15 µl
Phosphate-buffered saline	1 ml

Use serum from the animal in which the secondary antibody was produced.

Note: Prepare a new batch of solution every time.

Vectastain Elite ABC Kit (Vector Laboratories catalogue number: PK–6100)

Phosphate-buffered saline	2.5 ml
Reagent A	1 drop
Reagent B	1 drop

Note: Prepare the ABC solution 30 minutes before use.

Vector NovaRED Peroxidase Substrate Kit (Vector Laboratories catalogue number: SK–4800)

Distilled water	5 ml
Reagent 1	3 drops
Reagent 2	2 drops
Reagent 3	2 drops
H_2O_2 (included in the kit)	2 drops

Note: Prepare the NovaRED staining solution immediately before use.

Mayer's haematoxylin

Aluminium potassium sulfate	50 g
Distilled water	1000 ml
Haematoxylin	1 g
Sodium iodate	0.2 g
Glacial (100%) acetic acid	20 ml

Note: When the aluminium potassium sulfate is completely dissolved in the distilled water, add the haematoxylin.

Note: When the haematoxylin is completely dissolved, add the sodium iodate and acetic acid. Bring the solution to a boil very briefly. Filter, if necessary.

Note: Mayer's haematoxylin can be reused at least 10 times.

7.7.2 STAINING PROCEDURE

Note: Use a wet chamber for the trypsin pretreatment and for steps 6–12.

1. Deparaffinize the slides and hydrate with water.
2. Pretreatment step: The detection of many antigens can be improved by using pretreatment procedures that uncover hidden antigenic sites. Because of this, results obtained using different antigen-retrieval procedures should be compared if staining with a particular combination of antigen and antibody is not satisfactory without pretreatment.
   a. No pretreatment
      i. Continue to step 3.
   b. EDTA pretreatment
      i. Heat the slides in the EDTA working solution in a microwave until the liquid starts to boil.
      ii. Stop heating and wait for 5 minutes, leaving the slides in the microwave.
      iii. Repeat steps i and ii three times in total.
      iv. Allow the slides to cool for 20 minutes in the EDTA buffer.
      v. Slowly cool the slides by adding distilled water over a period of 5 minutes.
      vi. Go to step 3.
   c. Citrate buffer pretreatment
      i. Heat the slides in the citrate working solution in a microwave until the liquid starts to boil.
      ii. Stop heating and wait for 5 minutes, leaving the slides in the microwave.
      iii. Repeat steps i and ii two times in total.
      iv. Allow the slides to cool for 20 minutes in the citrate buffer.
      v. Slowly cool the slides down by adding distilled water over a period of 5 minutes.
      vi. Go to step 3.
   d. Trypsin pretreatment
      i. Cover the entire tissue section by adding a large drop of trypsin working solution to each tissue slide in a wet chamber.
      ii. Incubate for 30 minutes.
      iii. Go to step 3.

3. Wash the slides in phosphate-buffered saline for 1 minute; perform this step a total of three times.
4. Block endogenous peroxidase by placing slides in 0.3% H_2O_2 for 20 minutes.
5. Wash the slides in phosphate-buffered saline for 1 minute; do this step a total of three times.
6. Cover the entire tissue section by adding a large drop of blocking solution to each slide; wait 30 minutes. **Note:** Usually 100 µl of solution is sufficient for one tissue section. A liquid blocker pen should be applied around tissue sections.
7. Remove excessive blocking solution with tissue paper.
8. Add the appropriate dilution of the primary antibody diluted in 1° antibody dilution buffer; incubate for 1 hour at room temperature or as long as overnight at 1 °C depending on the antibody used.
9. Wash the slides in phosphate-buffered saline for 1 minute; repeat this step a total of three times.
10. Add biotinylated secondary antibody diluted in 2° antibody dilution buffer; incubate for 30 minutes.
11. Wash the slides in phosphate-buffered saline for 1 minute; repeat this step a total of three times.
12. Add the premixed ABC solution; incubate for 30 minutes. **Note:** Prepare the ABC solution 30 minutes before use.
13. Wash the slides in phosphate-buffered saline for 1 minute; repeat this step a total of two times.
14. Wash the slides in distilled water for 1 minute.
15. Add the freshly prepared NovaRED staining solution, and monitor the development of a brown–red precipitate under the microscope. **Note:** Development time for the precipitate can range from 30 seconds to 10 minutes depending on the antibody used.
16. Stop the reaction by rinsing the slides in distilled water.
17. Counterstain with Mayer's haematoxylin solution for 17 seconds.
18. Develop the colour by incubating the slides for 3 minutes in tap water (not in distilled water).
19. Rinse quickly in distilled water.
20. Dry the slides.
21. Mount slides in a resinous mounting medium.

