Report of a WHO–FIND consultative meeting on diagnostics for Buruli ulcer

Geneva, Switzerland, 21 November 2013

WHO/HTM/NTD/IDM/2014.2

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Introduction

A consultative meeting of the World Health Organization and the Foundation for Innovative New Diagnostics (WHO–FIND) on the diagnosis of Buruli ulcer (BU) was held at WHO headquarters on 21 November 2013. The agenda and list of participants are included in Annexes 1 and 2 respectively.

Background on Buruli ulcer

Infection with *Mycobacterium ulcerans* has been documented in more than 33 countries worldwide. The majority of cases are in Sub-Saharan Africa, although the disease also occurs in other regions such as South-East Asia, South America and Western Pacific. The causative organism of BU is *Mycobacterium ulcerans* but the modes of transmission have not been identified.

The following diagnostic tests are currently available for BU.

Direct smear

Ziehl–Neelsen stained smears are a rapid and simple way of confirming BU cases that can be performed at any facility capable of light microscopy. However, this method has a low sensitivity (40–60%).

Polymerase chain reaction (PCR)

PCR is currently the gold standard test for BU and targets the IS*2404* insertion element, which has multiple copies in the *M. ulcerans* genome. This test has high sensitivity and specificity for *M. ulcerans* infection (>90%) and can be performed on a number of different samples, such as fine needle aspirates (FNA) from pre-lesion nodules, swabs from ulcerous lesions and infected tissue. However, this technology requires specialist equipment, training and infrastructure that are only available in tertiary laboratories.

Culture

Culture on solid media at 30–33 ^{o}C is the only currently available method for detecting viable bacilli. However, *M. ulcerans* grows slowly on solid culture medium, requiring an average of 6 weeks to become positive but isolation can take much longer than that. It also requires the sophisticated infrastructure and technical skill required for mycobacterial culture and so is normally confined to tertiary laboratories.

Samples for BU

Before the use of antibiotics in the treatment of BU, surgically removed tissue was used as a diagnostic specimen. This was then replaced by tissue obtained by punch biopsy and for ulcerated lesions, swabs taken from the edge of the ulcerative lesion. For non-ulcerated lesions, samples obtained by FNA have now replaced punch biopsies, as these are less traumatic for the patient.

Treatment

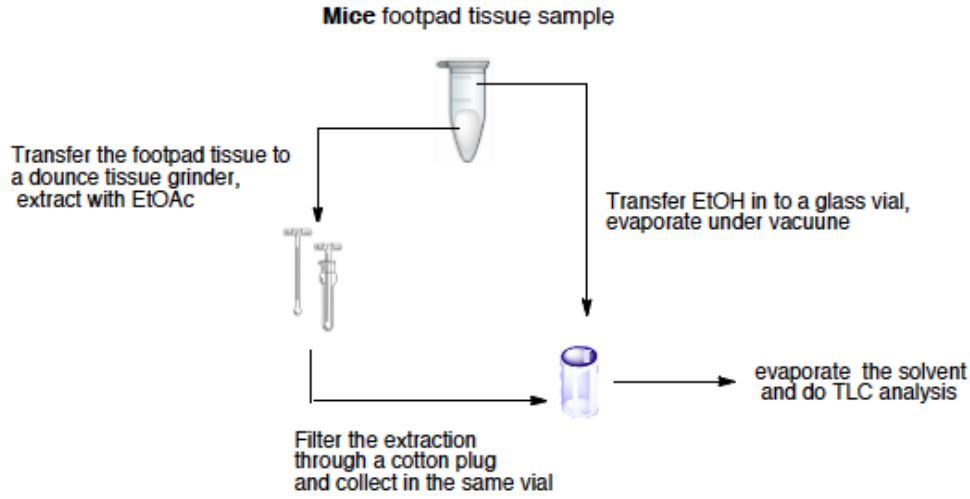
Treatment for BU used to be through debridement of the ulcerative tissue. However, since 2004 antibiotic therapy using rifampicin and streptomycin for 8 weeks has been introduced, and good treatment outcomes have been reported. Given the requirement to give streptomycin by injection, an alternative regimen is undergoing clinical trials using clarithromycin in place of streptomycin, and has shown great potential to replace the older treatment regimen.

Technologies under development

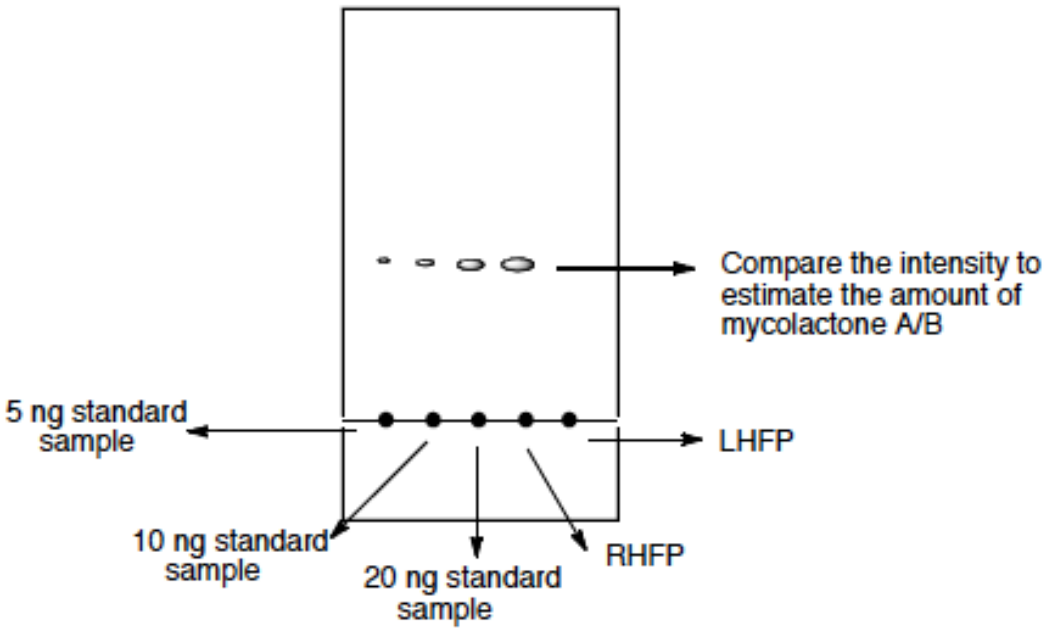
Detection of mycolactones by fluorescent thin layer chromatography (TLC)

