Laboratory Diagnosis of Buruli Ulcer: Challenges and Future Perspectives

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1 Introduction

The clinical presentation of Buruli ulcer (BU) is manifold and includes relatively unspecific, non-ulcerative manifestations such as nodules, papules, plaques, and edema, which may eventually progress to necrotic ulcers [1]. Early case detection and adequate treatment are essential to prevent the formation of large cutaneous lesions that are often associated with serious morbidity and permanent disability. While surgical resection of BU lesions has long been the only treatment option, demonstration of the efficacy of rifampicin against *M. ulcerans* in a mouse footpad model [2, 3] and of a combined regimen of rifampicin and streptomycin in a clinical trial [4], have shifted first-line treatment recommendations to antibiotic therapy. Routine implementation of the drug regimen consisting of rifampicin and streptomycin administered daily for 8 weeks, has greatly improved specific therapy and reduced the frequency of relapses [5–7]. However, considering the history of antibiotic resistance in other bacterial pathogens such as *M. tuberculosis* [8], concerns have arisen that inappropriate use of these antibiotics may lead to similar patterns of resistance in *M. ulcerans*. These concerns appear justified, as rifampicin, currently the only highly effective drug available for the treatment of BU, is also a major component in the treatment of other often co-prevalent mycobacterial infections such as tuberculosis and leprosy. Furthermore, streptomycin should be prescribed with caution, as long-term streptomycin toxicity in the form of a high incidence of persistent hearing loss has been documented in a follow up study of former BU patients who had received the combination therapy [9]. A new combination

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therapy with rifampicin and clarithromycin is currently being evaluated as first-line treatment of BU. Pre-treatment laboratory confirmation of clinically suspected BU cases has thus gained in importance; particularly because the differential diagnosis of skin conditions with similar manifestations is broad ([1, 10], http://www.who.int/neglected_diseases/resources/9789241513531/en/) (Fig. 1) and misclassification of clinically suspected cases seems to be more common than previously assumed [11].