7.8 RECOMMENDATIONS FOR STORING PARAFFIN BLOCKS AND SLIDES

To allow paraffin blocks and slides to be used for quality control or complementary studies, it is important to organize their storage. Using an identification number that is shared with the patient's file, and the samples on blocks and slides, allows each specimen to be easily identified.

7.8.1 STORING PARAFFIN BLOCKS

Paraffin blocks must be stored in a well organized manner – for example, they may be stored in chronological order by the date on which they were received. Storage systems should allow them to be retrieved easily; systems may include clearly labelled shelves or well organized and labelled boxes.

The temperature of the storage area must be low enough to avoid melting the paraffin (that is, <45 °C). The duration of storage should follow national recommendations (for example, in France they must be stored for 30 years).

7.8.2 STORING SLIDES

Slides also may be archived chronologically to allow easy retrieval. Different slides from the same patient may be stored together. Slides can be stored in the same place as paraffin blocks. However, after 10 years, slides are considered to have discoloured and should be destroyed, but new slides can be made from the paraffin block.

ANNEX 8:
QUALITY ASSURANCE

8.1 INTERNAL QUALITY CONTROL

Quality control (also known as QC) helps to ensure that the results produced by a laboratory are accurate, reliable and reproducible. A quality control analysis should be performed regularly and, to be effective, the process must be practical and readily included as part of each laboratory's standard reporting practices. All laboratory technicians are responsible for performing, recording and reporting results of quality control analyses.

Many components of quality control are either performed in conjunction with routine testing or as part of the regular management of the laboratory.

Quality control analyses usually focus on six areas:

- laboratory arrangement and administration;
- laboratory equipment;
- specimens and request forms;
- reagents;
- laboratory tests;
- recording and reporting.

8.1.1 LABORATORY ARRANGEMENT AND ADMINISTRATION

All laboratories must consider the following items when assessing how well their arrangements and administration function.

- Ensure that doors into the laboratory are always closed. Work areas, equipment and supplies should be arranged to aid in managing a logical and efficient workflow.
- Work areas should be kept free of dust. Benches should be cleaned at least once each day with an appropriate disinfectant.
- Laboratory procedures should comply with appropriate guidelines.
- Every procedure performed in the laboratory must be carried out following standard operating procedures.
- Written copies of standard operating procedures must be kept in the laboratory and be readily available.
- The laboratory supervisor must sign and write the date by any changes made to the procedures.
- Staff should have appropriate training, and their performance must be monitored regularly.
- In a laboratory performing PCR, a strict spatial separation should be maintained among extraction procedures, pre-PCR procedures, PCR procedures and post-PCR activities. (For more details, see the discussion of the three-room principle in ***Annex 6***, section 6.1).

8.1.2 LABORATORY EQUIPMENT

The following items should be considered in the laboratory's equipment.

- The operating manuals and cleaning instructions for all equipment must be readily available; this includes instructions and manuals for the microscope, balance, laminar flow cabinet, thermocycler, centrifuge, incubator, fridge and freezer.

- Records showing the service dates must be kept for all equipment.
- Equipment must be monitored regularly to ensure consistent performance.

8.1.3 SPECIMENS AND REQUEST FORMS

The following items should be considered in the laboratory's handling of specimens and request forms.

