Evaluation of Two Commercial Nucleic Acid Amplification Tests for Diagnosis of Extrapulmonary Tuberculosis from Lymph Nodes of Children in Tanzania

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Prof. Dr. Jörg Schibler
Dekan

To my late Parents
Fatehali and Sherbanu Bholla.
Although your time with us was short,
your legacies live on these 20 and 30 years later
and will continue to do so.

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ABBREVIATIONS

°C	temperature in degrees centigrade
22/23G	22/23 gauge
AFB	acid-fast bacilli
AKHD	The Aga Khan Hospital, Dar es Salaam
BAL	broncho-alveolar lavage
BCG	bacillus Calmette-Guerin
BRTC	Bagamoyo Research and Training Centre (Ifakara Health Institute)
cfu	colony-forming units
CI	confidence interval
CPA	cross-priming amplification
CRF	clinical record form
CRI	colorimetric redox indicator
CXR	chest X-ray/ radiography
ddH_2O	double distilled water
DNA	deoxyribonucleic acid
DR	drug resistance
DST	drug susceptibility testing
EasyNAT	Ustar EasyNAT™ TB IAD (Biotech) kit
EDCTP	European and Developing Countries Clinical Trials Partnership
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
EMB	ethambutol
EPTB	extra-pulmonary tuberculosis
et al.	“and others”
FBP	full blood profile or haemogram
FDC	fixed-dose combinations
FIND	Foundation for Innovative New Diagnostics
FNA	fine needle aspiration
FNAB	fine needle aspiration biopsy
FNAC	fine needle aspiration cytology
GC	growth control
HBCs	(TB) High Burden Countries
HCl	hydrochloric acid
HIV	human immunodeficiency virus
HRMA	high-resolution melting-curve analysis
IAD	isothermal amplification diagnostic (kit)

ICF	informed consent form
IGRA	interferon-gamma release assay
IHI	Ifakara Health Institute, Tanzania
INH	isoniazid or isonicotinic acid hydrazide
IRB	Institutional Review Board
LAM	Lipoarabinomannan
LED	light emitting diode
LJ	Lowenstein-Jensen
LoC	Lab-on-chip
LPA	line probe assay
LTBI	latent tuberculosis infection
MBL	mannose-binding lectin
MDR	multidrug resistant
MGIT	mycobacterial growth indicator tube (Becton Dickinson)
ml	milliliter
mM	millimole
mm^3	cubic millimeters
MODS	microscopic observation drug susceptibility assay
MTA	Material transfer agreement
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MUHAS	Muhimbili University of Health and Allied Sciences, Dar es Salaam
NA	nucleic acid
NAAT	nucleic acid amplification tests
NALC	N-acetyl-L-cysteine-sodium hydroxide
NaOH	sodium hydroxide
ng	nanogram
nm	nanometer
NRA	nitrate reductase assay
NTLP	National TB and Leprosy Programme
NTM	non-tuberculous mycobacteria
PAP	Papanicolaou (stain)
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pmol	picomole
POC	point of care
PTB	pulmonary tuberculosis
RIF/RMP	rifampicin/rifampin

rpm	revolutions per minute
rpoB	portion of the Mycobacterium tuberculosis gene encoding the beta subunit of RNA polymerase
RRDR	rifampin-resistance determining region
RTLC	Regional TB and Leprosy Programme Coordinator
SNSF	Swiss National Science Foundation
SOP	Standard Operating Procedure
Swiss TPH	Swiss Tropical and Public Health Institute
TB	tuberculosis
TDH	Temeke District Hospital, Dar es Salaam
TFE	tetrafluoroethylene
TST	tuberculin skin test
U	unit
μl	microliters
UV	ultra violet
VOC	volatile organic compounds
WGS	whole genome sequencing
WHO	World Health Organization
XDR	extensively drug-resistant
xg	relative centrifugal force (RCF) units in gravitational force
Xpert	Xpert® MTB/RIF (Cepheid) kit
X-ray	radiography
ZN	Ziehl-Neelsen

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“One child, one teacher, one book, and one pen, can change the world.”

Malala Yousafzai – 16year old Nobel Prize Winner, 12th July 2013

FRAMEWORK

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SUMMARY

Approximately one third of the global population is latently infected with tuberculosis (TB) and there are approximately 9.6 million new cases of TB disease per year resulting in 1.5 million deaths. Eleven percent of cases globally occur in children and 81% of the burden of TB disease is borne by the developing world and countries with emerging economies (BRICS). The African region accounts for 28% of new TB cases globally.

TB remains a significant public health concern globally, particularly amongst children and immunocompromised individuals. Diagnosis of childhood TB is an on-going challenge, as children usually do not present with the same signs and symptoms as adults, and are often misdiagnosed. Tuberculosis infection in children is seldom confirmed through sputum culture, as good sputum samples can rarely be collected. Only 15% of cases from children are sputum smear positive by acid-fast staining, and only 30%–40% are *Mycobacterium tuberculosis* (MTB) culture positive. Up to 40% of children present with extrapulmonary manifestations of TB disease. The most common manifestation is tuberculous lymphadenopathy. Good specimens for TB detection can be obtained from these cases through fine needle aspiration biopsy (FNAB), a cost-effective and practical out-patient procedure for obtaining specimens from enlarged superficial lymph nodes.

The conventional laboratory techniques that have been used globally for TB diagnosis are Ziehl-Neelsen (ZN) staining for acid fast bacilli (AFB) microscopy, culture and more commonly now LED microscopy, cytological determination with auto-fluorescence staining and molecular Xpert® MTB/RIF (Cepheid) detection. AFB-microscopy requires minimally 5000 AFB per millilitre of specimen to yield a consistently positive result and observation of between 100 and 300 microscopic fields in order to obtain accurate results. Culture for MTB is the gold standard diagnostic method but has a slow turnaround time and requires laboratory resources that are not available in most parts of the world.

Recent systematic reviews of studies evaluating commercially available nucleic acid amplification test (NAAT) technologies confirm very high specificity, with sensitivity approaching, but not reaching, that of culture. The

complexity and insufficient robustness of existing commercial NAAT protocols and their need for precision instruments, a high degree of technical support, and quality assurance make them unsuitable for most low-resource TB-endemic countries. In addition, none of these techniques have been fully validated for diagnosing TB in children and specifically not for extrapulmonary specimens.

In light of these challenges, there is promise in two technologies that have been developed and under evaluation over the last few years; the Xpert® MTB/RIF kit (Cepheid) for rapid detection of MTB and rifampicin resistance endorsed for use by the WHO; and the Ustar EasyNAT™ TB IAD kit (Ustar Biotechnologies, Hangzhou) for detection of MTB, selected by the WHO as a technology on assessment for use in TB endemic countries. In this study, their performance was assessed against the conventional laboratory diagnostic techniques of smear AFB microscopy, cytology and mycobacterial culture of fine needle aspirates from lymph nodes of children suspected of TB lymphadenopathy in Tanzania.

Age-defined clinical assessments were done for all 75 participants and TB treatment initiated based on these and/or laboratory diagnostic outputs. All laboratory diagnostic modalities were primarily assessed against TB culture as the conventional reference standard. As has been evidenced in earlier studies, the sensitivities for both smear microscopy and TB culture were very low in these extrapulmonary specimens. Lacking a true reference standard, composite reference standards (CRSs) were created to assess the performance of the test modalities under study. An alternative method for assessing diagnostic accuracy under these conditions is latent class analyses (LCA), which was utilized to further assess the performance of all diagnostic modalities in the study.

The overall outcomes of the project demonstrated that cytomorphology was a feasible and effective technique for detection of TB in lymph node aspirates (sensitivity: LCA 100%, specificity: LCA 94.7%) that may complement TB culture (sensitivity: LCA 74.5%, specificity: LCA 90.3%). Further, it was shown that Xpert (sensitivity: CRS 58% LCA 70.7%, specificity: CRS 93% LCA 94.2%) was superior in performance to EasyNAT (sensitivity: CRS 19% LCA 29.2%, specificity: CRS 100% LCA 100%) and ZN (sensitivity: CRS 14% LCA

19.1%, specificity: CRS 100% LCA 100%) analyses, respectively. Combining two or more tests significantly improved the diagnostic efficacy, but including either EasyNAT testing or ZN microscopy to a diagnostic algorithm that already had Xpert testing added no value.

These findings indicate that combining clinical assessment, cytology and Xpert MTB/RIF can provide for rapid and accurate diagnosis of childhood tuberculous lymphadenitis. Larger diagnostic evaluation studies on Xpert MTB/RIF would be required, to assess its use as a solitary initial test for tuberculous lymphadenitis in children.

INTRODUCTION

TUBERCULOSIS, EPIDEMIOLOGY AND CLINICAL MANIFESTATIONS

The *Mycobacterium tuberculosis* complex (MTBC), comprises of several mycobacterial species which cause tuberculosis (TB) in a variety of hosts: *M. tuberculosis*, *M. africanum*, *M. canetti* (all cause TB in humans), *M. microti* (infect voles), *M. bovis* (mainly infect cattle), *M. caprae* (infect sheep and goats), *M. pinnipedi* (infect seals and sea lions) and the vaccine strain Bacillus Calmette-Guérin (BCG) (Smith et al., 2006). More recently, two novel members of the MTBC were discovered through sequence analyses: *M. mungi* that infects banded mongoose in Botswana and; *M. suricattae* that infects meerkats in Southern Africa (Alexander et al., 2010; Dippenaar et al., 2015). *Mycobacterium tuberculosis* (MTB) is the main causative agent of human tuberculosis. It is a member of the family *Mycobacteriaceae*: aerobic bacilli (rod-shaped bacteria) with cell walls impervious to Gram staining (gram negative).

Approximately one third of the global population is latently infected with TB. There were an estimated 9.6 million new cases of TB disease globally in 2014, with 1.5 million deaths (WHO, 2015a). Between 10.4% and 11% of cases globally occur in children (WHO, 2015a; Perez-Velez and Marais, 2012) and 81% of the burden of TB disease is borne by the developing world and countries with emerging economies (Brazil, Russia, India, China and South Africa [BRICS]). The African region accounts for 28% of new TB cases (WHO, 2015a).

Tuberculosis remains one of the commonest causes of childhood mortality in the world. Recent modelling data suggest that over 650,000 children develop TB every year in the 22 countries with the highest TB burden (Dodd et al., 2014). The World Health Organization (WHO) estimated 1 million new childhood TB cases worldwide in 2014 (WHO, 2015a). Case reporting depends on a number of factors that vary per region and can range from 5% in low-burden countries to 20-40% in high-burden countries (Swaminathan and Rekha, 2010). Prevalence reports from a rural setting in Ethiopia showed that 46.3% of reported cases of TB were in children (<15yrs) (Ramos et al., 2010), while a study conducted in the Kilimanjaro Region of Tanzania

estimated the childhood TB burden to be 13% of the total burden of the country (Mtabho et al., 2010), making it one of the regions with the highest childhood TB prevalence in the world.

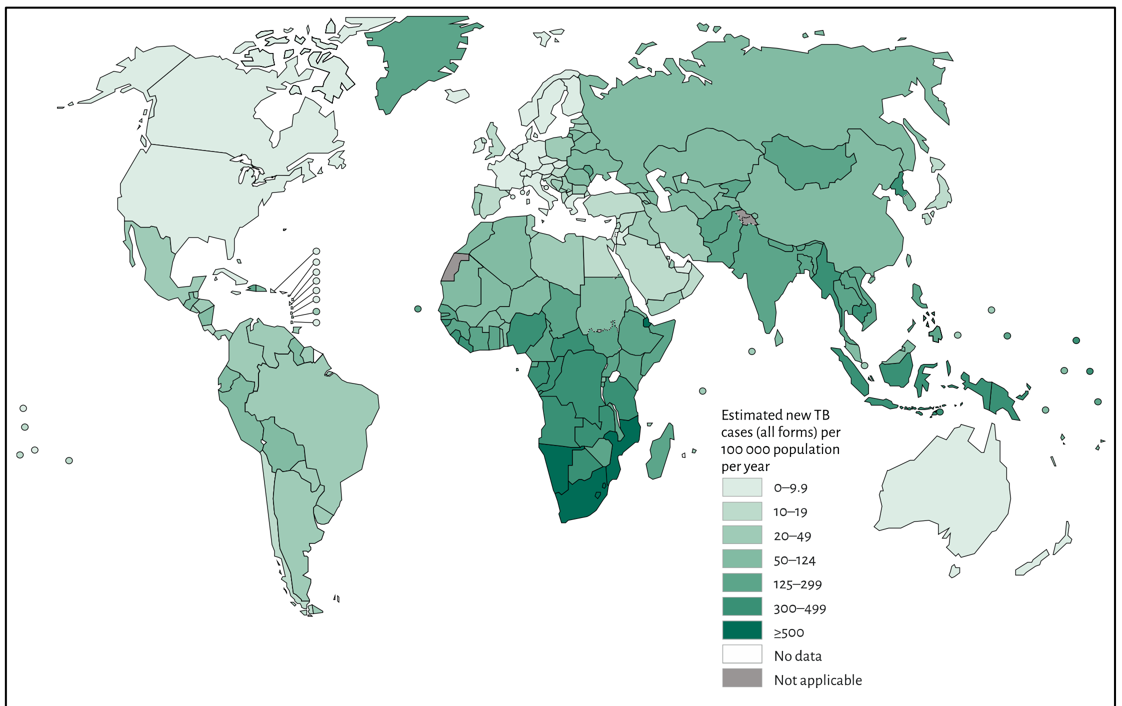

Figure 1: Estimated global TB incidence rates, 2014.
Source: Global Tuberculosis Report 2015, World Health Organization (WHO, 2015a).

The key mode of transmission of TB is inhaled aerosols containing MTB, formed when persons with pulmonary tuberculosis cough or sneeze. The primary site of infection in the lungs is known as a Ghon focus, usually visible on chest X-ray. In asymptomatic individuals, this may be an indicator of latent TB infection (LTBI). It is important to distinguish between infection and disease (active TB) in tuberculosis. The introduction and establishment of the bacterium in an individual constitutes infection while bacterial proliferation in the lungs or other tissues, conferring development of persistent and non-remitting symptoms, denotes active infection or disease. In TB disease, the bacteria replicate in macrophages and affect the surrounding tissue, causing abnormal cell death (necrosis). This occurs within structures known as tubercles in the lungs, also visible on X-ray examination.

Although TB primarily manifests in the lungs (pulmonary), it can affect multiple systems of the body outside of the lungs and chest cavity. This is known as extrapulmonary TB (EPTB) with a large variety of presentations and

manifestations in both adults and children. Though not well reported, the global incidence of EPTB in 2012 was 18% of all new cases (WHO, 2013a), with a potentially higher prevalence. Progression to death has been found to be significantly higher in EPTB patients than in pulmonary TB (PTB) patients. Immune suppression and old age has been shown to predispose patients to EPTB development (Fortún et al., 2014a).

Active TB in adults can develop either from recent infection or a reactivated LTBI, whereas in children it usually results from recent infection. It is found that children with TB disease usually have far fewer bacteria in their lungs (paucibacillary in nature) than in adults and it is rare for children to be the source of transmission to adults or other children (Logitharajah, 2008), although it is possible in older children or those with cavitating TB disease (Schaaf et al., 2007). Childhood TB is therefore often considered an indicator of TB transmission in the community in high endemic settings, with children older than 3 years being infected through contact within the community rather than through household contact (Marais et al., 2009).

IMMUNOLOGY OF TB INFECTION AND DISEASE

Once in the lungs the bacteria invade and replicate in alveolar macrophages. T-lymphocytes, B-lymphocytes and fibroblasts surround these macrophages, all of which aggregate to form granulomas. At this point, there may be one of three outcomes of TB infection. First, the host immunity may be able to eliminate the infection completely through successful phagocytosis. Second, by inhibiting fusion of phagosomes with lysosomes, MTB prevents its own digestion, thriving within macrophages, multiply within the lung and other tissues; developing disease. Third, the host immune response acts to prevent dissemination of the mycobacteria, which become dormant leading to LTBI (Ernst, 2012). As such 90% of people infected with MTB develop LTBI (Skolnik, 2011). These different outcomes of the infection are often not clear-cut and patients may present in transition states. The lifetime risk of developing active TB from LTBI (re-activation) in immune-competent adults is 10%. Re-activation occurs after several years, leading to clinical disease referred to as secondary infection.

CD4+ T-cells play a major role in the formation of granulomas, as they produce cytokines such as interleukin-12 (IL-12) and interferon gamma (IFN-γ), which are activating agents for macrophages. As CD4+ T-cells counts are usually low in advanced HIV infection, HIV positive patients have an increased risk (up to 40-fold) of being infected with TB (Brites and Gagneux, 2012). Accordingly, it has been found that LTBI patients co-infected with HIV have an incremental risk of reactivation, of 10% per year (Mainous and Pomeroy, 2010).

DISEASE PREVENTION

Since the development of the Bacillus Calmette-Guerin (BCG) vaccine (attenuated live bovine tuberculosis bacillus, *Mycobacterium bovis*) in the 1920's, there has been no other vaccine against TB endorsed for use in humans. BCG protects against miliary TB and TB meningitis in immune-competent children, but does not confer protection against primary infection or reactivation of latent pulmonary infection in both children and adults (WHO, 2004).

There have been some vaccine candidates in development since the year 2000. These candidates fall into four categories based on their expected modes of action. The first are prime vaccines, designed to replace the BCG vaccine, through their modification or genetic attenuation of MTB. The second are prime-boost vaccines, designed to improve the limited immunity conferred by the BCG vaccine. This is through transportation of MTB deoxyribonucleic acid (DNA) into human cells, where the DNA is transcribed into antigens, or by combining different MTB antigens with adjuvants that boost the body's natural immunity. The third are immunotherapeutic vaccines designed to shorten the timeframe of TB treatment regimens. The fourth are whole-cell vaccines constructed from inactivated mycobacteria related to MTB, such as *Mycobacterium vaccae*. The first TB vaccine efficacy trial since 1968, was on the prime-boost vaccine MVA85A. The trial, run in South Africa between 2009 and 2011, demonstrated that MVA85A did not confer significant added protection against TB to infants vaccinated BCG (Tameris et al., 2013).

Apart from vaccination, the focus of disease prevention should be the reduction of risk factors associated with TB. These factors include poor

ventilation, overcrowding, immune-compromising conditions and drugs, diabetes, smoking, alcohol abuse, and some social-economic and behavioural factors (Narasimhan et al., 2013).

TB DIAGNOSIS

In adults, common signs and symptoms of TB disease include fever, chills, night sweats, loss of appetite, weight loss, and fatigue. These are used in clinical assessment to guide treatment choices. Pulmonary TB usually presents with a Ghon focus and other lung changes that may be detected through chest radiography (X-ray). A productive cough is common in adults whereby sputum samples can be tested for MTB to diagnose TB in adults.

Children often do not present with these signs, symptoms or cough in primary TB infection and it is therefore difficult to diagnose. (Please refer to section: 'Disease Progression and Manifestations of TB in Children' for this information).

Laboratory based TB diagnostics

Diagnosis of latent TB infection:

About three to twelve weeks after primary infection with MTB, cell-mediated immune priming occurs in immune-competent patients. This immune memory persists through the person's lifetime, unless lost due to immune system dysfunction or old age. The **Mantoux** or tuberculin skin test (TST) is a delayed-type hypersensitivity reaction occurring through this immunity, to a purified protein derivative of a human-infective strain of *M. tuberculosis* injected intradermally. In clinical routine LTBI is defined as a positive *M. tuberculosis*-specific immune response (e.g. TST) in the absence of active TB disease. False-positive TST results have been observed in children inoculated with BCG up to 18 weeks prior to Mantoux testing, as well as those exposed to non-tuberculous or environmental mycobacteria (NTM), long after neonatal BCG vaccination (Farhat et al., 2006; Ota et al., 2006; Burl et al., 2010).

A negative TST result does not exclude the possibility of TB infection because many disseminated forms of TB, including miliary and meningitis, can induce anergy or absence of the normal immune response to the skin test. Up to 15% of children who have clinical TB have negative TST results. A false-negative

TST result has also been associated with recent measles infection, high-dose corticosteroid treatment, irradiation, other immunosuppressive therapy, or immune-compromising medical conditions such as HIV infection (Cruz and Starke, 2010).

Another method of testing for LTBI is the **interferon-gamma release assay (IGRA)**. Commercially available assays include the T-SPOT® TB (enzyme linked immunosorbent spot test, Oxford Immunotec, Oxford, UK) and the QuantiFERON® TB Gold (Cellestis, Carnegie, VIC, Australia). The antigens in these assays do not cross-stimulate BCG-primed lymphocytes and potentially overcome the problem of determining whether the condition is LTBI or a reaction to vaccination (Ewer et al., 2003; Liebeschuetz et al., 2004; Pai et al., 2004). Both TST and IGRAs however, do not distinguish between active and latent TB infection, but indicate exposure to TB (leading to the cell-mediated immune priming). The recommendation for diagnosis of LTBI is therefore; a positive TST result, epidemiologic information (exposure to a known source case) and a compatible clinical picture.

Diagnosis of active pulmonary and extrapulmonary TB:

Direct visualisation techniques:

Ziehl-Neelsen staining is the most widely available technique for the detection of TB in smears (sputum or other fluids) from samples of active TB patients. It is a special bacteriological stain used to identify acid-fast bacilli (AFB), mainly mycobacteria, as these organisms are not decolorized by the acid:alcohol mixture used in the technique once carbol-fuchsin stains the lipid-rich cell wall. It is therefore not specific to MTB. AFB microscopy requires minimally 5000 AFB per millilitre of specimen to yield a consistently positive result (Charnace and Delacourt, 2001). In addition, the procedure requires observation of between 100 and 300 microscopic fields in order to obtain accurate results (Somoskovi et al., 2001). Only up to 15% of cases of children are found to be sputum AFB smear positive (Eamranond and Jaramillo, 2001). Notwithstanding, adolescent children frequently develop sputum smear-positive adult-type disease and sputum microscopy has diagnostic value in this subset of children (10 to 14 years) (Marais et al., 2005).

Cytomorphology is quickly becoming one of the more reliable visual detection methods for identifying MTB (Mittal et al., 2011). Smears of host cells from the

focus of infection are stained using **Papanicolaou (PAP)** stain and the microscopic examination requires well-trained staff. The cell morphology is used as a proxy for the presence of AFB and has been found to be fairly accurate in indicating the presence of MTB (Wright et al., 2008).

Some microorganisms are able to re-emit light upon light excitation known as **autofluorescence**. One such organism is MTB which fluoresces under light-emitting diode (LED) lighting using specific filters within the cyan range of light (Kumar et al., 1998). A large study comparing routine PAP stained slides of samples from MTB infected lymph nodes and the same glass slides viewed under fluorescence microscopy, demonstrated that cytomorphology had 84.9% sensitivity in detecting presence of MTB, but had low specificity of only 50.9%. Autofluorescence demonstrated lower sensitivity of 65.9%, but improved specificity of 73.0%. On combining the two techniques, the specificity improved to 81.8% (Wright et al., 2004). MTB autofluorescence assays are relatively sensitive (<106 bacilli/ml of sputum can be detected). Moreover, the use of microplates allows for examination of only 200 microlitres of sputum per sample without a loss of sensitivity (Patino et al., 2008).

LED bulbs emit a very narrow spectrum of light and have a longer life span and lower energy requirements than the conventional mercury light bulbs used in microscopes. LED bulbs are also able to excite auramine and other commonly used **fluorescent stains** without the need of UV lighting (Marais et al., 2008; Annam et al., 2009b).

Growth-based detection techniques:

Bacterial culture remains the gold standard for TB detection in a variety of specimens from patients suspected of TB disease. The current WHO endorsed growth-based tests for TB have been standardized mainly for sputum specimens (Dorman, 2010) and these techniques are usually feasible only in reference laboratory or high-resource settings, not for resource-limited TB-endemic regions. They are expensive, require specialised training and appropriate laboratory infrastructure (biosafety level 3). Amongst others, they include culture in liquid media using MGIT (Becton Dickinson) and BacT/TB ALERT (BioMérieux). Liquid culturing is found to be more sensitive and faster in TB growth detection than solid media, but is prone to contamination.

Sputum TB culture positivity can be detected within 10 to 14 days of inoculation, but culture-negative reports require up to 6 weeks for confirmation.

Both solid and liquid media are used to assess drug susceptibility (and resistance) of cultured mycobacteria through drug minimum-inhibitory-concentration (MIC) determination. Colorimetric methods, such as the nitrate reductase assay (NRA) and colorimetric redox indicator (CRI) methods performed on cultures on solid media, reduce the time to detection compared to conventional MIC-based drug sensitivity testing (DST). These are being encouraged for use in resource-limited settings, as they are cost-effective and highly sensitive and specific for most of the first-line anti-TB drugs (Coban et al., 2014; Arentz et al., 2013).

TB disease in children is seldom confirmed through bacterial culture, as standard sputum samples can rarely be obtained from children less than 10 years of age. If sputum collection is successful, only 30%–40% of the cases are mycobacterial culture positive (Shingadia and Novelli, 2003; Planting et al., 2014). Culture also generally has low sensitivity in EPTB specimens (Khan et al., 2006; Trajman et al., 2008; Polesky et al., 2005).

The microscopic observation drug susceptibility assay (**MODS**) is a novel method of mycobacterial growth and drug susceptibility assessment, with good sensitivity and a mean-time-to-positivity of 8.24 days (Wikman-Jorgensen et al., 2014). It uses an inverted light microscope to rapidly detect 'spindle and cord formation' in selective broth culture that is indicative of mycobacterial growth (Moore et al., 2006). The assay is performed directly on sputum samples, which are difficult to obtain from children who also present with paucibacillary disease, therefore requiring further investigation into its use in paediatric specimens (Logitharajah, 2008).

<u>Volatile organic compounds, phage amplification and biomarkers:</u>

There are tools, still in the early phase of development, that have demonstrated potential for use in the clinical setting. The Breathalyzer screening test is designed to detect volatile organic compounds (**VOC**) from the breath of patients with active pulmonary TB. VOCs may comprise of metabolic biomarkers, from the MTB organism such as cyclohexane and

benzene derivatives, and from the infected host as products of oxidative stress such as alkanes and alkane derivatives. A study using gas chromatography or mass spectroscopy (breathalyzer screening) assessed the feasibility of this technology in diagnosing TB. The technique was 84% sensitive and 64.7% specific in detecting active pulmonary TB. It has potential use in clinical practice but is highly specialised and very expensive (Phillips et al., 2010). Cricetomys rats are being trained to detect TB in sputum specimens purportedly through their ability to identify VOCs being emitted from these specimens (Mgode et al., 2012; Reither et al., 2015), and more recently are potentially able to detect different genotypes of MTB (Mgode et al., 2015).

Phage amplification assays, that use bacteriophages (bacterial viruses) to detect the presence of viable MTB within 24 hours of specimen preparation, are promising for rapid TB detection in clinical respiratory specimens. The technology is based on mycobacteriophages replicating within live host mycobacteria in-vitro. The unabsorbed phages are inactivated using a virucidal solution and *Mycobacterium smegmatis* is mixed with the specimen on agar plates whereby progeny phages infect, replicate in and lyse the *M. smegmatis*, forming clear zones or plaques. These plaques on agar plates represent the number of viable tubercle bacilli in the original specimens. If there are no plaques, it indicates that there were no viable mycobacteria. There are currently limited studies in support of this technology (Prakash et al., 2009).

For **biomarkers** such as adenosine deaminase, interferon gamma and lysozyme (muramidase), further developments and studies are still required to determine their use and feasibility in TB detection (Dinnes et al., 2007).

<u>Diagnostic antibody and antigen detection techniques:</u>

With the inaccuracy, inaccessibility or expense of most of the technologies mentioned, serological tests hold the potential to pre-empt missed pulmonary and extrapulmonary TB diagnoses. Unfortunately, recent meta-analyses on studies from 1990 to 2010 have shown that all serology-based assays, including enzyme-linked immunosorbent based assays (ELISA) incorporating MTB specific antigens including lipoarabinomannan (LAM), have been highly inconsistent in sensitivity and specificity (Flores et al., 2011; Steingart et al.,

2011). Presently, the 2011 WHO recommendation that serological tests not be used for the diagnosis of pulmonary and extrapulmonary TB still stands, due to the highly inconsistent sensitivity and specificity of these tests and the low quality of studies conducted (WHO, 2011a).

Nucleic acid based technologies:

Polymerase chain reaction (PCR) and other nucleic acid based methods for TB detection have the advantage of speed (compared to culture) and sensitivity (compared to microscopy) (Boehme et al., 2007), including in paucibacillary specimens or those with minute amounts of nucleic acid. Pyrosequencing is a method of deoxyribonucleic acid (DNA) sequencing based on the "sequencing by synthesis" principle. It differs from Sanger sequencing, in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. A 2013 meta-analysis determined that pyrosequencing is a highly sensitive and specific tool that significantly reduces the time taken to test for rifampicin (RIF) resistance in TB (Guo et al., 2013), and more recently specifically in EPTB specimens for most first- and second-line drugs, although sensitivity in RIF resistance determination in this case was limited (67%) (Georghiou et al., 2016).

Whole genome sequencing (WGS), the complete DNA sequencing of an organism's genome at a point in time, is now seen as a new frontier for management of drug resistance in patients and even prediction of drug resistance mechanisms when developing new drug targets (Witney et al., 2015; WHO, 2015b). Due to the financial and technical investments required for most sequencing technologies, they are usually restricted to reference level and research laboratories.

High-resolution melting-curve analysis (HRMA) is gaining credence as a screening technology for drug-resistance mutations, without the need for specific probes, which has a low turnaround time and is potentially more cost effective than other nucleic-acid based technologies. A study on RIF resistance in MTB, using HRMA compared to a line-probe assay (LPA) that utilizes nucleic acid amplification technology and conventional MIC-based DST from culture, showed that HRMA has potential as a rapid screening method for drug resistance detection in TB (Malhotra et al., 2015). More

studies are needed before global health authorities can endorse HRMA-based tests for use in TB assessment.

Systematic reviews over the last decade have shown that nucleic acid amplification tests (NAAT) have significant potential in TB diagnostics. They are highly sensitive in pulmonary TB diagnosis and highly specific in extrapulmonary forms of TB (Dinnes et al., 2007) and some have been recommended for use alongside the conventional tests for TB (Ling et al., 2008). Swifter developments are therefore being encouraged for rapid and efficient NAAT based DSTs for TB (Wells et al., 2013).

Some commercial NAATs for TB detection currently available include the Roche LightCycler® Mycobacterium detection kit (Roche Applied Science, IN, USA), the Loopamp TB Detection (FIND, Switzerland/ Eiken, Japan), the Anyplex^{TM} TB series of assays (Seegene, Seoul, Korea), the Genedrive MTB/RIF ID (Epistem, United Kingdom), the ProbeTec Direct (DTB), the Pure LAMP (Eiken Chemical Company, Japan) and semi-automated ProbeTec ET system (Becton Dickinson, NJ, USA) and FluoroType MTB (Hain Lifescience, Germany), amongst others. These technologies have either been found to have significant flaws, have insufficient evidence to be recommended for use by expert review panels or are still under development (HIV i-Base/Treatment Action Group, 2015).

Commercial NAAT systems for TB detection in respiratory specimens, that are currently FDA approved, include the real-time PCR based COBAS TaqMan® MTB (Roche Molecular Systems, CA, USA) and the AMPLIFIED^{TM} *M. Tuberculosis* Direct Test including the enhanced AMPLIFIED *M. Tuberculosis* Direct Test (Gen-Probe, CA, USA) and BDProbeTec^{TM} (Becton Dickinson, NJ, USA) (Rie et al., 2010). These systems are in use in tertiary healthcare facilities or at reference laboratories, and do not fit the point-of-care (POC) diagnostic requirements of resource-limited TB-endemic settings.

WHO-endorsed nucleic acid based tools to date include the molecular line probe assays (LPAs) for first-line or multi-drug resistance (MDR) TB detection (GenoType® MTBDRplus [Hain Lifescience, Nehren, Germany], INNO-LiPA Rif. TB [Innogenetics, Gent, Belgium]) and the real-time PCR based Xpert® MTB/RIF ([GeneXpert] Cepheid, Sunnyvale CA) for TB detection and

assessment for rifampicin resistance (WHO, 2010, 2013a). Line probe assays use a PCR/hybridization technique to identify members of the MTBC, while simultaneously identifying drug-resistant strains by detecting the most common single nucleotide polymorphorisms (SNPs) associated with resistance. They have lower sensitivity when testing direct sputum, implicating the need to test culture isolates, increasing cost, specimen manipulation and the potential for cross contamination. The Xpert® MTB/RIF system is designed to test direct sputum specimens (refer to the section The Xpert® MTB/RIF test [Cepheid]), significantly shortening the time to detection. All the listed NAAT have been standardized for respiratory specimens.

Following a 2013 systematic review of the performance of Xpert® MTB/RIF, the WHO updated its recommendation for the use of Xpert® MTB/RIF on extrapulmonary specimens (WHO, 2013b). The Xpert® MTB/RIF was recommended for use in preference over conventional microscopy and culture for CSF specimens and as a replacement test as-needed, for conventional tools for specific non-respiratory specimens in both adults and children (WHO, 2013c). This update notwithstanding, the studies chosen for the systematic review were found to be very heterogeneous in sample processing and ‘representative patient spectrum’ amongst other limitations, and further studies with defined guidelines were encouraged (Denkinger et al., 2014).

TB TREATMENT AND DRUG RESISTANCE

TB exposure, bacillary load of the positive case and anatomic foci of infection, are key considerations in disease management for both adults and children.

Antibiotics against TB have been in development for less than a century. Streptomycin was first isolated in 1943 (Comroe, 1978) and isoniazid was synthesized in the early 20th century, but its activity against tuberculosis was first reported in the early 1950s (Rieder, 2009).

Figure 2 illustrates the different categories of anti-TB medications that have been developed over the years and their mechanisms of action. For many years, preventive treatment with isoniazid (INH) a nitroimidazole, for a period of 9 months had been recommended to reduce the risk of progression of LTBI into active disease (Wilkinson, 2000). More recently a combination of INH and

rifapentine (an inhibitor of bacterial RNA synthesis) for a reduced duration of 3 months, has been suggested for high-risk groups, including children exposed to TB in households and HIV positive patients (Sterling et al., 2011).

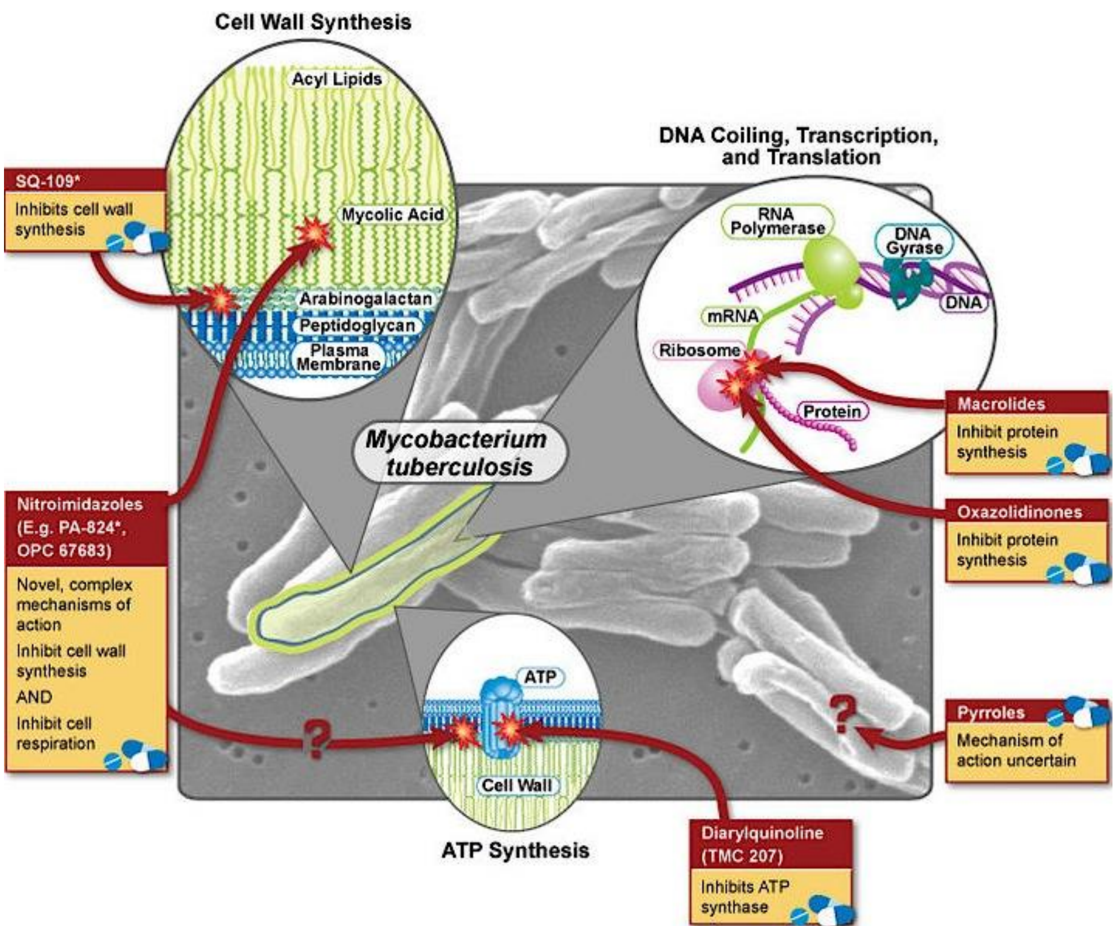

Figure 2: TB drug categories and their modes of action.
Source: https://www.niaid.nih.gov/sitecollectionimages/topics/tuberculosis/tb4.jpg

Table 1 defines: the anti-TB drug categories, drug-resistance profiles, TB patient categories based on treatment history, WHO-recommended regimens for TB treatment by category, and definitions of the different treatment outcomes. Fixed-dose combinations (FDC) are defined dose combinations of anti-TB drugs developed into single tablets, to reduce the pill burden, aimed at enhancing patient drug compliance.

During the Intensive phase of treatment, the drugs kill off TB bacilli in patients, with the patients usually becoming asymptomatic and non-infectious. The Continuation phase is usually longer, with the aim of eliminating any viable bacilli due to the fastidious nature of TB infection. Patients can therefore be

cured from TB infection, but there is significant anti-TB drug resistance being observed now in many settings worldwide.

