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

Geneva, Switzerland, 21 November 2013

WHO/HTM/NTD/IDM/2014.2

Table of Contents

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

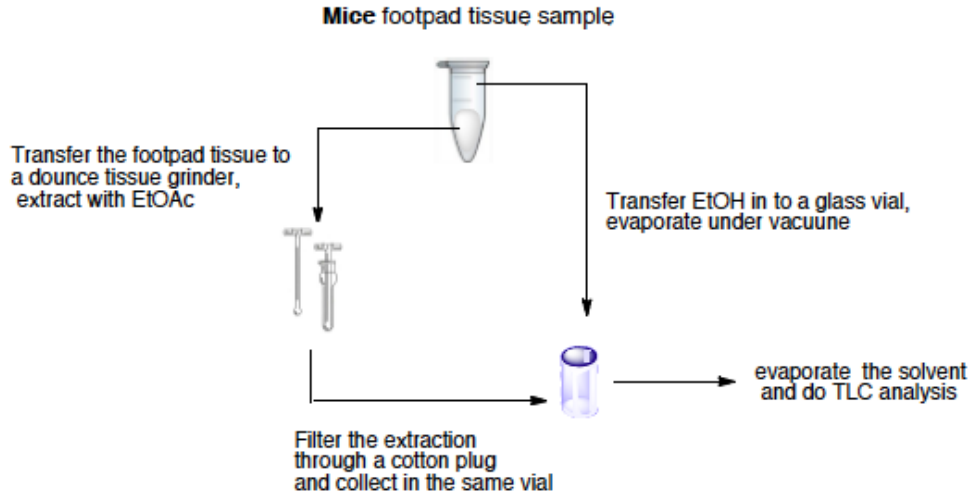
Treatment for BU used to be through debridement of the ulcerative tissue. However, since 2004 antibiotic therapy using rifampicin and streptomycin for 8 weeks has been introduced, and good treatment outcomes have been reported. Given the requirement to give streptomycin by injection, an alternative regimen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

Technologies under development

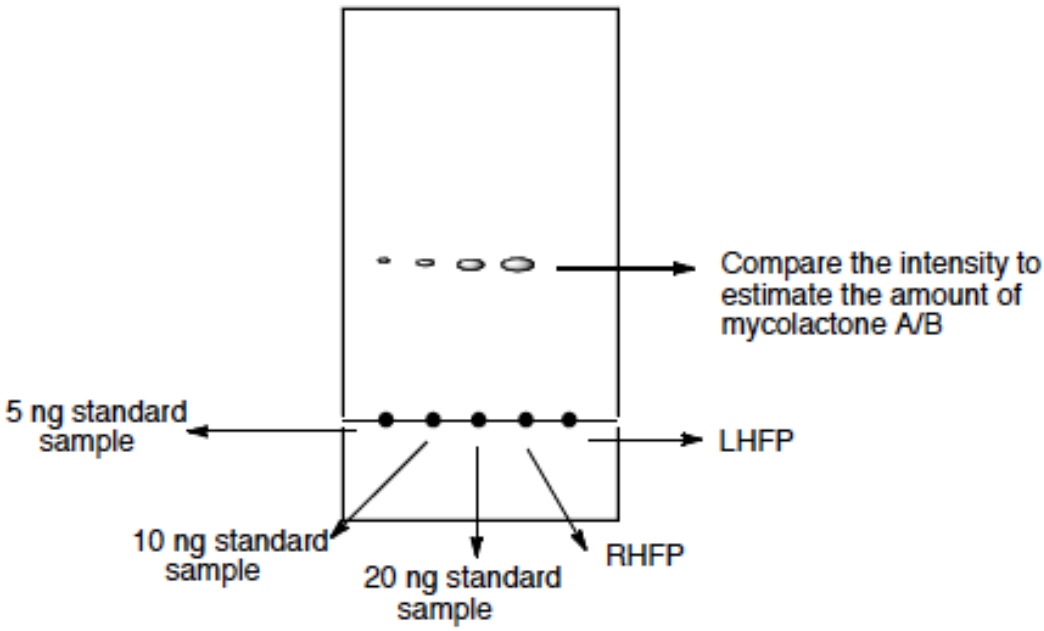
Detection of mycolactones by fluorescent thin layer chromatography (TLC)

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

Feasibility profiles to address each of the identified priorities were generated from the discussions. Criteria marked with a "–" indicate that this was not discussed during the meeting and so no value could be entered.