- Analyses should be performed only upon the written request of an authorized person.
- Analyses should not be performed when requested orally unless they are followed with a completed request form.
- Staff should insist that request forms are completed appropriately and specimens are labelled properly. This ensures that specimens are correctly associated with each patient.
- Ensure that specimens are rejected if they cannot be properly identified, are leaking, or are in broken containers. Request a repeat specimen.
- Ensure that staff record the date that specimens arrive in the laboratory. Staff should document on the request form any delays in the delivery of specimens.
- Staff must evaluate the quality of specimens. They must also record and monitor the number of specimens received by the laboratory.

8.1.4 REAGENTS

The following items should be considered in the laboratory's handling of reagents:

- All reagents should be labelled with their name, the date they were prepared, and the date they were first opened.
- If reagents were prepared in another laboratory, indicate the date they were received.
- Any material found to be unsatisfactory should be recorded. Remove the material from the laboratory immediately so it is not used.
- Keep only a 6-month supply of stock. Rotate stock to ensure that the oldest is used first.

8.1.5 LABORATORY TESTS

Internal quality control analyses should be part of the laboratory's regular functions and used to monitor the performance of all tests.

8.1.5.1 Direct smear examination

- Ensure that both positive controls and negative controls are included at least once each week in microscopy exams.
- The following types of slides should not be used as controls:
  - those where the positive control is not stained red;
  - those where the negative control remains red after decolourization;
  - those where the background has not been properly decolourized.
- Ensure that problems with control smears are resolved before patients' smear results are reported.
- Microscopy results should be compared with PCR results. Negative PCR specimens almost never yield positive smears because of differences in the sensitivity of the tests. A specimen that is negative by PCR but positive by a smear test may be the result of a false-negative PCR, a false-positive smear or, rarely, a lesion caused by a mycobacterium other than ***M. ulcerans*** (such as, ***M. tuberculosis, M. chelonae, M. marinum, M. haemophilum***).

8.1.5.2 Polymerase chain reaction (PCR)

- Ensure that both positive extraction controls (containing a very diluted bacterial suspension in molecular-grade water) and negative extraction controls (containing only molecular-grade water) are included in every DNA extraction batch.
- Ensure that positive PCR controls (containing a dilute solution of ***M. ulcerans*** genomic DNA, which in a gel-based PCR reaction would give rise to a weak band, or in a real-time PCR reaction would give a Ct value of 25–30) and negative PCR controls (containing only molecular-grade water) are included in every PCR run.
- If any of the controls produces an incorrect result, the entire extraction or PCR batch must be discarded and repeated.
- The following results are considered to be unacceptable for controls:
  - if the positive extraction control is negative, then there may be a problem with DNA extraction or PCR;
  - if the negative extraction control is positive, then there has been contamination during DNA extraction or PCR;
  - if the positive PCR control is negative, then there has been a problem during PCR;
  - if the negative PCR control is positive, then there has been contamination during PCR.
- Problems with DNA extraction controls or PCR controls must be resolved before patients' samples are analysed and their results reported.
- Ensure that PCR results are compared with the results of microscopy and culture. A specimen that is negative by PCR but positive by smear or culture could be the result of a false-negative PCR or a false-positive smear or culture. Positive smears or cultures may also be caused by the presence of another mycobacterial species in the lesion (such as, ***M. tuberculosis***, ***M. chelonae***, ***M. marinum***, ***M. haemophilum***).
- Ensure that false-negative results caused by inhibition of the PCR reaction are excluded by performing each conventional PCR reaction in duplicate. In this system, the first tube contains only the sample. The second tube contains the sample to which purified ***M. ulcerans*** DNA has been added. If the tube to which the purified DNA was added (the inhibition-control tube) is negative, then the PCR reaction was inhibited. In real-time PCR, a commercially available TaqMan exogenous internal positive control is incorporated into each reaction to ensure that a negative result for a particular sample is truly IS***2404*** negative and not caused by PCR inhibitors. Inhibition in clinical specimens can often be overcome by repeating the PCR using a 1:10 (or 1:100) dilution of the extracted DNA sample.
- Ensure that any contamination in the laboratory is followed up promptly. Monitoring contamination in PCR laboratories is done by systematically using negative controls for all PCR activities, and by annually analysing samples exposed to the environment. For this purpose, six microtubes with 400 µl 1x Tris plus EDTA buffer are placed opened inside the safety cabinets of the pre-PCR room (room 1) and of room 2, and on the bench of the room with the thermocycler (room 3) for about 24 hours – that is:
  - two tubes are placed in the PCR-mix cabinet (room 1);
  - two tubes are placed in the DNA-extraction cabinet (room 2);
  - two tubes are placed in the room with the thermocycler (room 3);
  - from each of the six tubes, 5 µl is analysed by PCR.