Table 1: Definitions, categories and regimens of TB treatment

Definitions	
First-line TB drugs	Isoniazid (INH), rifampicin (RMP), ethambutol (EMB), parazinamid (PZA), streptomycin (STR)
Second-line TB drugs	Fluoroquinolones (e.g. moxifloxacin) and the injectible drugs amikacin, kanamycin, capreomycin
Pansusceptible TB	Susceptible to all 5 first-line TB drugs
Types of Resistance	
Monoresistant TB	Resistant to a single drug of the first-line treatment
Poly drug-resistant TB	Resistant to more than one of the first-line treatment drugs (other combinations than INH and RMP)
Multi drug-resistant TB	Resistant to at least RMP and INH, the two main first-line treatment drugs
Extensively drug-resistant TB	MDR TB with additional resistance to a fluoroquionolone and to at least one of the second-line injectible drugs (amikacin, kanamycin and/or capreomycin)
Treatment Outcomes	
Cured	A pulmonary TB patient with bacteriologically confirmed TB at the beginning of treatment who was smear- or culture-negative in the last month of treatment and on at least one previous occasion.
Treatment completed	A TB patient who completed treatment without evidence of failure BUT with no record to show that sputum smear or culture results in the last month of treatment and on at least one previous occasion were negative, either because tests were not done or because results are unavailable.
Treatment Failure	A TB patient whose sputum smear or culture is positive at months 5 or later during treatment
Died	A TB patient who dies for any reason before starting or during the course of treatment
Defaulted/Lost to follow-up	A TB patient who did not start treatment or whose treatment was interrupted for 2 consecutive months or more
Relapse	A TB patient previously declared cured or treatment completed and is again diagnosed with sputum smear or culture positive tuberculosis

Categories and Regimens of TB treatment					
		Drug Regimen and Duration			
		Intensive phase		**Continuation phase**	
Category	**Definition**	**Drugs**	**Duration**	**Drugs**	**Duration**
Category I (Category III)*	All new TB patients, defined as never having taken TB treatment before or for less than 1 month	RMP INH EMB PZA	2 months, daily (FDC)	RMP INH	4 months, daily (FDC)
Category II	All retreatment cases (relapse, treatment after default, treatment after failure);	RMP INH EMB PZA STR	3 months, daily (FDC) ________ 2 months, daily	RMP INH EMB	5 months, daily (FDC)
Category IV	MDR-TB cases, failures	second-line treatment with fluoroquionolones and injectibles			18 - 24 months

**Category III patients are defined as category I patients but with a thrice a week instead of daily treatment regimen.*
This treatment category classification is no longer recommended by WHO as it can increase DR levels in undetected MDR cases if repeatedly put on Cat egory II treatment. However, in countries with no readily available DST and or second-line treatment the above classifications are still used.

Source: Doctoral thesis of Serej Ley (Ley, 2014), sources of information: World Health Organisation.

Under sub-optimal drug concentrations, micro-organisms can mutate (nucleic acid changes at the genetic level) to avoid drug action, known as drug

resistance (DR). There are several situations that can lead to patients having sub-optimal drug concentrations in the body mainly; poor compliance to treatment regimens by patients on anti-TB therapy, often through inadequate treatment guidance or supervision by healthcare personnel, or intermittent drug supply; interactions with other drugs the patients are taking that compete with the mechanism of action of the anti-TB drugs and; the metabolizer phenotype (the range of metabolizers [enzymes that change the constitution of a consumed drug] that a patient presents with based on their genetic make-up) of a patient (Marc et al., 2005; Garcia-Martin, 2008).

In the case of MTB, drug resistance develops exclusively through chromosomal mutations, in particular, single-nucleotide polymorphisms (SNPs) sequentially accumulating (Ramaswamy and Musser, 1998), (O'Malley and Melief, 2015). Over the years, mutations associated with DR in MTB have been identified in several genes including *katG*, *gyrA* and the promoter region of *mabA*/*inhA* operon. The target of rifampicin for example, is the *rpoB* gene in MTB coding for the β-subunit of RNA polymerase. There is an 81bp region in this gene wherein point mutations associated with 95% of RIF resistance cases have been identified, and are now used as markers to identify RIF resistance in clinical specimens (Helb et al., 2010).

The prevalence of drug resistance in a community guides treatment timeframes and regimens (Perez-Velez and Marais, 2012). Two definitions of MTB drug resistance are particularly relevant for patient management: multidrug-resistant (MDR) TB with strains resistant to both isoniazid (INH) and rifampicin (RIF); and extensively drug-resistant (XDR) TB, with additional acquisition of resistance to any member of the quinolones and at least one of the following second-line anti-TB injectable drugs: kanamycin, capreomycin or amikacin. Bedaquiline, a diarylquinoline anti-TB drug developed in 2012, is in phase III clinical trial stage investigating its safety and efficacy. WHO issued interim policy guidelines in 2013 for its use in treatment of MDR-TB, once all other drug are found resistant (WHO, 2013d). Delamanid, a nitroimidazole is in phase III clinical trial stage for treatment of MDR-TB as well (RESIST-TB, 2015). Unfortunately, there is already a case presenting with drug resistance to both these drugs (Bloemberg et al., 2015). The study emphasizes the need for the use of appropriate companion drugs when bedaquiline and delamanid

are used. Carbapenems, which include ertapenem, imipenem and meropenem, are also used in MDR and XDR TB treatment regimens and relatively well tolerated (Sotgiu et al., 2016). There has been a recent WHO guideline update for a MDR TB treatment regimen that is shorter (9-12 months), with the aim of cost effectiveness and improved patient compliance (WHO, 2016).

DISEASE PROGRESSION AND MANIFESTATIONS OF TB IN CHILDREN

Table 2 is an excerpt from a review by Perez-Velez and Marais (2012) summarizing disease manifestations, age-related risk for disease progression, immune characteristics, tuberculin skin test (TST) and interferon gamma release assay (IGRA) presentations, during the different phases of disease progression in untreated childhood TB. This is with a proviso that the same trends may not be seen in immune-compromised children.

Age is the most important variable that determines risk of progression to disease in immune-competent children. Primary infection before 2 years of age frequently progresses to serious disease within the first 12 months, without significant prior symptoms. If these children present with any symptoms, they are fairly non-specific including fever, anorexia, and weight loss, with or without cough. Primary infection between 2 and 10 years of age rarely progresses to disease, and if there is progression, it is associated with significant clinical symptoms which represent a window of opportunity in which to establish a clinical diagnosis. Primary infection after 10 years of age frequently progresses to adult-type disease including cough, fever, night sweats, haemoptysis, and weight loss (Marais B. J., 2004).

If there is a successful immune response to primary infection, asymptomatic healing can occur within four to six weeks of initial exposure to MTB. During this time erythema nodosum, a hypersensitivity response may occur on the skin. Tuberculosis can be cured after primary infection, or the disease may manifest, or become dormant (LTBI) from which reactivation (endogenous) may occur, resulting in post-primary tuberculosis. Endogenous reactivation occurs when dormant bacteria (confined in macrophages but not killed) establish infection when the child becomes severely malnourished or

immuno-compromised while exogenous re-infection occurs through fresh contact with TB, usually in areas of high TB prevalence.

Table 2: Clinical syndromes associated with tuberculosis in children

Clinical syndromes associated with tuberculosis in children*					
Disease phase & timing	**Clinical syndrome**	**Group at greatest risk**	**Immuno-pathogenesis**	**Results on TST and IGRA**	**Manifestations on imaging**
Primary infection					
Incubation, 0-6 wk	Asymptomatic	All ages	No adaptive immunity	Negative	None
Immune conversion, 1-3 mo	Self-limiting symptoms (mild, viral-like); hypersensitivity reactions (fever, erythema nodosum, phlyctenular conjunctivitis)	All ages	Acquisition of adaptive immunity	Generally positive; infection may be lifelong; no test for reinfection	Transient hilar or mediastinal lymphadenopathy detected in 50-70% of cases; transient Ghon focus usually not detected
Early disease progression†					
2-6 mo	Uncomplicated lymph node disease	<10 yr of age	Inadequate innate immunity, adaptive immunity, or both	Generally positive‡	Hilar or mediastinal lymphadenopathy without airway involvement; Ghon focus without cavitation
	Progressive Ghon focus	<1 yr of age severely compromised immune system	Inadequate innate immunity, adaptive immunity, or both	Generally positive‡	Ghon focus with visible cavitation
	Disseminated (miliary) disease, tubercular meningitis, or both	<3 yr of age or severely compromised immune system	Inadequate innate immunity, adaptive immunity, or both	Generally positive‡	Discrete lung nodules (1-2 mm in diameter) on chest film, hepatosplenomegaly, retinal lesions with hydrocephalus, basal meningeal enhancement, brain infarcts or tubeculomas on CT of the head
4-12 mo	Complicated lymph node disease (airway compression, expansile caseating pneumonia, infiltration of adjacent anatomical structures [bronchus, esophagus, pericardium, phrenic nerve])	>1 yr of age	Exuberant lymph node responses, with inadequate innate immunity, adaptive immunity, or both	Generally positive‡	Hyperinflation, atelectasis, or collapse of lung; expansile consolidation of segment or entire lobe; tracheoesophageal or bronchoesophageal fistula; pericardial effusion; hemidiaphragmatic palsy
	Pleural disease (exudative effusion, empyema in rare instances, or chylothorax with ductus thoracicus infiltration)	>3 yr of age	Hypersensitivity response to tuberculoprotein	Generally positive‡	Effusion, sometime large, usually in one lung; pleural thickening and loculations detected on ultrasonography
	Peripheral lymphadenitis (most frequent extrathoracic disease manifestation, usually in the neck)	1-10 yr of age	Inadequate local control	Generally positive‡	Ultrasonography usually not needed, but may reveal matting and adjacent soft-tissue edema

Late disease progression §					
8-24 mo	Adult-type pulmonary disease (difficult to differentiate among primary infection, reactivation, and reinfection; reactivation may occur >20yr after initial infection)	>10 yr of age, but can occur in children as young as 8 yr of age	Overly aggressive innate immunity, adaptive immunity, or both	Generally positive‡	Apical cavities in one or both lungs; minimal or no lymph-node enlargement (previously referred to as postprimary tuberculosis)
1-3 yr	Osteoarticular disease (e.g. spondylitis, arthritis, osteomyelitis)	>1 yr of age	Inadequate local control¶	Generally positive‡	Periarticular osteopenia, subchondral cystic erosions, narrowing of joint space
>3 yr	Urinary tract (kidney, ureter, bladder) disease	>5 yr of age	Inadequate local control¶	Generally positive‡	Renal calcifications, cavitation, hydronephrosis, calyceal dilatation, ureter stricture

* Adapted from Wallgren (1948) and Lincoln and Sewell (1963) (Wallgren, 1948), (Lincoln and Sewell, 1963). Age ranges, risk groups, and timelines are intended to provide general guidelines only; children infected with the human immunodeficiency virus are particularly susceptible to tuberculosis and may present with atypical features. For the phases of disease, the times shown are the interval between initial exposure and the onset of the disease phase. IGRA denotes interferon γ-release assay.
† At least 90% of disease manifestations occur within 12 months after infection.
‡ Because test results may be negative in immunocompromised patients, a negative result cannot be used to rule out infection.
§ Late disease progression is generally rare, but adult-type pulmonary disease is common in adolescents.
¶ In cases of inadequate local control, manifestations are usually restricted to the local focus of disease, although disease can disseminated from any active focus.

Source: Perez-Velez and Marias (2012)

TB disease in children can manifest in the lungs as a Ghon focus, pneumonia, pleural effusion, or lobar collapse, especially of the middle lobe (Brock's syndrome). Lobar collapse develops from substantial hilar lymphadenopathy (lymph enlargement) causing obstruction and compression of bronchi. Twenty to forty percent of children with TB disease present with extrapulmonary (outside the lungs) manifestations, while 10–30% present with both pulmonary and extrapulmonary manifestations concurrently (Cruz and Starke, 2007). The percentage presenting with EPTB goes up further to 50% in young and/or HIV co-infected children (Newton et al., 2008). Extrapulmonary and miliary tuberculosis occur mostly following primary infection rather than after reactivation. Miliary TB is defined by its millet-seed like appearance and can affect a variety of organs, including the lungs. Risk of progression to miliary TB or tuberculous meningitis depends on age and immune status (Marais et al., 2006a), with infants and children co-infected with HIV being at highest risk.

The most common extrapulmonary manifestations of TB in children include; tuberculous lymphadenitis - mostly superficial (67%), tuberculous meningitis (13%, occurring most often in infants and toddlers), pleural TB (6%), miliary TB (5%), and skeletal TB (4%) (Cruz and Starke, 2010). Tuberculous

lymphadenitis (lymph node infection) may be mediastinal, intra-abdominal or peripheral. Peripheral lymphadenitis is typically superficial and often presents as a non-specific swelling which may be mistaken for malignant in nature. The lymph nodes usually measure 2 to 4 cm and lack the classical inflammatory characteristics of pyogenic nodes (Anunobi et al., 2008). Cervical is the most common presentation of superficial tuberculous lymphadenitis in children. Other sites include anterior cervical, posterior triangle, submandibular, and supraclavicular nodes. Untreated lymph nodes may caseate (necrosis in which tissue is changed into a dry mass resembling cheese), spread to nearby organs and tissues, and lead to formation of sinus tracts.

Generally, diagnosis of TB in children is complicated by non-specific clinical presentations. Fever and cough may be observed in infants, but in children between 5 and 10 years of age, symptoms are rarely observed indicative of TB disease as in adolescents and adults. In lymphatic disease, systemic symptoms occur only in 50% of children, abnormal chest radiographs are seen in approximately 33% of children and most are positive on TST testing, which is not confirmatory for active TB infection (Cruz and Starke, 2010). Interpreting radiological features of TB lymphadenitis with certainty is also difficult.

SPECIMEN COLLECTION METHODS IN CHILDHOOD TUBERCULOSIS

Collection of bacteriologic specimens is a major challenge in cases of suspected paediatric TB.

Pulmonary specimens

Sputum collection for TB diagnosis is usually not feasible in young children as they tend to have paucibacillary disease and are also unable to expectorate. Adolescent children between the ages of ten and fourteen years frequently develop sputum smear-positive adult-type disease and obtaining good quality sputum specimens from this group is more likely (Marais et al., 2005). **Sputum induction** is a specialised technique that requires nebulisation and suction instruments. In some studies this method has been shown to have significantly more bacterial yield than gastric aspirates from children of all ages (Zar et al., 2005; Hatherill et al., 2009). Sputum induction

can be done as an outpatient procedure but its use outside of hospital settings is limited since infection control measures need to be in place.

More recently the **Lung Flute,** a plastic device made by Medical Acoustics (USA) and a non-invasive method for sputum collection, is gaining popularity for rapid diagnosis of pulmonary TB. Collection of own sputum is facilitated by creation of vibrations in the lungs when the individual exhales into the flute, which helps to loosen and liquefy sputum in the alveolar cavities (Fujita et al., 2009). Further studies are needed on sputum yield by this method, both in adults and in children, for TB diagnosis.

Sputum is commonly swallowed, particularly when a person is as rest. **Gastric aspiration** is therefore a plausible alternative for sputum collection. It is an invasive procedure, not easily performed on an outpatient basis. It requires prolonged fasting and sample collection over three consecutive days, which makes it less feasible for low-income settings due to the hospitalization requirement. An alternative that is less invasive and may be performed on an outpatient basis is **nasopharyngeal aspiration**. This method does not require fasting and has a comparable bacterial yield to gastric aspiration.

The **string test** involves a nylon string being swallowed by the child, so that it remains in situ for 4 hours absorbing swallowed sputum, with the trailing string being retained outside the mouth. It is less invasive than gastric aspiration and is tolerated well in children of 4 years and older (Chow et al., 2006). The string test was shown to have superior sensitivity compared to induced sputum in one study (Vargas et al., 2005). Another means of obtaining bacteriologic respiratory specimens is **broncho-alveolar lavage** (BAL), an intricate procedure recommended for intubated patients and those who require diagnostic bronchoscopy.

Extrapulmonary specimens

Urine and **stool** samples are generally obtained non-invasively, but due to their nature, confer limited bacterial load based on the focus of disease. Collection of **blood** specimens in children is not only challenging, especially in infants and emaciated patients, but is also only useful in disseminated TB disease or when using antigen/antibody detection assays, which are currently not recommended for use (WHO, 2011a). **Bone marrow aspiration** is an

invasive procedure that requires well trained personnel, elaborate equipment and stringently sterile conditions (Marais, 2006). **Lumbar puncture** for cerebrospinal fluid has similarly stringent requirements, is an invasive procedure that requires hospitalisation and usually has a low mycobacterial yield.

Fine needle aspiration biopsy (FNAB) is a robust and simple technique using a fine needle to aspirate bodily fluids, including from lymph nodes, that can be performed in any outpatient setting, with minimal side-effects, providing creditable bacteriologic diagnostic value (Marais et al., 2006b). It is an important tool and often the only way to diagnose extrapulmonary TB in children (Cotton et al., 2004). The value of FNAB in diagnosis of mycobacterial lymphadenitis in adults is well documented (Samaila and Oluwole, 2011; Nayak et al., 2003; Kocjan and Miller, 2001). Although there are few studies on FNAB in children (van Wyk et al., 2011; Balaji et al., 2009; Wright et al., 2008; Bruijnesteijn van Coppenraet et al., 2004), one study comparing FNAB against gastric aspirates and induced sputum in children with pulmonary TB and peripheral lymphadenopathy, showed that FNAB had superior diagnostic yield and significantly reduced time-to-diagnosis (Wright et al., 2009a). Compared to **core needle biopsy** that uses a larger needle to obtain tissue samples, the risk of sinus or fistula formation, permanent damage to nerves or seeding of tumours (if present) along the needle track for FNAB is minimal (DeMay, 1996). As an inexpensive outpatient procedure, FNAB is easy to perform in resource-limited settings and may pre-empt the infection risks that accompany alternative specimen collection procedures.

PROMISING NAATS FOR EPTB DIAGNOSIS IN CHILDREN

The complexity and insufficient robustness of many existing commercial NAAT protocols, their requirement for precision instrumentation, quality assurance, high levels of investment and technical support make them unsuitable for most resource-limited TB-endemic settings. Additionally, the technical skills required have resulted in variable performance, even in experienced molecular laboratories whether in low- or high-resource settings. Furthermore, none of these techniques have been fully validated for diagnosing TB in children and specifically not for extrapulmonary specimens.

In light of these challenges, there is promise in two technologies that have been developed and under evaluation over the last several years; the Xpert® MTB/RIF kit (Cepheid) endorsed for use by the WHO as the initial diagnostic test in individuals suspected of MDR-TB or HIV/TB (WHO, 2010); and the Ustar EasyNAT™ TB IAD kit (Ustar Biotechnologies, Hangzhou) selected by the WHO as a category 1: commercialized/-isable stage innovative technology on assessment for use in TB endemic countries (WHO, 2011b).

The Xpert® MTB/RIF test (Cepheid)

This is a commercially available semi-quantitative assay designed to detect *M. tuberculosis* complex DNA directly from human clinical sputum specimens. It integrates and automates sample processing and simultaneous detection of MTB complex and rifampicin (RIF) resistance. This is done through real-time PCR using five overlapping probes that are complementary to the 81 base pair rifampicin-resistance determining region (RRDR) of the *M. tuberculosis rpoB* gene (Figure 3). There is an internal PCR amplification control probe for SPC (*Bacillus globigii*) that determines if PCR has taken place. The system indicates an "invalid" result should this probe not bind, indicating PCR inhibition.

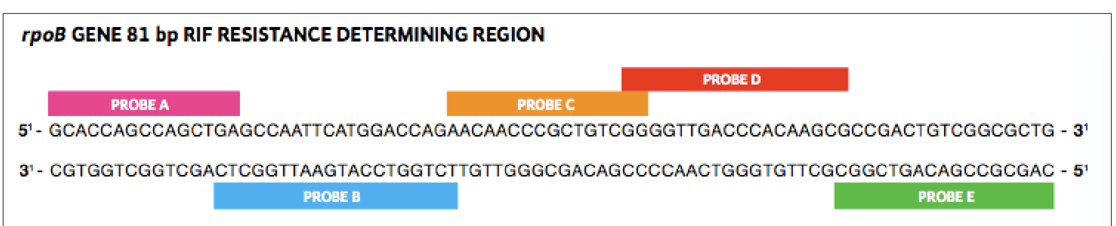

Figure 3: Illustration of the 5 probes used in the Xpert® MTB/RIF assay.
Source: Xpert® MTB/RIF (Cepheid) brochure (0089-02): the 5 probes targeting the *rpoB* gene of *M. tuberculosis* (Cepheid, 2009).

The five test-specific probes are designed to bind to the wild-type (non mutated) MTB *rpoB* sequence. Should any one of the probes do not bind; it is an indication of a mutation along that probe binding site and therefore RIF resistance. The PCR amplification process is hemi-nested, and the amplified target is detected in real time by six colour fluorescent molecular beacons, one for each probe (Figure 4). Continuous optical monitoring allows the software to automatically stop the reaction as soon as the target is detected, further accelerating time to results. MTB detection is reported as 'very low',

‘low’, ‘medium’ or ‘high’ in concentration and RIF resistance reported as ‘detected’ or ‘not detected’.

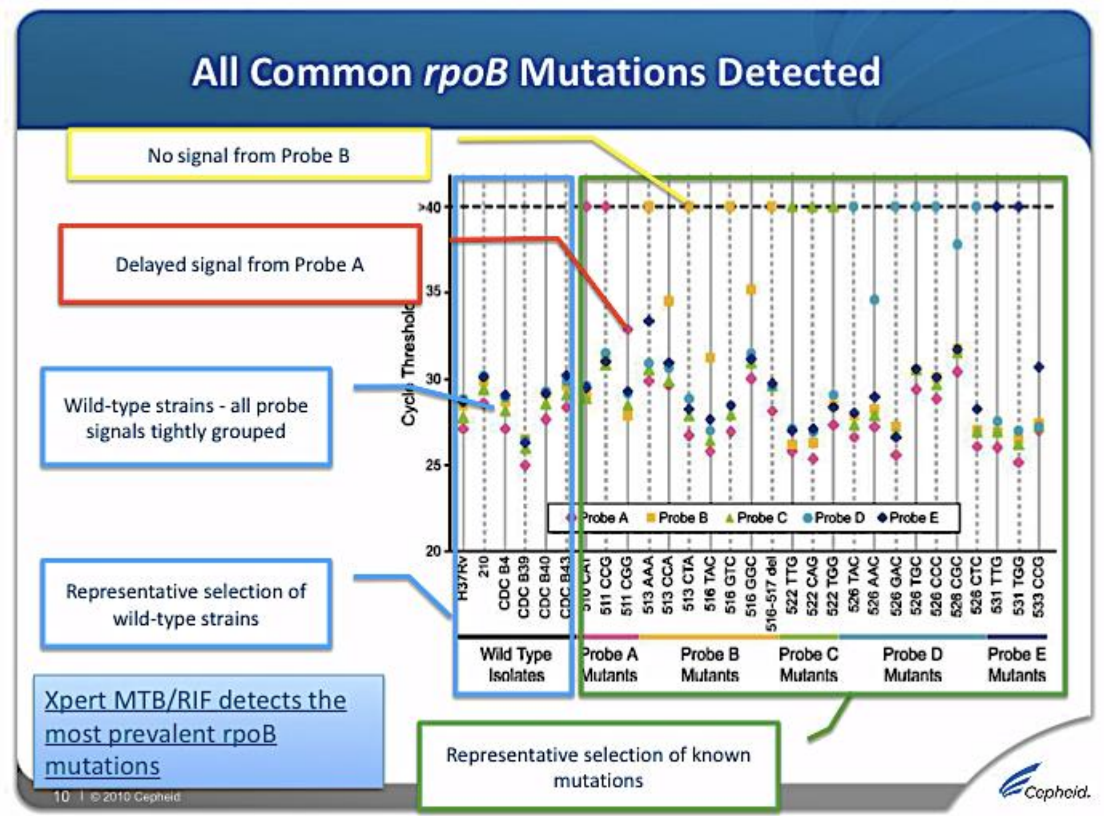

Figure 4: Xpert® MTB/RIF six colour fluorescent molecular beacon detection for MTB and RIF resistance.
Source: Cepheid Xpert MTB/RIF Training presentation, Soundiram 2012, p.10: http://www.moleculartb.org/gb/pdf/ppt/14_SYMP_ISoundiram_Xperttraining_2902.pdf
Reported as MTB DETECTED if at least two of the five *rpoB* probes are bound within two cycles of each other. A delay of beacon detection (more than 2 cycles) indicates a weak mutation, while the absence of beacon detection (no probe binding/no signal from probe) indicates a strong mutation.

As the system fully integrates and automates sample extraction, amplification and detection in one cartridge, it eliminates the need for amplicon manipulation and thus the risk of cross-contamination of samples (Banada et al., 2010). Low-skilled technicians can perform the assay, with a hands-on of 2 minutes; shorter than that for smear microscopy or culture. The test turnaround time is less than 2 hours, similar to smear microscopy and many times faster than culture-based techniques and it requires minimal training.

The assay sensitivity is many times higher than that of smear microscopy (10,000 cfu/ml) and close to that of liquid culture (10–100 cfu/ml). The test is also highly specific to MTBC detection and there is no cross reaction with non-tuberculous mycobacteria. The assay detects mutations in the RRDR

which occur in 95 to 98% of all known rifampicin-resistant MTB strains (Helb et al., 2010). Through concerted efforts by the Foundation for Innovative New Diagnostics (FIND) over the recent years, GeneXpert technology is now available for sputum TB detection and rifampicin resistance testing in the public sector of 116 of the 145 TB high burden countries eligible for concessional pricing (WHO, 2015a).

A systematic review was done of the performance of the Xpert assay, for pulmonary tuberculosis and rifampicin resistance detection on pulmonary specimens in adults, from 2009 until February 2013. The assay was found to have a pooled sensitivity of 98% in smear-positive, culture-positive specimens and as an initial test replacing smear microscopy it had a pooled sensitivity and specificity of 89% and 99%, respectively (The Cochrane Collaboration and Steingart et al., 2014).

The number of studies on the performance of Xpert in detecting TB in extrapulmonary specimens, with sufficient sample size, is limited. The pooled sensitivity and specificity from a systematic review done in 2013 of eleven identified studies was 84.9% and 92.5%, respectively (WHO, 2013b). Some of these studies had a small sampling of specimens that were aspirates from lymph nodes and were not specific on the number of children included in the study, if at all. Of the analyses done on lymph nodes, excluding case-control studies, the pooled sensitivity and specificity were found to be 89% and 90%, respectively. One study on lymph node aspirates specifically, with less than 15% of the samples being from children, showed a high sensitivity and specificity for detection of TB (96.7% and 88.9% respectively) (Ligthelm et al., 2011). The heterogeneity in performance characteristics across the different extrapulmonary sample types was found to be substantial and the data available on lymph node aspirate analysis by Xpert in children were very limited (WHO, 2013b). There is therefore value in further studying the performance of Xpert MTB/RIF in extrapulmonary specimens, specifically lymph node aspirates, in children.

Some of the limitations of the Xpert® MTB/RIF assay are that it needs to be more robust in regard to its requirement for continuous electrical power and should be more cost effective, so as to be considered the ultimate solution for TB diagnosis in TB endemic, resource-limited settings.

The Ustar EasyNAT™ TB IAD (Biotech)

The Ustar EasyNAT™ TB Isothermal Amplification Diagnostic test (Biotech) is a commercially available kit for the qualitative detection of MTB in human samples. The technique involves manual sample processing and post PCR procedures. The assay is a nucleic acid amplification and hybridization technique that uses cross primers (double crossing) through a cross-priming amplification (CPA) technology from an isothermal DNA amplification system by Ustar Biotechnologies Co., Ltd., Hangzhou, China.

Using multiple cross-linked primers (six to eight primers) targeting the *gyrB* gene of *M. tuberculosis*, a DNA target sequence is amplified at a constant temperature of 63 or 65°C (Figure 5). The denaturing and annealing of DNA molecules occurs dynamically. Using multiple displacement and cross primers, PCR products are generated as interval tandem repeats of the target region based on the *H37Rv* sequence (*BX842572.1, NT 5582-5789*) of TB.

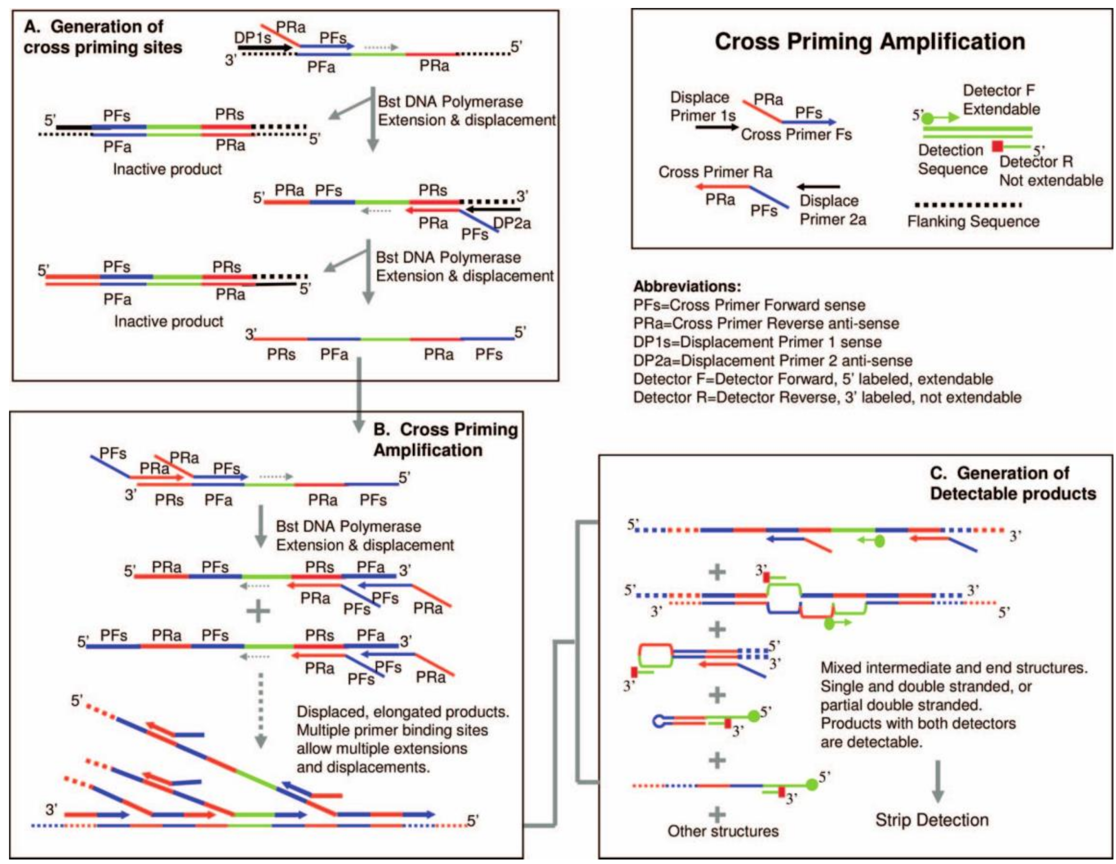

Figure 5: Illustration of the Ustar EasyNAT™ TB isothermal cross-priming DNA amplification technology.
Source: Fang et al. 2009.

Generation of cross-priming sites first occurs in the presence of Bst DNA polymerase through extension and displacement activity. Multiple primer binding sites are created which allow for multiple extensions and displacements, which occur at the cross-priming amplification stage. Mixed intermediate and end structures are then present to which the forward and reverse detector sequences bind to create single, double-stranded or partial double-stranded sequences (Fang et al., 2009).

Detection occurs when the strands created through detector sequences, bind to sites on detection strips provided (Figure 6). The lateral flow detection strips are housed individually in sealed plastic devices to prevent leakage of amplicons. Results are visualized as bands on the test strips; appearance of both control and test bands indicate presence of MTB, while appearance of only the control band indicates the test is valid, but that there is no MTB or the amount of MTB DNA is below the detection limit.

In a study conducted on 180 clinical sputum specimens, the sensitivity of EasyNAT in smear and liquid culture-positive specimens was 96.9%, and that from smear-negative but liquid culture-positive specimens was 87.5%. The specificity of the assay in culture-negative specimens from patients with other pulmonary diseases was 98.8% (Fang et al., 2009).

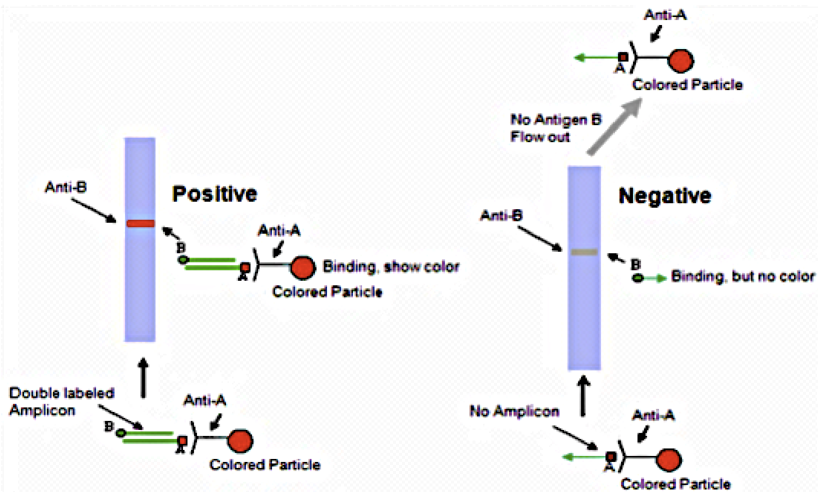

Figure 6: Principle of strip detection in the Easy NAT^{TM} system.
Source: Ustar Biotechnologies (Hangzhou) Ltd (2013):
http://www.bioustar.com/Upload/files/videos/Ustar%20TB%20Flyer1.pdf

More recently, Ustar Biotechnologies (China) have developed three isothermal based NAATs for the detection of TB, as well as rifampicin and isoniazid resistance-conferring mutations (WHO, 2013a). A multicentre study conducted in China evaluated this TB detection kit, which is based on single crossing CPA technology targeting the IS6110 region of the MTB genome, and is multiplexed with an internal amplification control reaction (Ou et al., 2014). The results were promising but not as sensitive and specific as the double CPA *gyrB* gene targeting EasyNAT^{TM} IAD kit (Fang et al., 2009).

The double CPA *gyrB* gene targeting EasyNAT^{TM} IAD kit (2009) kit was considered a tool to assess for detection of TB in lymph node aspirates in my study. The assay does not require a precision instrument. It does require some training, a centrifuge and a water bath or a heating block, making it a more viable tool to use in TB-endemic low-resource settings and the time to results is just over 2 hours.

STUDY RATIONALE

Direct smear microscopy of sputum samples, a diagnostic tool with poor sensitivity (missing over half the cases) and lack of differentiation between drug-sensitive and drug-resistant TB, remains the primary method of TB diagnosis in most low to middle-income TB-endemic countries. Culture of MTB is the gold standard diagnostic method, but has a slow turnaround time and requires laboratory resources that are not available in the majority of resource-limited TB-endemic settings. The paucibacillary nature TB infection in most children makes it difficult to diagnose through sputum smear microscopy and culture, and good quality samples can rarely be obtained from young children (WHO, 2013a).

Childhood tuberculosis continues to be one of the most important public health issues facing health systems in TB-endemic countries. One of the major priorities identified in a review of new and improved tuberculosis diagnostics, is the development of a rapid test for childhood TB that will not depend on sputum specimen testing, but preferably other point-of-care rapid detection techniques (Pai et al., 2010). The refinement of existing tools and development and testing of new tools for the diagnosis of childhood TB and EPTB continues to be the recommendation to-date (Kik et al., 2014).

At the time at which this study was being initiated (2012), some of the developments highlighted in this review (2010 to date) had not been realised. Despite these developments and the success of the Xpert MTB/RIF assay, a very recent systematic review of the Xpert MTB/RIF assay for diagnosis of extrapulmonary tuberculosis, still indicated the need for further studies availing primary and secondary-level health facility data, specific to TB-endemic regions, and more specifically, an improved representative patient spectrum, in this case children (Denkinger et al., 2014). The high sensitivity and specificity of the Xpert and EasyNAT kits for detecting TB in sputum specimens, as well as their ease of use by non-specialist personnel, were reasons for their assessment in the detection of TB in lymph node aspirates from children suspected of TB in Dar-es-Salaam.

STUDY OBJECTIVE

To evaluate the use of two nucleic acid amplification tests: the Xpert® MTB/RIF (Cepheid) automated PCR and the Ustar EasyNAT^{TM} TB IAD Kit (Biotech) for the diagnosis of TB lymphadenitis in cytological aspirates from children seen at a district hospital in Tanzania and comparing these new techniques with established FNAB methodologies: Ziehl-Neelsen (ZN) staining, cytological determination and liquid culture.

STUDY SIGNIFICANCE

The study was a feasibility assessment of the two diagnostic kits; Xpert® MTB/RIF and Ustar EasyNAT^{TM} TB IAD for diagnosis of childhood TB lymphadenitis at district public health level in Tanzania. Ease-of-use, utility in relation to sample type and collection methods, and robustness were therefore investigated.

The NAATs were expected be at least as sensitive, if not more sensitive than the gold standard and/or the other standard methods in use for diagnosing TB lymphadenitis. This was with a vision to enhance the diagnostic algorithm, and in so doing; improve the management of TB disease in children. Other pertinent data were also accrued, such as gender- and age-specific trends of TB and HIV infection. The study also contributes to childhood TB diagnostic literature, aiding in policy development.

STUDY APPROACH

RECRUITMENT SITES

1. Temeke District Hospital, Dar es Salaam (TDH)
2. Ifakara Health Institute, Bagamoyo Research and Training Centre (IHI-BRTC)

RECRUITMENT PROCESS

The project was established at the TB clinic of the district hospital and convenience sampling was done; children attending the hospital facilities, having symptoms per the inclusion criteria, were referred to the study. All children suspected of having TB lymphadenitis, following criteria defined in the "Guidance for national tuberculosis programmes on the management of tuberculosis in children" (WHO, 2006) for diagnosing TB in children, were recruited following informed consent.

Inclusion Criteria

- Signed written consent or witnessed oral consent in the case of illiteracy of the parent/guardian, before undertaking any study related activity
- Assent provided by the child if more than 7 years of age
- Child of more than 8 weeks, but less than 16 years of age
- Child with lymphadenopathy (defined as palpable lymph nodes ≥ 1 cm) persisting for more than 4 weeks despite a course of oral antibiotics
- Child with strong clinical suspicion or microbiological confirmation of mycobacterial infection in combination with lymphadenopathy (defined as palpable lymph nodes ≥ 1 cm)

Exclusion Criteria

TB treatment in the past year (anti TB drugs taken within the last 12 months).

ETHICAL CONSIDERATIONS

The parent or legal guardian of the participant was appropriately informed about the study processes, including possible benefits, risks and inconveniences. Consent was obtained from them and the child (if more than

7 years of age) to proceed with assessment of the child in determining potential TB lymphadenitis and consequent fine needle aspiration (FNA) of palpable nodes.

Do no harm

- The procedures performed in the study, including FNA, were performed professionally and with all infection control measures in place (**Appendix 13**)
- The information solicited in this study was secure and confidential and was coded for purposes of the research. Barcode labels (without patient details) were used on all data sheets and specimen containers (**Appendix 15**). Only authorized medical personnel at the study site had access to participant names for purposes of treatment and follow-up.

The direct benefit to study participants was; enhanced free diagnostic assessment for TB disease, compared to the routine national programmatic level assessment.

The kits tested in this study were either endorsed for use (Xpert® MTB/RIF), or endorsed for assessment (Ustar EasyNAT™ TB IAD) by the WHO for TB endemic countries. The study was an evaluation of laboratory diagnostic tools. TB treatment decisions were not made primarily based on the results from the Xpert® MTB/RIF (Cepheid) or Ustar EasyNAT™ TB IAD (Biotech) assays under evaluation, but on the basis of routine clinical and laboratory methods, including clinical work-up following the National TB and Leprosy Programme (NTLP) guidelines, TST, FNA AFB smear, cytomorphology and TB culture results.