Mycolactones are important compounds in the pathogenesis of BU but are weakly antigenic. Efforts have focused on direct detection of mycolactones specific to *M. ulcerans* in tissue but have not been investigated in other samples such as FNA. A workshop in 2010 in Accra, Ghana, showed that the technique was feasible but gave variable results in patient samples, and intra-laboratory reproducibility was poor. Samples also need to be stored and shipped in ethanol to maintain sensitivity of assay (due to action of punitive esterases) and there is no drop in sensitivity after 3 weeks. Using tissue from the footpads of infected mice, a drop in the amount of mycolactones was detected after 2 weeks with very little being detected after 5 weeks of treatment. Samples from uninfected mice gave no signal.

The procedure used is illustrated below.



The reading of the TLC is outlined below.



A weak band was detected in clinical control samples, indicating a potential for false-positivity in a practice setting. Using PCR as the gold standard, this method had a sensitivity of 68% and a detection limit between 2–8 µg/ml. However, Folch's technique was used to extract mycolactones rather than the methodology outlined above.

Antigen capture

Identifying *M. ulcerans*-specific antigens has proved difficult due to a high degree of antigenic cross-reactivity. The most promising results have so far used

polyclonal rabbit and monoclonal mouse IgG raised against antigen D, a highly expressed cell surface protein of *M. ulcerans*. The detection limit for the recombinant protein in an ELISA format was <1 ng/ml; however, sensitivity of the assay needs to be improved further (currently comparable to microscopy when using a small portion of material from swabs).It has been tested against other mycobacteria (*M. tuberculosis*, *M. bovis*, etc.) and there was no detectable cross-reactivity.

Attempts are being made to further improve sensitivity by changing the test format and adopting signal amplification steps, such as the Tyramide system.

Mycolic acids and their esters

Mixtures of mycolic acids are unique between mycobacterial species and therefore have the potential to identify different mycobacteria. Natural mycolic acids are immunogenic even in HIV-positive individuals. Using synthetic mycolic acid antigens to detect antibodies in serum samples for tuberculosis has shown that different antigens give different responses. The best antigens give a sensitivity of 88% and a specificity of 85%. Using two antigens in a traffic-light system gives 100% sensitivity and 91% specificity, although this needs to be validated using a larger number of samples. A paper-based sensor has been developed to allow visual interpretation of results.

Loop mediated isothermal amplification (LAMP)

LAMP has been used in the molecular diagnosis of a number of diseases, such as influenza, malaria, human African trypanosomiasis (sleeping sickness) and tuberculosis. For BU, the primers were redeveloped to align the sensitivity of the LAMP assay with the standard TaqMan PCR and also to reduce the time of the reaction. Sensitivity is now the same as for Real Time PCR using swabs and tissue samples.

Use of crude DNA extraction methods (boiling vs Qiagen) greatly decreased the sensitivity of LAMP. Use of a syringe with a membrane for binding DNA performed better but still had a reduced sensitivity in comparison with the Qiagen extraction method.

Mycolactone assay based on its binding to the Wiskott–Aldrich syndrome proteins (WASP)

WASP/N-WASP are members of a family of scaffold proteins involved in the remodelling of the actin cytoskeleton. Mycolactone has been shown to bind the proteins in vitro, and activate them by preventing auto-inhibition. This results in impaired integrity of mycolactone-injected skin. It has been demonstrated that biotinylated mycolactone binds dose-dependently to recombinant domains of WASP/N-WASP. This assay can be used to assess the presence of mycolactone quantitatively, by measuring the displacement of the biotinylated derivative

from plastic-coated WASP/N-WASP domains. It currently works with purified mycolactone but not in the presence of serum components.

Amphiphilic biomarkers for mycolactone

Detection targets often bind to proteins and lipids in the host and are hidden from traditional detection. Mycolactones are known to form high affinity conjugates with the WASP family of proteins, thus potentially reducing the concentration of the unbound form in samples. Conjugates may therefore be a better target for detecting infection with *M. ulcerans*. This approach has proven successful in the detection of lipoarabinomannan as a marker for tuberculosis, lipopolysaccharide for *Escherichia coli* and phenolic glycolipid-1 for *M. leprae.*

The best initial targets for diagnostic development of mycolactone are likely to be WASP and High Density Lipoprotein. Specific high affinity reagents could be developed using sets of recombinant monocolonal antibodies raised against the target conjugate using yeast and phage display. These antibodies can then be sorted by flow cytometry and subsequently affinity matured. Detection can be automated in the field using phospholipids and Self-Assembled Monolayer sensors.

Identifying unmet needs in BU diagnosis

The meeting identified two priorities (in order of importance):

1) A diagnostic test for the early detection of BU in symptomatic patients with sufficient positive predictive value to put patients on appropriate treatment.

2) A screening test at the primary or community level for symptomatic patients with ulcer.

Feasibility profile