8.1.5.3 Cultures

- Contamination rates should be verified monthly. If these are higher than 5%, the sterility of the reagents, media and the laminar-flow cabinet should be evaluated. The quality of specimens may also influence contamination rates, as well as the decontamination method used (for example, the oxalic acid methods yield less contamination than the modified Petroff method).
- Compare culture results with PCR results. Specimens that are positive by culture but negative by PCR suggest that the cultures are false-positives, the PCR results are false-negatives, or that a mycobacterium other than ***M. ulcerans*** has been cultured.

8.1.5.4 Histopathology

- Controls should be included to check the effectiveness of all staining reagents. The specific control tissues to be used are detailed in ***Annex 7***.

8.1.6 RECORDING AND REPORTING

- Ensure that results are sent out as soon as they have been validated. Monitor any delays in the time it takes to process specimens or delays in delivering results, and note them on the report form.
- Analyse results each month to detect any changes that may indicate a problem.
- All results should be recorded using a standard format in a laboratory register.
- All records should be retained for at least 2 years.

8. 2 EXTERNAL QUALITY ASSESSMENT

The intent of an external quality assessment (also known as an EQA) is to help laboratories identify errors and improve their practices in order to perform better. Taking part in an external quality assessment should not be seen as a threat, but rather as an opportunity to strengthen skills. Most laboratory technicians want to provide accurate testing. If the laboratory does well during an external quality assessment, then staff should feel reassured that they are contributing to the diagnosis and control of Buruli ulcer.

Three methods can be used to evaluate a laboratory's performance:

- an onsite evaluation;
- panel testing;
- blinded rechecking of results.

External quality assessments of microscopy are organized by national tuberculosis control programmes; Buruli ulcer programmes should collaborate with these programmes to ensure that standards for microscopy used to diagnose Buruli ulcer are maintained.

For PCR, the Institute of Tropical Medicine (Antwerp, Belgium), a WHO collaborating centre, regularly organizes external quality assessments consisting of proficiency testing that uses a panel of coded specimens. This involves sending a test panel of negative and positive samples to national laboratories. Panel testing offers the opportunity for laboratories to compare their performance with others, and may reassure technicians that they can attain the same results as other technicians.

Buruli ulcer clinical and treatment form – new case

BU 01

Health facility: _____
Name of health worker treating patient: _____

Date of clinical diagnosis or admission (dd/mm/yy): ____/____/____
Date of complete healing (dd/mm/yy): ____/____/____

Name of patient: _____ ID#: _____ __
Address (village or town): _____ District: _____
Province/Region/State:_____ Country: _____

Age (years): _____ Sex: ☐ Male ☐ Female
Weight (kg): _____ Profession:_____

CLINICAL HISTORY AT DIAGNOSIS

Duration of illness before seeking care (weeks): _____
Use of traditional treatment: ☐ Yes ☐ No
Limitation of movement at any joint: ☐ Yes ☐ No
Previous treatment with streptomycin: ☐ Yes (duration in days: ___) ☐ No

REFERRED BY :

☐ Self-referral ☐ Former patient
☐ Family member ☐ Schoolteacher
☐ Health worker ☐ Other (specify) :
☐ Village health worker _____