Any sputum test results available (based on sample production) were considered, as required for routine diagnostic evaluation according to NTLP from patients suspected of having TB. Specimens were stored in the TB CHILD specimen bank, in a confidential manner using a number or coding system. A Material Transfer Agreement (MTA) was issued by the Medical Research Coordinating Committee of the National Institute for Medical Research (NIMR) of Tanzania, for the TB CHILD project, to enable shipment of isolates that may need to be sequenced at the Swiss Tropical and Public Health Institute (Swiss TPH), for quality control. Participants were compensated for travel and time according to the site's research protocol.

Dissemination of study outcomes was done through scientific presentation to the Regional TB and Leprosy Coordinator (RTLC) and NTLP staff involved in the project, and involvement of key collaborators as authors in scientific publication. The information obtained from this study was based on good clinical Practice (GCP) and good clinical laboratory practice (GCLP) and therefore reliable when appropriately analysed.

STUDY ADMINISTRATION

This study was conducted as a scientific cooperation between the Ifakara Health Institute (IHI) Tanzania, Bagamoyo Research and Training Centre (BRTC) Tanzania, the Aga Khan Hospital, Dar-es-Salaam (AKHD) Tanzania, and the Swiss Tropical and Public Health Institute (Swiss TPH) Basel, Switzerland.

DATA STORAGE

Participant codes, related clinical and medical history, TST and chest x-ray (CXR) results, follow-up clinical and medical information and all laboratory results have been stored confidentially at BRTC and Swiss TPH.

SAMPLE SIZE

In the proposal for this study, in determining the target sample size for the study, a sample size estimation formula was adopted from Carley *et al* 2005 (Carley et al., 2005) for a confidence interval of 0.05 (Table 3). There was no prevalence information for extrapulmonary TB in children for Tanzania, therefore as per standard statistical protocol; an estimated prevalence of 50% was used. The sensitivity targets were determined from earlier studies on each kit (Boehme et al., 2010; Naidoo, 2010; Fang et al., 2009). There is still no clear information on the prevalence of TB in children in Tanzania (van Leth, 2013).

There were delays in initiation of the study and staffing issues at the paediatric and adult outpatient clinics of TDH that caused lapses in recruitment of participants for the study. Thereafter, screening of all children for superficial lymph nodes by a study-recruited nurse lead to recruitment of

all children aged 8 weeks to 16 years, suspected of having TB lymphadenitis. The target sample size could not be achieved due to the aforementioned challenges, but extension of the recruitment period to 12 months (April 2012 until March 2013) enabled recruitment of 79 participants in total. Follow-up of each participant was done 5 to 12 months after recruitment (median: 5 months); the last group of participants recruited were reviewed in mid-August 2013.

Table 3: Sample size estimation

Sensitivity target	Confidence interval (CI)	Required minimum group size	Estimated prevalence among TB suspects	Required TB cases
Xpert® MTB/RIF 90%	0.05	140	0.5	280
Ustar ™ TB IAD kit 90%	0.05	140	0.5	280

STUDY WORKFLOW

A. Information talks with legal guardian

B. Obtaining Informed Consent: *Informed Consent Form* (ICF) (**Appendix 1**) additionally assent from child seven years to 16 years of age: *Assent for Minors Form* (**Appendix 2**)

C. Obtaining contact information on the participant: *Contact Information Form* (**Appendix 3**)

D. Medical history on enrolment: *Medical History Enrolment Form* (CRF) (**Appendix 4**)

E. Clinical examination: *Clinical Examination Form* (CRF) (**Appendix 5**)

F. Participant sent for chest X-ray (CXR): *X-ray CRF* (**Appendix 6**)

G. Sputum collection for routine smear microscopy (done by NTLP at TDH laboratory or other facility). Result of smear microscopy recorded on *Medical History Enrolment Form* (CRF) (**Appendix 4**) when received

H. Clinical assessment done using NTLP TB Score Chart for children under 6 years of age (**Appendix 7**) or following criteria defined in the "Guidance for national tuberculosis programmes on the management of tuberculosis in

children" (WHO, 2006) for children 6 years and older. When a participant scored ≥7 in the score chart (<6 years of age) or was recommended for treatment after clinical assessment (≥6 years of age) they were referred to the NTLP TB treatment programme, where they were started on the appropriate anti-TB therapy.

I. Procedures for specimen collection were followed in the order illustrated in Figure 7 and Table 4 and as described in the "Procedures" section below.

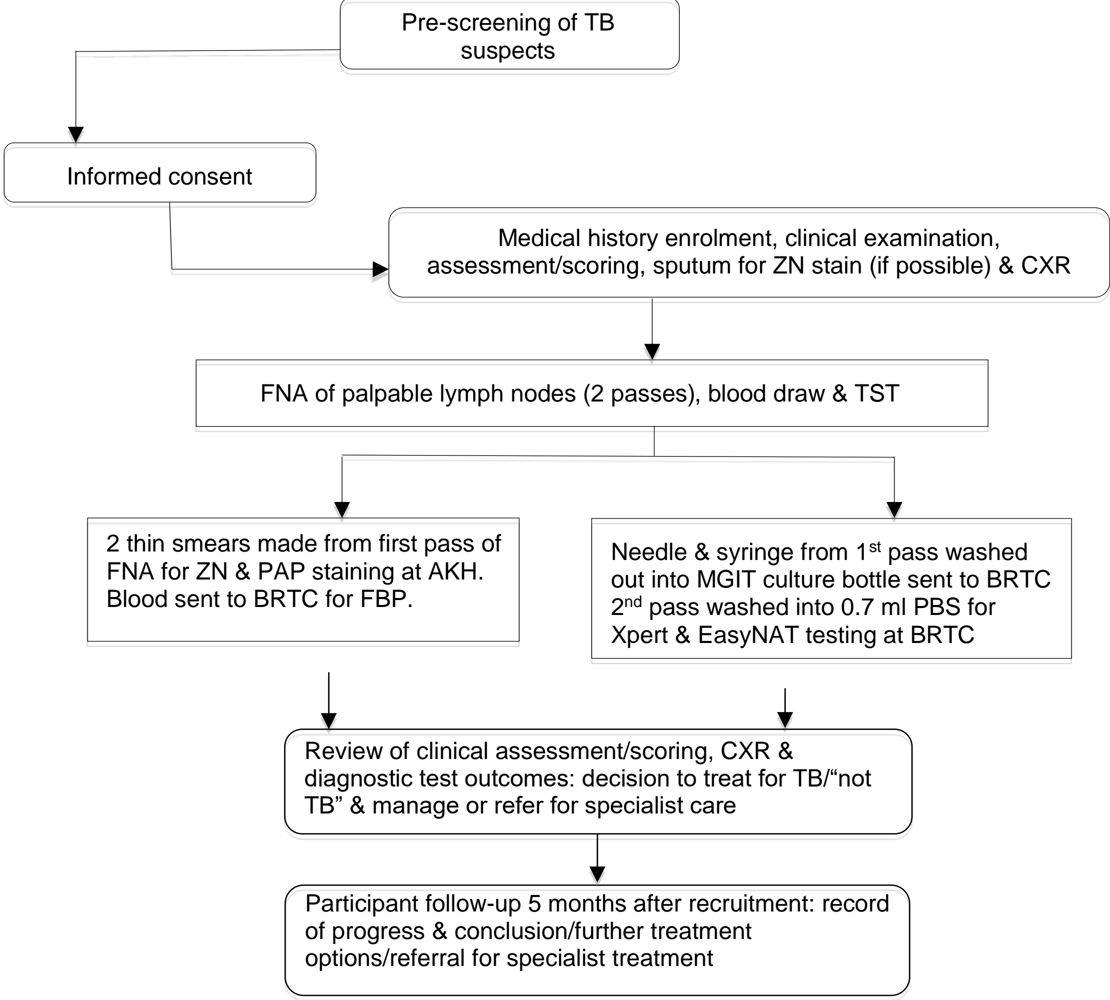

Figure 7: Flow chart of work-up and follow-up of participants

J. Participant management: NTLP officers at the TDH TB clinic were informed of all study outputs, including clinical assessments and laboratory results, as soon as they were reported. All participants recommended for TB treatment based on clinical assessment (including chest X-ray results)

and/or laboratory test outcomes were either managed at the TDH TB clinic or were issued detailed referral letters for management at NTLP clinics of their convenience. Newly diagnosed HIV-co-infected participants were also referred to the TDH HIV clinic for registration and appropriate management. All participants deduced as not having TB, after clinical assessment and receipt of laboratory results, were referred to relevant clinics (surgical, medical or HIV) for further management. Diagnosis and medication information was recorded on the *Diagnosis and Medication form* (CRF) (**Appendix 10**) during the first and follow-up visits, as needed.

K. All participants, both suspected and not suspected of having TB, were followed up five to twelve months after recruitment to record clinical outcomes and treatment success. Medical history was taken on the follow-up visit and the *Medical History Follow Up Form* (CRF) (**Appendix 11**) was filled out accordingly.

Table 4: Participant work-up and follow-up checklist

Activity	Day 1	Day 2	Day 4	Month 5
Screening / eligibility criteria	X			
Informed consent (& assent)	X			
Record of contact information	X			
Medical History	X			X
Clinical Examination	X			
CXR	X			
TST application		X		
Sputum collection - morning (if possible)		X		
Blood collection (full blood profile, HIV test)		X		
Sputum collection – spot (if possible)		X		
FNAB collected for:		X		
ZN		X		
Cytology		X		
Culture		X		
Xpert		X		
EasyNAT		X		
TST reading			X	
Review of available findings & treatment recommendation			X	X
Diagnosis and medication assessment			X	X

PROCEDURES

A trained clinical officer performed the following procedures per participant:

1. Fine needle aspiration biopsy (FNAB) and recorded the data on a *FNA CRF* form (**Appendix 8**)
2. Venous blood collection for full blood profile analysis (FBP or haemogram), HIV rapid testing, or HIV polymerase chain reaction (PCR) testing (for children ≤18 months of age) and recorded the data on a Universal sample transfer form (**Appendix 9**)
3. Tuberculin Skin Test (TST) intradermal injection and recorded on a TST log (**Appendix 12**). Examination of the TST site after 48 to 72 hours entering results into the *Clinical Examination Form* (**Appendix 5**).

Fine needle aspiration biopsy (FNAB) procedure (Wright et al., 2009b)

As per guidelines in SOP: Temeke_EPTB CHILD_FNA_01 (**Appendix 13**)

Materials:

- non-sterile gloves
- alcohol swabs
- 22 or 23 G cutting needles
- 10 ml disposable plastic syringes
- glass cytology slides (ground edges and frosted label end)
- 95% ethanol
- 100% methanol

The procedure was explained to the parent or guardian and child of 7 years and older. The parent or guardian was asked to remain with the child and to assist with restraining the child during the procedure.

Procedure:

- When there were multiple nodes at one site, the largest superficial node was chosen for FNA. Multiple sites of enlarged lymph nodes per participant were also aspirated when present, and the slides labelled accordingly.
- The skin on and around the lymph node was cleaned well with an alcohol swab and the mass immobilised using the forefinger and thumb. The

needle was positioned to access the entire mass and to avoid passing through muscles and major blood vessels.

- The needle was inserted firmly and no more than 1 ml suction was applied constantly throughout the procedure. The needle tip was moved back and forth or up and down "cutting" within the boundaries of the target in a fan-like fashion through the mass.
- Suction was released and the needle withdrawn once material was seen in the hub of the needle. On some occasions, there was no visible material in the hub of the needle, which then was seen on flushing onto slides and was usually sufficient for MGIT inoculation.
- Two needle passes were performed, each to aspirate as much fluid (if any) from the mass as possible. A fresh needle and syringe were used per pass.
- The parent or guardian was then asked to apply pressure to the puncture wound using dry sterile gauze.

Smear preparation:

- From the first FNAB pass the needle was removed from the syringe, 10 ml of air pulled into the syringe and the needle reattached. The air was used to express the material in the needle onto glass slides, ensuring the needle and slides were not in contact.
- A second glass slide was placed facedown on the first, the material allowed to spread gently and keeping both slides together, they were slid gently apart to create smears.
- Whenever possible, 2 smears were prepared for cytological assessment by soaking immediately in 95% ethanol and 2 smears were air dried for 5 minutes then soaked in 100% methanol for ZN staining.
- The slides were packaged and transported to AKHD laboratory at room temperature as per the TEMEKE_EPTB_CHILD_FNAB_Transport_01 protocol (**Appendix 14**).

MGIT tube inoculation:

- Using the same needle and syringe, an aliquot of liquid media (MGIT liquid culture enriched with MGIT PANTA antibiotic/growth supplement [Becton

Dickinson] less than 30 minutes prior to inoculation with specimen) was drawn into the syringe and expelled back into the MGIT tube, thereby using the culture medium to rinse out the syringe and needle in a sterile manner (**Appendix 16**: Procedure for Preparation and inoculation of MGIT Tubes).

- Inoculated MGIT tubes were transported to BRTC within 24 hours according to the TEMEKE_EPTB CHILD_FNAB_Transport_01 protocol (**Appendix 14**) along with a Specimen Transfer form Mycobacteriology (**Appendix 17**) each with details of the FNA collection.

Nucleic acid test sample preparation:

- The needle and syringe from the second FNAB pass per participant was rinsed with 0.7ml phosphate buffered saline (PBS) pre-prepared and sterilized in 2ml screw-cap tube. These were then transported in a cooler box along with Specimen Transfer Form for Molecular Testing (**Appendix 18**), ensuring the specimens were not packed in direct contact with the ice packs according to TEMEKE_EPTB CHILD_FNAB_Transport_01 protocol (**Appendix 14**).

Blood collection

Materials:

- Safety Needles, 22g or less
- Butterfly needles 21g or less
- Syringes
- Blood collection vacuum tubes designed to draw a predefined volume of blood
    - 2ml red-top clot activator tube
    - 4ml purple-top EDTA tube
- Tourniquet
- Individually packaged 70% isopropyl alcohol wipes.
- 2x2 Gauze or cotton balls.
- Sharps disposal container
- Bandages or tape

Procedure:

A standard protocol for venipuncture was followed (BRTC_CLN_001_V02: Collection of blood samples and transport to BRTC and Kingani Laboratory) to collect blood from each participant:

- 1ml of blood in a clot activator tube for rapid HIV testing at TDH TB Clinic
- 2ml of blood in EDTA for FBP stored and transported to BRTC at 2-8°C with a Universal Blood sample transfer form (**Appendix 9**)
- Only for participants ≤18 months of age; 4ml of blood was collected in EDTA anticoagulant and transported at 2-8°C immediately to Muhimbili University of Health and Allied Sciences (MUHAS) Laboratories, Dar es Salaam, with a Universal sample transfer form (**Appendix 9**) for HIV PCR analysis.

Mantoux/Tuberculin Skin Testing

Materials:

- Cotton swab
- Distilled water
- 1ml insulin syringe with needle
- Tuberculin PPD RT/23 2 TU vial (Statens Serum Institut Reg. No. BOT0600874) at room temperature

Procedure:

As per the protocol: TST administration and reading (**Appendix 12**), one upper forearm of each participant was cleansed with cotton wool wet with distilled water, allowed to dry and two units (0.1 ml) of purified protein derivative containing a human-infective strain of MTB antigen (Tuberculin PPD RT/23) injected intradermally. The parent or guardian was then informed not to wash the area until the TST was read, between 48 and 72 hours after the injection.

Reading:

An induration (a raised part of skin and not the whole reddened area) of at least 1mm high and more than 5mm in diameter, identified between 48 and 72 hours after injection, indicated a positive reaction.

A positive result indicated sensitivity to tuberculosis and could mean that the child either:

- Had active tuberculosis - often strongly positive: >15 mm, but can be negative*
- Had their BCG vaccine prior to TST - positive, but often <15mm
- Had latent tuberculosis - had tuberculosis in the past

For appropriate interpretation, TST results were reviewed along with the clinical assessment or scoring.

It is known that TST results are often negative in HIV co-infected children, and weakly positive (5-10 mm) in children exposed to *environmental mycobacteria which is not clinically significant unless the participant is immune-deficient (Logitharajah, 2008).

LABORATORY ANALYSIS SITES

Following the Ministry of Health and Social Welfare, and the National Aids Control Programme guidelines of Tanzania, staff at the TDH TB clinic performed rapid HIV testing for every participant. Test kits used were Alere Determine HIV 1&2 Ag/Ab Combo as first-line and Uni-Gold™ Recombigen® HIV-1/2 as confirmatory. The results were recorded in the HIV test register at TDH TB clinic. HIV PCR testing for samples from children 18 months and younger was done at the MUHAS laboratory, reported on their official report form and filed in relevant participant folders at TDH TB clinic. All HIV results were also recorded in the Diagnosis and Medication CRF (**Appendix 10**) per participant. Full blood profile analysis was done at BRTC and reported as a print out from the equipment that was filed in each participant folder.

Cytological smear processing, ZN staining and microscopic assessment by a pathologist were done at AKHD and reported on FNA CRF form (**Appendix 8**). Culture, DST and speciation was performed at BRTC according to the 'Preparation and inoculation of MGIT and Results interpretation' protocol (**Appendix 19)** and reported on a MGIT Culture and Speciation Report Form.

With the establishment of Xpert testing at IHI Mwananyamala TB Clinic (from October 2012), half the FNA-in-PBS specimen (~0.35ml) was analysed immediately on collection. The remaining specimen aliquots, and all specimens collected prior to October 2012, were frozen in a 2-8°C freezer for

not longer than 12 hours. These were then transported to BRTC at 2-8°C, where they were frozen at -20°C, for batch analysis to be done at a later date. Any “invalid/error” Xpert tests at Mwananyamala were re-tested from frozen (-20°C) FNA-in-PBS specimens at BRTC (stored for EasyNAT testing). Xpert results were printed in standard-format from the analyser and filed in participant folders.

One batch of the frozen (-20°C) FNA-in-PBS specimens was analysed using the EasyNAT assay in late December 2012. Specimens collected between December 2012 and March 2013 were analysed using EasyNAT in May 2013. EasyNAT results were reported on the Ustar Result Form (**Appendix 24**) and filed in participant folders.

FNA LABORATORY ANALYSES

All FNA specimens were assessed through AFB microscopy, cytological analysis, liquid (MGIT) culture, Xpert® MTB/RIF (Cepheid) and Ustar EasyNAT™ TB IAD analysis as described below.

Ziehl-Neelson staining and AFB microscopy

At the AKHD laboratory, heat-fixed FNA smears were flooded with 1% carbol fuchsin stain (1g basic fuschsin, 5g phenol, 10ml absolute ethanol [Rankem, India] per 100ml stain) and gently heated with a flame to encourage steaming for 5 minutes without allowing the stain to boil or dry. The slides were then washed and decolourized using 25% sulphuric acid (Rankem, India) for 1 minute or until the smear was light pink in colour. After washing, Methylene blue stain (Rankem, India) was used to counter stain the smear for 2 minutes, the slides washed and dried, and cover slips mounted. The smears were examined at 1000x magnification for 300 fields using light microscopy. The results were reported on **Appendix 8**: FNA CRF, as either “AFB seen” or “AFB not seen”.

Cytomorphological analysis

A classic Papanicolaou (PAP) stain comprises of 5 dyes in 3 solutions, used to differentially stain the components of cells to help distinguish between superficial, intermediate, parabasal and metaplastic cells. Papanicolaou staining was done at AKHD laboratory by first hydrating the ethanol-fixed

slides in reducing concentrations of ethanol for 2 minutes each (70% and 50% ethanol) and tap water for 2 minutes. The slides were then soaked in haematoxylin stain (Quimica Clinica Aplicada, SA) for 4 minutes, rinsed in tap water before differentiating in acid:alcohol for 30 seconds and rinsed in tap water again. They were then dehydrated with absolute alcohol for 30 seconds and soaked in orange G stain (Quimica Clinica Aplicada, SA) for 1 minute. Absolute alcohol was used for 50 seconds to rinse the slides which were then soaked in EA50 stain (Quimica Clinica Aplicada, SA) for 2 minutes. The slides were rinsed in absolute alcohol for 50 seconds, cleared in xylene (Quimica Clinica Aplicada, SA) for 30 seconds and a cover slip mounted with DPX mountant (DePeX® BDH, Prolabo, VWR, Ireland). Slides were then scrutinized under 1000x magnification with a light microscope.

The cytomorphological pattern in TB lymphadenitis FNA can vary greatly; from nonspecific lymphadenitis or suppurative acute lymphadenitis, to necrosis with or without granuloma formation or granuloma alone. The pattern may also vary based on the immune status of the patient, where a small amount of amorphous necrosis and occasional giant cells may be present in immune competent cases including epithelioid histiocytes and epithelioid granulomata in a background of reactive lymphocytes and plasma cells. In immune compromised patients with TB, smears commonly show abundant "dirty" necrosis with neutrophils and cellular debris being prominent (Cibas and Ducatman, 2009; Wright et al., 2008). In my study, the presence of granuloma and/or necrosis was reported as "consistent with mycobacterial infection".

Due to the variety in cytomorphological presentation, and the fact that other organisms such as fungal infections may present a similar morphological picture, aforementioned AFB staining was performed and reported on all aspirates in this study, irrespective of the cytological diagnosis. The slide (smear) quality, cytomorphology and diagnosis were reported on **Appendix 8**: FNA CRF. The diagnoses were reported as "confirmed TB lymphadenitis" (AFB present), "suspected TB lymphadenitis" (cytomorphology consistent with mycobacterial infection) or "other" which was then specified (including suspicion of malignancy).

MGIT culture, speciation and drug susceptibility testing (DST)

The Mycobacteria Growth Indicator Tube (MGIT) is a 16 × 100 mm round bottom tube containing 7ml of modified Middlebrook 7H9 Broth base (Becton Dickenson, Cockeysville, MD). A fluorescent compound, sensitive to the presence of oxygen dissolved in the broth, is embedded in silicone at the bottom of each tube. A large amount of dissolved oxygen in the non-inoculated medium quenches emissions from the compound and only a little fluorescence can be detected. A growth supplement and an antibiotic mixture (to prevent growth of other non-MTB organisms) is added according to standard protocol, to make a complete medium for mycobacterial growth, just prior to inoculation with specimen (Siddiqi and Rusch-Gerdes, 2006). When there is bacterial growth in the broth, respiration from these microorganisms consumes the oxygen and the BACTEC MGIT 960 System instrument is able to detect the increase in fluorescence in the tube.

Tubes in the BACTEC MGIT 960 System are continuously incubated at 37°C and monitored by the system every 60 minutes for increasing fluorescence. A tube is flagged as positive when it contains approximately 105 to 106 colony-forming units per millilitre (cfu/ml), determined by algorithms programmed into the system. Culture tubes which remain negative for a minimum of 42 days and which show no visible signs of positivity are considered negative for bacterial and mycobacterial growth (Siddiqi and Rusch-Gerdes, 2006).

In my study, each FNA specimen inoculated MGIT tube was incubated at 37°C for 8 weeks or until flagged positive on the instrument. No growth on the instrument after 8 weeks was reported as "negative for MTB". If flagged positive, the growth from the tube was plated on LJ medium (BBL^{TM} Becton Dickenson, Cockeysville, MD) for identification and blood agar medium to assess for bacterial contaminants (growth other than MTB). If there was growth on blood agar medium, the MGIT growth was decontaminated and re-inoculated in a fresh MGIT tube. If positive once again on blood agar medium, the specimen was reported as "contaminated" (**Appendix 19**: *MGIT Culture & Reporting SOP*).

If a sample was negative for blood agar growth and positive for AFB on ZN stain from MGIT culture, a TBCheck MPT64 assay (HAIN Lifescience GmbH)

was used to confirm MTB complex. Molecular speciation for MTB complex and detection of resistance to INH and RMP was performed using the GenoType MTBDRplus assay (HAIN Lifescience GmbH) (**Appendix 20**: *MTB speciation, INH & RMP resistance detection*). The specimen was also processed using GenoType MTBC (HAIN Lifescience GmbH) to identify the MTBC species, including *M. bovis BCG*.

Specimens that were negative for MTB complex were further analysed using LJ medium and GenoType Mycobacterium CM/AS assays (HAIN Lifescience GmbH) to determine if the growth was any of the 24 most common non-tuberculous mycobacteria (NTM) or 19 additional NTM species and reported as NTM ("negative for MTB").

In the case of indeterminate results from the LJ media and GenoType systems, DNA extracts of MGIT culture were sent for DNA sequence analysis to the Emerging Bacterial Pathogens Unit, WHO Supranational Reference Laboratory for Tuberculosis (SRL-TB), Division of Immunology, Transplantation and Infectious Diseases, San Raffaele Scientific Institute, Milan, Italy. If organisms other than MTB or NTM were identified, for example Nocardia, then the specimen was reported as "contaminated".

Laboratory report forms (LRF) no 2, 5, 6 and 7 were used to report MGIT culture, speciation and DST results as processed (**Appendix 21**).

Xpert® MTB/RIF (Cepheid) Assay

Xpert testing was performed on each FNA-in-PBS specimen following the Xpert MTB/RIF test for FNA protocol (**Appendix 22**). Half of the FNA-in-PBS sample per participant (approximately 0.35ml) was treated with a sodium hydroxide and isopropanol-containing reagent (Sample Reagent) in a 2:1 ratio (SR:sample) for 15 minutes. The treated sample was transferred, using a demarcated (defined volume) disposable pipette, into a single-use plastic cartridge ready-manufactured with preloaded liquid buffers and lyophilized reagent beads. The cartridge was then loaded into the GeneXpert instrument where sample processing, DNA extraction and hemi-nested real-time PCR were automated.

GeneXpert PC 21/12/12 09:39:04

Test Report

Patient ID*:
Sample ID: Xpert M 171212123930
Test Type: Specimen
Sample Type: FNA

Assay Information

Assay	Assay Version	Assay Type
Xpert MTB-RIF Assay G4	5	In Vitro Diagnostic

Test Result: MTB DETECTED VERY LOW;
Rif Resistance NOT DETECTED

Test and Analyte Result
No fields selected

User:			
Status:	Done	Start Time:	17/12/12 12:40:27
Reagent Lot ID*:	10914	End Time:	17/12/12 14:21:17
Expiration Date*:	14/07/13	Module Name:	A1
Cartridge S/N*:	205392312	Module S/N:	612768
S/W Version:	4.0b	Instrument S/N:	706600
Notes:	Used 0.3ml sample		
Error Status:	OK		

Errors
<None>

Tech. Initial/Date Supervisor Initial/Date

* indicates that a particular field is entered using a barcode scanner

For In Vitro Diagnostic Use Only.

GeneXpert® Dx System Version 4.0b Page 1 of 1

Figure 8: Sample of an Xpert® MTB/RIF report

Results were generated (Figure 8) through the system software programmed algorithm and reported as MTB NOT DETECTED or MTB DETECTED (if at least two of the five *rpoB* probes are bound within two cycles of each other) (Figure 4). Semi-quantitative estimates of MTB concentration were reported in the cases of MTB DETECTED as either LOW, MEDIUM or HIGH based on cycle number at threshold (Ct) values from the real-time monitoring of amplification. As the samples were collected from participants suspected of having TB, it was not unreasonable to assume the presence of active *M. tuberculosis* infection, in the case of a positive assay.

The five *rpoB* probes are designed to bind to the wild type sequence in the region where the most prevalent *rpoB* mutations occur. They do not bind to mutant sequences; therefore the assay detects mutations based on the absence of probe molecular beacon signals. If there was a 3.5 cycle or greater difference in the cycle threshold between the earliest and latest *rpoB* signals (delay) then this was also reported as RIF resistance (Helb et al., 2010; Blakemore et al., 2010).

Ustar EasyNAT^{TM} TB IAD (Biotech) Assay

EasyNAT testing was performed following the Ustar EasyNAT TB IAD Assay Protocol (**Appendix 23**) on each FNA-in-PBS specimen (remaining after Xpert analysis). Sample preparation involved a boiling and chemical extraction protocol. Up to 1ml of the FNA-in-PBS specimen was centrifuged in a 1.5ml centrifuge tube >10 minutes at >10,000rpm. The supernatant was discarded and 40µl of DNA extract solution added to the pellet, vortexed to mix well and incubated at 95-100°C for 10 minutes. The tube was cooled at room temperature for 5 minutes and centrifuged at >10,000rpm for >5 minutes. The supernatant was either immediately analysed as the amplification template for the next step, or stored at -20°C for not more than one week until analysed.

For the amplification and hybridization step, Reaction Mix (glassified) tubes were labelled; one per specimen and one for the negative and positive control each (as recommended to be done for every test batch). Fifteen microlitres of Resuspension Buffer were added into each tube (containing lyophilized reaction mix) and kept at room temperature for 2-3 minutes to allow complete dissolution. Four microlitres of ddH_2O was added to the tube for the negative control, 4µl of positive control into the positive control tube and 4µl amplification template per specimen into each of the specimen-labelled tubes. The contents of each tube were individually mixed thoroughly using the micropipette on application of each template, and the tube lids closed never to be opened again.

The tubes were centrifuged for 3 to 5 seconds at >4,000rpm and incubated at 63°C for 60 minutes. At the end of the incubation period, each tube was placed individually into device cartridges (without opening the tube lids).

These were then inserted into detection devices and the device lids snapped shut, at which point the reaction tubes were automatically pierced to release the PCR product onto the lateral-flow detection strip. Results were read visually from the strips, 10 to 30 minutes after placing the tubes in the detection devices (Figure 9).

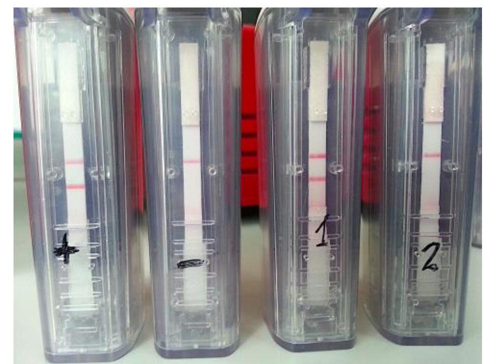

Figure 9: Strip detection for TB using EasyNAT^{TM} TB IAD strips and cartridges

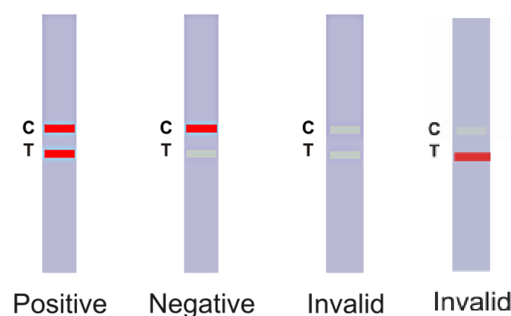

Figure 10: Interpretation of strip detection in EasyNAT^{TM} TB IAD

A valid result was one that showed a clear control band (PCR was successful) with a test band for presence of MTB or control positive, and no test band in the absence of MTB (Figure 10). A negative control showed only the control band.

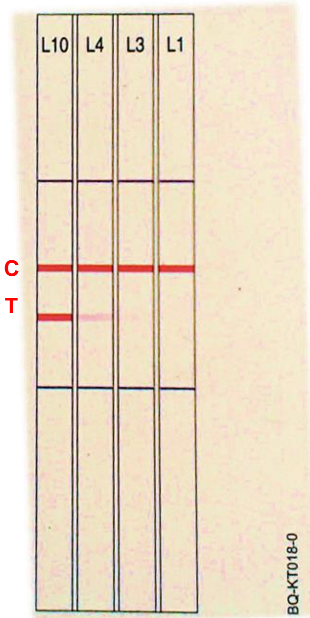

Figure 11: Intensity scale, EasyNAT^{TM} TB IAD insert (Ustar Biotechnologies, 2009).
C indicates the control band and T the test band.

Specimen positivity for TB was determined by the intensity of the test band in relation to an intensity scale provided in the kit (Figure 11). A test band with intensity of L4 and above was considered positive for MTB, while intensity of less than L3 was considered negative for MTB. Repeat testing was done to obtain conclusive results when the test-band intensity was between L3 and L4. Results read after 30 minutes were not valid (Ustar Biotechnologies, 2009). EasyNAT results were reported on USTAR Result Form (**Appendix 24**).

STATISTICAL ANALYSES

Four participants were excluded from the analyses because of incomplete data due to; death after recruitment, loss of contact, relocation and subsiding lymph node more than 4 weeks after general antibiotic treatment, respectively. All FNA diagnostic outputs, clinical assessment scores and composite reference standards created, were analysed for sensitivity, specificity and Cohen's kappa (κ) using Stata software version 12.1 (StataCorp, College Station, Texas, USA). Latent Class Analysis (LCA) was performed on all FNA diagnostic outputs (**Appendix 25**), using Statistical Analysis Systems (SAS) Software version 9.2 SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

EVALUATION OF XPERT® MTB/RIF AND USTAR EASYNAT™ TB IAD FOR DIAGNOSIS OF TUBERCULOUS LYMPHADENITIS OF CHILDREN IN TANZANIA: A PROSPECTIVE DESCRIPTIVE STUDY

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Evaluation of Xpert® MTB/RIF and Ustar EasyNAT™ TB IAD for diagnosis of tuberculous lymphadenitis of children in Tanzania: a prospective descriptive study

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Abstract

Background: Fine needle aspiration biopsy has become a standard approach for diagnosis of peripheral tuberculous lymphadenitis. The aim of this study was to compare the performance of Xpert MTB/RIF and Ustar EasyNAT TB IAD nucleic acid amplification assays, against acid-fast bacilli microscopy, cytology and mycobacterial culture for the diagnosis of TB lymphadenitis in children from a TB-endemic setting in Tanzania.

Methods: Children of 8 weeks to 16 years of age, suspected of having TB lymphadenitis, were recruited at a district hospital in Tanzania. Fine needle aspirates of lymph nodes were analysed using acid-fast bacilli microscopy, liquid TB culture, cytology, Xpert MTB/RIF and EasyNAT. Latent class analysis and comparison against a composite reference standard comprising "culture and/or cytology" was done, to assess the performance of Xpert MTB/RIF and EasyNAT for the diagnosis of TB lymphadenitis.

Results: Seventy-nine children were recruited; 4 were excluded from analysis. Against a composite reference standard of culture and/or cytology, Xpert MTB/RIF and EasyNAT had a sensitivity and specificity of 58 % and 93 %; and 19 % and 100 % respectively. Relative to latent class definitions, cytology had a sensitivity of 100 % and specificity of 94.7 %.

Conclusions: Combining clinical assessment, cytology and Xpert MTB/RIF may allow for a rapid and accurate diagnosis of childhood TB lymphadenitis. Larger diagnostic evaluation studies are recommended to validate these findings and on Xpert MTB/RIF to assess its use as a solitary initial test for TB lymphadenitis in children.

Keywords: Mycobacterium tuberculosis, Lymphadenitis, Childhood, Fine needle aspiration, Nucleic acid amplification, Xpert MTB/RIF, Ustar EasyNAT, Culture, Cytology, Ziehl-Neelsen

Background

Paediatric tuberculosis (TB) contributes considerably to the global burden of TB disease. The World Health Organization (WHO) reported that of the 9.6 million incident cases in 2014, an estimated 10.4 % (1 million) were children [1]. Other groups however, estimated the TB incidence for the same year, as between 200,000 and 970,000 new cases [2–4]. The large variation of those estimates illustrates the difficulties in assessing the disease burden in children, as recording and reporting of paediatric cases is often imperfect and ultimate confirmation of TB diagnosis is still a challenge.

Reasons for the difficulties in diagnosing TB in children are manifold: respiratory specimens from younger children are difficult to obtain, *Mycobacterium tuberculosis* (*M. tuberculosis*) is often not detectable in paucibacillary paediatric specimens and host biomarkers are not yet in place to reliably identify children with TB disease [5, 6]. Moreover, symptoms and radiological features are

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non-specific and overlap with those of other diseases, such as pneumonia, malnutrition or HIV-associated diseases. The tuberculin skin test (TST), which indicates TB infection, can guide clinicians in their decisions, but does not confirm TB disease [7]. Hence, better diagnostic tests for both pulmonary and extrapulmonary paediatric TB are urgently needed.

Extrapulmonary TB (EPTB) constitutes up to half of all paediatric TB cases, predominantly in children of young age and with HIV infection [8, 9]. The most common manifestation of EPTB in children is tuberculous lymphadenitis (TB lymphadenitis) in about two thirds of EPTB cases, followed by central nervous system involvement (meningitis), pleural, disseminated and skeletal TB [9]. TB lymphadenitis may be mediastinal, intra-abdominal or, as was the focus of this study, peripheral. This form is typically superficial, and often presents as a non-specific swelling which may be mistaken for malignant in nature [10]. Previous studies in East-African adults show that peripheral lymphadenitis is frequently caused by non-tuberculous mycobacteria or *M. bovis* [11, 12]; data on these etiologies in East-African children, however, are still scarce [13, 14].

Fine needle aspiration biopsy (FNAB), an inexpensive, easy-to-perform outpatient procedure, has become the diagnostic approach of choice for children with persistent lymphadenitis and is suited for primary health care [15–17]. Diagnostic microscopy is used for cytology, for smears after Ziehl-Neelsen (ZN) staining, and for autofluorescence detection on Papanicolaou stained smears. If combined, these three modalities have a reasonably high sensitivity and specificity compared to culture [18]. However, neither culture nor microscopy-based diagnostic approaches are considered a gold standard for TB lymphadenitis. These tests are still rarely used in peripheral facilities of resource-poor settings: culture is complex and implicates a high biosafety level; interpretation of cytology requires advanced technical expertise and small laboratories are often not equipped with fluorescence or light-emitting diode (LED) microscopes.

Automated and isothermal nucleic acid amplification tests (NAAT), designed for use at low biosafety levels, could offer promising alternatives for point-of-care diagnosis of TB lymphadenitis.

Xpert® MTB/RIF (Cepheid, Sunnyvale, USA) is an automated, cartridge-based NAAT for the detection of *M. tuberculosis* complex and rifampicin drug-resistance from sputum and other specimens, with a turnaround time of less than two hours [19, 20]. A WHO policy statement from 2013 formulated recommendations to guide the use of the test for pulmonary and extrapulmonary TB in adults and children. Based so far only on very low evidence, Xpert has been conditionally recommended as a replacement test for usual practice (including conventional microscopy, culture or histopathology) for testing specific non-respiratory specimens, including lymph nodes [21].

The EasyNAT™ TB Isothermal Amplification Diagnostic Kit (short form: EasyNAT; Ustar, Biotechnologies Co. Ltd, Hangzhou, China) is an assay that uses isothermal cross-priming amplification technology for the qualitative detection of *M. tuberculosis* [22]. The tool was approved for detection of pulmonary TB by the China Food and Drug Administration in 2014 [23]. Up to now, no studies have been published on EPTB diagnosis using EasyNAT.

The aim of our study was to compare the diagnostic performance of Xpert MTB/RIF and EasyNAT with acid-fast bacilli (AFB) microscopy, cytology and mycobacterial culture for the diagnosis of TB lymphadenitis in children from a TB-endemic setting in Tanzania.

Methods

Recruitment and clinical assessment

This prospective study was carried out from April 2012 until August 2013 at the Temeke District Hospital in Dar es Salaam, Tanzania. Children older than 8 weeks and younger than 16 years of age, suspected of having TB lymphadenitis as defined by WHO [24], were recruited. Inclusion criteria were: one or more palpable lymph nodes of 1 cm or more persisting for more than 4 weeks in-spite of oral antibiotic therapy and a strong clinical suspicion or microbiological confirmation of mycobacterial infection. Children who had received TB treatment in the preceding 12 months were excluded from the study. All participants were clinically assessed and treated according to current versions of the Tanzanian National TB and Leprosy Program (NTLP) guidelines [25, 26]. For children up to 6 years of age, the clinical assessment was based on the NTLP clinical scoring chart. Results from the evaluated diagnostic approaches also had an impact on treatment decisions.