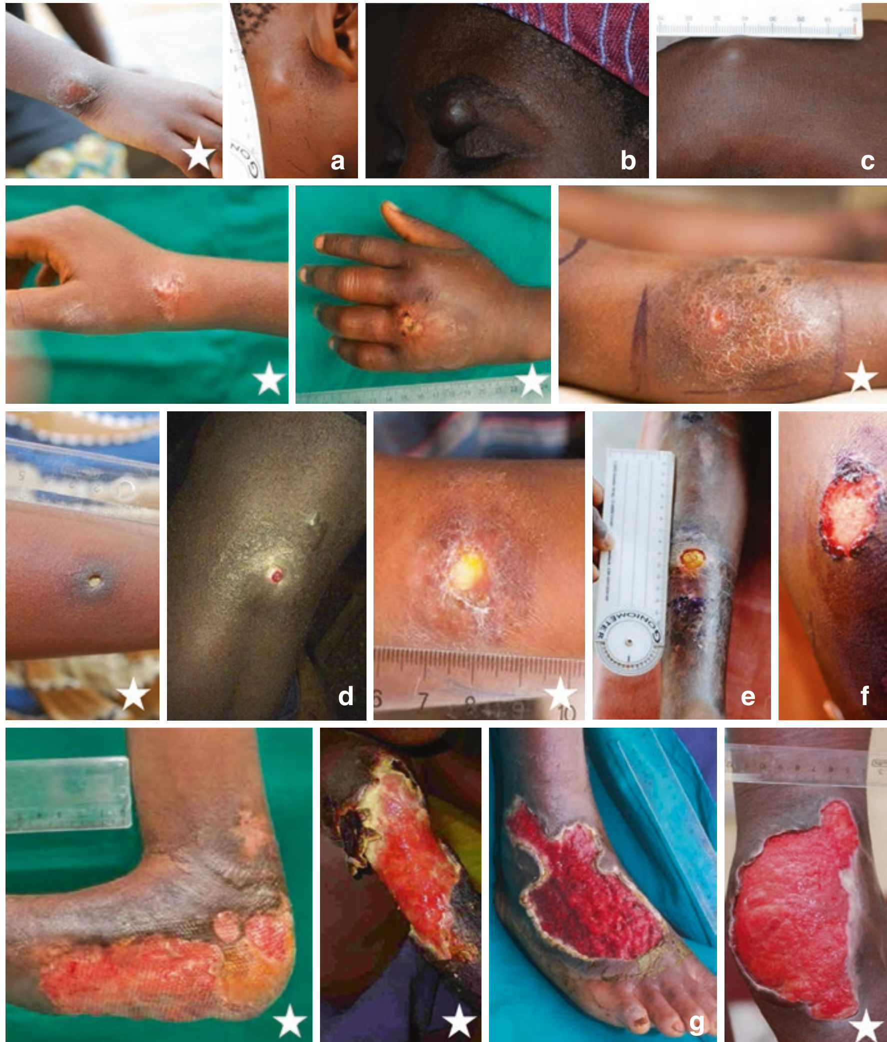

Fig. 1 Differential diagnosis of BU. Infection with *M. ulcerans* can cause a variety of clinical manifestations, including both non-ulcerative and ulcerative forms. The differential diagnosis of BU thus comprises a wide spectrum of other skin conditions with a similar appearance. Shown are BU lesions (marked by a star) and non-BU lesions ((**a**) swollen lymph node; (**b**) lipoma; (**c**) ganglion; (**d**) cutaneous tuberculosis; (**e**) sickle cell necrosis; (**f**) burn; (**g**) snake bite). Pictures provided by Markus Schindler, Thomas Junghanss and Moritz Vogel are included

For a detailed description of the currently available laboratory techniques and procedures for the detection of *M. ulcerans* the reader is referred to the WHO manual *"Laboratory Diagnosis of Buruli ulcer"* edited by Françoise Portaels [12]. Laboratory tests routinely used for the detection of *M. ulcerans* infections include microscopic detection of acid fast bacilli (AFBs) in stained smears from clinical specimens and DNA detection by PCR targeting the *M. ulcerans*-specific insertion sequence (IS) element IS*2404*. Histopathological analysis of sections from the affected tissue and primary cultivation of the mycobacteria require sophisticated infrastructure and can only be performed by specifically trained personnel [12]. Furthermore, cultivation of the extremely slow growing mycobacteria takes several weeks to months and is thus impractical to aid pre-treatment diagnosis.

Of the established tests for the detection of *M. ulcerans*, IS*2404* PCR has proven to be the most sensitive and specific, if performed according to demanding quality assurance schemes [13, 14]. Therefore, it is currently considered the diagnostic gold standard. However, data on the sensitivity and specificity of IS*2404* PCR as well as of the other reconfirmatory tests are difficult to interpret from available comparative studies. Indeed, many studies reporting on the test performance of *M. ulcerans* detection evaluated sensitivity and specificity by comparing the respective tests to clinical diagnosis, which has limited accuracy, even when performed by experienced health staff. The sensitivity of microscopy—the only test that can be performed at district hospital level—has been reported to be relatively low. In BU endemic countries with resource-rich healthcare systems and good laboratory infrastructure, such as Australia and Japan, PCR-based diagnosis is routinely done, whereas in resource-poor settings, logistical challenges and high costs often impede rapid PCR-based laboratory diagnosis at centralized reference laboratories. As a consequence, the diagnosis of BU at remote health facilities is often based on clinical judgment only. A simple and rapid point-of-care diagnostic test for BU is therefore of urgent need. Following recommendations of WHO, a diagnostic test suitable for application in developing countries should meet the so called ASSURED [15] criteria of being Affordable, Sensitive, Specific, User-friendly, Robust and rapid, Equipment free, and Deliverable to the end user. This chapter highlights current approaches as well as future prospects for the diagnosis of BU at district hospitals and the primary healthcare level, where the majority of BU patients are diagnosed to date.

2 Currently Available Laboratory Diagnostic Tests

2.1 Specimen Collection and Reference Standards

Before the introduction of antibiotic therapy in 2004 [16], the only treatment option for BU was surgical excision of the lesions, with or without subsequent skin grafting. Laboratory reconfirmation was often done retrospectively by analyzing specimens excised during surgery [17–19]. After 2004, when the importance of pre-treatment reconfirmatory laboratory diagnosis became more broadly recognized, alternative sources for diagnostic specimens included punch biopsies obtained from non-ulcerative lesions and swab samples taken from the undermined edges of

ulcerative lesions, where the bacterial load is typically higher than in the core of a lesion. However, due to the invasiveness of punch biopsies, a consensus has been reached that in the interest of the patients, the method should be limited to special circumstances such as the establishment of differential diagnosis, investigations on paradoxical reactions or the reconfirmation of suspected treatment failure [12, 20]. Since 2007, a less invasive technique referred to as fine-needle aspiration (FNA), which can be performed at all healthcare levels and on both non-ulcerative lesions and ulcers with scarred edges that hinder the collection of swabs, has gradually replaced the use of punch biopsies for routine laboratory confirmation. The current recommendation for the collection of diagnostic specimens is thus to take swab and FNA samples from ulcers and non-ulcerative lesions, respectively. Recommendations for storage and transport conditions (e.g. media and optimal temperatures) for different types of samples are detailed in the aforementioned WHO manual "*Laboratory diagnosis of Buruli ulcer*" [12].

IS*2404* PCR-based tests performed at reference centres have become a gold standard for the diagnosis of BU. While the accuracy of new diagnostic tests for *M. ulcerans* infection should therefore be assessed by a comparison to results obtained by PCR, the true accuracy of PCR assays is in turn difficult to evaluate, as it can only be compared to imperfect reference standards that have both limited specificity such as clinical diagnosis [10], or limited sensitivity, such as microscopy [21] and culture [22].