CLINICAL FORMS

☐ Nodule (N) ☐ Plaque (Q)
☐ Oedema (E) ☐ Ulcer (U)
☐ Osteomyelitis (O) ☐ Papule (P)

CATEGORIES

☐ **Category I**: A single lesion ≤ 5 cm in diameter
☐ **Category II**: A single lesion 5–15 cm in diameter
☐ **Category III**: A single lesion >15 cm in diameter, multiple lesions, lesions at critical sites, osteomyelitis

LOCATION OF LESION(S)

☐ Upper limb (UL) ☐ Abdomen (AB) ☐ Buttocks and perineum (BP)
☐ Lower limb (LL) ☐ Back (BK) ☐ Thorax (TH) ☐ Head and neck (HN)

CRITICAL SITES

☐ Eye ☐ Breast ☐ Genitalia

LABORATORY CONFIRMATION

Specimen(s) collected: ☐ **Yes** ☐ **No -** Date first specimen(s) taken (dd/mm/yy): ___/___/___

Specimen(s) type(s): ☐ Swab ☐ Fine needle aspiration (FNA) ☐ Biopsy

Results				
☐ ZN:	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ PCR:	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*
☐ Histo:	☐ *Positive*	☐ *Negative*	☐ *Positive*	☐ *Negative*

TREATMENT TYPE (Tick all applicable): ☐ Dressings ☐ Antibiotics ☐ Surgery (dd/mm/yy): ____/____/____ ☐ POD (prevention of disability)

DOSAGES **Rifampicin: _____ (mg)** **Streptomycin:_____ (g)** **Other (name): _____ : _____ (mg)**

Cross out each day (X) after administering the antibiotics; if antibiotics are not taken, indicate with the symbol Ø

Month \ Day	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	Total Doses

TREATMENT OUTCOME

☐ 1a: Antibiotic treatment completed ☐ 2a: Healed without surgery ☐ 3a: Healed without limitation of movement at any joint ☐ 4: Referred for further treatment
☐ 1b: Antibiotic treatment **not** completed ☐ 2b: Healed with surgery ☐ 3b: Healed with limitation of movement at any joint ☐ 5: Lost to follow-up ☐ Died

9.2. BU 03: REQUEST FOR LABORATORY CONFIRMATION OF A BURULI ULCER CASE

BU 03

REQUEST FOR LABORATORY CONFIRMATION OF A BURULI ULCER CASE

I. GENERAL INFORMATION

Name of treatment facility:_____

Name of health worker requesting examination:_____

Name of patient: _____ ID#:_____

Age (yrs):_____ Sex: ☐ M ☐ F

Address (village or town): _____ District: _____

Classification: ☐ New ☐ Recurrent

Clinical form: ☐ Nodule (N) ☐ Plaque (Q) ☐ Oedema (E) ☐ Ulcer (U) ☐ Osteomyelitis (O)

Date of specimen collection (dd/mm/yy): ___/___/___

Type of specimen: ☐ Swab ☐ Fine needle aspiration (FNA) ☐ Biopsy

II. REASONS FOR REQUESTING LABORATORY CONFIRMATION

Type of examination(s)	☐ ZN	☐ PCR	☐ Culture	☐ Histopathology

Reasons

☐ Diagnosis of a new case

☐ *Follow-up of a patient during antibiotic treatment (weeks of antibiotic treatment: _____)

☐ Diagnosis of a recurrent case (end of last antibiotic treatment (date or month: _____)

☐ Follow-up of a patient after antibiotic treatment

III. RESULTS

	ZN	PCR	Culture	Histopathology
Date (dd/mm/yy): ___/___/___				
Date (dd/mm/yy): ___/___/___				

Comments:_____

_____

Name of laboratory scientist providing the results:_____

Name of laboratory:_____

Date:_____

* May include patients who do not respond to treatment as expected

ANNEX 10:
GUIDANCE ON SAMPLING TECHNIQUES FOR LABORATORY CONFIRMATION OF *MYCOBACTERIUM ULCERANS* DISEASE (BURULI ULCER)

BACKGROUND

Advances in the clinical management of ***Mycobacterium ulcerans*** disease (Buruli ulcer) have shifted options for treatment from surgical management to combination antibiotic therapy. Antibiotic treatment with rifampicin and streptomycin, or rifampicin plus other oral regimens, has made it possible to offer decentralized treatment where previously only hospital-based surgical treatment was available. As a result of these advances, the number of surgical interventions has been reduced (in 2012, about 40% of patients were treated without surgery), and recurrences of the infection have fallen to almost zero.