At enrolment, clinical assessment comprised chest X-ray in a frontal view, TST, full blood count and HIV testing with pre- and post-test counselling. The HIV rapid testing algorithm followed national guidelines [27]. HIV DNA nucleic acid test was done for children between 8 weeks and 18 months of age, while for older children antibodies to HIV were detected using rapid tests or Enzyme Immunoassays. FNAB was performed on all participants. Expectorated sputa were collected from children with productive cough and sent for AFB microscopy to the district laboratory. At a follow-up visit (time after recruitment: 5–12 months; median 5 months), participants were assessed for treatment outcomes and referred for further treatment, if necessary.

Palpation-guided fine needle aspiration biopsy

Palpation-guided FNAB was carried out by trained medical officers on palpable regional lymph nodes. FNAB

was performed with a 23-gauge needle attached to a 10 mL syringe. The skin around the lymph node was cleaned with an alcohol swab, the child was held firmly and the node was stabilized while aspiration was performed, with not more than 2 mL of suction.

Two smears were prepared from the first aspirate. One smear was immediately fixed in 95 % ethanol solution for Papanicolaou staining for cytological assessment and the other was air-dried and fixed in 100 % methanol for ZN staining. The residue in needle and syringe was rinsed in mycobacterial growth indicator tube (MGIT) medium for mycobacterial culture with standard PANTA antibiotic/growth supplement (Beckton Dickinson, Sparks, USA) [28]. The MGIT tube was transported at ambient temperature to the laboratory for incubation and further processing.

From the same lymph node, a second aspiration was performed and rinsed into 0.7 ml sterile phosphate buffered saline (PBS), pre-prepared in 1.5 ml polypropylene tubes. These were transported to the laboratory at 4-8 ° C for NAAT testing. Laboratory personnel involved in Xpert MTB/RIF and EasyNAT testing were blinded to other results.

Laboratory diagnosis

FNA microscopy/cytology

The pathologist reported ZN stained smears as; "AFB seen" or "no AFB seen". Cytology results were categorised as "confirmed mycobacterial lymphadenitis" (visualization of AFB), "suspected mycobacterial lymphadenitis" (signs of TB necrosis and granuloma consistent with mycobacterial infection), nonspecific "reactive lymphadenopathy" or "suspicion of malignancy" [29].

FNA TB culture

The inoculated MGIT tubes were incubated at 37 °C for a maximum of 8 weeks. Positive MGIT cultures were tested for contamination on blood agar. MPT64 antigen and molecular tests as needed (GenoType® MTBDR*plus* system and GenoType MTBC, Hain Lifescience GmbH, Germany) were used for confirmation of *M. tuberculosis* complex and identification of MTBC species, including *M. bovis* BCG. Drug susceptibility testing (DST) was done as described in the BACTEC MGIT 960 product insert (Becton Dickinson) [30]. Non-tuberculous mycobacteria (NTM) were assessed using a reverse line-probe assay (GenoType® Mycobacterium CM/AS, Germany).

FNA Xpert MTB/RIF testing

One half of the aspirate in PBS (0.2 to 0.7 ml) per participant was filled up to 2.2 ml with Xpert MTB/RIF sample reagent and incubated for 15 min, prior to dispensing 2 ml of the mixture into a Xpert MTB/RIF. The cartridge was loaded into the Xpert MTB/RIF instrument for automated sample and real-time polymerase chain reaction (RT-PCR) processing. The instrument reported the presence or absence of *M. tuberculosis* complex, a semiquantitative estimate of *M. tuberculosis* complex concentration (high, medium, low, and very low) and the presence or absence of rifampicin resistance.

FNA EasyNAT testing

The other half of the aspirate in PBS was processed according to the EasyNAT testing protocol for specimens other than sputum [31]. Very viscous or purulent specimens were first treated with liquefying buffer provided, according to the protocol for sputum specimens. The DNA extracted for each sample was then prepared for amplification and incubated at 63 °C for 60 min [31]. Detection of amplified products was performed on a lateral flow strip, housed in an enclosed sealed plastic device, to prevent leakage of amplicons. Results were visualized after 20 to 30 min. Appearance of the control band alone indicated that the test was valid but that there was no *M. tuberculosis*, or that the amount of *M. tuberculosis* DNA was below the detection limit. In conjunction with a visible control band, positivity was determined by the intensity of the test band in relation to an intensity scale provided [31].

Statistical analysis

Sensitivity, specificity and Cohen's Kappa [32] were calculated for each and for all diagnostic modalities combined with culture and cytology as single reference standards, and with culture and cytology combined (*M. tuberculosis* positive when either was positive and *M. tuberculosis* negative when both were negative), as a composite reference standard (CRS). The calculations were performed only for patients without missing data for the respective modalities. Kappa (k) of more than 0.6 indicated substantial to almost-perfect agreement with the reference standard. Latent class analysis (LCA), a statistical method for finding subtypes of related (latent) cases from multivariate categorical data, was performed on all the FNAB diagnostic outcomes. For each diagnostic modality, two classes were identified, 1 for TB positive and 2 for TB negative. Blank cells indicated inconclusive outcomes or missing data. The LCA provided estimates of the probability of *M. tuberculosis*-presence or *M. tuberculosis*-absence per case, based on the data input. Additionally, a mixed logistic regression model with a 5-dimensional binary outcome defined by the results of the 5 test modalities was applied. The model included a random subject intercept and assigned each subject the empirical Bayes estimate of this random effect as a TB-score, which was then correlated with each binary test. Analyses were done using SAS version 9.2 (SAS Institute

Inc., Cary, NC, USA) and Stata version 12.1 (StataCorp, College Station, Texas, USA).

Results

A total of 79 children were recruited into this study; 4 children were excluded from analysis because of incomplete data due to death after recruitment, loss of contact, relocation and subsiding lymph node more than 4 weeks after general antibiotic treatment. During the recruitment phase, a standard 6-month anti-TB regimen was started in 57 participants based on NTLP paediatric TB scoring (for children <6 years of age) or clinical assessment (for children ≥6 years of age) and in 6 participants based solely on laboratory test results, i.e. cytology, Xpert MTB/RIF or culture. Participants who did not receive TB treatment ($n = 12$) were reviewed by the internal medicine, surgery or oncology departments. At the follow up visit, the lymphadenitis was resolved in 33/34 children who had completed the anti-TB therapy and 3/6 children who completed alternative therapy (surgical intervention, fungal or antibiotic treatment). Figure 1 displays the patient flow and clinical outcomes.

More boys than girls were recruited (61 % vs. 39 %). The HIV prevalence was 20 %. Only 2 participants, 4 and 11 years of age, had a history of TB. At recruitment one of the three children with additional productive cough was found to be sputum AFB positive. Table 1 summarizes the participant characteristics.

The frequency of results from the different diagnostic techniques on FNAB is shown in Table 2. In summary, cytology results in 18 children (24 %) were consistent with mycobacterial infection including 3 children with ZN-positive lymphadenitis. *M. tuberculosis* was identified in 7 children (9 %) by culture. *Mycobacterium bovis* BCG was isolated from 2 HIV negative children (3 %) who were 3 and 4 months of age. Xpert MT/RIF was positive in 14 (19 %) and EasyNAT in 5 (7 %) participants. There were no invalid Xpert MTB/RIF or EasyNAT results. No resistance was detected by Xpert MTB/RIF or phenotypic DST.

Figure 2 displays the correlation between the different laboratory testing modalities reporting cases as positive for TB. The culture results comprise both samples with confirmation of *M. tuberculosis* ($n = 7$) and *M. bovis* BCG ($n = 2$). In 25 children, the diagnosis 'TB lymphadenitis' was confirmed by at least one of the diagnostic modalities; of which 12 cases were positive by a single diagnostic modality alone.

The performance of each laboratory diagnostic modality was assessed against culture as the reference standard for TB. Due to the low positivity and high contamination rates for culture in this study, cytology was considered

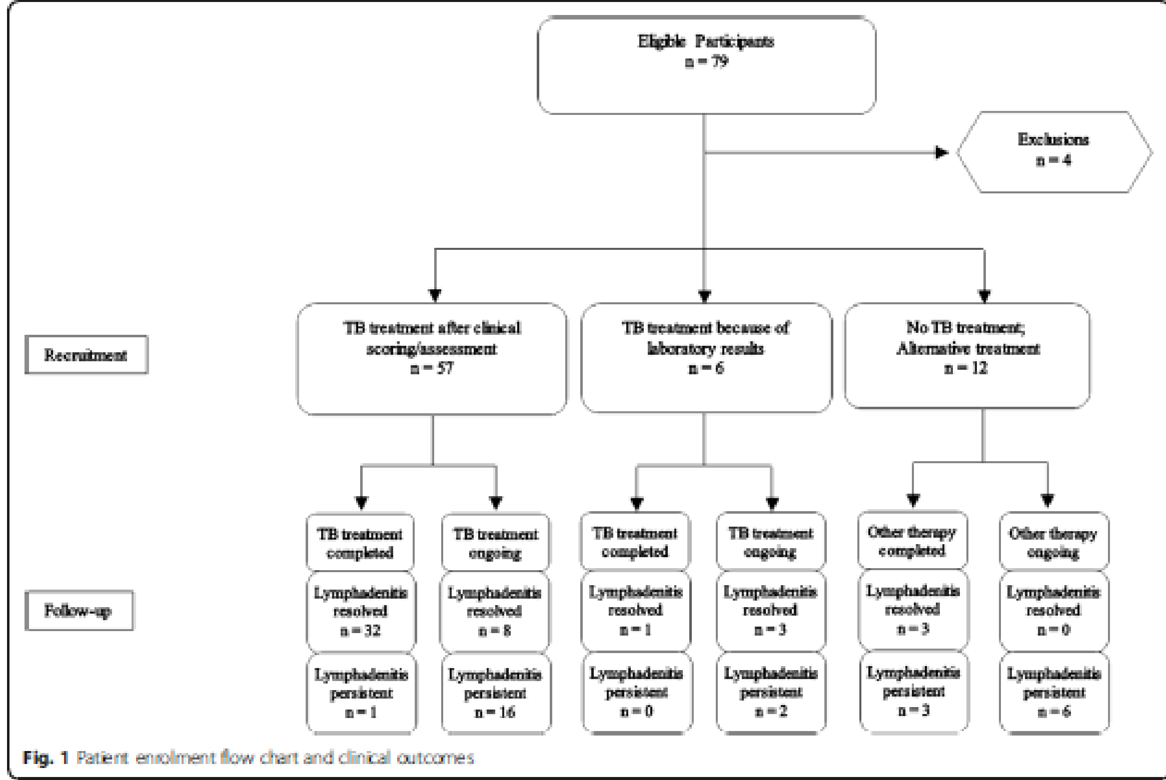

Fig. 1 Patient enrolment flow chart and clinical outcomes

Table 1 Participant characteristics

Characteristics at recruitment	Children included in study ($N = 75$) n (%)
Age (years)	
0 to ≤ 2	14 (19 %)
2 to ≤ 6	30 (40 %)
6 to ≤ 10	16 (21 %)
10 to ≤16	15 (20 %)
Gender	
Male	46 (61 %)
HIV status	
HIV test positive	15 (20 %)
HIV test negative	54 (72 %)
Unknown/missing/not done	6 (8 %)
TB contact in last 12 months	
Yes	18 (24 %)
General clinical condition	
Good	55 (73 %)
Reduced	15 (20 %)
Ill	5 (7 %)

Table 2 Frequency of laboratory findings on FNAB

Diagnostic modalities	Children included in study ($N = 75$) n (%)
Cytology	
Consistent with mycobacterial infection[a]	18 (24 %)
Reactive lymphadenopathy (negative)	39 (52 %)
Malignancy/suspected malignancy (negative)	4 (5 %)
Unsuitable for analysis	14 (19 %)
Missing	-
Ziehl Neelsen microscopy	
Acid fast bacilli-positive	3 (4 %)
Negative	71 (95 %)
Missing slide	1 (1 %)
Culture	
M. tuberculosis	7 (9 %)
M. bovis BCG	2 (3 %)
Negative	29 (39 %)
Non-tuberculous mycobacteria (negative)	1 (1 %)
Contaminated	36 (48 %)
Missing	-
Xpert MTB/RIF	
Positive	14 (19 %)
Negative	56 (74 %)
Missing result	5 (7 %)
EasyNAT	
Positive	5 (7 %)
Negative	70 (93 %)
Missing	-

[a] Consistent with mycobacterial infection' includes confirmed TB (AFB seen) and/or suspected TB (morphological features consistent with TB infection – caseating necrosis and granuloma)

as an alternative reference standard. Additionally, a composite reference standard, combining culture and cytology results, was used to assess the performance of the diagnostic tests (Table 3). Xpert MTB/RIF detected TB lymphadenitis with a sensitivity and specificity of 56 and 96 %, 69 and 93 %, 58 and 93 % against a reference standard consisting either of culture, or cytology, or culture and/or cytology respectively. The corresponding sensitivity and specificity values for EasyNAT were 22 and 100 %, 22 and 100 %, and 19 and 100 %. The category "any diagnostic modality" aimed to assess the performance of a diagnostic algorithm including all the modalities except those used as reference standard. The sensitivity of "any diagnostic modality" were either equal or superior compared to single tests, while the specificity of "any diagnostic modality" at 93 % was below specificity values of some single tests.

The agreement of each diagnostic modality with the appointed reference standard (Cohen's Kappa – k) is also described in Table 3. Xpert MTB/RIF, compared to EasyNAT, showed a better agreement with TB culture (k = 0.58 vs. 0.31), cytology (k = 0.64 vs. 0.29) and the CRS (k = 0.55 vs. 0.24). ZN microscopy had a poor agreement with the three reference standards.

For LCA two 'latent classes' were generated: class 1 for TB-positive (prevalence of TB lymphadenitis in the study population) with a probability of 22.8 % and class 2 for TB-negative with a probability of 77.2 %. The probability of a positive test result given membership to class 1 (i.e., the sensitivity) was high for cytology (100 %), culture (74 %), Xpert MTB/RIF (71 %), but low for EasyNAT (29 %) and ZN (19 %). Conversely, the probability of a negative test result given membership to class 2 (i.e., the specificity) was high for all 5 tests, with 100 % for EasyNAT and ZN, 95 % for Cytology, 94 % for Xpert MTB/RIF and 90 % for culture.

The mixed logistic regression model returned a TB-score for each patient. The correlation of this score with each binary test showed that cytology performed best with a correlation of $r = 0.87$, followed by culture and Xpert MTB/RIF at par ($r = 0.82$), EasyNAT ($r = 0.60$) and ZN ($r = 0.42$).

Positive laboratory test results were analysed in relation to results of the paediatric score chart for children below 6 years of age and to clinical assessment for the older children (Table 4). By assessing the agreement to score or clinical assessment, the true-positive rates for all of the five modalities ranged between 5 and 26 % and false-positive rates between 8 and 33 %.

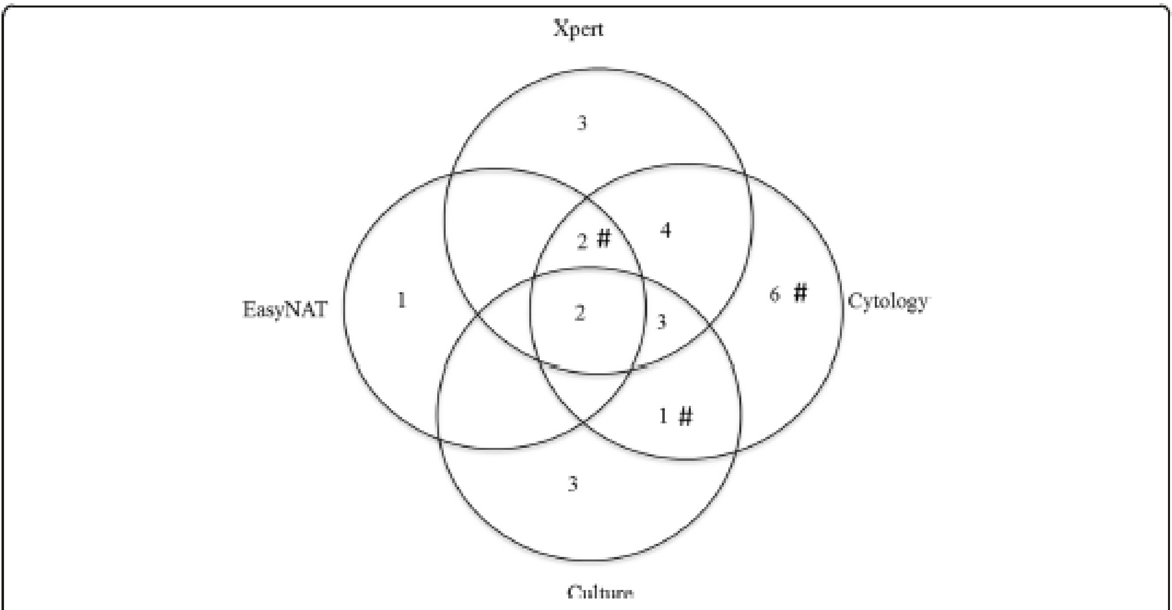

Fig. 2 Venn diagram of TB positive results from all diagnostic modalities for FNAB. Diagram shows positive results from culture (including *M. tuberculosis* and *M. bovis* BCG), Cytology, Xpert MTB/RIF and EasyNAT. The 3 AFB positive ZN smears are indicated by #

Discussion

We assessed the performance of Xpert MTB/RIF and of EasyNAT for the diagnosis of paediatric TB lymphadenitis, using culture or cytology as single reference standards and a combination of both as a composite reference standard. The results differed considerably between the two NAATs under evaluation. Xpert MTB/RIF performed with a modest sensitivity and good specificity, while the sensitivity of EasyNAT was comparatively poor, with excellent specificity. Both Xpert MTB/RIF and EasyNAT may have been negative in some cases of lymphadenitis caused by NTM infection, which is frequently seen in East Africa [11], but might be undetected in our study due to the high proportion of contaminated cultures. These results were consistent with a Cohen's kappa statistic, which showed a superior agreement of Xpert MTB/RIF with TB culture, cytology and the CRS compared to EasyNAT.

As an alternative to the CRS approach, a latent class analysis was applied to estimate the sensitivity and specificity of diagnostic tests by linking multiple test results to the same latent categories. This latent class model was used because of the imperfect or missing reference standard [33]. The LCA revealed superior sensitivity estimates for Xpert MTB/RIF compared to EasyNAT (71 % versus 29 %). Conversely, the specificity estimates for EasyNAT were to some extent higher than those for Xpert MTB/RIF (100 % versus 94 %). Interestingly, LCA indicated an excellent sensitivity (100 %) and good specificity (95 %) for cytology.

Table 3 Test performance and agreement of diagnostic modalities

	Culture as reference standard					Cytology as reference standard					Composite reference standard: culture and/or cytology				
	Sensitivity[a]		Specificity[a]		Cohen's Kappa	Sensitivity[a]		Specificity[a]		Cohen's Kappa	Sensitivity[a]		Specificity[a]		Cohen's Kappa
	n/N		*n/N*		k	*n/N*		*n/N*		k	*n/N*		*n/N*		k
Cytology	6/9	67 %	22/24	92 %	0.60	-	-	-	-	-	-	-	-	-	-
Culture	-	-	-	-	-	6/8	75 %	22/25	88 %	0.60	-	-	-	-	-
ZN	1/9	11 %	24/25	96 %	0.10	3/18	17 %	43/43	100 %	0.22	3/21	14 %	41/41	100 %	0.18
Xpert	5/9	56 %	26/27	96 %	0.58	11/16	69 %	38/41	93 %	0.64	11/19	58 %	40/43	93 %	0.55
EasyNAT	2/9	22 %	30/30	100 %	0.31	4/18	22 %	43/43	100 %	0.29	4/21	19 %	46/46	100 %	0.24
Any diagnostic modality	6/9	67 %	28/30	93 %	0.62	18/18	100 %	40/43	93 %	0.89	13/21	62 %	38/41	93 %	0.58

[a]Calculated only for patients without missing data

Table 4 FNAB laboratory diagnoses compared to clinical assessment

		Paediatric score <6 years of age (n = 44)		Clinical assessment ≥6 years of age (n = 31)	
		Score ≥7 (anti-TB therapy recommended)	Score <7 (anti-TB therapy *not* recommended)	Anti-TB therapy recommended	anti-TB therapy *not* recommended
	n	n = 38	n = 6	n = 19	n = 12
Positive culture	9	5 (13 %)	1 (17 %)	3 (16 %)	-
Positive cytology	18	9 (24 %)	2 (33 %)	5 (26 %)	2 (17 %)
Positive Xpert	14	5 (13 %)	2 (33 %)	5 (26 %)	2 (17 %)
Positive EasyNAT	5	2 (5 %)	-	2 (11 %)	1 (8 %)
Positive ZN	3	2 (5 %)	-	1 (5 %)	-

Percentages in columns for 'anti-TB therapy recommended' are true-positive rates
Percentages in columns for 'anti-TB therapy *not* recommended' are false-positive rates

A systematic review and meta-analysis of 13 studies described a pooled sensitivity and specificity of 83.1 and 93.6 % for the Xpert MTB/RIF assay with TB culture as a reference standard for the diagnosis of lymph node TB [34]. This publication ultimately informed WHO policy and has led to recommendations on the use of Xpert MTB/RIF, over established tests, for diagnosis of TB in lymph nodes in adults and children [35]. An additional meta-analysis which also examined Xpert MTB/RIF against TB culture for the diagnosis of TB lymphadenitis showed a slightly better pooled sensitivity (87 %) and a similar specificity (92 %) [36]. A hospital-based South African FNAB study in 72 children by *Coetzee* et al. reported a sensitivity of 80 % and a specificity of 93.8 % against a CRS (cytology suggestive of mycobacterial disease with visualization of the bacillus and/or positive TB culture) [37]. In contrast to mentioned meta-analyses which focused primarily on adults and the evaluation on children from South Africa, our Xpert MTB/RIF study in children showed substantially lower estimates for sensitivity, but similar estimates for specificity. The limited sensitivity of Xpert MTB/RIF is most likely caused by the paucibacillary nature of lymphatic TB manifestations in children [38] or by suboptimal rinsing of needle passes [37].

The findings underscore the need for larger and representative paediatric accuracy studies for the diagnosis of mycobacterial lymphadenitis by Xpert MTB/RIF and other NAATs. In the future, more sensitive, point-of-care NAAT, which are also able to perform resistance testing, are required to make an impact over current modalities with regard to mortality caused by paediatric and extrapulmonary TB, as described in a recent modelling study [39].

Until now, EasyNAT, a manual assay based on isothermal cross-priming amplification, has only been evaluated for the detection of pulmonary TB in adults [40, 41]. These two studies reported sensitivity for *M. tuberculosis* detection against culture of 84.1 and 66.7 %, and specificity of 97.8 and 100 %. Sensitivity in these studies was further reduced when only smear-negative but culture-positive pulmonary TB cases were tested (59.8 and 10 %) which indicates a suboptimal limit of detection that probably could explain the poor sensitivity with our paediatric patients, where low density of mycobacteria is expected in lymph nodes.

A major limitation of this study was the relatively high proportion of invalid test results; i.e. 19 % of the samples sent for cytology were not suitable for analysis and 48 % of the TB cultures were contaminated. Consequently, the comparison between diagnostic modalities was often restricted to a small number of participants with valid results. The high contamination rate negatively impacted the absolute number of positive TB cultures and therefore reduced the value of TB culture as a reference standard in FNAB.

The greatest promise of NAATs for the detection of TB in lymph nodes of children is fast and accurate diagnosis, combined with simultaneous resistance testing. The incomplete overlap of the different diagnostic modalities suggests that neither Xpert MTB/RIF nor EasyNAT currently seem to be appropriate as a single NAAT to diagnose TB lymphadenitis in children.

Conclusions

Based on our findings, a promising diagnostic algorithm for suspected childhood TB lymphadenitis seems to consist of the combination of clinical assessment according to WHO guidelines [17], cytology and Xpert MTB/RIF. However, larger studies in children from different settings are needed to prove this diagnostic concept. EasyNAT seems to be not suitable for diagnosis of TB lymphadenitis in children. At high healthcare levels, TB culture might be a valuable method for differentiation between *M. tuberculosis* and *M. bovis* BCG, which is important for management of patients with lymphadenitis, but its broader use is restricted due to the length of time needed to get results, technical complexity, high costs and biosafety requirements.

Abbreviations

AFB, acid-fast bacilli; BCG, Bacillus Calmette–Guérin; CRS, composite reference standard; DNA, deoxyribonucleic acid; DST, Drug susceptibility testing; EPTB, extrapulmonary tuberculosis; FNAB, fine needle aspiration biopsy; HIV, human immunodeficiency virus; LCA, latent class analysis; LED, light-emitting diode; MGIT, mycobacterial growth indicator tube; NAAT, nucleic acid amplification tests; NIMR, National Institute for Medical Research; NTLP, National TB and Leprosy Program; NTM, non-tuberculous mycobacteria; PBS, phosphate buffered saline; RT-PCR, real-time polymerase chain reaction; Swiss TPH, Swiss Tropical and Public Health Institute; TB, tuberculosis; WHO, World Health Organization; ZN, Ziehl-Neelsen

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Availability of data and materials

The study data will not be shared, as this was not part of the study/analysis plan, and therefore not included in the informed consent form.

Authors' contributions

MB (PhD student, Medical Parasitology-Infection Biology, Swiss TPH), HPB (Head of Medical Parasitology and Infection Biology, Swiss TPH) and KR (Group Leader - Clinical TB Research Group, Swiss TPH) designed the study. MB, NK (Regional TB and Leprosy Coordinator) and EM (TB and HIV Officer) were overseeing enrolment and patient care. HC (Histopathologist, Muhimbili University of Health and Allied Sciences, Dar es Salaam, Tanzania), LJ (Head of TB Laboratory, Bagamoyo Research and Training Centre, Tanzania), MS (Manager of TB Laboratory, Bagamoyo Research and Training Centre, Tanzania) and MB were responsible for the different laboratory procedures. TRC (Biostatistician, Swiss TPH), MB and KR did data management and analysis. MB, HPB and KR wrote the draft of the report. All authors contributed to data collection, interpretation of data, and revision of the article. All authors have read and approve of the final version of the manuscript.

Competing interests

Ifakara Health Institute purchased the Xpert® MTB/RIF and EasyNAT™ kits at trial prices for research purposes. The Cepheid and Ustar Biotechnologies companies were not involved in the design, analysis and writing of this manuscript. None of the authors received financial compensation for this work.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Ethical approval was obtained from the Institutional Review Board of the Ifakara Health Institute and the Medical Research Coordinating Committee, National Institute for Medical Research (NIMR), Tanzania. Participants were enrolled after written informed consent from a parent or legal guardian. In case of illiteracy, informed oral consent was attested by an independent witness and documented on a paper-based, dated, signed and/or thumb-printed consent form. Additionally, children of 7 years or more provided written assent for participation.

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GENERAL DISCUSSION

Tuberculosis is treatable and usually curable. Unfortunately, the greatest obstacle in managing the disease globally, still is the lack of effective, accessible point-of-care (POC) testing for all forms of TB, leading to challenges in treatment aimed at curbing disease progression, onward transmission and death. Between 2007 and 2011, there was a surge of WHO recommendations for diagnostic systems, in a concerted effort to improve TB diagnosis and management. The methods recommended at the time were:

- liquid culture media, such as the mycobacterial growth indicator tube automated platform (WHO, 2007)
- rapid species identification, such as with the Capilia rapid speciation test to distinguish TB from non-tuberculous mycobacteria (WHO, 2007)
- line probe assays for rapid detection of multi-drug resistant (MDR) TB, such as the GenoType MTBDRplus assay (WHO, 2008)

These technologies are suited for reference laboratory, research institution and private sector healthcare facilities, based on their infrastructural and resource requirements, and therefore cannot function as POC tests for use at low-resource TB-endemic primary care settings.

During the same period (2007-2011), the techniques recommended by the WHO, particularly for low- and middle-resource settings were:

- same-day diagnosis by microscopy (WHO, 2011c)
- fluorescence microscopy (WHO, 2011d) and
- non-commercial culture and DST methods (WHO, 2011e)
- the automated GeneXpert MTB/RIF real-time PCR NAAT (WHO, 2011f), updated (WHO, 2013c)

By the end of 2014, 69% of the TB high burden countries (HBCs) reported that national policy indicated the use of Xpert MTB/RIF as the initial diagnostic test for people at risk of drug-resistant TB, and 60% reported that national policy indicated its use as the initial diagnostic test for people living with HIV (WHO, 2015a). Unfortunately, even with improved access of the Xpert system to HBCs through efforts from the Foundation for Innovative New Diagnostics (FIND), wide-scale implementation of Xpert technology has only occurred in

South Africa, with other HBCs still relying heavily on smear microscopy (Pai and Schito, 2015). Some of the reasons for this stunted roll-out may be the high cost of the instrument and that the system was not designed for lower tiers of the healthcare system, based on the need for stable electrical power and on-site calibration. Therefore, there is still dire need for an effective POC test to impact TB management globally. The WHO has yet to recommend a new TB diagnostic test since the Xpert recommendation of 2010 (updated 2013).

INNOVATIONS IN THE FIELD

There have not been more recent meta-analyses or systematic reviews from TB review groups on commercially available NAATs for the diagnosis of TB, other than those for the diagnosis of TB lymphadenitis (Daley et al., 2007) and pulmonary tuberculosis (PTB) in respiratory specimens (Ling et al., 2008). The results of these reviews showed inconsistent outcomes for use of NAATs in diagnosing TB lymphadenitis and cautioned on the limitations of the review, while for respiratory specimens the analysis recommended for improved sensitivity of NAATs before they may be considered in place of conventional tests. Notwithstanding, there have been several recent systematic reviews evaluating the Xpert MTB/RIF NAA system. One such review was on the effectiveness of Xpert in diagnosing pulmonary tuberculosis and rifampicin resistance in adults, wherein further research was recommended into the use of the Xpert system in TB programmes in high TB burden settings (The Cochrane Collaboration and Steingart et al., 2014). Another review was on Xpert use for the diagnosis of EPTB, wherein Xpert was recommended over conventional tests for diagnosis of TB in lymph nodes and other tissues, and as the preferred initial test for diagnosis of TB meningitis (Denkinger et al., 2014).

Despite these positive reviews, Xpert technology still does not impact the on-going disease management challenges globally, of latent TB infection (LTBI), MDR and extensively drug-resistant (XDR) TB detection. Towards improving the impact on TB management Cepheid is designing the ‘Omni’, a new platform to specifically address many of the logistical constraints faced in microscopy centres, whilst using cartridges already in use for the current

GeneXpert® platforms (WHO, 2015b). Other developments in Cepheid technology are the Xpert MTB/RIF 'Ultra' and 'XDR'. The Ultra is an upgrade over the Xpert MTB/RIF system by way of: the sample-processing cartridge that doubles the amount of purified DNA delivered to the PCR reaction; four newly-designed probes that replace the earlier five probes that detect mutations in the *rpoB* gene; additional real-time MTB detecting probes targeting the IS6110 and IS1081 sequences; high-resolution melt-curve detection generating specific fluormetric fingerprints for each probe binding to its amplified complement allowing for better discrimination of allelic variance within each probe region; and optimization of cartridge fluidics and PCR cycling. It was reported in a February 2015 Conference on Retroviruses and Opportunistic Infections (CROI) presentation, that from an on-going trial the Xpert MTB/RIF Ultra was found to have a much better TB-detection sensitivity than Xpert MTB/RIF and was comparable to culture, increasing the potential for TB detection in smear-negative patients. With a better RIF-resistance specificity than Xpert MTB/RIF, the Ultra assay will also be able to provide more reliable RIF resistance detection (Alland et al., 2015; WHO, 2015b). The Ultra assay is scheduled to be evaluated in 2016 as a potential replacement for conventional diagnostic culture (WHO, 2015a). The XDR assay is being designed to detect isoniazid (INH), fluoroquinolone (FLQ) and aminoglycoside (AMG) resistance, the latter two being important second-line anti-TB drugs (WHO, 2015b).

Following the double CPA *gyrB* gene targeting EasyNAT^{TM} TB IAD kit production, there have only been two evaluation studies of this kit (Fang et al., 2009; Mhimbira et al., 2015) and no further endorsement from the WHO for its use in TB detection, other than trial status (WHO, 2011b). Since 2009, Ustar Biotechnologies (China) have developed three more isothermal cross-priming amplification (CPA) based kits for detection of TB, and rifampicin as well as isoniazid resistance-conferring mutations (WHO, 2013a). The only trial evaluating one of these new kits targeting the IS6110 sequence of MTB (TB detection), demonstrated lower sensitivity and specificity outputs than those of the earlier EasyNAT kit targeting the *gyrB* gene (Ou et al., 2014). This status renders none of the new Ustar developments eligible for recommendation by the WHO.

A development that has potential solutions for some key limitations of the NAATs under investigation in my study, is the TBDx system being developed by the Keck Graduate Institute of Applied Life Sciences, Claremont, California, in conjunction with Claremont BioSolutions, Upland, California, USA and Ustar Biotechnologies (Hangzhou), amongst others (WHO, 2015b). The pertinent limitations from my study were: the need for stable electrical power and the cost of the Xpert system (instrument cost of $17,500 and $9.98 per test for subsidy-eligible TB-endemic countries) (Foundation for Innovative New Diagnostics, 2012); and the extensive hands-on time for DNA extraction and the need for separate amplification and detection platforms for the EasyNAT system.

In the initial development of the TBDx system, Roskos et al. (2013) employed isothermal LAMP and comparative Exponential Amplification (EXPAR) reactions to detect MTB genomic DNA, coupled with nucleic acid lateral flow (NALF) technology (similar to the EasyNAT system) for visual endpoint detection, into a single prototype cartridge. Sample preparation was not incorporated into the cartridge a the time (Roskos et al., 2013). The current model uses a derivative of Claremont BioSolutions's PureLyse® technology (a novel solid-phase extraction in a microbead beater, that does not require chaotropic salts or organic solvents) within the amplification and detection cartridge, to capture MTBC cells, wash, lyse and elute the DNA, from liquefied and disinfected sputum. The eluted DNA is used to dissolve CPA specific reagents (similar to the EasyNAT assay) for an MTBC assay. Immunochromatographic strip detection is used and scored visually. All on-board liquid handling processes within the cartridge are operated through inexpensive electrolytic pumps (epumps). The TBDx is scheduled to be ready for use, by the second quarter of 2018, with the current cost estimated at US$ 150 for the instrument (no onsite calibration required) and under US$ 8 per test cartridge (WHO, 2015b).

One other development of key interest is the VerePLEX Biosystem by STMicroelectronics (Geneva, Switzerland), marketed by Veredus Laboratories (Republic of Singapore), for rapid diagnosis of MDR TB. It is a lab-on-chip (LoC) technology, involving single disposable devices comprising microfluidic PCR and microarray modules. Processing and detection are semi-automated,

with sensitivities of over 97.8% for MTBC, rifampicin and isoniazid resistance detection (mutations on rpoB, katG and inhA genes) in clinical isolates and smear-positive specimens, when assessed against Sanger sequencing and GenoType® MTBDRplus (Hain Lifescience, Nehren, Germany) based detection (Cabibbe et al., 2015). This technology offers promise for a fast and simple diagnostic tool for MDR in referral laboratories.

There are several similar developments in the TB diagnostics arena that are set to be launched over the next few years. They will all require evaluation and clinical-performance assessment before they are recommended for use in TB-endemic low-resource settings (WHO, 2015b).

Extrapulmonary Tuberculosis

In the field of EPTB detection by NAATs, a 2014 study of the Amplified MTB Direct test (AMTD; Gen-Probe Inc); a rapid isothermal technique for amplification of TB 16S-rRNA from urine of patients with EPTB, found the technique useful in early detection of EPTB with a sensitivity of 70%, but not as effective in diagnosing pulmonary TB (18%) (Fortún et al., 2014a).

With the aim of augmenting information on biomarkers for EPTB detection, the same group studied the serum of patients with EPTB. Levels of interferon-gamma, tumour marker Ca-125, adenosine deaminase, chemokine ligand-9 and mannose-binding lectin (MBL) were determined from patients with PTB, EPTB and health individuals. Levels of all the biomarkers were significantly higher in the PTB and EPTB cases than in the control cases. There were no significant differences in levels between the PTB and EPTB cases with the exception of MBL, which were significantly higher in patients with EPTB than in patients with PTB. Some of the biomarkers are not specific to MTB infection, but they do present as acute phase reactants and may be utilized to confirm MTB infection, while MBL may specifically be assessed to distinguish between PTB and EPTB (Fortún et al., 2014b).

CONTRIBUTIONS, STATISTICAL PECULIARITIES AND LIMITATIONS OF MY STUDY

Fine needle aspiration biopsy

Studies have demonstrated that fine needle aspiration cytology has significant benefits in aiding in the diagnosis of TB in resource-limited settings (Mittal et al., 2011; Wright et al., 2009a). In my study, 24% of the FNA specimens were positive for TB by cytology, with 19.6% being unsuitable for analysis compared to 12.5% in a similar study (Wright et al., 2008). There are several factors that could have influenced the success or failure of detecting the presence of TB in FNAB specimens. One key consideration is the naturally uneven proliferation of TB within lymph nodes and the paucibacillary nature of TB in lymphadenitis and EPTB. The site of FNAB may not have been the point of greatest TB proliferation, potentially leading to the patient being erroneously declared negative for TB disease, in the absence of other disease markers (Vadwai et al., 2011).

Considerations for future studies to maximize the yield of MTB in FNAB specimens, would be the use of the "bleach method", initially used to concentrate sputum specimens to improve direct microscopy (Wilkinson and Sturm, 1997). In this method, the specimens are incubated in NALC (N-acetyl-L-cysteine-sodium hydroxide) for 15 minutes and centrifuged at 3000g for 15 minutes, after addition of phosphate buffered saline, and the sediment retained for analysis (Tadesse et al., 2015; Annam et al., 2009a).

Mycobacterial culture

Mycobacterial culture positivity from lymph node aspirates was low in my study (12%). Earlier publications have also shown that the performance of TB culture in FNAB and extrapulmonary specimens is poor (Al-Nakeeb et al., 2013; Trajman et al., 2008; Khan et al., 2006; Chakravorty et al., 2005). A high proportion of the specimens being contaminated (48%) may have impacted culture positivity outcomes. A key source of contamination could have been the opening of the MGIT tubes during inoculation. There have been studies on transport media that potentially lower TB culture contamination rates through FNA inoculation without removal of lids in the field (Wright et al., 2010). These transport bottles contained 1 ml Middlebrook

7H9 broth (with 0.2% glycerol and 0.05% Tween 80 added) in 10 ml headspace glass vials, sealed with 20 mm TFE/Sil Septa and 20 mm aluminium open top seals, prepared in a laminar flow cabinet and autoclaved. Such considerations should be made in order to lower contamination rates when implementing FNA TB culture analyses in the field.