2.2 IS*2404* PCR: The Current Gold Standard

In many mycobacterial species so called insertion sequence (IS) elements have been identified, representing suitable targets for PCR-based detection assays [23–26]. IS sequences are typically characterized by species-specificity and the presence of multiple copies within one genome, facilitating a highly specific and sensitive detection of the respective pathogens. In 1997, Ross et al. provided a milestone for the PCR-based diagnosis of BU by identifying an *M. ulcerans*-specific repetitive DNA fragment [27], which was later characterized in detail and designated IS*2404* [28]. The high copy number of IS*2404* (between 150 and more than 200 copies per genome in *M. ulcerans* isolates from Australia and Africa) and of another IS, referred to as IS*2606* (63 to 98 copies per genome in *M. ulcerans* isolates from Australia and Africa) [29], predestine their application as targets for sensitive PCR amplification tests for *M. ulcerans*. High specificity of developed PCR assays targeting IS*2404* was indicated by the lack of IS*2404* PCR positivity among a wide range of other mycobacterial species [28, 30, 31]. The design of real-time quantitative PCR (qPCR) assays targeting IS*2404* [32, 33] has been another milestone in BU diagnostics development and has replaced conventional gel-based PCR as the routine method for laboratory confirmation of BU in many diagnostic and research laboratories [14]. Besides an increased sensitivity of *M. ulcerans* detection, major advantages of the qPCR assay are the reduced risk of contamination, and an improved turnaround time.

Nevertheless, like any molecular test, the highly sensitive IS*2404* PCR—whether performed as gel-based test or as a qPCR assay—is prone to contamination leading to false positive test results. Cross-contamination of samples with *M. ulcerans* genomic DNA may occur during sample collection or processing. However the most common problem is carryover of PCR product from previous amplification reactions. For instance, in a study describing histopathological features of BU, specimens obtained from cases with filarial nodules and a keratin cyst without any histopathological indication for BU, tested positive in a nested IS*2404* PCR [34]. To ensure accuracy of PCR assay results, it is thus essential to strictly adhere to the three-room principle necessitating one room for preparing the PCR mix, a second room for processing the samples and addition of template DNA in a PCR cabinet, and a third room for PCR amplification. Moreover, the accuracy of PCR is also endangered by false negative test results. In the study mentioned above, IS*2404* PCR was negative for a number of histology-confirmed BU patients [34]. If clinical diagnosis of BU appears convincing, but PCR results are negative, it is recommended to collect and test a new set of samples to verify laboratory test results. False negative testing can result from a low concentration of *M. ulcerans* DNA in lesion specimens, a poor DNA extraction efficiency, low PCR sensitivity, and/or the presence of PCR inhibitors.

Thus strict adherence to good clinical laboratory practices and implementation of quality assurance protocols are necessary to generate reliable IS*2404* PCR results. In 2008, the Technical Advisory Group of the WHO Global BU Initiative therefore recommended the establishment of an external quality assessment program (EQAP) for the PCR-based detection of *M. ulcerans* in clinical and also in environmental samples. This system was implemented and coordinated by WHO Collaborating Centres for BU (the Institute of Tropical Medicine (ITM) in Belgium for clinical samples and the Victorian Infectious Diseases Reference Laboratory in Australia for environmental samples) [14]. For the proficiency testing of clinical samples, coded specimens with known content were distributed by ITM to national reference and research laboratories that were asked to process the samples using DNA extraction and PCR procedures they usually apply for the detection of *M. ulcerans* DNA. Two rounds of clinical EQAP revealed a marked diversity in the quality of *M. ulcerans* DNA detection between laboratories. In the two assessment rounds, only 36% and 31% of the participating reference laboratories had more than 90% concordant results with the proficiency panels. 64% and 38% reported false positive and 55% and 81% false negative results, respectively. These data demonstrate the need for continued internal and external quality assurance [14]. Transport of samples from remote endemic areas to central reference laboratories, ensuring sample integrity and timely return of results, represents another major challenge for the African BU control programs.

In summary, PCR targeting IS*2404* is a sensitive and specific diagnostic laboratory test for *M. ulcerans*, but necessitates a well-equipped laboratory infrastructure, specifically trained laboratory staff and strict quality control. Recent advances in isothermal amplification and PCR product detection technologies have reduced equipment needs, but not staff requirements.