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

Health service level (minimum requirement): secondary (district hospital) level

Reagent performance

Criteria	Target spec. minimum	Target spec. optimum
Heat stability	Stable when stored for up to a year at 30 ^{0}C	Stable when stored for up to 5 years at 40 ^{0}C
Shelf-life	1 year	5 years

Assay performance

1. Analytical and diagnostic assay performance			
Criteria	**Target spec. minimum**	**Target spec. optimum**	**How measured? Standard?**
Sensitivity	90%*	As reference standard	Against IS*2404* PCR
Specificity	80%*	As reference standard	Against IS*2404* PCR
Potential to screen for other disease	No	Yes	–

* Values are provisional as no consensus was reached.

2. Assay workflow		
Criteria	**Target spec. minimum**	**Target spec. optimum**
Sample type	Fine needle aspirates	Non-invasive sample
Sample preparation	1 or 2 simple steps	None
Sample volume	100µl	–
Time to result	2 hours	15 minutes
Throughput	10 per day	–

3. Assay design		
Criteria	**Target spec. minimum**	**Target spec. optimum**
Type of analysis	Yes/No answer	–
Reading system	Simple equipment	Visual
Equipment required	Simple equipment	None
Infrastructure required	Basic district hospital laboratory (benches, electricity, running water etc.)	None
End-user profile	Qualified laboratory staff	Health worker (nurse, clinical officer, etc.)
Minimum training requirement	1 day	–
Cost	–	–

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

Health service level (minimum requirement): primary (health centre) level

Reagent performance

Criteria	Target spec. minimum	Target spec. optimum
Heat stability	Stable when stored for up to a year at 30 ^{0}C	Stable when stored for up to a 5 years at 40 ^{0}C
Shelf life	1 year	5 years

Assay performance

1. Analytical and diagnostic assay performance			
Criteria	**Target spec. minimum**	**Target spec. optimum**	**How measured? Standard?**
Sensitivity	90%*	As reference standard	Against IS*2404* PCR
Specificity	95%*	As reference standard	Against IS*2404* PCR

* Values are provisional as no consensus was reached.

2. Assay workflow		
Criteria	**Target spec. minimum**	**Target spec. optimum**
Sample type	Lesion swabs	Non-invasive sample
Reading system	Simple equipment	Visual
Sample preparation	1 or 2 simple steps	None
Sample volume	–	–
Time to result	2 hours	15 minutes
Throughput	–	–

3. Assay design		
Criteria	**Target spec. minimum**	**Target spec. optimum**
Type of analysis	Yes/No answer	–
Equipment required	Simple equipment	None
Infrastructure required	Primary level health post (no electricity, no running water)	None
End user profile	Health worker (nurse, clinical officer etc.)	Community volunteer
Training requirement	1 day	–
Cost	–	–

Outcomes

The following actions were agreed, with progress to be reported within the next 6 months.

Test	Action identified	Responsible person
Mycolactone detection	Test human samples (including FNA) by fluorescent TLC method after extraction with EtOAc instead of Folch's procedure to see if this excludes nonspecific bands	Steve Sarfo
Mycolactone detection	Develop WASP binding method using amphiphilic markers and phospholipid bilayer	Caroline Demangel
LAMP	Improve and simplify DNA extraction method	Tony Ablordey/ Zablon Njiru
Antigen capture	Optimize sensitivity and test on FNA samples	Katharina Röltgen
Mycolic acid antibodies	Arrange for human serum samples from BU patients and controls to be tested for specific antibodies to BU mycolic acids	Mark Baird and Richard Philips

Annexes

Annex 1: Agenda

WHO-FIND consultative meeting on diagnostics for Buruli ulcer

21 November 2013

WHO Headquarters, Room M-205

Agenda

	Chair: Mark Wansbrough-Jones	
09.00 – 10.30	Self-Introduction *(15 mins)*	All
	Welcome remarks *(10 mins each)*	Jean Jannin, WHO Catharine Boehme, FIND
	Buruli ulcer diagnosis and treatment *(15 mins)*	Kingsley Asiedu
	Overview of FIND's diagnostics development pipeline and expected outcome of the meeting *(15 mins)*	Joseph Ndung'u
10:30 – 10:45	***Coffee break***	
10:45 – 12:00	Mycolactone detection method – progress to date *(15 mins each)*	Yoshito Kishi Paul Converse Richard Phillips
	Development of a point-of-care antigen capture assay *(15 mins)*	Katharina Röltgen
	Discussions on gaps and future prospects - mycolactone method and antigen capture assay *(30 mins)*	All
12:00 – 12:45	LAMP diagnostic method – progress to date *(15 mins each)*	Anthony Ablordey Zablon Njiru
	Discussions on the gaps in diagnostics and future prospects DNA test *(15 mins)*	All
13:00 – 14:00	***Lunch***	
14:00 – 14:30	Other presentations *(10 mins each)*	Mark Baird Caroline Demangel Basil Swanson
14:30 – 15:30	Testing indication and required test attributes Draft target product profile Unmet needs beyond scope of FIND activities	All
15:30 – 15:45	***Coffee break***	
15:45 – 16:15	Plans to advance product development including partnerships	All
16:15 – 17:00	Wrap-up and closure	Chair
Reception	**Hosted by FIND**	

Annex 2: List of participants

External

Dr Anthony Ablordey, Noguchi Memorial Institute for Medical Research, Accra, Ghana – Tel: +233 275 652 022, E-mail: AAblordey@noguchi.ug.edu.gh

Professor Mark Baird, School of Chemistry, Bangor University Bangor, Gwynedd
LL57 2UW, United Kingdom – Tel: +44 1248 382375/2734,
E mail: m.baird@bangor.ac.uk.