Confirmation of cases using laboratory methods – such as PCR and direct smear examination – has become a central part in the overall management of the disease. Although cultures are not essential for diagnosing the disease or the immediate clinical management of patients, identifying treatment failures and recurrences of infection may require the detection of viable bacilli. Cultures may also be necessary if drug-resistant strains of ***M. ulcerans*** emerge.

In many countries where Buruli ulcer is endemic, 70–100% of patients present with ulcerative lesions, and 0–30% present with nonulcerative lesions. Since 2007, excellent progress has been made in using the fine-needle aspiration (FNA) to collect samples from patients who have been clinically diagnosed but have nonulcerative lesions. Until then, punch biopsy was preferred over the more invasive surgical biopsy for obtaining samples from nonulcerative lesions. Punch biopsy is used in a few countries, mainly for research purposes. Today, FNA is used in a number of countries to obtain specimens for laboratory confirmation of the disease. Punch biopsy is not the first choice for obtaining a sample, although it may be used in limited special circumstances, such as those indicated below.

Although surgical treatment is performed less often, surgery may still provide an opportunity to obtain samples for laboratory analysis.

METHODS OF DIAGNOSIS

Four methods are commonly used for laboratory confirmatory of ***M. ulcerans*** disease: direct smear examination, PCR, culture and histopathology. The advantages and disadvantages of these techniques are summarized below.

METHODS OF DIAGNOSIS

Method	Advantages	Disadvantages
Direct smear examination	• Easy to perform at local level • Does not require expensive materialsand equipment • Provides rapid results • Can be used with samples from swabs, fine-needle aspiration and biopsy	• Low sensitivity (<60%) • Needs trained personnel • Does not distinguish between viable and nonviable organisms • Needs external quality assurance
PCR for IS*2404*	• Results fairly rapid • Can be used with samples from swabs, fine-needle aspiration and biopsy • High sensitivity and specificity (>90%)	• Requires a sophisticated laboratory • Expensive to perform • Needs trained personnel • Requires strict quality control
Culture of *M. ulcerans*	• Can be used with samples from swabs, fine-needle aspiration and biopsy • Only method that can distinguish between viable and nonviable organisms • Can be used to monitor patients'response to antimycobacterial treatment	• Requires a sophisticated laboratory • Needs trained personnel • Results take at least 6–12 weeks • Low sensitivity (20–60%) • Not useful for immediate patientmanagement
Histopathology	• Sensitivity is about 90% • Results fairly rapid • Useful in establishing differentialdiagnosis, and monitoring unexpected response to treatment	• Requires a sophisticated laboratory • Expensive to perform • Needs trained personnel • Requires invasive procedure (that is,biopsy)

SAMPLING TECHNIQUES

Three techniques are used to collect specimens: swabs, FNA and biopsy (punch or surgical). Specimens may be used for routine diagnosis and the clinical management of patients, as well as for research.

1. ROUTINE CLINICAL MANAGEMENT AND CASE-FINDING

Swabs and FNA are simple procedures that can be undertaken at any level (at community centres, health centres, hospitals) during routine management or case-finding activities.

1.1 SWABS

Specimens obtained by swabs should be taken from the undermined edges of a clinically diagnosed Buruli ulcer. Physicians or experienced health workers can perform this technique. In general, most patients present with ulcers, so this technique can be used in most settings. However, every effort should be made to minimize the pain and bleeding that may occur during the procedure; health workers who perform swabs should be properly trained.