2.3 Detection of AFBs by Microscopy: A Test for the Primary Healthcare Level with Limited Sensitivity

At the primary healthcare level, where most of the BU patients are diagnosed to date, microscopic detection of AFBs in direct smears from lesion specimens using a conventional light microscope is the only available confirmatory test for *M. ulcerans* infection. This method relies on one of the characteristic properties of mycobacteria, namely their ability to form deeply coloured complexes with arylmethan dyes in phenol water such as carbol fuchsin, carbol crystal violet or carbol auramine O, that usually resist de-colorization by acidic ethanol (acid-fastness) [35]. For tuberculosis, sputum smear microscopy has for decades been the most widely used tool for the laboratory diagnosis in low- and middle-income countries. The most commonly used staining technique for the diagnosis of different mycobacterial infections is the so called Ziehl-Neelsen (ZN) method based on carbol fuchsin. When compared with IS*2404* PCR, the reported sensitivity rates of direct smear microscopy for the detection of *M. ulcerans* ranged between 26% and 67%.[1] The efficacy of the detection of small numbers of AFBs depends strongly on technical skills of the microscopist and on quality of the microscopic equipment. While being a simple and cost-effective first-line test for BU in resource-constrained settings, limited sensitivity even in the hands of well-trained personnel is a serious drawback. Furthermore, misdiagnosis of cutaneous tuberculosis as BU based on microscopy may occur [40], as staining is not specific for *M. ulcerans*.

Although fluorescence microscopy based on auramine O staining has several advantages over light microscopy using ZN staining, its widespread use has long been hindered by the need for a more expensive fluorescence microscope. However, the recent advent of low-cost ultrabright light emitting diodes (LED) has enabled the development of simple and affordable fluorescence microscopes [41, 42]. Advantages of fluorescence microscopy include not only a simpler, quicker, and cheaper staining procedure, but also the possibility to screen slides with a lower power objective lens, which may improve sensitivity. For the diagnosis of tuberculosis, fluorescence microscopy seems to be as specific and more sensitive than light microscopy [43]. However, in a study on the detection of *M. ulcerans*, no significant difference in sensitivity between the two microscopic detection methods was observed [38].

2.4 Considerations on the Accuracy of IS*2404* qPCR and AFB Detection by Microscopy: Direct Comparison of the Two Techniques

A comparative analysis of IS*2404* qPCR and smear microscopy conducted under optimal laboratory conditions using well-characterized lesion specimens from BU patients, showed that the amount of *M. ulcerans* DNA in extracts from the samples

[1] Twenty-six percent (11/43 punch biopsies) [36], 34% (344/1020 swab, FNA and tissue specimens) [11], 56.1% (69/123 swabs) [21], 58.4% (45/77 swab and FNA specimens) [37], 59.4% (66/111 tissue specimens) [38], 64.6% (822/1273 tissue specimens) [39], 67% (83/124 swab and FNA specimens) [22].

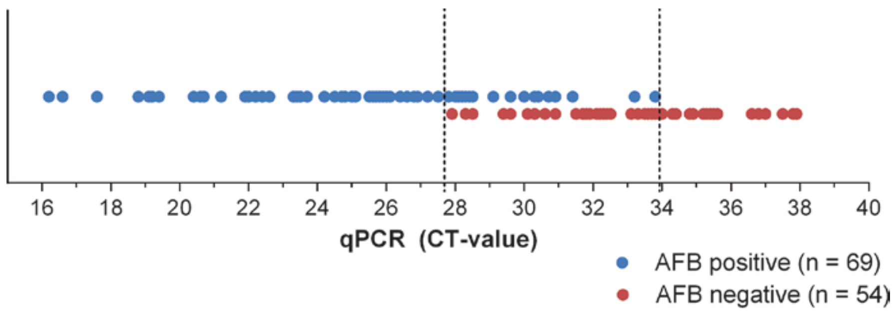

Fig. 2 Correlation between IS*2404* qPCR Ct values and microscopic AFB detection. A total of 123 IS*2404* qPCR-positive swab samples from BU lesions were analyzed by smear microscopy after ZN staining. While AFBs were detected in 69 (blue dots) of the 123 samples (56%), no AFBs were found in 54 (red dots) of the 123 samples (44%). AFBs were detected in all specimens having a qPCR Ct <27.8, whereas all specimens having a qPCR Ct >33.8 were microscopy-negative [21]

correlated well with the probability of finding AFBs [21]. While AFBs were detected in all IS*2404* qPCR highly positive samples, the probability of microscopy-positivity decreased for specimens with lower DNA content. Samples with qPCR cycle threshold (Ct) values above 34 were consistently negative (Fig. 2). A reasonable explanation for these results is certainly the higher sensitivity of qPCR as compared to smear microscopy. However, failure to detect even a single AFB in 54 IS*2404*-positive swab smears after careful screening of entire microscopy slides using a high power objective lens [21], may also raise concerns about false positive qPCR results. Even if qPCR assays are performed under strict quality control, false positive results with high Ct values may emerge through cross-contamination of samples with minimal amounts of *M. ulcerans* DNA in the process of sample collection (such as healthcare workers or other personnel handling several patients and samples without changing their gloves). In this context, the number of qPCR cycles that should be performed and which Ct values should be considered meaningful may have to be reconsidered.