Dr Paul Converse, Johns Hopkins University, Baltimore, United States of America – Tel: +1 410-502-8236, E-mail: pconver1@jhmi.edu

Dr Caroline Demangel, Unité d'Immunobiologie de l'Infection, CNRS URA1961, Département d'Immunologie, Pasteur Institute of Paris, Rue du Dr Roux, 75 015 Paris, France – Tel : + 33 1 40 61 30 66. E-mail: demangel@pasteur.fr

Dr Chris Gwenin, School of Chemistry, Bangor University Bangor, Gwynedd LL57 2UW, United Kingdom – Tel: +44 1248 382375/3741,
E mail: c.d.gwenin@bangor.ac.uk

Professor Yoshito Kishi, Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138, United States of America – Tel: +1 617 495 4679 / +1 617 496 5455, E-mail: kishi@chemistry.harvard.edu / quilty@chemistry.harvard.edu

Dr Zablon Njiru, Murdoch University, School of Professional Health, Peel Campus Mandurah, WA 6210, Australia – Tel: +61 958 255 04/ +61 404 522 785, E-mail: z.njiru@mucst.ac.ke / z.njiru@murdoch.edu.au

Dr Richard Phillips, Division of Infectious Diseases, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, United Kingdom – E-mail: rodamephillips@gmail.com

Dr. Katharina Röltgen, Department of Medical Parasitology and Infection Biology, Swiss Tropical and Public Health Institute, Socinstr. 57, CH 4002 Basel, Switzerland. Tel: +41 61 28 48 249, Fax: +41 61 28 48 101, E-mail: Katharina.Roeltgen@unibas.ch

Dr Thomas Spangenberg, Rue des Charmilles, 34, Geneva, Switzerland – E-mail: tomspangenberg@yahoo.fr

Dr Basil Swanson, Los Alamos National Laboratory, Los Alamos National Laboratory, P.O. Box 1663, Los Alamos, NM 87545, United States of America – Tel: +1 505 667 5814, E-mail: basil@lanl.gov

Dr Mark Wansbrough-Jones, Division of Infectious Diseases, St George's Hospital Medical School, Cranmer Terrace, London, SW17 0RE, United Kingdom – Tel: +44 20 8725 5831. E-mail: wansbrou@sgul.ac.uk / wansbrough-jones.2@blueyonder.co.uk

WHO

Dr Kingsley Asiedu, Department of Control of Neglected Tropical Diseases (NTD), World Health Organization , Avenue Appia 20, 1211 Geneva 27, Switzerland – Tel: +41 22 791 2803/2498, Email: asieduk@who.int

Dr Jean Jannin, Innovative & Intensified Disease Management (IDM), Department of Control of Neglected World Health Organization, Avenue Appia 20, 1211 Geneva 27, Switzerland –Tel: +41 22 791 3779/3878, Email: janninj@who.int

Dr Andrew Ramsay, Special Programme for Research & Training in Tropical Diseases (TDR), World Health Organization, Avenue Appia 20, 1211 Geneva 27, Switzerland – Tel: +41 22 791 1545, E-mail: ramsaya@who.int

FIND

Dr Sylvain Bieler, Foundation for Innovative New Diagnostics, Avenue de Budé 16, 1202 Geneva, Switzerland – Tel +41 22 710 27 81, E-mail: sylvain.bieler@finddiagnostics.org

Dr Catharine Boehme, Foundation for Innovative New Diagnostics, Avenue de Budé 16, 1202 Geneva , Switzerland – Tel +41 22 710 93 16, E-mail: catharina.boehme@finddiagnostics.org

Professor Joseph Ndung'u, Foundation for Innovative New Diagnostics, Avenue de Budé 16, 1202 Geneva, Switzerland – Tel +41 22 710 93 11,
Email: Joseph.Ndungu@finddiagnostics.org

Dr Mark Perkins, Foundation for Innovative New Diagnostics, Avenue de Budé 16, 1202 Geneva, Switzerland – Tel +41 22 710 05 92, E-mail: mark.perkins@finddiagnostics.org