BCG disease

Two cases of *Mycobacterium bovis* (BCG) (22% of MTB culture positives) were reported in this study, isolated from FNAB of lymph nodes of HIV-negative participants. Disseminated BCG disease is a rare complication of BCG vaccination and is usually associated with immune deficiency. Local complications from vaccination and regional BCG lymphadenitis however, have been reported in immune-competent BCG recipients ranging from 0.5% to 17.6% (Braun and Cauthen, 1992). A more recent study of BCG-infected infants reported an incidence as high as 24% of BCG disease in immune-competent infants and linked this to a year 2000 change in vaccine policy from use of a Tokyo-strain BCG vaccine administered percutaneously to a Danish-strain BCG vaccine administered intradermally (Hesseling et al., 2006). Wright et al. (2008) also report an incidence of 23% BCG infection of lymph nodes from the MTB culture positive cases seen in that study.

Nucleic acid testing

The minimal hands-on time, automated extraction and modular design of the Xpert system allowed for random testing of fresh FNA samples. Due to the protracted extraction and PCR preparation protocols of the EasyNAT assay, specimen analysis was done on stored (-20ºC) FNA samples in batches, for purposes of time and resource efficiency. There was no significant difference in performance observed between fresh and stored sample analyses, between and within the two NAATs.

The two NAAT under study differed significantly in their nucleic acid extraction protocols, which could have contributed to the vast distinction in performance of each assay. The Xpert process involved capture of MTB on a filter, sonication to break open the cells and elution of the nucleic acid material, all

fully-automated within the cartridge. The EasyNAT process was manual and involved the use of sodium hydroxide (NaOH) for decontamination and liquefaction in the case of pus-like FNA specimens, which were then pelleted and washed twice. There may have already been nucleic acid (NA) loss at this stage. For standard FNA samples and further to the pelleting step for purulent samples, there was an Extraction Solution step at the end of which the supernatant was saved for processing. Here too there was potential loss of NA material. Aliquots of these extracts were then used in the PCR mix, which further diluted the NA content. Based on these distinctions in protocols and the consideration that lymph node proliferation by TB is paucibacillary in nature, loss of NA material through extraction processes may have played a significant role in the sensitivity of the tests; the Xpert performing significantly better than the EasyNAT method.

Regarding alternative options for DNA extraction in the EasyNAT protocol, the package insert had a “Cautions” page with a point (No. 4) stating: “We provide nucleic acid extraction solution inside the kit for you to extract nucleic acid. However, you can also choose other reliable products for nucleic acid extraction”. As the performance of the EasyNAT kit specifically was being tested, the extraction protocol provided in the instructions-for-use was adhered to. Only in a more recent kit insert, not included in the kits received during the project, EasyNAT Ustar Biotechnologies suggested that the DNA extraction protocol might be performed using an alternative method that they provide, not requiring centrifugation. The alternative method is based on boiling-lysis, binding of the material to a lysis buffer, washing with an ethanol--containing buffer and elution, all using a syringe method. The syringe method may improve NA yield and in so doing, the sensitivity of the EasyNAT assay.

There have been further developments in MTB-DNA stabilization, transport without need for cold-chain, and nucleic acid extraction and concentration technologies for sputum, which may be adapted for use on FNAB specimens. The OMNIgene® SPUTUM by DNA Genotek Inc. (Ottawa, Canada; a wholly owned subsidiary of Orasure Technologies Inc.; USA) liquefies and decontaminates samples, allowing for transport without refrigeration. These samples may be used for microscopy, culture including MGIT, Xpert® MTB/RIF and other molecular detection methods testing for TB. The prepIT®

MAX technology by the same company, is a chemical-lysis and DNA ethanol-precipitation method that circumvents the need for mechanical lysis. Samples first treated with OMNIgene® SPUTUM can be subsequently processed with this kit to concentrate nucleic acids for further analysis. A direct-to-PCR method and direct integration into automated extraction systems are also under development. DNA Genotek Inc. is working with the STOP-TB Partnership, WHO, Foundation for Innovative New Diagnostics (FIND) and the Global Laboratory Initiative (GLI) to determine appropriate paths forward for technical evaluations of these technologies and to facilitate adoption by countries receiving donor funds (WHO, 2015b).

The PrimeStore Molecular Transport Medium® (PS-MTM®) by Longhorn Vaccines & Diagnostics (USA) is another product under consideration specifically for NAAT solutions (Daum et al., 2014a). It contains chemical denaturants that permit lysis of MTB cells with subsequent stabilization of MTB DNA, allowing for transportation without cold chain, and found suitable in one study for PCR analysis within 7 days of collection (Daum et al., 2014b).

To-date, the Xpert kit instructions do not have a protocol for processing of non-respiratory specimens. Although the EasyNAT kit instructions do not particularly mention lymph node aspirates, they have specified processes for sample preparation and DNA extraction from fluid non-respiratory specimens, which were followed for FNA samples in my study. The focus here therefore, is on the deviation from protocol for Xpert testing, of FNA in my study. The instructions direct for the use of Sample Reagent (SR [NaOH and isopropanol]) in a ratio of 2:1 for expectorated sputum samples and 3:1 for re-suspended sputum sediments. In my study, the specimens collected were FNA washed into 0.7ml phosphate-buffered saline (PBS). Half this volume per specimen was used for Xpert analysis (the other half for EasyNAT testing), to which SR was added to a total volume of just over 2mls. Depending on the total specimen volume available, the range of specimen volumes used in Xpert analysis was 0.2ml (in the case of repeat analysis) to 0.7ml. A large proportion of cases had a SR to sample ratio of 5:1 (1.8ml:0.35ml), while the remaining ranged between 10:1 and 2:1. There was no evident correlation between decreased sample volume (decreased ratio) and test positivity,

although there appeared to be a positive correlation between purulent samples and test positivity.

With reference to the current WHO Xpert MTB/RIF Implementation Manual based on a 2014 systematic review of studies using Xpert for diagnosis of extrapulmonary TB, there is no recommendation for the processing of lymph node aspirates, only for lymph node biopsies or tissues (WHO, 2014a; Denkinger et al., 2014). Studies in the systematic review included those by Vadwai (2011), wherein they added SR to the specimen to a total volume of 2ml for CSF, while Ligthelm (2011) increased sample volume to 0.7ml with PBS and then added SR (SR to sample ratio being 3:1) (Vadwai et al., 2011; Ligthelm et al., 2011). Moure (2012) added 3ml (2ml SR and 1ml sediment) to the cartridge, which is not desirable as this volume exceeds the recommended volume for the cartridge, while Malbruny (2011) concentrated all specimens except CSF and had a SR to sample ratio of 3:1 (Moure et al., 2012; Malbruny et al., 2011).

CSF specimens are fluid, and similar to lymph node aspirates, are paucibacillary in TB infection and often with small aspiration volumes. The WHO Xpert MTB/RIF Implementation Manual recommendations for CSF are based on sample volume availability, whereby concentration of specimens by centrifugation is recommended for volumes of 5ml and more, while an equal volume of SR is recommended for samples of 1 to 5ml. Most relevant, is the recommendation for sample volumes of 0.1 to 1ml, wherein adding SR to the whole sample to a final volume of 2 ml, is recommended. All of the mixture is then added directly to the Xpert MTB/RIF cartridge. Accordingly, my study followed the most appropriate sample processing protocol for Xpert testing for FNA specimens.

Statistical analyses

The use of a composite reference standard (CRS) to assess the accuracy of diagnostic kits has been highly recommended by Denkinger et al. (2014) and Naaktgeboren et al. (2013), while options for the use of CRS, latent class analysis (LCA) and other accuracy measures have been categorized well by Reitsma et al. (2009): (Denkinger et al., 2014; Naaktgeboren et al., 2013; Reitsma et al., 2009).

Composite Reference Standard

Examples of studies that have used CRS to assess the performance of Xpert MTB/RIF on lymph node specimens are Tadesse et al. (2015 – CRS: culture and/or smear microscopy) Ligthelm et al. (2011 - CRS: culture and histology), Tortoli et al. (2012 - CRS: culture, histology and improvement with anti-TB therapy), Vadwai et al. (2011 – CRS: culture, radiology and improvement with anti-TB therapy), Van Rie et al. (2013 – CRS: culture, histology and smear with species identification) and Zeka et al. (2011 – CRS: culture, histology and radiology): (Tadesse et al., 2015; Ligthelm et al., 2011; Tortoli et al., 2012; Vadwai et al., 2011; Van Rie et al., 2013; Zeka et al., 2011). With culture as the conventional gold standard and cytology performing well in my study, analyses were done with "culture and/or cytology" as the CRS, to assess the performance of the other diagnostic modalities. Concordance to the reference standard of each modality went down, compared to that with either culture or cytology as the reference standard. This outcome using a CRS, may suggest a more credible assessment of the modalities, which demonstrated highest concordance to the CRS by Xpert, followed by EasyNAT and ZN microscopy.

Latent Class Analysis

Mycobacterial culture positivity of FNAB in my study was low (12%). This made culture an uncertain reference standard against which to assess the performance of alternative TB diagnostic tools. The true TB status of participants could not be confirmed without AFB visualization (microscopic) or MTB growth. Clinical assessment has previously been shown to overestimate the incidence of TB disease (Fox et al., 2013) and similarly in my study, it does not correlate well with diagnostic outcomes. Resolution of lymphadenitis of alternative infectious aetiologies other than TB, through use of TB therapy, is not uncommon (Low, 2009; Rayner and Munckhof, 2005). Resolution of lymphadenopathy could therefore not be used as a proxy for TB diagnosis.

In cases of imperfect or missing reference standards, latent class analysis (LCA) has been recommended to assess the efficacy of diagnostic tools (Reitsma et al., 2009; Denkinger et al., 2014). There are no published studies using LCA to assess the accuracy of Xpert to-date. There are limited studies evaluating EasyNAT, none of which employ CRS or LCA for accuracy estimation.

I used LCA to analyse the performance of all the diagnostic modalities used to test FNAB in my study. LCA was used to create a “true TB status” per participant, based on probability statistics applied to the diagnostic outputs from my study (**Appendix 25**). The 100% sensitivity output from LCA of cytology was possibly an overestimation, due to the wide spread of cytology positives over positive cases calculated by LCA. This propensity for over-estimation through LCA has previously been identified (Pouillot et al., 2002; Alonzo and Pepe, 1998; Vacek, 1985). Beyond this limitation, LCA outputs for the other diagnostic modalities correlated well with the prior statistical analyses; demonstrating superiority of cytological analysis over culture for TB detection in FNA of lymph nodes and that there was added value in the use of Xpert in a diagnostic algorithm.

A 2014 systematic review on the use of Xpert for the diagnosis of extrapulmonary TB, demonstrated a very low proportion of non-interpretable results by Xpert overall (1.2%) and concluded that Xpert was highly sensitive in diagnosing TB lymphadenitis, both from tissue and aspirate samples (Denkinger et al., 2014). The review also stipulated that culture was an imperfect reference standard for TB lymphadenitis and recommended the use of CRS and LCA for accuracy estimation of diagnostic tests. No distinction was found between TB detection by Xpert on lymph node specimens from HIV-positive and HIV-negative participants. Also pertinent was the need, as identified from the review, for more studies on the use of Xpert for extrapulmonary specimens in a primary or secondary care TB-endemic setting, as was the case in my study. The review was used to guide a WHO recommendation for the use of Xpert over conventional tests for diagnosis of TB in lymph nodes in adults and children (WHO, 2014a).

The overall statistical outcomes of my study demonstrated that cytomorphology is a feasible and effective technique for detection of TB in lymph node aspirates, that may complement TB culture where available, and that Xpert was superior in performance to EasyNAT and ZN analyses respectively. Adding either of the latter 2 tests to a diagnostic algorithm that already included Xpert testing would have no added value. Smear microscopy is often the only test available in many TB endemic settings, but there has been substantial progress in availing Xpert testing in these resource-limited

countries and the technology is gaining endorsement as a key diagnostic tool particularly on varied specimen types in paediatric TB detection (Raizada et al., 2015).

SIGNIFICANT OUTPUTS AND CONCLUSION

Nucleic acid based methods for TB detection have the advantage of speed and sensitivity, particularly in specimens with minute amounts of nucleic acid. Currently, there are several tests in late-stage development or on-track for evaluation by the WHO, including several NAATs, antibody/antigen-detection based (including LAM in urine), VOC based, and automated-imaging based technologies for TB diagnosis and/or DST (HIV i-Base/Treatment Action Group, 2015). Some of these are newly developed while others are second- and third-generation developments from technologies already recommended. With the exception of very few of these test candidates (dipstick for LAM in urine, the Xpert MTB/RIF Ultra, stronger supporting evidence for GenoType MTBDRsl and PURE-LAMP) it is envisaged that "no test under development is likely to be on the market with policy endorsements within the next three to five years" (WHO, 2014b).

Two of the key recommendations by the HIV/HCV/TB 2015 Tuberculosis Diagnostics Pipeline Report were:

- To "Improve the quality of research studies, e.g., for follow-on NAA technologies, which have the potential to be cheaper, more portable, and more accessible than Xpert MTB/RIF, but for which evidence of their effectiveness has been sorely lacking" and
- To "Intensify investments in comparative studies of new TB diagnostics and algorithms to optimize the use of current and emerging approaches in all important settings"

My study was able to address a substantive component of these recommendations including assessing the performance of a cheaper alternative NAAT to the Xpert (**Appendix 26**) and has contributed to the overall pool of knowledge.

Through concerted efforts by the Foundation for Innovative New Diagnostics (FIND) over the recent years, GeneXpert technology is now available in the public sector of 116 of the 145 TB high burden countries eligible for concessional pricing (WHO, 2015a). With the protocol defined in my study, performing Xpert analysis on lymph node FNA is very feasible for the diagnosis of TB lymphadenitis in children.

A significant outcome of my study was the value of FNA cytology in detection of TB in lymph nodes, which performed better than Xpert testing. Having basic and cheap sample collection and transportation requirements, introducing this test modality into TB-endemic low-income settings is very plausible, requiring investment mainly in training for cytomorphological analyses (Mittal et al., 2011).

From my study, a functional diagnostic algorithm for suspected childhood TB lymphadenitis would be the combination of clinical assessment according to WHO guidelines (WHO, 2014c), with lymph node cytomorphology, and Xpert MTB/RIF, a fast NAAT detecting TB and rifampicin resistance. EasyNAT appears unsuitable for diagnosis of tuberculous lymphadenitis in children. There is still significant value in TB culture for purposes of confirming TB infection and for drug susceptibility testing; the challenge remains its availability at the grassroots level in low-income endemic settings.

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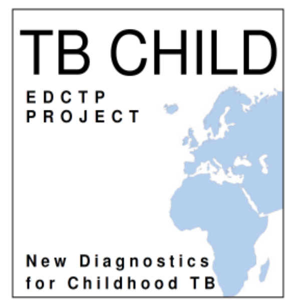
Appendix 1: Informed Consent Form EPTB CHILD v1.0 English 02.06.11



Ifakara Health Institute (IHI)
Bagamoyo Research and Training Centre (BRTC)
P.O. Box 74
Bagamoyo
United Republic of Tanzania

INFORMATION SHEET

TO THE PARENTS OR LEGAL GUARDIANS OF CHILDREN SUSPECTED TO HAVE TB.

ADDITIONALLY, AN ASSENT FORM WILL BE MADE AVAILABLE FOR CHILDREN > 7 YEARS AGE AND SIGNED BY THE MINOR IF HE/SHE UNDERSTANDS THE CONTENT AND AGREES TO THE STUDY

STUDY TITLE:
EVALUATION OF Xpert^{TM} MTB/RIF (GeneXpert) AND Ustar IAD TB (Biotech) ON CYTOLOGICAL ASPIRATES FOR DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN CHILDREN

Name of patient: ____

Date: ____

Study ID Number: ____

Thank you for taking time to read this information. Your child is invited to participate in a research study on new diagnostics for tuberculosis (TB). Before you decide, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Feel free to ask us if there is anything that is not clear or if you would like more information. You are completely free to choose whether or not you wish to take part. Please take your time to decide.
We have selected your child because he/she is suffering from symptoms which are likely to be caused by TB, but can also be caused by another disease. We invite you to you read this form and ask any questions you may have before agreeing to study.
This study is being conducted by Ifakara Health Institute (IHI)/Bagamoyo Research and Training Centre (BRTC) in cooperation with different research institutes in Africa and Europe. The study is funded by the European & Developing Countries Clinical Trials Partnership (EDCTP) and European Governments.
The study protocol has been approved by the IHI Institutional Review Board and the Medical Research Coordination Committee (MRCC) / NIMR of Tanzania.

Study Purpose

TB is difficult to diagnose, in children even more than in adults. The purpose of this study is to find out whether **new diagnostic methodologies** are more precise in detecting childhood TB than the existing approaches. We hope that the newly developed tests will diagnose TB in an easier and more reliable way than the current widely used methodologies and might also help to monitor TB treatment.

Who can take part?

Children older than 8 weeks and younger than 16 years of age who are suspected having TB of parts of the body where there are swollen glands known as lymph nodes and have not been treated for TB in the past 12 months can take part in this study. Whether or not your child takes part in the study is entirely up to you, and you do not have to give a reason if you don't want to be in the study. If you decide that your child does not take part your child will not have a disadvantage. If you decide that your child takes part in the study, but change your mind later, you will be free to withdraw from the study at anytime without giving a reason. If you withdraw from the study it will not affect the medical care of your child.

Study Procedures

In the first days of our study the following will be carried out: Initially, we will examine your child thoroughly and will ask questions about his/her medical history and your household. Furthermore, we will **collect sputum** [maximum 2x5 ml] for diagnosis of TB. In addition, we will **draw** blood [3 ml] for standard investigations (e.g. blood cell count, HIV tests and CD4 count).

A standard **tuberculin skin test** will be performed in each participating child. Additionally, **x-ray** of the chest and probably of other parts of the body will be necessary. **Biopsy** of enlarged lymph nodes and the **collection of other samples** as described in the sample donation form will be essential to confirm or rule out the diagnosis.

Your child will have a test for HIV. This is recommended for everyone suspected having TB and is required for this study. Before and after testing an experienced counsellor will explain the tests and the results and will give support to your child and the family. In case your child is tested positive, a second confirmatory test will be employed. If this test confirms that your child is HIV positive, we will strongly recommend to attend the Care and Treatment Centre and probably anti-retroviral therapy will be provided there.

If we confirm, that your child has or most likely has TB, immediate TB treatment will be recommended according to the national guidelines of Tanzania. There will also be cases where a **preventive TB treatment** (e.g. less than 5 year old child with contact with smear positive adult) has to be recommended. The definite decision for TB treatment will always be the responsibility of the Physician of the Paediatric Department and the local coordinator of the national TB control programme. Any other disease will also be treated by the Paediatric Department of our collaborating hospital according to national guidelines.

We will carry out one **follow-up visit** after 5 months, which will include clinical examination and medical questionnaire. All procedures will be explained by the physician in charge to you and your child.

Risks of Study Participation

The study has the following risks: Taking the blood will cause some pain. Venous puncture and biopsy might lead in very rare case to infection or bleeding, which will be treated immediately. Adherence to strict hygiene regulations and standard operational procedures will minimize this risk. Participating in the study and thereafter revealing your child's health status might be psychologically disturbing for you and your child. We have a trained counsellor to help in these difficult moments.

Benefits of Study Participation

There is no monetary benefit in this study. However, your child will be quickly and reliably diagnosed and actively monitored during the observation period using advanced diagnostics in a standardised way. The community will benefit from the information that will be obtained from this study. The collected data will help to improve TB diagnostics for children and treatment monitoring in the future.

Study Costs/Compensation

There will be no costs for you if your child participates in the study. You will not get paid for participation. However you will be compensated for time, travelling and inconvenience by 7,000 Tsh for each of visit.

Confidentiality

The records of this study will be kept private. In any publications or presentations, we will not include any information that will make it possible to identify your child as a subject. The records for the study may, however, will be reviewed by the participating research institutes with appropriate regulatory oversight. If any study data will be transmitted via the Internet, files are encrypted and password-protected to guarantee privacy.

Voluntary Nature of the Study

Participation in this study is voluntary. Your decision whether or not your child will participate in this study will not affect your current or future relations with the Temeke District Hospital, the Aga Khan Hospital, the Ifakara Health Institute (IHI) / Bagamoyo Research and Training Centre (BRTC) or any decisions made for medical care.

Contacts and Questions

The researchers clinically conducting this study are: Ms. Maira Bholla (PI) and Dr. Nuruddin Lakhani (Co-Investigator). You may ask any questions you have now, or if you have questions later, you are encouraged to contact them at the Aga Khan Hospital, Dar-es-Salaam / Temeke District Hospital.

Ms. Maira Bholla (PI) Phone: 0684600599	Dr. Fredrick Haraka (Co-PI) Phone: 0754 539 673 Dr. Nuruddin Lakhani (Co-Investigator) Phone: 0783004301

You will be given a copy of this form to keep for your records. You do not have do decide immediately about your participation in the study. For your consideration 24 hours are given.

Informed Consent Form

Study Title: **EVALUATION OF Xpert^{TM} MTB/RIF (GeneXpert) AND Ustar IAD TB (Biotech) ON**

CYTOLOGICAL ASPIRATES FOR DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN CHILDREN

Principal Investigator: **Ms. Maira Bholla**

Name of patient: ____

Study ID Number: ____

I confirm that I have read and understand the information sheet, dated 2 June 2011 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	!	--------
I understand that the participation of my child is voluntary and that I am free to withdraw at any time, without giving any reason, without medical care or legal rights being affected.	!	--------
I understand that participation in the trial will require my child to have an HIV test. The result of this will be kept confidential and will not be given to me or my child without my permission. I have been given a chance to think it over and to ask questions.	!	--------
I understand that sections of any of my child's medical notes and data collected during the study may be looked at by responsible individuals of the research consortium conducting this trial, from regulatory authorities or from the hospital, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	!	--------
I agree that my child takes part in the above study	!	--------

All 5 boxes must be initialled or marked for the consent to be valid.

________________________ Name of Parent/Guardian	________________ Date	________________________ Signature or thumb print of Parent/ Guardian
________________________ Name of Witness (if appropriate)	________________ Date	________________________ Signature
________________________ Name of Person taking consent (if different from researcher)	________________ Date	________________________ Signature
________________________ Researcher	________________ Date	________________________ Signature

When completed 1 copy for patient; 1 copy for researcher site file; 1 copy (original) to be kept in patient's medical notes

Sample Donation Form

Study Title: **EVALUATION OF Xpert™ MTB/RIF (GeneXpert) AND Ustar IAD TB (Biotech) ON CYTOLOGICAL ASPIRATES FOR DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN CHILDREN**

Site Principal Investigator: **Ms. Maira Bholla**

Name of patient: ______________________________________

Study ID Number: ______________________________________

Information:
Your samples (blood, sputum and biopsy samples of lymph nodes) will be kept in the TB CHILD specimen bank of our institute for up to ten years, after which they will be destroyed. They will be identified only by a code number, and only the project leaders will have access to the code. In future, we may use the sample for other scientific studies solely related to tuberculosis. The samples can be shipped to cooperating research institute for special investigations on TB research.

Statement:
I voluntarily donate the required samples to the above mentioned study. The samples will be used for TB research, which might be unknown at this point of time.

____

Printed Name of parent/guardian

____

Signature of parent/guardian (or fingerprint)

____

Date

____

Printed Name of Witness

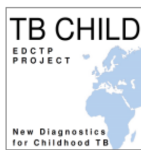
____

Signature of Witness

____

Date

Appendix 2: Assent form EPTB CHILD v1.0 English 02.06.11



Ifakara Health Institute (IHI)
Bagamoyo Research and Training Centre (BRTC)
P.O. Box 74
Bagamoyo
United Republic of Tanzania

ASSENT FOR MINORS

AN ASSENT FORM WILL BE MADE AVAILABLE FOR CHILDREN > 7 YEARS AGE AND SIGNED BY THE MINOR IF HE/SHE UNDERSTANDS THE CONTENT AND AGREES TO THE STUDY

Study Title: **EVALUATION OF Xpert™ MTB/RIF (GeneXpert) AND Ustar IAD TB (Biotech) ON CYTOLOGICAL ASPIRATES FOR DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN CHILDREN**

Site Principal Investigator: **Ms. Maira Bholla**

Name of patient: ____

Study ID Number: ____

Who are we?
We are doctors and scientist from Temeke District Hospital, Aga Khan Hospital Dar es Salaam / Ifakara Health Institute (IHI) / Bagamoyo Research and Training Centre (BRTC).

Why are we meeting with you?
We want to tell you about a study that involves children like yourself. We want to see if you would like to be in this study too.

Why are we doing this study?
We want to find out why you are sick and want to help you. In the same moment we are also looking at an illness called tuberculosis. It is up to now very difficult to find out if a child is suffering from this illness. This study will hopefully help us and other doctors to diagnose and control tuberculosis more easily and to help many children in future.

What will happen to you if you are in the study?
After explaining the study to your parents and you, we will ask questions about your health and examine you. Furthermore, x-ray and other examinations will be done. These examinations will be explained to you as they are done. And we want to collect sputum, draw blood and other body fluids from you at different times of the study.

What are the good things and bad things that may happen to you if you are in the study?
The good thing about this study is that we are using very good methods to diagnose your illness and to help you. Besides, many children will benefit in future from the results of this study.
The bad thing is that in this study, we want to draw blood several times and body fluids at least once from you. That might be a bit painful.

Do you have to be in the study?
No you don't. No one will get angry or upset with you if you don't want to do this.
Just discuss it with you parents and tell us if you don't want to be in the study. And remember, you can change your mind later if you decide you don't want to be in the study anymore.

Do you have any questions?
You can ask questions at any time. You can ask now. You can ask later. You can talk to me or you can talk to someone else at any time during the study.

If you want to be in the study, sign your name on the line below:

Signature of Child: ____ Date: ____________

Signature of Witness: ____ Date: ____________

Signature of PI or Designee: ______________________________________ Date: ____________

Appendix 3: Contact Information v1.1, 14.04.11

CONTACT INFORMATION FORM

-not to be entered into the database-

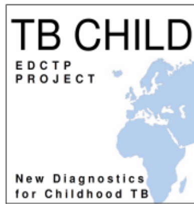
Study ID	Date of Interview (DD/MM/YYYY)
|___|___|___|___|___|-|___|___|-|___|___|___|	|___|___| |___|___| |___|___|___|___|
Initials of Participant	Date of Birth (DD/MM/YYYY)
|___|___|___| *(initials of first name, middlename, sure name)*	|___|___| |___|___| |___|___|___|___| or Age Years: |___|___| Months: |___|___|

Contact information

Phone number(s) of patient (or guardian)	
Home address (House and plot no.; town/village) Neighbour's name	
Well-known sign near your house	
Next pharmacy / drug store	
Name of close friend / relative	
Phone number(s) of close friend / relative	

Version 1.1, dated 14th April 2011 Page 1 of 1

Appendix 4: Medical history enrolment v1.1, 14.04.11



MEDICAL HISTORY AT ENROLMENT

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|	Date of Interview (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___|
Initials of Participant |___|___|___| (*initials of first name, middlename, sure name*)	Date of Birth (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___| or Age Years: |___|___| Months: |___|___|

1. TB contact

Has the participant had any known TB contact in the last twelve months?

1☐ yes 2☐ no 9☐ unknown

If no or unknown, please go to 'present symptoms'

If yes, please specify:	1☐same household *specify:* ______________ 2☐neighbour 3☐work place 4☐school 5☐other *specify:* ______________
Was this TB contact	1☐sputum smear positive 2☐sputum smear negative 9☐unknown
Was this TB contact treated for TB?	1☐yes 2☐no 9☐ unknown

2. Present symptoms

Has the participant got any of the following symptoms at the moment? *(multiple answers possible)*			Duration of the last episode: 1 = 1-7 days 2 = 8-14 days 3 = 15-27 days 4 = 1-3 months 5 = > 3 months
Cough	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Fatigue/lethargy	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Wheezing	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Difficulty in breathing	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐

MEDICAL HISTORY AT ENROLMENT

Fever over last month	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Chest pain	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Night sweat	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Haemoptysis	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Enlarged lymphnodes	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Skin changes	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Abdominal pains	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Weight loss over last month	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐
Other symptom *Please specify:* ____________________________		1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐

3. Usual social and functional activities (only for children <u>less than 16 years</u> of age)

Social interaction *(according to age)*	1☐ normal (responds well to friends, family, community) 2☐ limited ability to socialize/respond 3☐ child does not/is not able to socialize/respond
Play activities *(according to age)*	1☐ normal play activities 2☐ limited/reduced plays activities 3☐ child does not/is not able to play
Learning tasks *(according to age)*	1☐ normal *(same as other kids)* 2☐ has difficulties to learn/concentrate/follow in school *(delayed compared to other kids same age)* 3☐ child does not/is not able to learn or concentrate
Milestones *(according to age)*	1☐ normal 2☐ mild to moderate delay 3☐ severely delayed

4. Past medical history

How often has the participant been for medical treatment in the past year?	1☐never 2☐1-3x 3☐4-6x 4☐7-9x 5☐>9x	
What were the main reasons for medical treatment in the past year? *(multiple answers possible)*	1☐no medical treatment 2☐cough 3☐diarrhoea 4☐malaria	5☐failure to thrive (only in children) 6☐others,*specify*____________________________ 9☐unknown
Was participant hospitalized in the past year?	1☐no 2☐yes, 1x 3☐yes, >1x 9☐unknown	*<u>If yes</u>, specify reason(s) for hospitalisation:*

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

MEDICAL HISTORY AT ENROLMENT

Is the participant currently hospitalized?	1☐yes 2☐no	*If yes, specify reason(s) for hospitalisation:*	
Has the participant received any blood transfusion in the last year?	1☐yes 2☐no 9☐unknown		
Have any of the participant's relatives ever been tested for TB?	1☐yes 2☐no 9☐unknown		
Has the participant ever been tested for TB?	1☐yes 2☐no 9☐unknown	If yes, when was the last TB test?	1☐During the last 3 months 2☐During the last 12 months 3☐Other___________________
If yes, which tests were performed? *(multiple answers possible)*	1☐clinical examination 2☐chest X-ray 3☐sputum 4☐TST 5☐others, *specify* ____ 6☐no tests done 9☐unknown		
Has the participant ever been diagnosed of having TB?	1☐yes 2☐no 9☐unknown	If yes, when was the participant diagnosed of having TB?	1☐during the last 3 months 2☐during the last 12 months 3☐other___________________
Is a member of the family known to be HIV positive?	1☐yes 2☐no 9☐unknown	If yes, specify: *(multiple answers possible)*	1☐father 2☐mother 3☐brother or sister 4☐grandmother or grandfather 5☐uncle or aunt 6☐any other relative 7☐other ________________
Has the participant ever had a **negative** HIV test?	1☐yes 2☐no 9☐unknown	If yes, when was the last HIV negative test?	1☐during the last 3 months 2☐during the last 12 months 3☐other___________________
Was the participant ever diagnosed of having HIV	1☐yes 2☐no 9☐unknown	If yes, when was the participant first tested being HIV positive?	1☐during the last 3 months 2☐during the last 12 months 3☐other___________________
Did the participant ever receive a BCG vaccination?	1☐yes 2☐no 9☐unknown	If yes:	1☐ during the last month 2☐ during the last 3 months 3☐ > 3 months ago

5. Medication history

Did the participant receive any medication during the last 3 months?	1☐yes 2☐no 9☐unknown *(if no or unknown, skip the next 2 questions)*
If yes, what kind of treatment was this?	1☐antibiotic, *please specify name:* ___________________________________ 2☐TB treatment 3☐ARVs 4☐other, *please specify:* ____

Study ID |___|___|___|___|___|___|-|___|___|-|___|___|___|

MEDICAL HISTORY AT ENROLMENT

Does the participant currently take any medication?	1☐yes, *please specify name:* ______________________ 2☐no 9☐unknown		
Did the participant ever get medication for TB	1☐yes 2☐no 9☐unknown *(if no or unknown, skip the next 3 questions)*		
If yes, when did the participant last have TB treatment?	1☐four to twelve months ago 2☐more than one year ago 3☐never got TB treatment 9☐unknown		
If yes, did the participant complete any previous TB treatment?	1☐yes 2☐no 9☐unknown		
If yes, how long did the participant take the TB treatment?	1☐<1 month 2☐1-3 month 3☐3-6 months 4☐6-9 months 5☐more than 9 months 6☐never got TB treatment 9☐unknown		
Was the participant ever started on medication for HIV?	1☐yes 2☐no 9☐unknown		
If yes, does the participant currently take any HIV medication?	1☐yes 2☐no 9☐unknown		
If yes, did the participant ever discontinue HIV medication?	1☐yes 2☐no 9☐unknown	*If yes, specify reasons for dis-continuation:*	1☐do not believe that treatment helps 2☐lack of medical supply/access 3☐side effects 4☐too many tables 5☐child could not take treatment 6☐other:

6. Nutrition (only for children less than 16 years of age)

Is the child breastfed?	1☐yes 2☐no 9☐unknown	If yes, is the child	1☐exclusively breastfed 2☐breasted with supplemental food
How many meals does your child have every day?	1☐1 meal 2☐2 to 3 meals 3☐more than 3 meals 9☐unknown		
Do you have enough food to feed your child?	1☐yes, always 2☐most of the time 3☐often lack of food at home 9☐don't know		

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

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MEDICAL HISTORY AT ENROLMENT

Which of the following food does your child get at least three times per week? *(multiple answers possible)*	1☐ ugali 2☐ rice 3☐ bananas 4☐ potatoes 5☐ vegetables 6☐ fruits 7☐ chicken 8☐ meat 10☐ fish 11☐ eggs 12☐ milk 13☐ others, specify ______________ 9☐ unknown
If the child gets milk, where does it come from? *(multiple answers possible)*	1☐ own cattle 2☐ cattle in village/neighbourhood 3☐ milk powder 4☐ pre packed/pasteurised from shop 9☐ unknown
Do you boil your milk before consuming?	1☐ yes 2☐ no 9☐ unknown
Has the child got close contact to cattle or any other life stock?	1☐ yes 2☐ no 9☐ unknown

7. Comments

Name of Clinician/Interviewer	Signature	Date
Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

Study ID |___|___|___|___|___|___|-|___|___|-|___|___|___|

Version 1.1, dated 14th April 2011

Appendix 5: Clinical examination v1.1, 14.04.11

CLINICAL EXAMINATION FORM

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|	Date of Interview (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___|
Initials of Participant |___|___|___| (*initials of first name, middlename, sure name*)	Date of Birth (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___| or Age Years: |___|___| Months: |___|___|

Physical Examination / Measurements *(anthropometric data will be calculated automatically)*

Weight |___|___|, |___| kg	Height |___|___|___|, |___| cm
Temperature (ear) |___|___|, |___| °C	Mid upper arm circumference |___|___|, |___| cm *for children under 6 years of age*
Respiratory rate ________ / min	Pulse rate ________ / min
TST 1☐ applied TST 2☐ not applied *If TST apllied please give the following information:* Date of test (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___| Result *(48-72h)* |___|___| mm	Blood pressure |___|___|___| / |___|___|___| mmHg

General Appearance

General condition	1☐ good 2☐ reduced 3☐ ill 4☐ critically ill	
Pallor/anaemia	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
Dehydration	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
Malnutrition	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe

CLINICAL EXAMINATION FORM

Jaundice	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
Cyanosis	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
Clubbing	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
In pain/discomfort	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe
Oedema	1☐ yes, 2☐ no	*if yes, please specify:* 1☐mild 2☐moderate 3☐severe *please specify location:* 1☐feet 2☐hands 3☐face 4☐generalised
Others	1☐ yes, 2☐ no	*if yes, please specify* ____

Lymphatic system

				checked
Enlarged lymphnodes	1☐yes 2☐no	*if yes, please specify: (multiple answers)*		
		1☐ Cervical	1☐right 2☐left 3☐both	☐
		2☐ Sub mandibular	1☐right 2☐left 3☐both	☐
		3☐ Nuchal	1☐right 2☐left 3☐both	☐
		4☐ Supra clavicular	1☐right 2☐left 3☐both	☐
		5☐ Axillar	1☐right 2☐left 3☐both	☐
		6☐ Inguinal	1☐right 2☐left 3☐both	☐
		7☐ Others ______________	1☐right 2☐left 3☐both	☐

Size of biggest lymphnode	1☐ < 1 cm 2☐ 1 cm to < 2cm 3☐ 2 cm to < 3 cm 4☐ 3 cm and more
Main characteristic of lymphnodes *(multiple answers possible)*	1☐ indurated 2☐ soft 3☐ painful 4☐ not painful 5☐ matted 6☐ Other, *please specify* ____

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

CLINICAL EXAMINATION FORM

Respiratory system

Any abnormal finding	1☐yes 2☐no *If yes, please answer the following questions:*			checked
Dyspnoea *(multiple answers possible)*	1☐yes 2☐no	1☐alar flare	1☐mild 2☐moderate 3☐severe	☐
		2☐chest in-drawings	1☐mild 2☐moderate 3☐severe	☐
		3☐use of auxiliary breathing muscles	1☐mild 2☐moderate 3☐severe	☐
		4☐other, *please specify*	1☐mild 2☐moderate 3☐severe	☐
Percussion abnormal *(multiple answers possible)*	1☐yes 2☐no	1☐dull	1☐right 2☐left 3☐both	☐
		2☐hyper sonant	1☐right 2☐left 3☐both	☐
		3☐others *please specify:* ________________________________	1☐right 2☐left 3☐both	☐
Auscultation abnormal *(multiple answers possible)*	1☐yes 2☐no	1☐transmitted sounds	1☐right 2☐left 3☐both	☐
		2☐crepitations	1☐right 2☐left 3☐both	☐
		3☐wheezes	1☐right 2☐left 3☐both	☐
		4☐pleural rub	1☐right 2☐left 3☐both	☐
		5☐reduced air entry	1☐right 2☐left 3☐both	☐
		6☐prolonged expiration	1☐right 2☐left 3☐both	☐
		7☐others *please specify:* ________________________		☐
Chest deformity	1☐yes 2☐no	*if yes, please specify:*		
Other abnormalities	1☐yes 2☐no	*if yes, please specify:*		

Cardiac system

Any abnormal finding	1☐yes 2☐no *If yes, please answer the following questions:*	
Heart sounds abnormal	1☐yes 2☐no	1☐ decreased heart sounds 2☐ galloping 3☐ others, *please specify:* ____________________________________

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

CLINICAL EXAMINATION FORM

Murmurs	1☐yes 2☐no	1☐ systolic murmur 2☐ diastolic murmur	grade: ________ / 5 grade: ________ / 5
Jugular veins abnormally distended	1☐yes 2☐no		
Other abnormalities	1☐yes 2☐no	*if yes, please specify:* ____________________	

Abdominal system

Any abnormal finding	1☐yes 2☐no *If yes, please answer the following questions:*	
Abdominal pain/ tenderness	1☐yes 2☐no	1☐ general 2☐ located *(specify)* ____________________
Distension	1☐yes 2☐no	
Ascites	1☐yes 2☐no	
Palpable mass	1☐yes 2☐no	*If yes, specify location* ____________________
Hepatomegaly	1☐yes 2☐no	*If yes, specify:* _______cm below rib cage
Splenomegaly	1☐yes 2☐no	*If yes, specify:* _______cm below rib cage
Bowel sounds abnormal	1☐yes 2☐no	1☐ Hyperactive 2☐ Decreased 3☐ Others *please specify:* ____________________
Renal angle tenderness	1☐yes 2☐no	1☐right 2☐left 3☐both
Other abnormalities	1☐yes 2☐no	*if yes, please specify:*