2.5 Histopathology and Cultivation of *M. ulcerans*: Research Tools Rather than Diagnostic Tests

The pathogenesis of BU is primarily driven by the unique polyketide-derived macrolide exotoxin mycolactone of *M. ulcerans*, which causes apoptosis in mammalian cells [44]. In early non-ulcerative nodular stages of the infection the epidermis remains intact, but contiguous coagulative necrosis is found in the lower dermis. If mycolactone-mediated destruction of the subcutaneous tissue extends, the dermis and epidermis overlying foci of early *M. ulcerans* infection eventually degenerate. This leads to the formation of ulcers with undermined edges and a necrotic slough in the base. If mycolactone-mediated progression of tissue necrosis spreads mainly laterally, patients may develop extended non-ulcerative plaque or edematous forms of the disease [1].

Macroscopic features are thus changing as BU lesions evolve, whereas the progressive contiguous coagulative necrosis of the dermal and subcutaneous adipose tissue with dead adipocytes appearing as characteristic "ghost" outlines [45, 46] are considered BU-specific histopathological hallmarks that can be found in both pre-ulcerative (nodules, plaques, edema) and ulcerative stages. Necrosis of the subcutaneous tissue and necrotic collagen in the dermis seem to be the best histopathological predictors of *M. ulcerans* disease [34], while other features, like epidermal hyperplasia, destruction of blood vessels and interstitial edema complete the picture. Vasculitis and mineral deposits have been observed mainly in specimens from African BU patients [46]. In active lesions, extracellular clusters of AFBs are typically located in deep layers of the necrotic subcutaneous fat tissue [21]. Due to an uneven distribution of the clusters [21], AFBs may not be found in all parts of an active BU lesion. While there is a remarkable lack of inflammatory leukocytes in the necrotic centre of the lesions, a belt of inflammatory cells surrounding the necrotic core can be observed already in early lesions [47].

For routine diagnosis of BU in the endemic African countries, histopathological analysis of tissue samples is impractical, as it necessitates sophisticated technology as well as specifically trained and highly experienced personnel. Moreover, adequate tissue specimens obtained from the centre of a non-ulcerative lesion or from the edge of an ulcer by invasive sampling techniques are required. Therefore, histopathological analyses are not routinely used for diagnosing BU in Africa, but rather for the establishment of a differential diagnosis such as for suspected cases of squamous carcinoma secondary to BU [48, 49], or as a research tool to monitor treatment success [50] or paradoxical reactions [51].

Culture of the causative organism of an infectious disease is a mainstay of bacteriological diagnosis, not least because it provides information on the viability of the pathogen and allows for drug susceptibility testing. However, the extremely long generation time of *M. ulcerans*—colonies appear only after more than 2 months of incubation [18, 39]—excludes the application of cultivation for a pre-treatment confirmation of the clinical diagnosis. Moreover, successful cultivation of *M. ulcerans* depends on well-trained personnel and a complex laboratory infrastructure, only available at a few reference centres in the BU endemic African countries. Primary cultivation of *M. ulcerans* is thus performed mainly for research purposes, such as the monitoring of the efficacy of new treatment modalities [4, 6] as well as the distinction between paradoxical reactions and treatment failures [52, 53]. In addition, *M. ulcerans* isolates have been used for molecular epidemiological studies [29, 54, 55] and are used for the surveillance of the potential emergence of drug resistance.

2.6 From Theory into Practice: Diagnosis of BU in Resource-Constrained Endemic Countries

BU mainly affects impoverished populations living in remote, rural areas of West and Central Africa with only limited access to health facilities. Patients often have to travel long distances to reach a BU treatment facility. Primary or district level

health facilities, where the majority of BU patients present are usually not capable of performing PCR-based analyses. At best, direct microscopy of ZN-stained swab smears is performed, a test with limited sensitivity yielding a high proportion of false negative results. Specimens for PCR reconfirmation are usually stored and transported in bulk to reference laboratories, leading to delayed delivery of diagnostic test results. Major delays in diagnosis and late initiation of treatment may on the other hand distract patients from returning to facilities of the formal health system. To avoid the dropout of patients, antibiotic therapy is often initiated upon the clinical diagnosis.

As the routine use of IS*2404* PCR in resource-constrained countries is also limited by high costs, more cost-effective stepwise approaches starting with the local microscopic analysis of swab smears for the presence of AFBs and—if this initial test is negative—subsequent testing by PCR at a reference laboratory have been proposed [37, 56]. However, the right balance between saving costs and taking the risk of false positive microscopy results has yet to be evaluated, as patients with cutaneous tuberculosis may receive the short 8 week course of antibiotics, if the treatment decision is made only on the grounds of a positive microscopy result [40].