1.2 FINE-NEEDLE ASPIRATION

Fine-needle aspiration (FNA) is mainly used to obtain samples from clinically diagnosed nonulcerative lesions (such as nodules, plaques and oedemas). This technique is necessary in up to 30% of patients (depending on the setting), and is simple enough to be used more widely in the field. FNA may also be used for ulcerative lesions where it is difficult to take swabs because the edges are healing. Only experienced physicians or experienced health workers should perform FNA; continuing training and regular supervision should be provided to health workers to improve their skills.

Extreme care should be taken when FNA is performed around the head and neck area (especially around the eyes), and the genitalia. Where necessary, an expert clinician should perform FNA in order to minimize any unintended damage to important organs or structures.

WHO recommends that a maximum of two swabs or two FNA samples be taken from each lesion, depending on the experience of the person taking the sample.

Sampling may need to be repeated if the results of PCR are negative despite a strong clinical diagnosis.

1.3 BIOPSY (PUNCH OR SURGICAL)

Samples obtained from swabs or FNA for confirmation are sufficient in most cases. Punch biopsy or surgical biopsy may be used when the diagnosis is in the direct interests of the patient (for example, when swabs and FNA have been tried unsuccessfully or abandoned). Surgical biopsy may be preferable when larger diagnostic specimens are required for histopathological analyses (in case of suspected cancerous changes or when pathologies other than Buruli ulcer are suspected).

INDICATIONS AND SUGGESTED SAMPLING TECHNIQUES

Indication	Suggested sampling technique
1. To establish the differential diagnosis of Buruli ulcer	Surgical or punch biopsy
2. To investigate the cause of a paradoxical reaction (see section 4.7.7 of this manual for additional information)	Surgical or punch biopsy
3. To determine whether treatment has failed despite successful administration of high-quality antibiotics	Surgical or punch biopsy
4. To establish whether cancerous changes have occurred	Surgical or punch biopsy
5. In clinical trials, to reconfirm the clinical diagnosis using at least two laboratory methods, and to evaluate disease process and therapeutic efficacy	Surgical or punch biopsy

Collecting specimens

Biopsies should be carried out by a trained physician or an experienced health worker who has examined the patient and decided that a biopsy is the only option for obtaining a sample.

- Samples for histopathological analysis (or microbiological analysis) should be taken from a single biopsy rather than multiple punch biopsies.
- Biopsies should not be performed on lesions on the face (for cosmetic reasons) and other critical sites (for example, on the head and neck and near the genitalia).
- Punch biopsies should be performed only in settings where the risk of infection can be minimized, and where facilities are available to manage profuse bleeding.
- All necessary steps should be taken to minimize the patient's pain and discomfort.

2. RESEARCH

Many of the techniques described above and the conditions under which they will be chosen for use also apply to decisions made for research. In exceptional cases or where ethically sound justifications have been made (such as why routine samples or procedures cannot be used or where there is a need to answer critical research questions), explanations should be detailed in the research protocol. Information (about the procedure and the intended use of the samples) should also be provided to patients and included in the consent form. Single biopsies should be taken only by experienced clinicians. Punch biopsies should not be performed on the face, neck and near the genitalia.

Protections for patients taking part in research are described and follow international standards; these standards should not differ from country to country. Therefore, it is essential that those working as part of the Buruli ulcer community develop their own code of ethics to uphold such standards of medical practice within their research work.

Samples for research are still possible. Researchers should be assured that opportunities for obtaining samples from patients to conduct research are available and should be explored.

Histopathology (and microbiology)

Debridement or limited excision of tissue from necrotic ulcers or lesions during or after antibiotic treatment provides an opportunity to obtain samples for research.

Cultures

Adequate cultures are possible even if taken from the swabs of 70–100% of patients with ulcerative lesions. Further work is needed to determine how many cultures can be obtained from FNA samples. It is not necessary to obtain cultures from every patient or lesion as long as there is no evidence for the emergence of antibiotic-resistant *M. ulcerans* strains.