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

CLINICAL EXAMINATION FORM

Eyes *(multiple answers possible)*

Any abnormal finding	1☐yes 2☐no	*If yes, specify:*	*checked*
		1☐Conjunctivitis	☐
		2☐Phlyctenular conjunctivitis	☐
		3☐Keratitis/Keratoconjunctivitis	☐
		4☐Corneal opacity/scarring	☐
		5☐Cataract	☐
		6☐Trachoma	☐
		7☐others *please specify:* ________________________	☐

Oro-pharynx *(multiple answers possible)*

Any abnormal finding	1☐yes 2☐no	*If yes, specify:*	*checked*
		1☐Oral thrush	☐
		2☐Inflamed pharynx/tonsils	☐
		3☐Angular cheilitis	☐
		4☐Stomatitis/gingivitis	☐
		5☐Nasal discharge	☐
		6☐Foetor ex ore (bad breath)	☐
		7☐others *please specify:* ________________________	☐

Ears *(multiple answers possible)*

Any abnormal finding	1☐yes 2☐no	*If yes, specify:*	*checked*
		1☐Retro-auricular swelling	☐
		2☐Inflamed ear canal/ear drum	☐
		3☐Ear discharge	☐
		4☐others *please specify:* ________________________	☐

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

CLINICAL EXAMINATION FORM

Uro genital system *(Please ask respondent if the child has any of the following. Only examine if problems are reported)*

Any abnormal finding	1☐yes 2☐no	*If yes, specify:*	*checked*
		1☐Penile/vaginal discharge	☐
		2☐Penile/vaginal sores	☐
		3☐Swelling/redness of glans penis/vagina	☐
		4☐others *please specify:* ________________________	☐

Skin

Any abnormal finding	1☐yes 2☐no *If yes, please answer the following questions:*	
Dryness / eczema	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Erythema nodosum	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Impetigo	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Tinea	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Scabies	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Ulcerations	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Urticaria	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Herpes simplex	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Herpes zoster active	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Herpes zoster scar	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe
Kaposi sarcoma	1☐yes 2☐no	*please specify:* ____
Other skin abnormality	1☐yes 2☐no	1☐mild 2☐moderate 3☐severe *please specify:* ____________________________________

Nervous system

Any abnormal finding	1☐yes 2☐no *If yes, please answer the following questions:*
Alert	1☐yes 2☐no

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

CLINICAL EXAMINATION FORM

Lethargic/drowsy	1☐yes 2 ☐no	*if yes, specify:* 1☐mild 2☐moderate 3☐severe
Unconscious	1☐yes 2 ☐no	
Confused	1☐yes 2 ☐no	*if yes, specify:* 1☐mild 2☐moderate 3☐severe
Mentally retarded	1☐yes 2 ☐no	*if yes, specify:* 1☐mild 2☐moderate 3☐severe
Abnormal muscle tone	1☐yes 2 ☐no	*If yes, specify:* 1☐hypertone location ____________ 2☐hypotone location ____________
Neck-stiffness	1☐yes 2 ☐no	
Anterior fontanelle	1☐closed / normal 2☐bulging 3☐sunken	

Skeletal system

			checked
Any abnormal finding	1☐yes 2 ☐no	*If yes, specify:*	
		1☐Joint pains location: ____________	☐
		2☐Swelling of joint location: ____________	☐
		3☐Joint deformity location: ____________	☐
		4☐others *please specify:* ____________________	☐

Name of Clinician	Signature	Date
Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

Appendix 6: X-ray v1.1, 14.04.11

TB CHILD
EDCTP PROJECT
New Diagnostics for Childhood TB

X-RAY FORM

Study ID	Date of Birth (DD/MM/YYYY)	or Age
|__|__|__|__|__|-|__|__|-|__|__|__|	|__|__| |__|__| |__|__|__|__|	|__|__|
Initials of Participant	**Date of x-ray (DD/MM/YYYY)**	
|__|__|__| *(initials of first name, middlename, sure name)*	|__|__| |__|__| |__|__|__|__|	

Type of requested examination	1☐ chest x-ray 2☐ other, specify________________________	Short signature of clinician:	
Reason for x-ray	________________________________	**X-ray quality**	1☐ accepted 2☐ not accepted

A. Chest x-ray *If abnormal specify:*	1☐ normal		2☐ abnormal
Consolidation	1☐ left	2☐right	3☐ bilateral
Ghon focus	1☐ left	2☐right	3☐ bilateral
Infiltration	1☐ left	2☐right	3☐ bilateral
Cavitation	1☐ left	2☐right	3☐ bilateral
Micronodules <7mm	1☐ left	2☐right	3☐ bilateral
Macronodules ≥ 7mm	1☐ left	2☐right	3☐ bilateral
Bronchiectasis	1☐ left	2☐right	3☐ bilateral
Perihilar lymphadenopathy	1☐ left	2☐right	3☐ bilateral
Paratracheal lymphadenopathy	1☐ left	2☐right	3☐ bilateral
Tracheal compression	1☐ yes		
Bronchial compression	1☐ left	2☐right	3☐ bilateral
Pulmonary oedema	1☐ left	2☐right	3☐ bilateral
Pleural effusion	1☐ left	2☐right	3☐ bilateral
Calcification(s)	1☐ nodes 2☐ lung 3☐ pleura 4☐ pericardium		
Enlarged heart silhouette	1☐ yes		
other findings, specify:	________________________		

B. Other X-ray *If abnormal specify:*	1☐ normal 2☐ abnormal
________________________________	
________________________________	
________________________________	
________________________________	

A./B. Conclusion

1☐certain TB 2☐uncertain TB 3☐no TB

further specify:

____

____

Previous x-ray?	1☐ yes 2☐ no
Date of last x-ray (MM/YYYY)	|__|__| |__|__|__|__|
Comparison with last x-ray	1☐ normalized 2☐ improved 3☐ unchanged 4☐ aggravated 5☐ new finding

Name of Radiologist	Signature	Date
Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

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Appendix 7: NTLP TB Score Chart for children under 6 yrs Guidelines (2006 & 2012)

Score	0	1	2	3	4	Score
Duration of illness	< 2 weeks	2-4 weeks		More than 4 weeks		
Failure to thrive or weight loss	Weight gain		No weight gain		Weight loss	
TB contact	None	Reported, not proven		Proven EPTB, Smear +	Proven Smear +	
Malnutrition				Not improved after 4 weeks		
Chronic infant disease				Not improved after 4 weeks		
Frequency of illness		Recurrent		No response to antibiotics		
Chest x-ray				TB-suggestive features like infiltration, cavity, or hilar lymph nodes		
Lymph nodes				Cervical, sub-mandibular		
Swelling of bones or joints				Suggestive feature on x-ray		
Ascites			No abdominal mass	With abdominal mass		
Meningitis				Chronic CNS signs		
Angle deformity of the spine					X-ray feature	
TOTAL SCORE						

A score of **7 or more** indicates a high likelihood of TB. Refer the child for TB treatment.

Appendix 8: FNA Form v1.1, 14.04.11

FINE NEEDLE ASPIRATION (FNA) FORM

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|		**Date of Birth (DD/MM/YYYY)** |___|___| |___|___| |___|___|___|___|	**or Age** |___|___|
Initials of Participant |___|___|___| *(initials of first name, middlename, sure name)*	**Gender** 1☐ female 2☐ male	**Date of FNA (DD/MM/YYYY)** |___|___| |___|___| |___|___|___|___|	

Reason for FNA - Short history		Name&Signature -clinician:
Site of FNA (one form for one site)	1☐ cervical right 3☐ supraclavicular right 5☐ axillary right 7☐ inguinal right 2☐ cervical left 4☐ supraclavicular left 6☐ axillary left 8 ☐ inguinal left 9☐ other, specify:______________________________	
FNA technique	1☐ Palpation guided 2☐ Ultrasound guided 3☐ CT guided	
Total No. of slides	|___|___|	
Quality of slides	1☐ good, acceptable 2☐ poor, not acceptable, specify reason:______________________	
ZN staining	1☐ AFB seen 2☐ No AFB seen	
Autofluorescence	1☐ AFB seen, typical Mtb 2☐ AFB seen, not typical Mtb 3☐ No AFB seen	
Cytology	1☐ NL _ necrotising lymphadenitis 2☐ NGL _ necrotising granulomatous lymphadenitis 3☐ GL _ granulomatous lymphadenitis 4☐ NSL _ necrotising suppurative lymphadenitis 5☐ Reactive lymphadenopathy 6☐ Acute lymphadenitis/abscess 7☐ Suspected malignancy 8☐ Other, specify:______________________________	
	Description:	
Diagnosis	1☐ confirmed TB Lymphadenitis 2☐ suspected TB Lymphadenitis 3☐ Other, specify:______________________________	

Name of Pathologist	Signature	Date of investigation
Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

Appendix 9: Universal Blood Sample Transfer Form 12.12.11-V02

SPECIMEN TRANSFER FORM	BLOOD	universal

DISPATCH SECTION *Completed by the attending physician / nurse*

Specimen Unique Number	ATTACH LABEL
Patient initials	☐☐☐
Patient data	Date of birth ☐☐ day ☐☐ month ☐☐ year; sex ☐ female ☐ male

Tests requested by the physician		
	☐ HIV testing (rapid and ELISA)	☐ Detection of IP-10 in serum
	☐ CD4 count	☐ Micro RNA
	☐ TAM-IGRA	☐ Biochemistry
Signature ____________	☐ Full Blood Count	☐ Storage
	☐ Quantiferon Gold In Tube	☐ Other Specify: ______________

Specimen containers required				Number of containers collected			
QFT:	3x1ml ☐	EDTA	2 ml ☐	QFT:	3x1ml ☐	EDTA	2 ml ☐
CPDA:	9 ml ☐	Serum	4 ml ☐	CPDA:	9 ml ☐	Serum	4 ml ☐
	6 ml ☐		3 ml ☐		6 ml ☐		3 ml ☐
	4 ml ☐		2 ml ☐		4 ml ☐		2 ml ☐

Specimen taken	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time	
Attending physician / nurse	Name:	Signature:

TRANSPORT SECTION *Completed by the courier*

Specimen dispatched	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time	
Temperature of transport box [° C]		
Courier	Name:	Signature:

LABORATORY SECTION *Completed by the Lab. Techn. / Ass.*

Specimen received	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time
Temperature of transport box [° C]	

Specimen containers received				Details on blood condition (haemolytic, too little amount etc.)			
QFT:	3x1ml ☐	EDTA	2 ml ☐	QFT:	3x1ml	EDTA	2 ml
CPDA:	9 ml ☐	Serum	4 ml ☐	CPDA:	9 ml	Serum	4 ml
	6 ml ☐		3 ml ☐		6 ml		3 ml
	4 ml ☐		2 ml ☐		4 ml		2 ml

Did you have to contact study clinician / nurse because of problems with transport?	☐ yes ☐ no	Name of contact person:	Specify problems (*fill out reverse side*):
Laboratory staff	Name:		Signature:

Valid from: 14.06.2011
page 1 of 2

SPECIMEN TRANSFER FORM	BLOOD	universal

Specimen Unique Number	ATTACH LABEL

COMMENTS / CLARIFCATION:
(document details of telephone call, eg. time, solution of problem, etc.)

Laboratory staff: ______________________
Date & Signature

Attending physician / nurse: ______________________
Date & Signature

Valid from: 14.06.2011
page 2 of 2

Appendix 10: Diagnosis and Medication EPTB v1.2, 31.05.11

DIAGNOSIS AND MEDICATION FORM

Study ID |___|___|___|___|___|-|___|___|	
Initials of Participant |___|___|___| (*initials of first name, middlename, sure name*)	Date of Birth (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___| or Age Years: |___|___| Months: |___|___|

Modified for the sub-study: EVALUATION OF Xpert™ MTB/RIF (GeneXpert) AND Ustar® IAD TB (Biotech) ON CYTOLOGICAL ASPIRATES FOR DIAGNOSIS OF EXTRAPULMONARY TUBERCULOSIS IN CHILDREN

Study A: TB Diagnosis at enrolment *(to be filled at month 5)*	1☐ Group I (Smear-positive, Culture-positive for *M. tuberculosis*) 2☐ Group II (Smear-negative, Culture-positive for *M. tuberculosis*) 3☐ Group III (Smear-negative/positive, Culture-positive only for non-tuberculous mycobacteria) 4☐ Group IV (Smear-negative, Culture-negative, but clinically highly suspicious of having pulmonary TB due to clinical presentation and radiological findings at baseline and/or response to anti-TB therapy during follow up) 5☐ Group V (Smear-negative, Culture-negative but not matching with categories I-IV; indeterminate)	
TB Medication *(to be filled at month 5 and 9)* *(multiple answers possible)*	1☐ No TB treatment 2☐ category I or III (2RHZE/4RH), 3☐ category II (2SRHZE/1, RHZE/5, RH_3E_3) 4☐ reserve (second-line) TB drugs, *please specify names:* ____________________ ____________________ 5☐ other, *please specify names:* ____________________ ____________________	
Outcome status *(to be filled at month 5 and 9)*	After 5 months: 1☐ Ongoing 2☐ Resolved 3☐ Resolved with sequelae 4☐ Died	After 9 months: 1☐ Ongoing 2☐ Resolved 3☐ Resolved with sequelae 4☐ Died

Version 1.2, dated 14th April 2011 Page 1 of 5

DIAGNOSIS AND MEDICATION FORM

Tuberculosis:

<table>
<tr><td>Study B:
TB Diagnosis at enrolment
(to be filled at month 5)</td><td colspan="3">1☐ Definite tuberculosis: Symptoms suggestive of tuberculosis plus
• Clinical specimens positive for M. tuberculosis on liquid or solid culture

2☐ Highly probable tuberculosis: Symptoms suggestive of tuberculosis plus
• Chest radiograph indicative of active tuberculosis ("certain TB" confirmed by 2 independent reviewers)
• Histology/Cytology showing typical morphology
• Flourescent or acid fast bacilli on microscopy

3☐ Probable tuberculosis
• Cervical lymph node mass (greater than 2x2cm) with resolution on TB therapy
• Abdominal mass or ascites with abdominal lymphadenopathy on ultrasound scan and resolution on TB therapy
• Spinal gibbus (clinically & radiologically detectable angulation of the spine)
• Clinical picture of meningitis associated with CSF changes and/or CT scan findings consistent with TB meningitis (after other likely causes have been excluded)
• Suggestive symptoms, but CXR uncertain for active tuberculosis or discordance between independent reviewers; no alternative diagnosis established and complete symptomatic or radiographic resolution on TB treatment

4☐ No tuberculosis (controls)
• Alternative diagnosis established and TB workup negative
• Well-being established after 3-6 month follow-up without TB treatment

5☐ Indeterminate
• Any other possible combination of results and/or loss to follow up after recruitment</td></tr>
<tr><td>Date of TB Diagnosis:
DD/MM/YYYY</td><td colspan="3">|___|___| |___|___| |___|___|___|___|</td></tr>
<tr><td>Resolution date:
DD/MM/YYYY</td><td colspan="3">|___|___| |___|___| |___|___|___|___|</td></tr>
<tr><td>Spectrum of disease irrespective probability of diagnosis
(to be filled at month 5)
(multiple answers possible)</td><td>1☐ No TB (controls)</td><td>Intra-thoracic TB:
2☐ Ghon focus
3☐ Primary complex
4☐ Lymph node disease
5☐ Compression
6☐ Consolidation
7☐ Pleurisy
8☐ Disseminated
9☐ Adult-type</td><td>Extra-thoracic TB:
10☐ Peripheral lymphadenitis
11☐ Meningitis
12☐ Abdominal TB
13☐ Osteo-articular
14☐ Skin
15☐ others:
____________________</td></tr>
</table>

Study ID |___|___|___|___|___|-|___|___|

DIAGNOSIS AND MEDICATION FORM

TB Medication ***(to be filled at month 5)*** *(multiple answers possible)*	1☐ No TB treatment 2☐ category I or III (2RHZE/4RH), 3☐ category II (2SRHZE/1, RHZE/5, RH_3E_3) 4☐ reserve (second-line) TB drugs, *please specify names:* ____ ____ 5☐ other, *please specify names:* ____ ____
Outcome status	**After 5 months:** 1☐ Ongoing 2☐ Resolved 3☐ Resolved with sequelae 4☐ Died

HIV Infection

HIV Test	1☐ *HIV test positive* 2☐ *HIV test negative* 3☐ *HIV test result unknown* 4☐ *HIV test never done*	
Date of Test	|___|___| |___|___| |___|___|___|___|	*DD/MM/YYYY*
CD4 count	|___|___|___|___| cells/µl	
	|___|___| %	
CD4/CD8-ratio	|___| . |___|	
Date of CD4 cell count	|___|___| |___|___| |___|___|___|___|	*DD/MM/YYYY*
HIV Test (2)	1☐ *HIV test positive* 2☐ *HIV test negative* 3☐ *HIV test result unknown*	
Date of Test (2)	|___|___| |___|___| |___|___|___|___|	*DD/MM/YYYY*
CD4 count (2)	|___|___|___|___| cells/µl	
	|___|___| %	
CD4/CD8-ratio (2)	|___| . |___|	
Date of CD4 cell count (2)	|___|___| |___|___| |___|___|___|___|	*DD/MM/YYYY*

Study ID |___|___|___|___|___|-|___|___|

DIAGNOSIS AND MEDICATION FORM

HIV Test (3)	1☐ *HIV test positive* 2☐ *HIV test negative* 3☐ *HIV test result unknown*		
Date of Test (3)	|___|___| |___|___| |___|___|___|___|		*DD/MM/YYYY*
CD4 count (3)	|___|___|___|___| cells/µl		
	|___|___| %		
CD4/CD8-ratio (3)	|___| . |___|		
Date of CD4 cell count (3)	|___|___| |___|___| |___|___|___|___|		DD/MM/YYYY
HIV Classification	WHO: 1☐ grade I 2☐ grade II 3☐ grade III 4☐ grade IV 5☐ no HIV infection		*If 1-4 please specify HIV OI DIAGNOSIS WHO CODES:* ☐☐☐ ☐☐☐ ☐☐☐
Medication	*If ART given, please specify ART treatment according to code:* ☐☐ ☐☐ ☐☐ ☐☐	01 = Zidovudine (AZT) 02 = Stavudine (d4T) 03 = Lamivudine (3TC) 04 = Didanosine (ddI) 05 = Abacavir (ABC) 06 = Tenofovir (TDF) 07 = Nevirapine (NVP) 08 = Efavirence (EFV) 09 = Lopinavir/r (LPV/r) 10 = Nelfinavir (NFV) 11 = Saquinavir/r (SQV/r)	

Other Diagnoses:

Diagnosis:		
Date of Diagnosis:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Resolution date:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Further specification of diagnosis:		
Medication:		

Study ID |___|___|___|___|___|-|___|___|

DIAGNOSIS AND MEDICATION FORM

Diagnosis:		
Date of Diagnosis:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Resolution date:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Further specification of diagnosis:		
Medication:		

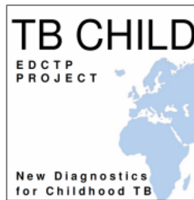
Diagnosis:		
Date of Diagnosis:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Resolution date:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Further specification of diagnosis:		
Medication:		

Diagnosis:		
Date of Diagnosis:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Resolution date:	|___|___| |___|___| |___|___|___|___|	DD/MM/YYYY
Further specification of diagnosis:		
Medication:		

Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

Study ID |___|___|___|___|___|-|___|___|

Appendix 11: Medical history follow up v1.1, 14.04.11



MEDICAL HISTORY AT FOLLOW UP

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|	Date of Interview (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___|
Initials of Participant |___|___|___| (*initials of first name, middlename, sure name*)	Date of Birth (DD/MM/YYYY) |___|___| |___|___| |___|___|___|___| or Age Years: |___|___| Months: |___|___|

1. TB contact

Has the participant had any known TB contact since the last visit? 1☐ yes 2☐ no 9☐ unknown	
If yes, please specify:	1☐same household *specify:* ________________ 2☐neighbour 3☐work place 4☐school 5☐other *specify:* ________________
Was this TB contact	1☐sputum smear positive 2☐sputum smear negative 9☐unknown
Was this TB contact treated for TB?	1☐yes 2☐no 9☐ unknown

2. Present symptoms

Has the participant got any of the following symptoms at the moment? *(multiple answers possible)*			Duration of the last episode: 1 = 1-7 days 2 = 8-14 days 3 = 15-27 days 4 = 1-3 months 5 = > 3 months	Is this a pre-existing symptom or new? 1 = new 2 = pre-existing ***If 2:*** 3 = same 4 = better 5 = worse
Cough	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Fatigue/lethargy	1☐yes 2☐no 9☐unknown	1☐continuous 2☐remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐

MEDICAL HISTORY AT FOLLOW UP

Wheezing	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Difficulty in breathing	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Fever over last month	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Chest pain	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Night sweat	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Haemoptysis	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Enlarged lymphnodes	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Skin changes	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Abdominal pains	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Weight loss over last month	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐
Other symptom *Please specify:* ________________________	1☐ yes 2☐ no 9☐ unknown	1☐ continuous 2☐ remittent	1☐ 2☐ 3☐ 4☐ 5☐	1☐ 2☐ 3☐ 4☐ 5☐

3. Usual social and functional activities (only for children less than 16 years of age)

Social interaction *(according to age)*	1☐ normal (responds well to friends, family, community) 2☐ limited ability to socialize/respond 3☐ child does not/is not able to socialize/respond
Play activities *(according to age)*	1☐ normal play activities 2☐ limited/reduced plays activities 3☐ child does not/is not able to play
Learning tasks *(according to age)*	1☐ normal *(same as other kids)* 2☐ has difficulties to learn/concentrate/follow in school *(delayed compared to other kids same age)* 3☐ child does not/is not able to learn or concentrate
Milestones *(according to age)*	1☐ normal 2☐ mild to moderate delay 3☐ severely delayed

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

MEDICAL HISTORY AT FOLLOW UP

4. Past medical history

How often has the participant been for medical treatment (outside of the study) since the last study visit?	1☐never 2☐1-2x 3☐>3x	
What were the main reasons for medical treatment since the last visit? *(multiple answers possible)*	1☐no medical treatment 2☐cough 3☐diarrhoea 4☐malaria	5☐failure to thrive (only in children) 6☐others,*specify*________________ 7☐others,*specify*________________ 9☐unknown
Was the participant hospitalized since the last visit?	1☐no 2☐yes, 1x 3☐yes, >1x 9☐unknown	*If yes, specify reason(s) for hospitalisation:*
Is the participant currently hospitalized?	1☐yes 2☐no	*If yes, specify reason(s) for hospitalisation:*
Has the participant received any blood transfusion since the last visit?	1☐yes 2☐no 9☐unknown	
Has the participant had any new known TB contact since the last visit?	1☐yes 2☐no 9☐unknown	
Is anyone of the participant's relatives presently on new TB treatment?	1☐yes 2☐no 9☐unknown	

5. Medication history

Did the participant receive any new medication since the last visit?	1☐yes 2☐no 9☐unknown
If yes, please specify: What was/is this treatment?	1☐antibiotic, *please specify name:* ____________________ 2☐TB treatment *please specify:* 3☐category I or III (2RHZE/4RH), 4☐category II (2SRHZE/1, RHZE/5, RH_3E_3) 5☐reserve (second-line) drugs, *please specify names:* ________________________ ________________________ 6☐other TB drugs, *please specify names:* ________________________ ________________________ 6☐ARVs 7☐other, *please specify:* ____________________

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

MEDICAL HISTORY AT FOLLOW UP

6. Questionnaire for Micro-RNA-assay (these questions refer to the moment of enrolment)

Was the patient pregnant at the moment of enrolment? (only for females in child bearing age)	1☐yes 2☐no 9☐unknown
Has the patient ever been a smoker?	1☐yes 2☐no
Did the patient ever use intravenous drugs?	1☐yes 2☐no
Did the patient ever undergo a transplantation?	1☐yes 2☐no
Has the patient ever been diagnosed with diabetes?	1☐yes 2☐no 9☐unknown
Has the patient ever been diagnosed with renal failure?	1☐yes 2☐no 9☐unknown
Has the patient ever been diagnosed with silicosis?	1☐yes 2☐no 9☐unknown
Has the patient ever been diagnosed with sarcoidosis?	1☐yes 2☐no 9☐unknown
Has the patient ever been diagnosed with cancer?	1☐yes 2☐no 9☐unknown

7. Comments

Name of Clinician/Interviewer	Signature	Date
Name of Data Clerk (1st data entry)	Signature	Date
Name of Data Clerk (2nd data entry)	Signature	Date

Study ID |___|___|___|___|___|-|___|___|-|___|___|___|

Version 1.1, dated 14th April 2011

Appendix 12: BRTC_TB CHILD_TST

Bagamoyo Research and Training Centre

TITLE: TB CHILD STUDY TST ADMINISTRATION AND READING	VERSION:01	PAGE: 1 - 6

SOP Code: BRTC_TBCHILD_005_V01	AREA: Reception, Clinical	
WRITTEN BY: Name: Klaus Reither Date: 22.03.11 Signature:	REVISED BY: Name: Christian Pohl Date: 27.03.11 Signature:	
APPROVED BY QA Name: Date: Signature:	AUTHORIZED BY UNIT LEADER: Name: Kefas Mugittu Signature:	Effective date:
ORIGINAL LANGUAGE: English		
TRANSLATED BY: Name Date Signature	TRANSLATION VERIFIED BY Name Date Signature	

CHANGES

DATE	CHANGE	REASON FOR CHANGE
	Creation	

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1. Purpose **3**

2. Definitions and Abbreviations **3**

3. Scope **3**

4. Responsibilities **3**

5. Procedures **4**

5.1 Patient Information **4**

5.2 TST administration **4**

5.2 Reading and recording results **5**

5.3 TST Log **5**

1. Purpose

The purpose of this SOP is to describe the procedure of TST administration and reading in the TB CHILD study. (Study title: Evaluation of new and emerging diagnostics for childhood tuberculosis in high burden countries (TB CHILD)).The skin test is used to evaluate participants for TB infection.

2. Definitions and Abbreviations

BDH: Bagamoyo District Hospital
BRTC: Bagamoyo Research and Training Centre
CRF: Case Record Form
IHI: Ifakara Health Institute
SOP: Standard Operating Procedure
TB: Tuberculosis
PPD: Purified Protein Derivative
TST: Tuberculin Skin test

3. Scope

This procedure applies to all research staff working at the Ifakara Health Institute - BRTC who is involved in TST administration and reading (e.g. medical and clinical officers, clinical trial assistants, nurse).

4. Responsibilities

The site Principal Investigator or the designated Project Leader will have overall responsibility for the procedures in the SOP. The Head of the Clinical Section or designated medical officers will supervise the procedures for this SOP. The delegated staff responsible to perform the procedures described will be included in the site Delegation Log

5. Procedures

5.1 Patient Information

- Inform the participant and/or guardian that TST is one of the procedures in assessing TB infection in the study
- Explain the procedure to the participant and/or guardian as detailed:
    - Tell parent that the procedure involves injecting a drug on the fore arm, it takes about 10 minutes, and is read after 48 - 72 hours, which will take again about 10 minutes.
    - Tell her/him the expected date of return visit will be explained by the study secretary.
    - Participant and/or guardian should not massage or wash the injection site.
    - Should not apply any oil, cream or similar substances at the injection site.
    - Explain to the participant and/or guardian that we expect the injection site to swell. This should not be a reason to get scared.
    - Let the participant and/or guardian know that in rare cases site may be itchy and at times blister. Should this happen, participant and/or guardian should report at the TB Research Clinic.
- Obtain the verbal consent for the procedure.

5.2 TST administration

- Prepare and set materials for the test/procedure
- Ensure aseptic techniques
    - Wash hands with soap
    - Clean the injection site with distilled water. In case alcohol is used to swab the skin; it must be allowed to evaporate before test is done.
- Prepare a tuberculin syringe (disposable 1ml syringe graduated in hundredths of millilitre) or similar syringe
- Use prepared syringe to withdraw 0.1 ml of the tuberculin
- On a firm, well-lit surface, expose the patient’s arm and slightly flex it at the elbow. Choose injection site the middle third of the inner part of the fore arm (preferably the left arm).
- The area selected should be free of any barriers to placing and reading the skin test such as muscle margins, heavy hair, veins, sores, or scars.

- The skin should be lightly stretched and the needle point will be inserted with the bevel upwards in the direction of the forearm into the superficial layer of the skin (intradermal). The plunger is not touched until the needle point has been satisfactorily inserted.
- The volume of exactly 0.1 mL is slowly injected, and the finger removed from the end of the plunger before the needle is withdrawn. The injection should raise a flat anaemic wheal with pronounced pits and a steep border.
- If there is significant leakage of tuberculin (at the onnection of the needle and syringe or because the needle was not appropriately inserted) or if the test was subcutaneous rather than strictly intradermal, the test is repeated on the opposite arm
- Leave the injection site uncovered
- Do not massage the area
- Record in the TST Log, the date and time of the injection and the location of the injection site.

5.2 Reading and recording results

- The test must be read 48 - 72 hours after being administered.
- Identify the injection site, by palpating the area for the induration.
- The basis of reading the skin test is the presence or absence of induration, which is a hard, dense, raised formation. This is the area that is measured.
- Sometimes the site has erythema, a reddening of the skin that can also have swelling. The erythema should NOT be measured.
- Measure the diameter of the induration (not erythema) transverse to the long axis of the arm.
- A soft, flexible, transparent ruler, calibrated in millimetres is used.
- Record the results in mm
- Record results in TST Log and Clinical Examination Form of the participant.

!

5.3 TST Log

Bagamoyo Research and Training Centre

Patient ID	Initials	Date of TST administration	Time of TST administration	Administered by:	Date of TST reading	Time of TST reading	Read by:	Measurement of induration (mm)	Adverse event

Appendix 13: Temeke EPTB CHILD FNA Procedure v1.0

The Aga Khan Hospital, Dar es Salaam

<table>
<tr><td colspan="2">TITLE:
EPTB CHILD STUDY
Procedure for Fine Needle Aspiration Biopsy of peripheral lymph nodes</td><td>VERSION:01</td><td>PAGE: 1 - 5</td></tr>
<tr><td>SOP Code:
TEMEKE_EPTB CHILD_FNA_ 01</td><td colspan="3">AREA: Reception, Clinical</td></tr>
<tr><td>WRITTEN BY:
Name Date
Signature</td><td colspan="3">REVISED BY:
Name Date
Signature</td></tr>
<tr><td>APPROVED BY QA
Name Date
Signature:</td><td colspan="2">AUTHORIZED BY UNIT LEADER:
Name: Signature:</td><td>Effective date:</td></tr>
<tr><td colspan="4">ORIGINAL LANGUAGE: English</td></tr>
<tr><td>TRANSLATED BY:
Name Date
Signature</td><td colspan="3">TRANSLATION VERIFIED BY
Name Date
Signature</td></tr>
</table>

CHANGES

DATE	CHANGE	REASON FOR CHANGE

Table of Contents

1. Purpose **3**

2. Definitions and Abbreviations **3**

3. Scope **3**

4. Responsibilities **3**

5. Procedures **3**

1. Purpose

The purpose of this SOP is to describe the procedure of fine needle aspiration biopsy of peripheral lymph nodes in the EPTB CHILD study. (Study title: Evaluation of Xpert MTB/RIF (GeneXpert) and Ustar IAD TB (Biotech) on cytological aspirates for diagnosis of extrapulmonary tuberculosis in children.

2. Definitions and Abbreviations

AKHD: Aga Khan Hospital, Dar es Salaam
BRTC: Bagamoyo Research and Training Centre
CRF: Case Record Form
FNAB: Fine needle aspiration biopsy
NAA: Nucleic acid amplification
PBS: Phosphate Buffered Saline
PI: Principal Investigator
SOP: Standard Operating Procedure
TB: Tuberculosis
TDH: Temeke District Hospital

3. Scope

This procedure applies to all research staff working at the Temeke District Hospital TB Clinic who are involved in fine needle aspiration biopsy procedures (e.g. medical and clinical officers, clinical trial assistants, nurse).

4. Responsibilities

The site Principal Investigator or the designated Project Leader will have overall responsibility for the procedures in the SOP. The Head of the Clinical Section or designated medical officers will supervise the procedures for this SOP. The delegated staff responsible to perform the procedures described will be included in the site Delegation Log.

5. Procedures

TB FNA procedure at TDH TB Clinic (Wednesdays)

RECEPTION:

Registration
The nurse at the TDH TB Clinic courteously welcomes the participant and registers the participant in the visit log after checking the ID card. The nurse asks if there are any changes of the contact information and makes a note where required. The nurse retrieves the participant study file and takes it to the doctor's room.

DOCTOR'S ROOM:

Medical history and clinical examination:
The medical officer or a designated person carries out a brief clinical examination to ensure that the participant does not require immediate medical attention or hospital admission. The findings are recorded on a source document not intended for data entry.
The medical officer in charge or a designated person takes the medical history and examines the participant briefly and systematically, and confirms the presences of lymph nodes, mass or swelling which needs the fine needle biopsy on that day.

Participant information:
The medical officer in charge or a designated person informs the participant and/or guardian that fine needle aspiration biopsy is a way to check a mass or a lump to determine the cause of the swelling and is an established way to assess TB infection. The medical officer in

charge or a designated person further explains that a small needle is put into the mass and gently moved back and forth while suction is applied and one or two drops of fluid are removed. That the most common complication is a slight bruising or tenderness of the area for a few days following the procedure and that infection at the site of biopsy is rare.
The FNA procedure is already described in the general consent form the participant has signed in the first visit. Additional oral consent is obtained from the participant/guardian after this specific information talk.

SAMPLE COLLECTION ROOM:

Preparations

The PI makes sure the following are available for the procedure:

Equipment

Needle	not larger than 22G (black)
Syringe	10ml
Pencil	
Slides	glass slides with ground glass edge
Fixative	95% ethanol and 100% Methanol in leak-proof containers to hold slides
MGIT	tubes PANTA prepared for culture
PBS	sterile 0.7ml per sample in 2ml polypropylene tubes for GeneXpert and Ustar tests

Anaesthesia

- No local anaesthetic to be used
- Sedatives for children like: i.v.Diazepam (Valium) or intranasal Midazolam (Dormicum)
- Topical anaesthetic (Emla cream) may be used

Positioning of participant

If possible lying down on examination couch, but for small supraclavicular nodes – sitting

FNA procedure

- Tell participant/guardian that the procedure involves injecting a needle in the enlarged lymph nodes
- Children who are agitated should receive sedative premedication and need to be monitored by using a pulse oximeter
- Clean skin on and around lymph node using alcohol swab
- After locating and stabilisation of the mass for biopsy and using careful palpation the needle is passed into the mass
- The largest superficial node in a particular group of lymph nodes is the best choice. Multiple sites of enlarged lymph nodes can be aspirated
- The target size and distance from overlying skin should be assessed by palpation. In small lesions (1cm) aim for the centre of the lesion. In very large lesions (greater than 5cm) the lymph node periphery is preferred, because there may be central necrosis. In medium sized lesions (2-4cm) two different areas, one to the side of the centre and another in the mirror image position of the previous aspiration should be targeted.
- Maintain 1-2 ml suction throughout aspirate. Once suction has been applied, the needle tip must be moved back and forth (or up and down) within the boundaries of the target.
- Aspirate using cutting motion until materials appears in hub of needle.
- Release suction (if required) before withdrawing needle.
- Always perform a minimum of 2 needle passes. Always use a sterile needle and syringe for each pass.
- Place cotton wool on insertion site and ask guardian or participant to apply pressure.

- Remove needle from syringe, introduce 5-10 ml air into syringe, re-attach needle and, holding needle onto syringe, use air by pushing the plunger swiftly through the syringe to express material in needle onto glass slide.

SMEAR PREPARATION:

- Slides will be labelled with participant initials, identifying number and site of aspiration if multiple sites are aspirated
- Each pass yields 2 slides
- Average aspirate should yield 4 slides per participant (2 for ZN and 2 for Papanicolau staining)
- To prevent splattering, the tip of the needle should rest on the slide closer to the frosted end of a clean slide
- The material in the needle is often expelled in form of a droplet onto the slide
- The slide is picked up by its frosted end between thumb and forefinger (of the non dominant hand) with the other fingers creating a firm platform beneath the slide
- A second clean slide (spreader) is held by its frosted end (in the dominant hand), its lower edge placed against the 1st slide at 45-degree angle proximal to the droplet
- The top slide is then lowered until it touches and covers the droplet and the two slides are flush
- The material is then spread in one swift motion by pulling the top slide along the entire length of the bottom slide
- Slides meant for papanicolau staining should immediately be put into 95% ethanol, while those for ZN should be air dried for at least 5 min and then put into 100% methanol
- Transport to the AKHD laboratory as per SOP: TEMEKE_EPTB CHILD_FNAB_Transport_01

FOR TB CULTURE:

- For TB culture, rinse the needle and syringe in TB medium (MGIT tube) prepared as per SOP: TEMEKE_EPTB CHILD_FNA_MGIT_001_V01 (Appendix 15) pre-labelled with the participants ID. Store tubes at **room temperature away from direct sunlight** and transport to BRTC as per SOP: TEMEKE_EPTB CHILD_FNAB_Transport_01

FOR NAA TESTS:

- The needle and syringe from a second FNAB pass should be rinsed in sterile 0.7ml phosphate buffered saline (PBS) in a 2ml screw-cap tube pre-labelled with the participants ID. Transport tubes to BRTC on ice as per SOP: TEMEKE_EPTB CHILD_FNAB_Transport_01

The medical officer fills the specimen transfer forms for each test (Appendix 8: FNA, CRF version 1.1, 14.04.11 for ZN and cytology; Appendix 13: Specimen transfer form - FNA MGIT; and Appendix 14: Specimen transfer form - FNA Molecular) and ensures that each form is appropriately labelled with ID stickers per patient (Appendix 16). The specimens are sent to the laboratory according to guidelines in Appendix 17: TEMEKE_EPTB CHILD_FNAB_Transport_01.

Appendix 14: TEMEKE EPTB CHILD FNAB Transport v1.0

The Aga Khan Hospital, Dar es Salaam

<table>
<tr><td colspan="2">TITLE:
Transportation and Submission of Fine Needle Aspiration Biopsy (FNAB) Specimen from Temeke District Hospital</td><td>VERSION:01</td><td>PAGE: 1 of 5</td></tr>
<tr><td>SOP Code:
TEMEKE_FNAB_ TRANSPORT _001_V01</td><td colspan="3">AREA: TB Clinic</td></tr>
<tr><td>WRITTEN BY:
Name: Maira Bholla
Date: 06/04/11
Signature:</td><td colspan="3">REVISED BY:
Name:
Date:
Signature:</td></tr>
<tr><td>APPROVED BY QA:
Name:
Date:
Signature:</td><td colspan="2">AUTHORIZED BY UNIT LEADER: Name:
Signature:</td><td>EFFECTIVE DATE:</td></tr>
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Name Date
Signature</td><td colspan="3">TRANSLATION VERIFIED BY:
Name Date
Signature</td></tr>
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1. Objective

To describe the safety procedures for packing, transporting and submitting FNAB specimens for diagnosis of tuberculosis

2. Definition

Abbreviations:

BRTC	-	Bagamoyo Research and Training Centre
FNAB	-	fine needle aspiration biopsy
MGIT	-	Mycobacterium Growth Indicator Tube
PBS	-	Phosphate buffered saline
SOP	-	Standard operating procedure
TB	-	Tuberculosis
TDH	-	Temeke District Hospital, Dar es Salaam

3. Scope

This document contains procedures for packing, transporting and submitting fixed slide samples, MGIT tubes for culture and frozen PBS tubes for molecular testing of FNAB to the designated laboratory for diagnosis of tuberculosis.