Until the early 2000s clinical specimens from suspected BU patients were primarily analyzed at international reference laboratories for retrospective confirmation of the clinical diagnosis. Although several BU endemic African countries including Benin, Cameroon, Central African Republic, Côte d'Ivoire, Democratic Republic of the Congo, Ghana and Togo have installed their own reference laboratories for the diagnosis of BU [12], the situation has remained very challenging with respect to both timely sample transport and quality assurance. Therefore, the development of a simple point-of-care diagnostic test for BU remains a major research priority [57].

3 Development of BU Diagnostics for District Hospital or Primary Healthcare Facility Level

In 2013, WHO together with the Foundation for Innovative New Diagnostics (FIND) convened a meeting of BU experts to review the need for BU diagnostics in the form of low-tech assays to be used in district hospitals and of simple, instrument-free rapid diagnostic tests (RDTs) that can be performed at the primary healthcare level. RDTs should be low-cost, simple to operate and read, stable, and yield results in a short period of time. They are expected to reduce the need for multiple healthcare visits, improve the chances that patients receive appropriate treatment, and can be highly cost-effective. Most available RDTs for neglected tropical diseases (NTDs) are based on immunoassays, including lateral flow, flow-through, agglutination, and dipstick test formats for antibody or antigen detection. Current strategies for the development of a BU RDT rely on antigen detection assays. For the diagnosis at district hospital level, options to detect *M. ulcerans* DNA by loop-mediated isothermal amplification (LAMP), antigens by enzyme-linked immunosorbent assay (ELISA) and mycolactone by fluorescence-based thin layer chromatography (f-TLC) are being evaluated.

3.1 LAMP: An Alternative for the Detection of *M. ulcerans* DNA

After its first description in 2000 [58], LAMP has gained attention as a rapid and cost-effective nucleic acid amplification method for the diagnosis of infectious diseases. Advantages of the LAMP technique over conventional PCR-based tests are lower technology requirements and simple read-out, as results can be read by the naked eye. However, this simple read-out does not allow for a distinction between the specific products and products of nonspecific amplification, posing a risk of false positive results [59], particularly when performed under suboptimal conditions [60]. Major improvements in LAMP test specificity have recently been reported by combining the technique with molecular beacons, targeting an internal sequence of the amplicon, thus allowing for a direct, specific detection of the expected product [61]. Nevertheless, significant training and infrastructure development is required to achieve acceptable performance of LAMP assays [60], making it suitable for district hospital settings, but not for the primary healthcare level.

In 2012, three different LAMP assays for the detection of *M. ulcerans* DNA were published. Sensitivity rates targeting either a sequence of the *M. ulcerans* virulence plasmid [62] or the multi-copy sequence IS*2404* [63, 64] were determined to be close to those of conventional IS*2404* PCR. No cross-reactivity was recorded with DNA of other closely related mycobacterial species [62–64]. However, the accuracy of these assays was assessed by testing only a very limited number of clinical specimens. As the potential occurrence of false positive results—compared to the conventional IS*2404* PCR gold standard—was reported [63] even when performed in well-equipped specialized laboratories, LAMP assays will have to be evaluated in decentralized settings, where the technique is intended to be applied. Initial results have shown that the extraction and purification of *M. ulcerans* DNA and the generation of isothermal conditions during the LAMP reaction remain major challenges for the application of LAMP assays under field conditions. Use of crude, non-purified DNA extracts and pocket warmers as heat source were shown to significantly decrease test sensitivity [64]. In order to overcome another shortcoming of the technique—the requirement of cold-chains for transport and storage of reagents—a dry-reagent LAMP assay targeting IS*2404* was developed. Validation of this assay with a limited number of qPCR-confirmed lesion specimens in a laboratory environment indicated a sensitivity and specificity comparable to that of conventional gel-based PCR [65].

Taken together, the LAMP method has the potential to be developed into a sensitive and simple test for BU at district hospital level. As the technique requires basic laboratory infrastructure for template preparation and generation of isothermal conditions, it is at its current stage of development not suitable for an application at the primary healthcare level.

3.2 Detection of Mycolactone by f-TLC: Struggling with the Complexity of Lipid Extracts

As the production of mycolactone appears to be restricted to clinically relevant *M. ulcerans* lineages and some other closely related *M. ulcerans* ecovars [66], the toxin is considered an ideal target for the diagnosis of BU. In addition to their diagnostic application, mycolactone-based tests also have the potential to be used for the monitoring of treatment success by measuring mycolactone levels as an indicator for the presence of viable *M. ulcerans* bacteria in treated BU lesions [67].

One strategy to detect mycolactone is by staining of mycolactone bands after TLC-based separation of lipids extracted from clinical specimens. After demonstration by TLC and mass spectrometry that mycolactone can principally be detected in extracts from tissue samples of patients with ulcerative and non-ulcerative BU lesions [68], the TLC method has gradually been optimized to facilitate its application in more peripheral laboratory settings. In order to overcome shortcomings of the technique such as low sensitivity and limited specificity due to the presence of other lipids with similar chromatographic behaviour in the lipid extracts, a boronate-assisted f-TLC was developed. Staining is based on the formation of cyclic boronates with the 1,3-diols present in the mycolactone variants produced by the human pathogenic *M. ulcerans* lineages [69]. It has been reported that with this technique, mycolactone could selectively be visualized using an ultraviolet lamp with a detection limit of 2 ng [69]. In a preliminary study, f-TLC was applied to a panel of IS*2404* PCR-positive samples from BU patients, returning 73.2% sensitivity and 85.7% specificity [70]. Implementation of the f-TLC method in hospital laboratories appears to be non-trivial and specific detection of mycolactone can be challenging due to background staining of co-extracted human lipids [71].

3.3 Serological Tests: Only Suitable for Seroepidemiological Studies

For serology-based diagnostic approaches, the identification of appropriate target antigens that are *M. ulcerans*-specific and at the same time capable of mounting strong immune responses in BU patients, but not in healthy individuals exposed to the pathogen is essential. Different strategies were used to find suitable antigens, but none of them led to the development of a diagnostic serological assay. While initial studies have reported that BU patients develop antibodies to antigens present in *M. ulcerans* culture filtrate, broad antigenic cross-reactivity among mycobacterial species complicated the design of a serological test specific for *M. ulcerans* infections. Thus it is not surprising that the same studies have also shown that sera from healthy control individuals living in BU endemic areas, as well as from tuberculosis patients, contained antibodies that recognized the *M. ulcerans* target antigens [72, 73].

Moreover, among *M. ulcerans*-specific antigens identified by comparative genomics, none enabled a distinction between BU patients and healthy control subjects living in the same BU endemic area [74]. Furthermore, from a panel of monoclonal antibodies (mAbs) generated against immunodominant *M. ulcerans* proteins, only those specific for the 18 kDa small heat shock protein (shsp)—revealed sufficiently limited interspecies cross-reactivity. However, this antigen is not suitable as target for a BU-specific serological test, as anti-shsp antibodies are also frequently found in sera of healthy individuals living in BU endemic areas [75]. This circumstance however has opened up the opportunity for seroepidemiological studies assessing the exposure of populations to *M. ulcerans* [76–78].

3.4 Detection of Mycolactone and *M. ulcerans* Proteins by Antigen Detection Assays: Prospects for the Development of an RDT

As the development of sero-diagnostic tests for BU has failed, current strategies to develop RDTs for BU rely on the detection of *M. ulcerans* protein antigens or mycolactone. The main advantage of assays utilizing antigen-antibody interactions is their potential to be converted into test formats that can be deployed at the primary healthcare level. In fact, for numerous infectious diseases developed ELISAs in the form of antigen capture assays have been converted or have the potential to be converted into point-of-care (lateral flow) diagnostic assays [79–82].

In the case of mycolactone as a capture assay target antigen, the lipid-like nature of this macrolide toxin, as well as its cytotoxic and immunosuppressive properties have long hampered the generation of specific antibodies that can be used for its detection. Recently, a new strategy to immunize mice with a protein conjugate of a non-toxic synthetic truncated mycolactone derivative facilitated the generation of mAbs specific for the upper side chain and part of the core structure of mycolactone [83]. By using the generated mAbs, a first prototype competition assay was designed, in which a mycolactone-specific mAb is used in combination with a mycolactone derivative as signaling molecule, to quantify the amount of mycolactone in a sample. While the assay showed excellent specificity, its sensitivity must be optimized to allow for the detection of mycolactone in clinical specimens. If non-competing pairs of anti-mycolactone mAbs can be generated, development of an antigen capture assay may become possible.

An ideal protein target antigen for the development of a diagnostic test for BU should (1) be highly expressed by *M. ulcerans*, (2) have no orthologs in other prevalent pathogenic mycobacteria and (3) be easily accessible through a cell surface location. These predefined criteria were shown to be met by the *M. ulcerans* protein MUL_3720 [84]. Immunization of mice with the recombinantly expressed MUL_3720 facilitated the generation of a panel of high affinity mAbs against this antigen. Tandems of non-competing MUL_3720-specific mAbs recognizing different epitopes were selected to enable the development of a highly specific MUL_3720 detection assay in a sandwich-ELISA format [84]. Preliminary analyses comparing

qPCR and ELISA results indicate that the MUL_3720 capture assay is highly specific. Optimization of the ELISA format potentially suitable for district hospitals is on-going to reach a sufficiently high sensitivity of antigen detection. As a next step towards an RDT, the application of the generated mAbs in a lateral flow assay format is being evaluated.