4. Responsibilities

The EPTB PI and clinicians at TDH TB clinic.

5. Procedures

5.1 General description:

This document details the method for packing, transporting and submitting FNAB specimens on slides, in MGIT tubes and in 2ml screw cap polypropylene tubes with PBS, to respective laboratories for diagnosis of tuberculosis.

5.2 Materials and Equipment required

- Permanent marker
- FNA requisition form for ZN and cytology (Appendix 8: FNA CRF version 1.1, 14.04.11)
- TB CHILD FNA Specimen Transfer Form Mycobacteriology (Version 1.0, valid from: 15.02.12) (Appendix 13)
- TB CHILD FNA Specimen Transfer Form Molecular Testing (Version 1.0, valid from: 15.02.12) (Appendix 14)
- Leak proof containers with screw cap for slide transport in alcohol
- Zip-lock bag or box or container indicating bio hazardous sign
- Black ink pen

5.3 Packing and transporting procedures of FNAB-slides:

- Ensure the collected slides of FNAB are marked clearly with the patients ID number and initials as well as other identifiers as required. If more than one site is sampled, the slide must clearly be marked with site information.
- Put the first slide immediately into the primary container containing fixative (95% ethanol) and other slides into 100% methanol after air-drying for about 5mins. The

optimal design feature of the container should be easily opened container, stay closed during transportation, made by shock resistant material and having enough room to prevent slides from adhering to one another or the container

- Then place a container with slides in fixative into a transporting bag/box/container to avoid leakage during transportation. FNA requisition forms (Appendix 8: FNA CRF version 1.1, 14.04.11) that accompany slides in fixative that should be placed in an outside pocket to avoid exposure to any leakage of fixative. **Note:** enclosure of slides in fixative into the transport bag or box or container indicating biohazardous contents is prudent whether it is delivered manually or by courier system
- Transport the FNAB slides in fixative following guidelines on transportation of clinical specimens
- Slides in fixative should be submitted in leak proof containers that protect against breakage and the slides clearly labelled with patient's ID and specimen site(s).

5.4 Packing and transporting procedures of FNAB-MGIT tubes:

- Ensure that all MGIT tubes are appropriately labelled with patient ID labels
- Ensure that MGIT tubes are not broken in any way and the caps are securely screwed on
- Inoculated MGIT tubes should be stored and transported to BRTC away from direct sunlight and at temperatures of above 8 degrees and well below 37 degrees centigrade
- FNA Mycobacteriology request forms (Appendix 13 Specimen transfer form - FNA MGIT) should accompany the specimens in a separate pocket in case of sample leakage

5.5 Packing and transporting procedures of FNAB-PBS tubes for molecular testing:

- FNAB-PBS tubes should be well labelled with patient ID label
- Immediately after sample addition to PBS, the tubes should be placed in a 2-8°C freezer for not longer than 12 hours.
- The PBS tubes should be transported to BRTC Laboratory frozen and on ice pack in a cooler box
- The FNA Molecular Testing requisition forms (Appendix 14: Specimen transfer form - FNA Molecular) should be packed separately as they will get wet due to the ice pack and condensation

5.6 Submission of FNAB specimens to the responsible laboratory

- The FNAB slides will immediately be delivered to AKHD laboratory after sample collection. A staff at AKHD laboratory will sign a copy of the list of specimens being delivered. The original list will be left at the AKHD laboratory and the copy retuned for filing at TDH TB Clinic.
- The inoculated MGIT tubes and PBS frozen tubes will immediately be delivered to Mwananyamala IHI TB Clinic for transportation to BRTC with the next scheduled transport. The last three rows of specimen labels (Appendix 16) per participant will be cut out and sent with the relevant specimens to BRTC. The driver will deliver the transportation box with MGIT and PBS tubes to BRTC Laboratory where the staff receiving will inspect the tubes and received them for forwarding the relevant section

of the laboratory for analysis. A list of specimens and forms delivered to BRTC will be signed and filed at BRTC.

6. References

1. NCCLS. Procedure for handling and transport of Diagnostic Specimens and Etiologic Agents-Third Edition: Approved Standard (1994). NCCLS document H5-A3 NCLS, 940 West Valley Road, Suite 1400 Wayne, Pensylvania19087-1898 USA, 1994.

2. NCCLS. Fine Needle Aspiration Biopsy (FNAB) Techniques; Approved Guideline Second Edition. NCCLS document GP10-A2 [ISBN 1-56238-000-0]. NCCLS, 940 West Valley Road, Suite 1400 Wayne, Pensylvania19087- -1898 USA, 2002.

Appendix 15: Labels FNA TB CHILD Study

#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)
#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)
#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)
#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)	#NAME? **340__-15-V01** FNA study (TB CHILD)
#NAME? **340__-15-V01S1** FNA study (TB CHILD)	#NAME? **340__-15-V01S1** FNA study (TB CHILD)	#NAME? **340__-15-V01S1** FNA study (TB CHILD)	#NAME? **340__-15-V01S1** FNA study (TB CHILD)	#NAME? **340__-15-V01S1** FNA study (TB CHILD)
#NAME? **340__-15-V01S2** FNA study (TB CHILD)	#NAME? **340__-15-V01S2** FNA study (TB CHILD)	#NAME? **340__-15-V01S2** FNA study (TB CHILD)	#NAME? **340__-15-V01S2** FNA study (TB CHILD)	#NAME? **340__-15-V01S2** FNA study (TB CHILD)
#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)
#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)	#NAME? **340__-15-V01B1** FNA study (TB CHILD)
#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)
#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)
#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)
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#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)	#NAME? **340__-15-V01T1** FNA study (TB CHILD)

Appendix 16: TEMEKE_EPTB CHILD_FNA_MGIT_001_V01

The Aga Khan Hospital, Dar es Salaam

<table>
<tr><td colspan="2">TITLE: Procedures for Preparation and inoculation of MGIT Tubes</td><td>VERSION: 01</td><td>PAGE: 1 of 4</td></tr>
<tr><td>SOP Code: EPTB CHILD_FNA_MGIT_001_V01</td><td colspan="3">AREA: Laboratory and FNA Room</td></tr>
<tr><td>WRITTEN BY:
Name . Date Signature
Maira Bholla</td><td colspan="3">REVISED BY:
Name Date
Signature</td></tr>
<tr><td>APPROVED BY QA:
Name Date
Signature</td><td colspan="2">AUTHORIZED BY UNIT LEADER:

Signature:</td><td>EFFECTIVE DATE:</td></tr>
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1. Objective

To describe the procedures for preparation and inoculation of MGIT with FNA

2. Definition

Abbreviations:

BRTC	-	Bagamoyo Research and Training Centre
EPTB	-	Extrapulmonary tuberculosis
FNA	-	Fine needle aspiration
MGIT	-	Mycobacterium Growth Indicator Tube
PI	-	Principal Investigator
SOP	-	Standard operating procedure
TB	-	Tuberculosis
TDH	-	Temeke District Hospital, Dar es Salaam

3. Scope

These procedures are applicable to the Clinician performing FNA and the PI or staff responsible for preparing MGIT tubes for inoculation at TDH.

4. Responsibilities

The PI of the EPTB Study and Clinician involved in FNA at TDH.

5. Procedures

5.1. Principle

A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected.

Tubes entered into the BACTEC MGIT 960 System are continuously incubated at 37 °C and monitored every 60 min for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive, i.e., the test sample contains viable organisms. An instrument positive tube contains approximately 10^5 to 10^6 colony-forming units per millilitre (CFU/ml). There is no direct correlation of biomass and Growth Unit (GU) at the time of instrument passivity. Culture vials which remain negative for a minimum of 42 days (up to 56 days) and those which show no visible signs of positivity are removed from the instrument as negatives and sterilized prior to discarding.

5.2. Safety measures

- Working with *Mycobacterium tuberculosis* grown in culture requires biosafety level 3 practices, containment equipment and facilities.
- All inoculated MGIT tubes have to be treated as bio-hazardous waste.
- In case of accident see safety SOP# BRTC_LAB_111_V01

5.3. Specimen

FNA material obtained as per SOP: Temeke_EPTB CHILD_FNA_01, is directly inoculated into MGIT tubes.

5.4. Equipment and Material

- Biological safety cabinet class II
- Refrigerator
- Pipette pump
- Sterile transfer pipettes

5.5. Procedure

Preparation for test

- Switch on the safety cabinet approximately 15 min before use.
- Label all MGIT tubes with the specimen number.
- Sort the samples according to ascending sequence of numbers.

Sample preparation

Consideration:

- o Prior to use, each MGIT tube should be examined for evidence of contamination or damage. Discard any tubes if they appear unsuitable.
- o Dropped tubes should be examined carefully. If damage is seen, the tube should be discarded.

- Reconstitute MGIT PANTA with 15 ml MGIT growth supplement. Mix until completely dissolved. This mixture is stable for 5 days if stored at 5 ± 3 °C.
- Aseptically add 0.8 ml of this enrichment to each MGIT tube prior to inoculation of specimen (preferably not more than 30 min before). Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.
- Unscrew the cap on the MGIT tube and with the needle and syringe from the first FNAB pass (after preparation of smears), aspirate approximately 1ml of MGIT culture medium and dispense it back into the liquid medium. Tightly recap the MGIT tube and mix well by inverting the tube several times.
- Store at room temperature and transport to BRTC within 24 hours as per TEMEKE_TB CHILD_FNAB_Transport_01.

5.6. Sources of error and interference factors

- The reconstitution of the MGIT PANTA with the MGIT Growth Supplement must be done under sterile conditions.
- For best results, the addition of Growth Supplement / MGIT PANTA Antibiotic Mixture should be performed just prior to specimen inoculation, preferably not more than 30 minutes before adding the FNA material.

6. References

1. BD BBL™ MGIT™ Instruction, Becton, Dickinson and Company, Sparks, Maryland, USA
2. Instruction BD BACTEC™ MGIT™ 960 System, BD Diagnostic Systems
3. MGIT 960 Operating and Maintenance Manual
4. Salman H. Siddiqi, Sabine Rüsch-Gerdes. Procedure manual For BACTEC™ MGIT 960™ TB System. 2006

Appendix 17: Specimen transfer form - FNA MGIT

TB CHILD **SPECIMEN TRANSFER FORM**	**Mycobacteriology**

DISPATCH SECTION *Completed by the attending physician / nurse*

Specimen Unique Number	ATTACH LABEL
Patient initials	☐☐☐
Patient data	Date of birth ☐☐ day ☐☐ month ☐☐ year; sex ☐ female ☐ male
Specimen taken	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time
location of biopsy (R / L) — Type of sample	☐ early morning sputum ☐ spot sputum ☐ induced sputum ☐ gastric lavage ☐ Urine ☐ Saliva ☒ FNA (Indicate location) ☐ Other Specify: ____________
Tests requested Requested by: ____________	☐ AFB microscopy ☐ Direct ☐ From sediment ☐ GeneXpert ☒ MGIT culture ☐ LJ culture ☐ Antigen identification (Capilia) ☐ Molecular identification (CM/AS) ☐ Molecular differentiation of M.tb complex (MTBC) ☐ Genotypic resistance testing (MTBDRplus) ☒ MGIT DST ☐ USTAR ☐ ☐
Attending physician / nurse	Name: Signature:

TRANSPORT SECTION *Completed by the courier*

Specimen dispatched	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time
Temperature of transport box [° C]	
Courier	Name: Signature:

TB CHILD **SPECIMEN TRANSFER FORM**	**Mycobacteriology**		
Specimen Unique Number	ATTACH LABEL		
LABORATORY SECTION *Completed by the Lab. Techn. / Ass.*			
Specimen received	day month year time :		
Temperature of transport box [° C]			
Condition of FNA sample	normal bloody mucoid other, specify:___________________________		
Volume of the FNA sample [ml]			
Did you have to contact study clinician / nurse because of problems with transport or specimens?	yes no	Name of contact:	Specify problems:*(fill out reverse side)*
Laboratory staff	Name:	Signature:	

COMMENTS / CLARIFICATION:

(document details of telephone call, eg. time, solution of problem, etc.)

____

____

____

____

____

____

____

Laboratory staff: ______________________________

Date & Signature

Attending physician / nurse: ______________________________

Date & Signature

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Version 1.0, valid from: 15.02.12

Appendix 18: Specimen transfer form - FNA Molecular

TB CHILD FNA **SPECIMEN TRANSFER FORM**	**Molecular Testing**

DISPATCH SECTION *Completed by the attending physician / nurse*	
Specimen Unique Number	ATTACH LABEL
Patient initials	☐☐☐
Patient data	Date of birth ☐☐ day ☐☐ month ☐☐ year; sex ☐ female ☐ male
Specimen taken	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time
location of biopsy (R / L) — Type of sample	☐ early morning sputum ☐ spot sputum ☐ induced sputum ☐ gastric lavage ☐ Urine ☐ Saliva ☒ FNA (Indicate location) ☐ Other Specify: ____________
Tests requested	☐ AFB microscopy ☐ Direct ☐ From sediment ☒ GeneXpert ☐ MGIT culture ☐ LJ culture ☐ Antigen identification (Capilia) ☐ Molecular identification (CM/AS) ☐ Molecular differentiation of M.tb complex (MTBC) ☐ Genotypic resistance testing (MTBDRplus) ☐ MGIT DST ☒ USTAR ☐ ☐
Requested by: ____________	
Attending physician / nurse	Name: Signature:

TRANSPORT SECTION *Completed by the courier*	
Specimen dispatched	☐☐ day ☐☐ month ☐☐ year ☐☐:☐☐ time
Temperature of transport box [° C]	
Courier	Name: Signature:

TB CHILD **SPECIMEN TRANSFER FORM**	**Molecular Testing**		
Specimen Unique Number	ATTACH LABEL		
LABORATORY SECTION *Completed by the Lab. Techn. / Ass.*			
Specimen received	day month year time		
Temperature of transport box [° C]			
Condition of FNA sample	normal bloody mucoid other, specify:___________________________		
Volume of the FNA sample [ml]			
Did you have to contact study clinician / nurse because of problems with transport or specimens?	yes no	Name of contact:	Specify problems:*(fill out reverse side)*
Laboratory staff	Name:	Signature:	

COMMENTS / CLARIFICATION:

(document details of telephone call, eg. time, solution of problem, etc.)

____

____

____

____

____

____

____

Laboratory staff: ______________________________

Date & Signature

Attending physician / nurse: ______________________________

Date & Signature

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Version 1.0, valid from: 15.02.12

Appendix 19: MGIT culture and Reporting

Bagamoyo Research and Training Centre

<table>
<tr><td colspan="2">TITLE: Procedures for Preparation and inoculation of MGIT and Results interpretation.</td><td>VERSION: 02</td><td>PAGE: 1 of 10</td></tr>
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<tr><td>WRITTEN BY:
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Jugheli, L</td><td colspan="3">REVISED BY:
Name Date Signature
Rutaihwa, L.</td></tr>
<tr><td>APPROVED BY QA:
Name Date Signature</td><td colspan="2">AUTHORIZED BY BRTC LEADER:
Mugittu, K.
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CHANGES

DATE	CHANGE	REASON FOR CHANGE
24.11.2011	Decontamination of contaminate MGIT vial according. Page 7	HighRif 2 Study specific changes

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1. Objective

To describe the procedures of preparation and inoculation of MGIT

2. Definition

Abbreviations:

BRTC - Bagamoyo Research and Training Centre
SOP - Standard operating procedure
MGIT - Mycobacterium Growth Indicator Tube
TB - Tuberculosis

3. Scope

These procedures are applicable to the Microbiology - TB section within BRTC.

4. Responsibilities

All trained Laboratory Personnel of BRTC Microbiology - TB section are responsible for the whole content of this SOP.

5. Procedures

5.1. Principle

A fluorescent compound is embedded in silicone on the bottom of 16 x 100 mm round bottom tubes. The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected.

Tubes entered into the BACTEC MGIT 960 System are continuously incubated at 37 °C and monitored every 60 min for increasing fluorescence. Analysis of the fluorescence is used to determine if the tube is instrument positive, i.e., the test sample contains viable organisms. An instrument positive tube contains approximately 10^5 to 10^6 colony forming units per milliliter (CFU/ml). There is no direct correlation of biomass and Growth Unit (GU) at the time of instrument passivity. Culture vials which remain negative for a minimum of 42 days (up to 56 days) and which show no visible signs of positivity are removed from the instrument as negatives and sterilized prior to discarding.

The BACTEC MGIT Growth Supplement is added to each MGIT tube to provide substances essential for the rapid growth of mycobacteria. Oleic acid is utilized by tubercle bacteria and plays an important role in the metabolism of mycobacteria. Albumin acts as a protective source. Catalase destroys toxic peroxides that may be present in the medium.

Growth of contaminating flora from the sample is reduced when supplementing the BBL MGIT broth base with BACTEC MGIT Growth Supplement/BBL MGIT PANTA antibiotic mixture prior to inoculation with a clinical specimen

5.2. Safety measures

- For the entire procedure a biological safety cabinet class II is needed.
- Working with *Mycobacterium tuberculosis* grown in culture requires biosafety level 3 practices, containment equipment and facilities.
- All inoculated MGIT tubes have to be treated as bio-hazardous waste.
- In case of accident see safety SOP# BRTC_LAB_111_V01

5.3. Specimen

Acceptable specimens types are digested and decontaminated clinical specimens (except urine), and sterile body fluids (except blood) using the NALC-NaOH method.

5.4. Equipment and Material

- Biological safety cabinet class II
- Bactec-MGIT 960 instrument
- Refrigerator
- Pipette pump
- Micro tube 2.0 ml, PP; (for performance and storage of extracted DNA from decontaminated sputum/pellet)
- Sterile transfer pipettes
- 1 ml plastic sterile pipette
- Rack
- Lockable container with submitted disinfectant (e. g. Dettol).
- Autoclave bag
- BBL MGIT Mycobacteria Growth Indicator Tubes 7 ml
- BACTEC MGIT 960 Supplement Kit

5.5. Procedure

Preparation for test

- Switch on the safety cabinet approximately 15 min before use.
- Label all MGIT tubes with the specimen number.
- Sort the samples according to ascending sequence of numbers.

Sample preparation

Consider:

- Prior to use, each MGIT tube should be examined for evidence of contamination or damage. Discard any tubes if they appear unsuitable.
- Dropped tubes should be examined carefully. If damage is seen, the tube should be discarded.

- Reconstitute MGIT PANTA with 15.0 ml MGIT growth supplement. Mix until completely dissolved. This mixture is stable for 5 days if stored at 5 ± 3 °C.
- Aseptically add 0.8 ml of this enrichment to each MGIT tube prior to inoculation of specimen (preferably not more than 30 min before). Do not add PANTA/enrichment after the inoculation of specimen. Do not store MGIT tube after the addition of enrichment/ PANTA.
- For each specimen, label a MGIT tube with the sample ID number
- Add 0.5ml of resuspended pellet generated as per SOP_LAB_098_VO1 to MGIT tube. Dispose of transfer pipette into the autoclave bag.
- Tightly recap the MGIT tube and mix well by inverting the tube several times.
- If contamination is detected the procedure is repeated with the remaining pellet, using the reverse side of MGIT Report Form (form# BRTC_LAB_099_APP01_V01).
- Leave the inoculated MGIT tube in the rack for 30 min.
- Tubes inserted into the BACTEC-MGIT 960 instrument will be automatically tested for the duration of the 42 day testing protocol

Results

Instrument Positive:

An instrument-positive sample is determined by the BACTEC MGIT 960 instrument and confirmed by an acid-fast smear.

i. Print an “Unloaded positive report” from the MGIT instrument with details of all instrument positive bottles. Add the corresponding ID number and sign off the print out. This print out must be kept in the unloaded positives folder. The MGIT will record the date the tube was flagged as positive and the number of days and hours taken to reach positivity.

ii. Simultaneously do the preparation for a ZN smear and the inoculation of a blood agar plate:
   - ✐ Using a sterile transfer pipette, remove an aliquot from the bottom of the tube (approx. 0.1 ml) for AFB stain preparation and blood agar plate inoculation.
     - o The slide and the blood agar plate should be labeled with the specimen number, and the abbreviation for this medium (“M”).
     - o Heat fix and stain the smear as for Ziehl-Neelsen staining protocol (see SOP# BRTC_LAB_097_V01).
     - o Examine the smear for the presence of acid fast bacilli (AFB). Describe AFBs i.e. typical, atypical and whether cording is seen and record the results in BRTC_LAB_099_APP01_VO1
     - o Incubate the inoculated blood agar plate along with the MGIT tube (using labeled rack `MGIT with inoculated BA`) in the incubator at 37 ± 2 °C for 48 hours.

These procedures will give the information that AFBs and a contamination are present or not.

iii. After 48 hours check the blood agar plate for bacterial contamination.

iv. Depending on the results of ZN stain and blood agar plate proceed as following noted in Table 2.

<table>
<tr><th>ZN stain</th><th>Blood agar plate</th><th>Action taken</th></tr>
<tr><td>AFB positive</td><td>No growth</td><td>Result is valid MGIT positive and should be reported on form# BRTC_LAB_099_APP01_V01.
Place the tube on the respective labeled racks for:
o Molecular Identification (see SOP# BRTC_LAB_101_V01)
o Inoculation (100 – 200 ! l) of LJ slope and incubate at 37 ± 2°C up to day 56 or up to there is any growth detectable. (Rack labeled with: `LJ Subcultures from first MGIT tube`). After detected growth the slope should be stored at a cool dark place.</td></tr>
<tr><td>AFB positive</td><td>Growth</td><td>MGIT tube is contaminated and is a mixed culture (AFB+).
Do the following:
· Transfer the entire MGIT broth into a 50 ml centrifuge tube.
· Add an equal quantity of 4% sterile NaOH solution.
· Mix well and let stand for 15-20 minutes, mixing and inverting the tube periodically.
· Add phosphate buffer pH 6.8 after 20 minutes up to 50 ml mark. Mix well.
· Centrifuge at least at 3000x g for 20 minutes.
· Pour off the supernatant fluid.
· Re-suspend the sediment in 0.5 ml of buffer and mix well.
· Inoculate 0.5 ml into a fresh MGIT tube supplemented with MGIT growth supplement/PANTA.
If the sample has already been retreated once and is still contaminated – this should be reported as contaminated</td></tr>
<tr><td>AFB negative</td><td>Growth</td><td>MGIT tube is contaminated
-Re-treat the stored sputum pellet (see SOP# BRTC_LAB_098_V01) and reinoculate a new MGIT and LJ slope.
If the sample has already been retreated once and is still contaminated – this should be reported as contaminated</td></tr>
</table>

ZN stain	Blood agar plate	Action taken
		For HighRif study decontaminate the content of MGIT vial as discribed in above section (ZN – AFBpos, BA – Growth)

ZN stain	Blood agar plate	Action taken
AFB negative	No growth	Possible false positive – need to confirm absence of AFBs o Redo the slide to double check for AFB o Look at the tube – is it turbid or could it be a true false positive (from chemical or pH problems) o Label the tube on top with the date of the final smear (in 3 days) to be performed and re-insert the tube into the MGIT instrument to allow further *M.tuberculosis* growth. After this 3 days repeat the AFB stain. If no AFBs are present, inoculate a labeled LJ slope (Lab.-Accession and study number – or screening number if pre-enrolment), Date of inoculation, and “M”). Put the LJ slope on the rack `LJ subculture – MGIT instrument pos., AFB neg.` and incubate this tube at 37°C for the next 4 weeks (put a respective label with the last incubation date on top). If AFBs are present, follow the instructions as described above. If AFBs are not present, no further action.

Instrument Negative:

At the end of six weeks incubation,

i. Print an “Unloaded negative report” from the MGIT instrument. Add the corresponding specimen number and sign off the print out.

ii. The print out must be kept in the ring binder “MGIT printouts - Instrument negative”. The sample will be reported as MGIT culture negative and the result is recorded on the form # BRTC_LAB_099_APP01_V01 – MGIT Result.

iii. The MGIT tubes will be discarded as biohazard waste.

5.6. Sources of error and interference factors

- Incorrect procedure of the decontamination and enrichment.
- The reconstitution of the MGIT PANTA with the MGIT Growth Supplement must be done under sterile conditions.
- For best results, the addition of Growth Supplement / MGIT PANTA Antibiotic Mixture should be performed just prior to specimen inoculation, preferably not more than 30 min before adding 0.5 ml of the concentrated specimen suspension
- Before insertion of the tubes into the instrument, tightly recap the tubes and mix well and leave untouched under the Safety cabinet at room temperature for approximately 30 min.
- Contamination of (or by) neighboring samples during the procedure.

5.7. Quality assurance

a. Decontamination procedure

1) Check of contamination rates: The weekly contamination rates for MGIT culture should be 3 – 8%.

b. Culture Media

1) Procedure: see SOPs# BRTC_LAB_114_V01, BRTC_LAB_112_V01.
2) Forms: see form# BRTC_LAB_114_APP01_V01, BRTC_LAB_112_APP01_V01
3) QA/QC check of blood agar plates.

5.8. Further handling of the samples after the investigation

Identification

All AFB positive cultures should be identified by MTBDRplus test and further by MTBC or Mycobacterium CM/AS tests.

Sample storage

AFB positive MGIT tube is to be stored at 36 ± 1 °C until the results of Molecular speciation by HAIN system, and/or the DST are known,

Sample disposal

The disposal of the sample containers into the autoclave bag is done by the responsible coworker.

6. References

1. BD BBL™ MGIT™ Instruction, Becton, Dickinson and Company, Sparks, Maryland, USA
2. Instruction BD BACTEC™ MGIT™ 960 System, BD Diagnostic Systems
3. MGIT 960 Operating and Maintenance Manual
4. Salman H. Siddiqi, Sabine Rüsch-Gerdes. Procedure manual For BACTEC™ MGIT 960™ TB System. 2006

Appendix 20: MTB speciation, INH & RMP resistance detection

Bagamoyo Research and Training Centre

<table>
<tr><td colspan="2">TITLE: Molecular Speciation of M.tb complex and detection of resistance to INH and RMP</td><td>VERSION: 02</td><td>PAGE: 1 of 15</td></tr>
<tr><td>SOP Code: BRTC_LAB_101_V02</td><td colspan="3">AREA: Laboratory</td></tr>
<tr><td>WRITTEN BY:
Name Date Signature
Jugheli, L.</td><td colspan="3">REVISED BY:
Name Date Signature
Rutaihwa, L.K
Mickson, S
Mswata, S.</td></tr>
<tr><td>APPROVED BY QA:
Name Date Signature</td><td colspan="2">AUTHORIZED BY BRTC LEADER:

Signature:</td><td>EFFECTIVE DATE:</td></tr>
<tr><td colspan="4">ORIGINAL LANGUAGE: ENGLISH</td></tr>
<tr><td>TRANSLATED BY:
Name Date Signature
Not applicable</td><td colspan="3">TRANSLATION VERIFIED BY:
Name Date Signature
Not applicable</td></tr>
</table>

CHANGES

DATE	**CHANGE**	**REASON FOR CHANGE**
21/10/2010	Added Figure 1	Added graphical explanation of DNA extraction
	Adjusted centrifugation speed on page 8.	The maximum speed of the centrifuge in BSL3 lab is 14680 g

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1. Objective

To describe the procedures of Molecular speciation of M.tb complex and detection of resistance to INH and RMP by GenoType MTBDRplus test.

2. Definition

Abbreviations:

BRTC	-	Bagamoyo Research and Training Centre
SOP	-	Standard Operating Procedure
INH	-	Isoniazid
RMP	-	Rifampicin

3. Scope

These procedures are applicable to the Microbiology and Molecular TB section within BRTC.

4. Responsibilities

All trained personnel of BRTC Microbiology and Molecular TB section are responsible for the whole content of this SOP.

5. Procedures

5.1 Purpose

The GenoType® MTBDR*plus* test is based on the DNA-Strip technology and permits the molecular genetic identification of the Mycobacterium tuberculosis complex and its resistance to rifampicin and/or isoniazid from cultivated samples or pulmonary smear-positive direct patient material

5.2 Principle

The identification of rifampicin resistance is possible by the detection of the most significant mutations of the *rpoB* gene (coding for the ! -subunit of the RNA polymerase).
For testing of high level isoniazid resistance, the *katG* gene (coding for the catalase peroxidase) is examined and for testing of low level isoniazid resistance, the promoter region of the *inhA* gene (coding for the NADH enoyl ACP reductase) is examined.
The whole procedure is divided into three steps:

- DNA isolation
- a multiplex amplification with biotinylated primers and
- a reverse hybridization.

The hybridization includes the following steps:

- chemical denaturation of the amplification products,
- hybridization of the single-stranded, biotin-labeled amplicons to membrane-bound probes,
- stringent washing,

- addition of a streptavidin / alkaline phosphatase (AP) conjugate, and
- AP mediated staining reaction.

A template ensures the easy and fast interpretation of the banding pattern obtained

5.3 Safety measures

- Samples from suspected patients and cultures isolated from those samples must always be handled under suitable safety conditions.
- Specimen treatment and sample preparation must be carried out in class II safety cabinet with an exception of heat inactivation.
- Before the heat inactivation step, samples must be centrifuged in an aerosol-tight rotor.
- Working with Mycobacterium tuberculosis grown in culture requires biosafety level 3 practices, containment equipment and facilities.
- Always wear suitable protective clothing and gloves.
- Observe the usual precautions for amplification set-up. It is essential that all reagents and materials used for DNA isolation and amplification set-up are free from DNases.
- The Denaturation Solution (DEN) contains <2 % NaOH and is irritating to eyes and skin
- The Substrate Concentrate (SUB-C) contains Dimethyl Sulfoxide and is irritating.

5.4 Specimen

- **Cultured material (liquid culture / solid culture)**
  - **Sample amount:**
    Liquid medium: 1.0 ml.
    Using too many bacteria for isolation may lead to inhibition of PCR. When densely grown cultures are used, the amount of starting material should be reduced.

  Solid medium: a loopful of bacteria.
  - **Sample keeping until the starting procedure:**
    Samples must be kept in incubator at 37 ± 2 °C.

- **Previously isolated DNA;** any DNA isolation procedure producing amplifiable MTBC-DNA from bacteria can be used

5.5 Equipment and Material

- GenoType MTBDR*plus*; HAIN LIFESCIENCE.
  A test kit contains:

  - Membrane strips
  - Primer Nucleotide Mix (PNM)
  - Denaturation Solution (DEN)

- Hybridization Buffer (HYB)
- Stringent Wash Solution (STR)
- Rinse Solution (RIN)
- Conjugate Concentrate (CON-C)
- Conjugate Buffer (CON-D)
- Substrate Concentrate (SUB-C)
- Substrate Buffer (SUB-D)
- Tray, evaluation sheet
- Manual, template

- HotStarTaq DNA Polymerase;
- Water, molecular biology grade, bi-distilled, sterilized;
- Distilled water.
- Safety cabinet biological class II
- Thermal cycler
- TwinCubator
- Water bath
- Vortexer
- Microcentrifuge (with angel rotor for 1.5 and 2 ml tubes)
- Heating block (for 1.5 and 2 ml tubes)
- Refrigerator
- Freezer
- Timer
- Pencil
- Tweezers
- Sterile centrifuge tubes, 50 ml,
- Sterile transfer pipettes, 3.5 ml;
- Rack for micro centrifuge tubes and PCR tubes
- Micro centrifuge tubes 1.5 ml.
- PCR tubes, 0.2 ml
- Absorbent paper
- Adjustable pipettes for 10, 20 200 and 1000 "1
- Disposable sterile pipette tips with filter;
- Disposable gloves
- Lockable container with submitted disinfectant (e. g. Kohrsolin)
- Autoclave bag.

5.6 Procedure

The whole procedure is divided into four steps:

1) DNA isolation from cultured material
2) Preparation of PCR master mix
3) a multiplex amplification with biotinylated primers and
4) a reverse hybridization.

a. Preparing measures

1) Switch on the safety cabinet approximately 15 min before use.
2) Sort the samples according to ascending sequence of numbers.
3) Label all tubes with specimen numbers.
4) Fill for each investigation material a min sheet and fill out the evaluation sheet (**BRTC_LAB_103_APP01_V01 and BRTC_LAB_103_APP02_V01**).
5) Heat a heating block to 95°C.

b. Sample preparation

Consider:

- The following steps have to be performed in the safety cabinet!
- The working area must be free from amplified DNA.
- It is crucial to heat samples to 95 – 105 °C for at least 15 – 20 min in orde to totally lyse cells and to inactivate vegetative bacteria from cultured material.

1) DNA isolation (see Figure 1): Preparing measures only for cultured material (from liquid or solid medium):

Raw material are mycobacteria grown on liquid (MGIT) or solid (LJ) medium.

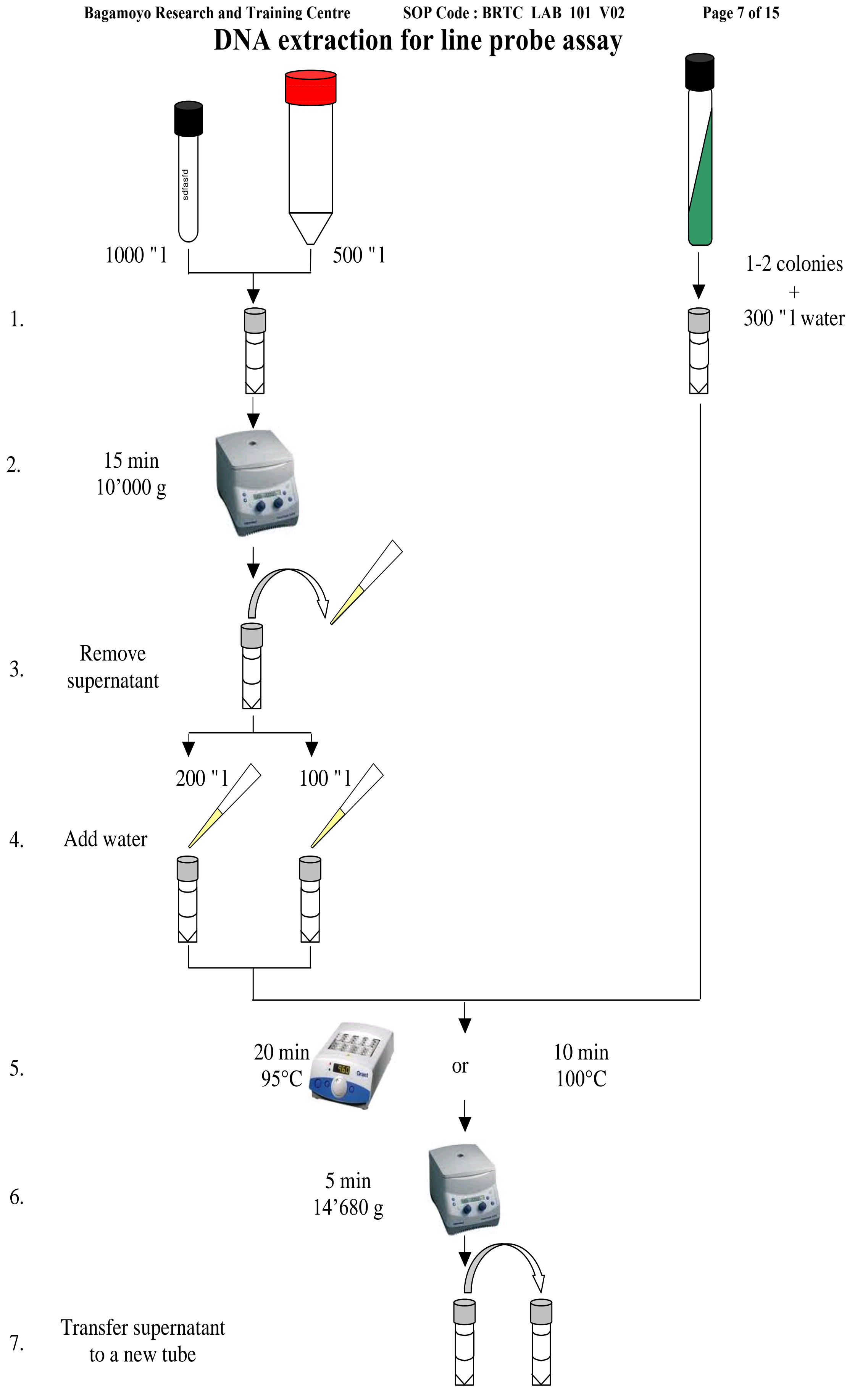
1a) **If using bacteria grown in liquid media:**

- ✂ Directly apply **1.0 ml** of the medium to a 2.0ml micro centrifuge tube. Using too many bacteria for isolation may lead to inhibition of PCR. When densely grown cultures are used, the amount of starting material should be reduced.
- ✂ Centrifuge for 15 min at 10000 x g (use aerosol protection hood!)
- ✂ Carefully remove the supernatant with transfer pipette ensuring that pellet remains in the tube
- ✂ Add 100 "l of water (molecular biological grade) (using a filtered pipet tip) and re-suspend the pellet completely by vortexing

1b) **If using bacteria grown in solid media:**

- ✂ Collect bacteria with a sterile inoculation loop and suspend in 300 "l water (molecular biology grade) (using a filtered pipet tip). Using too many bacteria for isolation may lead to inhibition of PCR. When densely grown cultures are used, the amount of starting material should be reduced.

DNA extraction for line probe assay



1c) **If using prior isolated DNA**, continue with step 4).

2) Incubate bacteria from 1a) and 1b) for 20 min in a heating block at 10(°C (95 – 105 °C).

3) Spin down for 5 min at full speed (14680 g).

4) Transfer supernatant to a new centrifuge tube, which is labeled with the sample ID number.

5) The working area has to be cleaned with Hypochlorite 0.5 % solution and alcohol 70 %

2) Preparation of PCR master mix:

Prepare the amplification mix (45 " l) in a "Pre-PCR" room (Media preparation room).

Important

- **To minimize chance of cross-contamination, all reagents and DNA extracts stored in 1.5-2.0 ml tubes should be vortexed and short-spun before use.**
- **Always wear "clean" gloves (not used to handle DNA samples) when handling reagents in Pre-PCR room**
- **Wear designated "clean" lab coat when preparing master mix**
- **PCR tubes to be handled with tweezers.**
- **Taq to be taken out of the freezer only when is to be added and immediately returned to the freezer afterwards.**
- **Taq to be added last when preparing the master mix.**

a. The amount needed is calculated by taking the number of samples to be analyzed + control samples. A negative control sample, for example, contains 5 " l of water (molecular biology grade) instead of DNA extract.

Table 1. Buffer-Master-Mix preparation depending on number of samples.

No. of samples	1	2	3	4	5	6	7	8	9	10	11	12	13*
PN-Mix	35†	70	105	140	175	210	245	280	315	350	385	420	455
PCR Buffer	5	10	15	20	25	30	35	40	45	50	55	60	65
$MgCl_2$	2	4	6	8	10	12	14	16	18	20	22	24	26
H_2O	3	6	9	12	15	18	21	24	27	30	33	36	39
HotStarTaq-Polymerase‡	0.2	0.4	0.6	0.8	1	1.2	1.4	1.6	1.8	2	2.2	2.4	2.6

* Always prepare master mix for one sample more then actual number of samples;

† All amounts in "l.

‡ HotStarTaq-Polymerase: 5 U/"l

b. The resulting Buffer-Master-Mix is mixed carefully by pipetting up and down and 45 "l is added to each PCR tube (samples + 1 negative control) for amplification.