4 Discussion

In spite of the availability of sensitive and specific IS*2404* PCR assays routinely performed in resource-rich BU endemic countries and implemented in several national reference laboratories in BU endemic African countries, there is still an urgent need for simple and accurate point-of-care tests for the diagnosis of BU at district hospital and at primary healthcare level. In addition to logistical challenges and delays in the transport of clinical specimens to reference laboratories, outcomes of external quality assessment programs have demonstrated major shortcomings associated with the routine application of the PCR tests [14]. Ideally, the diagnosis of BU should furthermore not depend on the availability of laboratory infrastructure or of specifically trained laboratory personnel and should be performed directly at the point-of-care, so that treatment can be started without delay.

Immunochromatographic detection of antigens or antibodies in a dipstick or lateral flow format constitute currently the core of commercially available point-of-care RDTs for infectious diseases [85]. Whereas attempts to design an antibody-based sero-diagnostic assay for BU have been equivocal [74–76], the recent identification of a target protein suitable for antigen-capture test formats, shows great promise [84]. In addition, mycolactone—the lipid-like molecule secreted by *M. ulcerans*—may represent an optimal target molecule for the development of a sensitive and specific antigen detection test [83]. The advantage of antigen-based detection assays is that they can be converted to technically simple, robust test formats, easily applicable even at the primary healthcare level. In contrast, prerequisites for most molecular amplification techniques are incompatible with the ASSURED guidelines for point-of-care diagnostics [15].

In early 2011, WHO endorsed the first "sample in—answer out" qPCR platform with fully integrated sample processing for the diagnosis of tuberculosis in low-resource settings [86]. The GeneXpert MTB/RIF, a molecular test that can detect both *M. tuberculosis* DNA in sputum samples and rifampicin resistance mutations, has been developed for use at district and sub-district levels in tuberculosis endemic countries. However, in view of the limited resources available for the control of BU, costs for the production and broad introduction of a GeneXpert diagnostic test for *M. ulcerans* would be disproportionate. Several studies have reported attempts to develop nucleic acid-based point-of-care tests for the detection of *M. ulcerans* by the LAMP procedure [62–64]. While LAMP amplifies DNA with high sensitivity and specificity, its application under field conditions has so far been limited. This is mainly due to technology requirements associated with LAMP, such as template preparation in field settings, production of kits with dried-down reagents, and

methods for unambiguous detection of amplification products [87]. If these prerequisites are met, the LAMP platform can potentially be progressed into a format for the detection of *M. ulcerans* at district hospital level.

Point-of-care tests are key components to improve global health, but only if they are rigorously evaluated, and effectively regulated [88]. Careful pre-implementation evaluation of the accuracy of novel diagnostic tests for BU is absolutely essential and a consensus has to be reached on the reference standard to which the performance of new diagnostic tests is compared. In view of the broad differential diagnosis of BU and reported misclassification of cases based on the clinical presentation of patients, a comparison to clinical diagnosis—as done in many previous studies—is not satisfactory. Instead, test performance should be evaluated by a comparison to the current qPCR reference standard. However, strict quality assurance of the qPCR assay used for evaluation of the tests has to be ensured. Clearly, technological innovation is not sufficient. After successful development and evaluation of a new point-of-care test for BU, decentralized implementation of the test will involve training programs and monitoring of the effectiveness of the new tests in decentralized settings, to ensure the accuracy of test results.

5 Outlook

In the past years, the number of BU cases reported annually in many African endemic countries has declined. This may partly be attributed to the establishment of effective national BU control programs and a reduction in transmission intensity. On the other hand, the intensity of disease surveillance and case search activities may have declined, resulting in an underestimation of the true disease burden. Limited funding for the control of BU can lead to a lack of awareness and a loss of local competencies among health workers. For instance, in a recent retrospective assessment of the diagnosis of BU in Ghana between 2008 and 2016, a gradual decline in the annual laboratory confirmation rate of clinically suspected cases from 52% in 2008 and 76% in 2009 to only 15% in 2016 was revealed, reflecting both the decline in BU incidence and the loss of clinical expertise [11]. Considering this scenario, as well as the broad differential diagnosis of BU, the availability of a reliable point-of-care test for the diagnosis of BU is crucially important. Apart from BU, several other NTDs present with skin manifestations, either as the primary or as an associated clinical condition. Many skin diseases have similarities in terms of their ability to cause long-term disabilities, reinforcement of poverty and geographical distribution. Collectively, these highly disabling and stigmatizing diseases constitute a great burden on the affected populations. Major deficiencies in our understanding of many of these diseases and a lack of tools to combat them necessitate further investment in research and development of control strategies. Integration provides an opportunity to leverage funding and to spearhead efforts for the development, optimization, and implementation of new diagnostic, therapeutic, and preventive tools for the simultaneous control of several co-endemic skin NTDs [89].

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