3) Amplification

Addition of DNA:

The DNA sample should be added in a separated area (PCR room). If you are working on frozen extracted DNA, perform a short spin before you continue with the following steps, to avoid a contamination.

c. Add 5 "l of the extracted DNA (or water for negative control) to the master mix

d. The tubes are transferred to the ThermoCycler and the respective programme is started. Duration: approximately 2.5 h.
The amplification profile at the ThermoCycler by using HotStarTaq-Polymerase is following:

Table 2. Amplification profile at the ThermoCycler

	Culture samples
Programme name	MTBDR-culture
15 min 95 °C	1 cycle
30 sec 95 °C 2 min 58 °C	10 cycles
25 sec 95 °C 40 sec 53 °C 40 sec 70 °C	20 cycles
8 min 70 °C	1 cycle

e. Amplification products can be stored at 4 °C to -20 °C.

4) Hybridization:

The hybridization is done in the “Post-PCR”.

Consider:

· Always wear gloves and lab coats when working in Post-PCR room. These gloves and coats should **NEVER** be used outside the Post-PCR room and should be either discarded (gloves) or washed (coats) in bleach;
· Take care not to spill solution into the neighboring wells;
· The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward;
· Using tweezers turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination.

Preparation:

- Prewarm the water bath to 42 °C (the acceptable range is 37°-45° C) and heat up solutions HYB and STR. The reagents must be free from precipitates (note, however, that solution CON-D is opaque. Mix if necessary.
- Warm the remaining reagents with the exception of CON-C and SUB-C to room temperature.
- Using a falcon tube, dilute Conjugate Concentrate (CON-C, orange) and Substrate Concentrate (SUB-C, yellow) 1:100 with the respective buffer (CON-C with CON-D, SUB-C with SUB-D) in the amounts needed. Mix well and bring to room temperature. For each strip, add 10 "l concentrate to 1 ml of the respective buffer. Dilute CON-C before each use. Diluted SUB-C is stable for 4 weeks if stored at room temperature and protected from light.

Performance:

i. Start the programme number 1 on the TwinCubator and pause to pre-warm the heating block.
ii. Dispense 20 µl of Denaturation Solution (DEN, blue) in a corner of each of the wells used.

iii. Add to the solution 20 µl of amplified sample, pipette up and down to mix well and incubate at room temperature for five minutes.

iv. Meanwhile, take strips out of the tube using tweezers and mark them with a pencil underneath the colored marker. This should be done on a paper towel. Always wear gloves when handling strips.

v. Carefully add to each well ml of pre-warmed Hybridization Buffer (HYB, green). Gently shake the tray until the solution has a homogenous color. Take care not to spill solution into the neighboring wells.

vi. Place a strip in each well. The strips must be completely covered by the solution and the coated side (identifiable by the colored marker near the lower end) must face upward. Using tweezers turn over strips which might have turned when immersed in the solution. Carefully clean tweezers after each use to avoid contamination. This also applies to all following steps.

vii. Place tray in shaking TwinCubator and incubate for 30 minutes at 45 °C.

viii. Completely aspirate Hybridization Buffer. For example, use a Pasteur pipette connected to a vacuum pump or simply pour off to a waste container and remove excess fluid by turning the tray upside down gently tapping on an absorbent paper.

ix. Add 1ml of Stringent Wash Solution (STR, red) to each strip and incubate for 15 minutes at 45°C in TwinCubator.

x. Work at room temperature from this step forward. Completely remove Stringent Wash Solution. Pour out Wash Solution in a waste container and remove all remaining fluid by turning the tray upside down and gently striking it on an absorbent paper. This also applies to all other wash steps.

xi. Wash each strip once with ml of Rinse Solution (RIN) for minute on TwinCubator (pour out RIN after incubation).

xii. Add 1 ml of diluted Conjugate (see above) to each strip and incubate for 30 minutes on TwinCubator.

xiii. Remove solution and wash each strip twice for minute with ml of Rinse Solution (RIN) and once for 1 minute with approx. 1 ml of distilled water (e.g. use wash bottle) on TwinCubator (pour out solution each time). Make sure to remove any trace of water after the last wash.

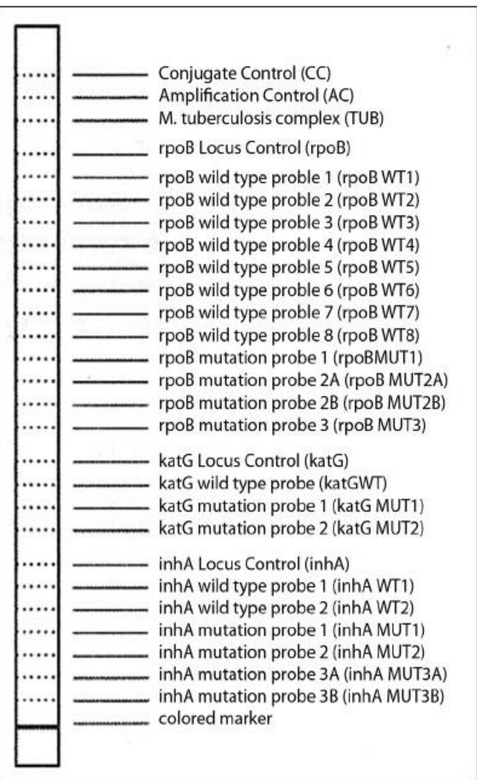
xiv. Add 1 ml of diluted substrate (see above) to each strip and incubate protected from light without shaking. Depending on the test conditions (e.g. room temperature), the substrate incubation time can vary between 3 and 20 minutes. Extended substrate incubation times can lead to increased background staining and might impair interpretation of the results.

xv. Stop reaction by briefly rinsing twice with distilled water.

xvi. Using tweezers remove strips from the tray and dry them between two layers of absorbent paper.

c. Evaluation and interpretation of results

- Paste the developed strips in the designated fields by aligning the bands CC and AC with the respective lines on the evaluation sheet (**BRTC_LAB_103_APP02_V01**).
- The supplied template also serves as an aid for evaluation and must be aligned with the bands CC and AC of the strip as well.
- Each strip has a total of 27 reaction zones (Figure 2).



- Figure 2. Reaction zones on the strip.Not all bands of a strip have to show the same signal strength.
- Determine the resistance status and note down in the respective column.
- Validation:
  - Conjugate Control (CC):
    A line must develop in this zone, documenting the efficiency of conjugate binding and substrate reaction.
  - Amplification Control (AC):
    When the test is performed correctly, a control amplicon generated during amplification will bind to the AC zone on the strip. A missing band therefore indicates mistakes during amplification set-up or the carry-over of amplification inhibitors with the isolated DNA. In case of a positive test result, the signal of the AC zone can be weak. In this case, however, the amplification reaction was performed correctly and the test does not have to be repeated.
  - *M.tuberculosis* complex (TUB):
    This zone hybridizes, as known, with amplicons generated from all members of the *M.tuberculosis* complex. If the TUB zone is negative, the tested bacterium does not belong to the *M.tuberculosis* complex and cannot be evaluated by this test system.
  - Locus Controls (rpoB, katG, inhA):
    The Locus Control zones detect a gene region specific for the respective locus and **must always stain positive**.

✂ Evaluation and Interpretation:
Wild type probes (WT) and Mutation probes (MUT):

- The wild type probes comprise the most important resistance areas of the respective genes. The strain tested is **sensitive** for the respective antibiotic, when all wild type probes of a gene stain positive (there is no detectable mutation within the examine regions).
- In case of a mutation, the respective amplicon cannot bind to the corresponding wild type probe. The absence of a signal for at least one of the wild type probes hence indicates a **resistance** of the tested strain to the respective antibiotic.
- Each pattern that deviates from the wild type pattern indicates resistance of the tested strain.
- The banding patterns obtained with the ***rpoB*** probes allows to draw a conclusion about a **rifampicin** resistance of the strain tested.
- The banding patterns obtained with the ***katG*** probes allows to draw a conclusion about a **high level isoniazid** resistance, the ***inhA*** probes a **low level isoniazid** resistance, respectively.

✂ Note the following special cases:

- There is a possibility that the specimen tested contains a heterogeneous strain. If, at investigation, this strain has developed only a partial resistance, one of the mutation probes as well as the corresponding wild type probe may appear.
- There is a possibility that the tested specimen contains more than one *M.tuberculosis* complex strain (due to mixed culture or contamination). If at least one of these strains harbors a mutation, one of the mutation probes as well as the corresponding wild type probe may appear.

✂ Evaluation examples (Figure 3):

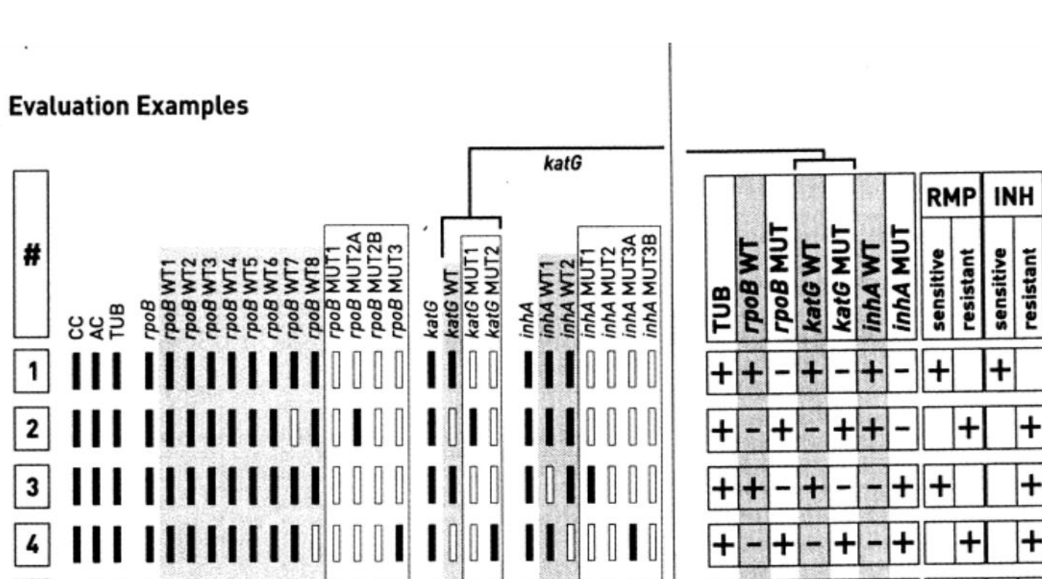


Figure 3: Examples for banding patterns and their evaluation with respect to RMP and/or INH resistance.

If all wild type bands display a signal, this is classified as positive and marked in the WT column of the respective gene as "+" (final result: sensitive to the respective antibiotic).

If **at least one of the wild type bands is absent**, this is classified as negative and marked in the WT column of the same gene as "-" (final result: **resistant** to the respective antibiotic).
Negative entries are only made to the mutation columns when none of the mutation bands display a coloration. If at least one of the mutation bands display coloration, this is classified as mutation-positive (final result: resistant to the respective antibiotic).

5.7 Sources of error and interference factors

- Overall weak or no signals (including Conjugate Control zone):
  - Room temperature too low or reagents not equilibrated to room temperature.
  - No or too little amount of CON-C and/or SUB-C used.
- Weak or no signals expect for Conjugate Control zone:
  - Quality and/or quantity of isolated DNA do not allow an efficient amplification. Check it in a 2 % agarose gel or try a different DNA isolation method.
  - Incubation temperature too high.
- No homogeneous staining:

  - Strips were not completely immersed during incubation steps.
  - Tray was not shaken properly.
- High background color:
  - CON-C and/or SUB-C used too concentrated.
  - Washing steps were not performed with the necessary care.
  - Wash solutions too cold.
- Unexpected result:
  - Wrong incubation temperature.
  - Hybridization Buffer and/or Stringent Wash Solution were not properly pre-warmed or mixed.
  - Contamination of isolated DNA and/or amplification agents with isolated and/or amplified DNA. In case amplification agents are contaminated a negative control sample also shows the respective banding pattern.
  - Contamination of neighboring wells by spillage during addition of Hybridization Buffer.
  - Depending on the amount of amplified DNA used and on the specific reaction conditions, a strong and fast color development may occur. In such cases, dis-continue the substrate incubation as soon as the signals are clearly visible in order to prevent the development of cross-hybridization bands.
  - No pure culture as starting material or more than one mutation in the tested strain.
  - Silent mutation in probe region.

5.8 Quality assurance

In order to validate the correct performance of the test and the proper functioning of reagents, each strip includes 5 control zones:

- a Conjugate Control zone to check the binding of the conjugate on the strip and a correct chromogenic reaction
- an Amplification Control zone to check for a successful amplification reaction
- three Locus Control zone (rpoB, katG, inhA) checking the optimal sensitivity of the reaction for each of the tested gene loci.

5.9 Further handling of the samples after the investigation

d. Sample storage

Store extracted DNA samples on -20°C.

6. References

a) HAIN LIFESCIENCE Manual, Nehren, Germany

Appendix 21: MGIT culture and speciation Report

Report form for MGIT culture

Bagamoyo Research and Training Centre

Specimen Unique Number	ATTACH LABEL
Patient initials	

RESULT SECTION – First MGIT tube - *Completed by the operator*			
Final concentration of NaOH used for decontamination	1%	1.5%	2%
Date of inoculation / Date started [1]	day month year	Initials ______	
MGIT tube No. [1]			
Instrument result [1]	pos.	day month year	Time to detection (TTD) / to positivity: Days . Hours
	neg.	day month year	Initial ______
If instrument positive: **Blood agar plate:**	done	not done	
Date of evaluation / test [2]:	day month year	Result pos. neg.	Initial ______
If instrument positive: **Ziehl-Neelsen stain:**			
Date of microscopy [2]	day month year	Result: AFB ! . pos. neg. cording typical atypical	Initial ______
Subculture on LJ slope	done not done – Reason(s):		Initial ______
Result of ZN stain from colonies on LJ	AFB pos. AFB neg.		Initial ______
Comments			
Lab. Supervisor *	*Date & Signature*		

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research | training | services

Report form for MTBC

Bagamoyo Research and Training Centre

Specimen Unique Number	ATTACH LABEL				
Patient initials					
RESULT SECTION					
DNA extracted from	Fresh sputum	Frozen Sputum	Stored pellet	Liquid Culture	Solid Culture
Date of DNA extraction	day month year			Initial ______	
Date of PCR	day month year			______	
Date of hybridisation	day month year			Initial ______	

Reaction zones	Visible bands	No visible bands
Conjugation Control (CC)		
Universal Control (UC)		
M. tuberculosis complex (MTBC)		

Reaction zones	Bands more or as intensive as UC	Bands less intensive than UC	No visible bands
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			

Report form for MTBC

Bagamoyo Research and Training Centre

Specimen Unique Number	ATTACH LABEL		
INTERPRETATION			
M.tuberculosis complex	**Yes**	**No**	**Not interpretable**
Species	***M.tuberculosis***	***M.bovis ssp bovis***	**Not interpretable**
	M.africanum	***M.bovis BCG***	
	M.microti	***M.bovis caprae***	
Comments			
Lab. Supervisor*	*Date*	*Signature*	

Report form for Mycobacterium CM

Bagamoyo Research and Training Centre

Specimen Unique Number	ATTACH LABEL		
INTERPRETATION			
Species	*M.tuberculosis* complex		
	M.avium	*M.chelonae*	*M.abscessus*
	M.fortuitum	*M.gordonae*	*M.intracellulare*
	M.scrofulaceum	*M.interjectum*	*M.kansasii*
	M.malmoense	*M.peregrinum*	*M.xenopi*
	M.marinum / M.ulcerans		Not interpretable
FURTHER ACTIONS			
Further speciation with **Mycobacterium AS** required:	**Yes**	**No**	
Comments			
Lab. Supervisor*	*Date*	*Signature*	

Report form for Mycobacterium AS

Bagamoyo Research and Training Centre

Specimen Unique Number	ATTACH LABEL		
INTERPRETATION			
Species	*M.simiae*	*M.mucogenicum*	*M.goodii*
	M.celatum	*M.smegmatis*	*M.genavense*
	M.lentiflavum	*M.heckeshornense*	*M.szulgai*
	M.phlei	*M.haemophilum*	*M.kansasii*
	M.ulcerans	*M.gastri*	*M.asiaticum*
	M.shimoidei		Not interpretable
Comments			
Lab. Supervisor*	*Date*	*Signature*	

Appendix 22: Xpert MTB/RIF test for FNA

Bagamoyo Research and Training Centre

<table>
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Maira Bholla</td><td colspan="3">REVISED BY:
Name Date Signature</td></tr>
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Name Date Signature</td><td colspan="2">AUTHORIZED BY BRTC LEADER:
Signature:</td><td>EFFECTIVE DATE:</td></tr>
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Not applicable</td><td colspan="3">TRANSLATION VERIFIED BY:
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Not applicable</td></tr>
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CHANGES

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1. **Purpose**

The Xpert MTB/RIF test for use with the Cepheid GeneXpert® System is a semi-quantitative nested real-time PCR in-vitro diagnostic test for: 1) the detection of *Mycobacterium tuberculosis* complex DNA in sputum samples or concentrated sediments prepared from induced or expectorated sputa that are either acid-fast bacilli (AFB) smear positive or negative; and 2) the detection of rifampin resistance associated mutations of the rpoB gene in samples from patients at risk for rifampin resistance. The MTB/RIF test is intended for use with specimens from untreated patients for whom there is clinical suspicion of tuberculosis (TB). Use of Xpert MTB/RIF for detection of *M. tuberculosis* (MTB) or determination of rifampin susceptibility has not been validated for patients who are receiving treatment for tuberculosis.

2. **Scope**

This procedure applies to all personnel working at the BRTC TB laboratories in Bagamoyo and Mwananyamala TB Clinic, Dar es Salaam.

3. **Principle**

The GeneXpert Dx System integrates and automates sample processing, nucleic acid amplification, and detection of the target sequences in simple or complex samples using real-time PCR and reverse transcriptase PCR. The system consists of an instrument, personal computer, barcode scanner, and preloaded software for running tests on collected samples and viewing the results. The system requires the use of single-use disposable GeneXpert cartridges that hold the PCR reagents and host the PCR process. Because the cartridges are self-contained, cross-contamination between samples is eliminated. For a full description of the system, see the GeneXpert Dx System Operator Manual. Xpert MTB/RIF includes reagents for the detection of tuberculosis and RIF resistance as well as a sample processing control (SPC) to control for adequate processing of the target bacteria and to monitor the presence of inhibitor(s) in the PCR reaction. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability.

The primers in the Xpert MTB/RIF assay amplify a portion of the rpoB gene containing the 81 base pair “core” region. The probes are able to differentiate between the conserved wild-type sequence and mutations in the core region that are associated with rifampin resistance.

4. **Safety measures**

- Treat all biological specimens, including used cartridges, as if capable of transmitting infectious agents. Because it is often impossible to know which might be infectious, all biological specimens should be treated with universal bio-safety precautions.
- Guidelines for specimen handling are available from the U.S. Center for Disease Control and Prevention and the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards).
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Wash hands thoroughly after handling specimens and test reagents. Follow your institution’s safety procedures for working with chemicals and handling biological samples.

5. **Specimen**

- Sputum specimens: a minimum of 1 ml of sputum per specimen should be processed. Specimens should be held at 2–8 °C prior to processing whenever possible. However, if necessary the specimens can be stored at a maximum of 35 °C for ! 3 days and at 4 °C for 4-10 days

- Sputum sediments obtained after the decontamination process using the NALC-NaOH method: at least 0.5 ml of resuspended sediment should be available to run Xpert MTB/RIF. If they are not immediately processed for Xpert MTB/RIF, store re-suspended sediments at 2-8°C not for more than 12 hours.
- Fine Needle Aspiration (FNA) specimens collected in 0.7ml phosphate buffered saline (PBS) should be held at 2-8°C for not more than 48 hours. If the volume of the specimen is still 0.7ml, then 0.35ml of the specimen should be available to run for Xpert MTB/RIF. If they are not immediately processed for XpertMTB/RIF, store FNA PBS specimens at -20 °C for not more than 4 months.

6. **Sources of error and interference factors**
- Use of non-respiratory specimens such as blood, CSF, stool or urine
- Specimens processed by methods other than NALC-NaOH
- Use of an Xpert MTB/RIF cartridge that has been open for more than 30 minutes. Do not open a cartridge until you are ready to perform the test.
- Use of the cartridge 7 days after opening the package because its stability is reduced.
- Use of a cartridge that has been dropped or shaken after you have added the treated sample.
- Use of a cartridge if it appears wet or if the lid seal appears to have been broken.
- Use of a cartridge that has a damaged reaction tube.
- Use of reagents or cartridges that have passed the expiration date.
- Reusing of spent cartridges. Each single-use Xpert MTB/RIF cartridge is used to process one test.
- Specimens with obvious food particles or other solid particulates.
- Processing more than four samples at one time
- Specimens not held at the indicated temperatures (see Section 5).

7. **Materials: Reagents / test components**

<u>Materials Provided</u>

The Xpert MTB/RIF kit (CGXMTB/RIF-10) contains sufficient reagents to process 10 patient or quality control specimens. The kit contains the following (as per kit instruction sheet):

- Xpert MTB/RIF cartridges with integrated reaction tubes (10 units)
- Bead 1 (freeze-dried) (2 per cartridge)
    - Primers
    - Probes
    - KCl
    - $MgCl_2$
    - HEPES, pH 8.0
    - BSA (bovine serum albumin)
- Bead 2 (freeze-dried) 2 per cartridge
    - Polymerase
    - KCl
    - $MgCl_2$
    - dNTPs
    - HEPES, pH 7.2
    - BSA (bovine serum albumin)
- Bead 3 (freeze-dried) 1 per cartridge
    - Sample Processing Control (SPC) approx. 2000 non-infectious sample preparation control spores (Bacillus globigii)
- Reagent 1 (Tris Buffer, EDTA, and surfactants) (4 mL per cartridge)
- Reagent 2 (Tris Buffer, EDTA, and surfactants) (4 mL per cartridge)
- Sample Reagent (Sodium Hydroxide and Isopropanol) (10 x 8 mL bottles)

- Sterile disposable transfer pipettes (12 units)

Materials Required but Not Provided

- GeneXpert Dx System equipped with GX2.1 software (catalog number varies by configuration): GeneXpert instrument, computer, barcode wand reader, and Operator Manual
- Printer (See GeneXpert Dx System Operator Manual for compatibility guidelines)
- Sterile screw-capped specimen collection containers
- Lab gown and disposable Gloves
- Labels and/or indelible labeling marker
- Sterile pipettes for sample processing
- Discard jar containing appropriate liquid disinfectant

8. Production of reagents, solutions etc.

- **Storage conditions and shelf life:**
  - Store the Xpert MTB/RIF cartridges and reagents 2 ± 28 °C
- **Manufacture regulations:** not applicable.

9. Devices, instruments and aids (listing)

- Safety cabinet biological class II
- Vortexer
- Timer
- GeneXpert Dx System equipped with GX2.1 software (catalog number varies by configuration): GeneXpert instrument, computer, barcode wand reader, and Operator Manual
- Sterile transfer pipettes, 3.5 ml; SARSTEDT; Catalog no. 86.1172.001 / 840 pcs
- Micro tube 1.5 ml, PP; SARSTEDT; Catalog no. 72.62.105 / 1000 pcs
- Sterile Falcon tubes (16.5x120 mm); 15 ml; BD; Catalog no. 352096 / 500 pcs
- Barcode labels for cartridge identification
- Lockable container with submitted disinfectant
- Autoclave bag
- Refrigerator
- Rack for Falcon tubes of 15 ml
- Disinfectant for surface and skin
- Disposable gloves.

10. Procedure

a. Procedure – Sputum Sediments

- Label each Xpert MTB/RIF cartridge with the sample ID (write on the side of the cartridge or affix ID label). Note: Do not put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge.
- Transfer at least 0.5 ml of the total resuspension pellet to a conical, screw-capped tube for the Xpert MTB/RIF using a sterile transfer pipette. Alternatively, the entire sample may be processed in the original tube.
- Store re-suspended sediments at 2–8 °C if they are not immediately processed for Xpert MTB/RIF. Do not store for more than 12 hours.
- Add 1.5 ml of Xpert MTB/RIF Sample Reagent (SR) to 0.5 ml of resuspended sediment sample using a sterile transfer pipette and shake vigorously 10 – 20 times. Note: One back-and-forth movement is a single shake.
- Incubate the specimen for 15 minutes at room temperature. At one point between 5 and 10 minutes of the incubation, again shake the specimen vigorously 10 – 20 times. Samples

should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample.

b. Procedure – Expectorated Sputum Samples

- Label each Xpert MTB/RIF cartridge with the sample ID (write on the side of the cartridge or affix ID label). Note: Do not put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge.
- For each of the samples; unscrew lid of original leak-proof sputum collection container; add Sample Reagent 2:1 (v/v) to sample, replace the lid, and shake vigorously 10 - 20 times. Note: One back-and-forth movement is a single shake.
- Incubate for 15 minutes at room temperature. At one point between 5 and 10 minutes of the incubation again shake the specimen vigorously 10 – 20 times. Samples should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample.

c. Procedure – Fine Needle Aspirate Samples in PBS

- Label each Xpert MTB/RIF cartridge with the sample ID (write on the side of the cartridge or affix ID label). Note: Do not put the label on the lid of the cartridge or obstruct the existing 2D barcode on the cartridge.
- Use fresh FNA in PBS specimen or thaw out frozen FNA in PBS and mix thoroughly by inverting several times. Spin down specimen in centrifuge at low speed for 30 seconds to remove specimen residue on lid of 1.5 ml tube. Transfer at least 0.35 ml of the FNA in PBS to a conical, screw-capped tube using a sterile transfer pipette. If more than 0.7ml specimen available, transfer at least 0.5ml of the FNA in PBS to the conical, screw-capped tube.
- Store remaining FNA sample at -20 ºC if not immediately used for another test.
- Add 1.8 ml of Xpert MTB/RIF Sample Reagent (SR) to the 0.35 ml of PBS specimen or make up the original specimen volume to just over 2ml with SR (i.e. 1.6ml for 0.5ml specimen) using a sterile transfer pipette and shake vigorously 10 – 20 times. Note: One back-and-forth movement is a single shake.
- Incubate the specimen for 15 minutes at room temperature. At one point between 5 and 10 minutes of the incubation, again shake the specimen vigorously 10 – 20 times. Samples should be liquefied with no visible clumps of sputum. Particulate matter may exist that is not part of the sample.

d. Procedure - Preparing the Cartridge

- Using the sterile transfer pipette provided in the Xpert kit, aspirate the liquefied sample into the transfer pipette until the meniscus is above the minimum mark (approximately 2ml). Do not process the sample further if there is insufficient volume.
- Open the cartridge lid. Transfer sample into the open port of the Xpert MTB/RIF cartridge. Dispense slowly to minimize the risk of aerosol formation.
- Close the cartridge lid. Make sure the lid snaps firmly into place. Remaining liquefied sample may be kept for up to 12 hours at 2 – 8 ºC should repeat testing be required.
- *Important*: Be sure to load the cartridge into the GeneXpert Dx instrument and start the test within 30 minutes of preparing the cartridge.

e. Starting the Test

Before you start the test, ensure that the system is equipped with the GX2.1 software, and the Xpert MTB/RIF assay is imported into the software). This section lists the basic steps of running the test. For detailed instructions, see the GeneXpert Dx System Operator Manual.

- Turn on the computer, and then turn on the GeneXpert Dx instrument.
- On the Windows® desktop, double-click the GeneXpert Dx shortcut icon.
- Log on to the GeneXpert Dx System software using your user name and password.

- In the GeneXpert Dx System window, click Create Test. The Scan Cartridge Barcode dialog box appears.
- Scan the barcode on the Xpert MTB/RIF cartridge. The Create Test window appears. Using the barcode information, the software automatically fills the boxes for the following fields: Select Assay, Reagent Lot ID, Cartridge SN, and Expiration Date.
- In the Sample ID box, scan or type the sample ID. Make sure you type the correct sample ID. The sample ID is associated with the test results and is shown in the "View Results" window and all the reports. Type in initial sample volume and any comments about the nature of the sample (pus/blood stained etc) into the comments.
- Click Start Test. In the dialog box that appears, type your password.
- Open the instrument module door with the blinking green light and load the cartridge.
- Close the door. The test starts and the green light stops blinking. When the test is finished, the light turns off.
- Wait until the system releases the door lock at the end of the run, then open the module door and remove the cartridge.
- Dispose of used cartridges in the appropriate specimen waste containers according to your institution's standard practices.

f. Results

The results are interpreted by the GeneXpert DX System from measured fluorescent signals and embedded calculation algorithms and will be displayed in the "View Results" window. Lower Ct (cycle number at threshold) values represent a higher starting concentration of DNA template; higher Ct values represent a lower concentration of DNA template.

A. MTB DETECTED (MTB target DNA is detected)

- MTB Detected—The MTB result will be displayed as High, Medium, Low or Very Low depending on the Ct value of the MTB target present in the sample. Table 1 lists the Ct value ranges for the displayed MTB results.

Table 1. MTB result name and Ct value range

MTB (result according to the concentration of DNA template)	Cycle number at threshold (Ct) range
High	<16
Medium	16-22
Low	22-28
Very Low	>28

A.1 EVALUATION OF RIFAMPICIN SUSCEPTIBILITY

This result will be displayed only in MTB DETECTED results and will be on a separate line from the MTB DETECTED result (see above)

- Rif Resistance DETECTED; a mutation in the rpoB gene has been detected that falls within the valid delta Ct setting.
- Rif Resistance INDETERMINATE; the MTB concentration was very low and resistance could not be determined.
- Rif Resistance NOT DETECTED; no mutation in the rpoB gene has been detected.

- SPC— NA (not applicable); SPC signal is not required since MTB amplification may complete with this control.
- Probe Check—PASS; all probe check results pass.

B. MTB NOT DETECTED (MTB target DNA is not detected, SPC meets acceptance criteria)

- MTB NOT DETECTED—MTB target DNA is not detected
- SPC— Pass; SPC has a Ct valid range and endpoint above the endpoint minimum setting.
- Probe Check—PASS; all probe check results pass.

C. RIF NOT DETECTED (RIF target DNA is not detected, SPC meets acceptance criteria).

- RIF NOT DETECTED—RIF target DNA is not detected
- SPC— Pass; SPC has a Ct valid range and endpoint above the endpoint minimum setting.
- Probe Check—PASS; all probe check results pass.

D. INVALID (Presence or absence of MTB cannot be determined, repeat test with extra specimen. SPC does not meet acceptance criteria, the sample was not properly processed, or PCR is inhibited)

- MTB INVALID—Presence or absence of MTB DNA cannot be determined.
- **SPC—FAIL**; MTB target result is negative and the SPC Ct is not within valid range.
- Probe Check—PASS; all probe check results pass.

E. ERROR

- MTB—NO RESULT
- SPC—NO RESULT
- **Probe Check—FAIL***; one or more of the probe check results fail.
  *If the probe check passed, the error is caused by a system component failure.

F. NO RESULT

- MTB—NO RESULT
- SPC—NO RESULT
- Probe Check—NA (not applicable)

G. REASONS TO REPEAT THE ASSAY (Repeat the test using a new cartridge or initiate alternate procedures if one of the following test results occurs):

- An INVALID result indicates that the SPC failed. The sample was not properly processed or PCR was inhibited.
- An ERROR result indicates that the Probe Check control failed and the assay was aborted possibly due to the reaction tube being filled improperly, a reagent probe integrity problem was detected, or because the maximum pressure limits were exceeded or there was a GeneXpert module failure.
- A NO RESULT indicates that insufficient data were collected. For example, the operator stopped a test that was in progress.

11. Data reporting

The result of each sample will be printed from the report generated by the proprietary software.

12. Quality assurance / quality control

(Each test includes a Sample Processing Control (SPC) and probe check (PCC).

a. **Sample Processing Control (SPC)—**Ensures the sample was correctly processed. The SPC contains non-infectious spores in the form of a dry spore cake that is included in each cartridge to verify adequate processing of MTB. The SPC verifies that lysis of MTB has occurred if the organisms are present and verifies that specimen processing is adequate. Additionally, this control detects specimen associated inhibition of the real-time PCR assay. The SPC should be positive in a negative sample and can be negative or positive in a positive sample. The SPC passes if it meets the validated acceptance criteria. The test result will be "Invalid" if the SPC is not detected in a negative test.

b. **Probe Check Control (PCC)**—before the start of the PCR reaction, the GeneXpert Dx System measures the fluorescence signal from the probes to monitor bead rehydration, reaction-tube filling, probe integrity and dye stability. Probe Check passes if it meets the assigned acceptance criteria.

13. References

a. BD BBL™ MGIT™ Instruction, Becton, Dickinson and Company, Sparks, Maryland, USA

b. BD BACTEC™ MGIT™ 960 System AST Instructions, BD Diagnostic Systems

c. MGIT 960 Operating and Maintenance Manual

d. GeneXpert Xpert® MTB/RIF (CGXMTB/RIF-10) Product Instruction Sheet 300-7810 Rev. A, April 2009

Appendix 23: Ustar EasyNAT TB IAD Assay

Bagamoyo Research and Training Centre

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Maira Bholla</td><td colspan="3">REVISED BY:
Name Date Signature</td></tr>
<tr><td>APPROVED BY QA:
Name Date Signature</td><td colspan="2">AUTHORIZED BY BRTC LEADER:
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CHANGES

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1. Purpose

The Ustar EasyNAT TB Isothermal Amplification Diagnostic (IAD) Kit is an accurate, simple, rapid and cross-contamination proof nucleic acid detection kit. It is intended for the qualitative detection of *Mycobacterium tuberculosis* (MTB) in human samples, and as a clinical diagnostic test for TB infection. The reaction mix in this kit is in a glassified state (a form of drying and crystallization making them stable at room temperature).

2. Scope

This procedure applies to all personnel working at the BRTC TB laboratory

3. Principle

The Ustar EasyNAT TB IAD kit is a nucleic acid amplification and hybridization technique using cross-priming amplification (CPA) technology from an isothermal DNA amplification system. Using multiple cross-linked primers (six to eight primers) targeting the gyrB gene of *M. tuberculosis* a DNA target sequence is amplified at a constant temperature of 63 or 65°C. Using multiple displacement and cross primers, PCR products are generated as interval tandem repeats of the target region based on *H37Rv* sequence (*BX842572.1, NT 5582-5789*).

Generation of cross-priming sites first occurs in the presence of Bst DNA polymerase through extension and displacement activity. Multiple primer binding sites are created which allow for multiple extensions and displacements, which occur at the cross-priming amplification stage. Mixed intermediate and end structures are then present with which bind the forward and reverse detector sequences to create single, double-stranded or partial double stranded sequences. Strip detection then occurs when these strands created through detector sequences bind to sites on the detection strips. The detection of amplified products is performed on a lateral flow strip housed in an enclosed, sealed plastic device to prevent the leakage of amplicons. Results are visualized as bands on the test strips present for positive samples and absent for negative results (Fang, Li et al. 2009).

4. Safety measures

- Treat all biological specimens, including used cartridges, as if capable of transmitting infectious agents. Because it is often impossible to know which might be infectious, all biological specimens should be treated with universal precautions.
- Guidelines for specimen handling are available from the U.S. Center for Disease Control and Prevention and the Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards).
- Wear protective disposable gloves, laboratory coats and eye protection when handling specimens and reagents. Wash hands thoroughly after handling specimens and test reagents. Follow your institution's safety procedures for working with chemicals and handling biological samples.

5. Specimens

- The specimens should be sputum, cerebrospinal, pleural, peritoneal, pericardial, ascites, and synovial fluid, but not saliva.
- Specimens may be stored up to 48 hours at 2°C to 8°C before testing. Keep the specimens at -20°C or colder for long-term storage.

6. Production of reagents, solutions etc.

Kit storage

- Component Box I can be transported at room temperature. However for long-term storage, the Sputum Liquefying Solution **MUST** be kept at 2°C to 8°C.
- Component Box II can be transported at ambient temperature. However for long-term storage, Reaction Mix (Glassified state) **MUST** be kept at 4°C and others MUST be kept at 2°C to 8°C
- ALWAYS keep Component Box II in its original box at -20°C, and Device at 2°C to 30°C.
- The Detection Device should be kept sealed until use.
- Do not freeze-thaw Box II repeatedly.
- The reagents should be returned to the said temperatures after each use.
- By following the storage instruction closely, the kit should be stable until the assigned expiry date.

Manufacture regulations: not applicable.

7. Sources of error and interference factors

Strict adherence to the assay procedure will ensure optimal assay performance. Any procedure deviation may lead to aberrant results.

- Do not use reagents from different lots.
- Use of USTAR cartridge that has been open after 30 minutes. Do not open a cartridge until you are ready to perform testing.
- Use the cartridge after 7 days opening the package because its stability is reduced.
- Use of a cartridge that has been dropped or shaken after you have added the treated sample.
- Use of a cartridge if it appears wet or if the lid seal appears to have been broken.
- Use of a cartridge that has a damaged reaction tube.
- Use of reagents or cartridges that have passed the expiration date.
- Insufficient lighting may cause false result reading.
- Reusing of spent cartridges. Each single-use USTAR tube and cartridge is used to process one test.
- Specimens and reagents should be held at the indicated temperature (see Section 5 and 6).

8. Warnings and precaution information

- For professional use only.
- For in vitro diagnostic use only.
- Please refer to the product labeling for information on potentially hazardous components.
- Reagents in this kit should not be health hazardous if used according to instruction.
- TB is an air-borne bacterium. Ensure necessary protection throughout the whole testing process.
- Follow laboratory procedures strictly when working with specimens.
- In case of an accident or contact with eyes, rinse immediately with plenty of water and seek medical attention.
- Seek immediate physician attention if contaminated materials are ingested or come in contact with open lacerations, or other breaks in the skin.
- All human source materials used in the preparation of the positive control has been tested and found to be non-reactive. Since no test method can give complete assurance of the absence of viral contamination, all reagents and human specimens should be handled as potentially infectious.

- Dispose of all specimens, nucleic acid detection devices and other materials after use as bio-hazardous waste.
- Do not pipette by mouth.
- The Sputum Liquefying Solution contains sodium hydroxide. Handle reagent carefully to avoid spills on work surface. Clean up spills with absorbent paper and water.
- Optimal assay performance requires STRICT ADHERANCE to the assay procedures described in these instructions for use. Any procedure deviation may lead to aberrant results.

9. Material: Reagents / test components

Materials Provided

Component Box I

Item	Quantity	Storage
· Detection Device and Cartridge (1pc each, packed in individually sealed aluminum pouch with desiccant)	20 devices	Store at 2°C to 30°C
· Sputum Liquefying Solution (125ml)	1 bottle	Store at 2°C to 8°C
· Instructions For Use	1 copy	
· Intensity Scale	1 copy	

Component Box II

Item	Quantity	Storage
· Reaction Mix (glassified state)*	20 tubes	Store at -20°C
· Resuspension Buffer (350ml)	1 tube	
DNA Extraction Solution (1ml)	1 tube	
· Mineral Oil (500ml)	1 tube	
· ddH$_2$O (180ml)	1 tube	
· Positive Control (40ml)	1 tube	

* The Reaction Mix (glassified state) has no label, for better fitting into the PCR machine or isothermal heating block.

Materials Required but Not Provided

- Micropipette and disposable tips
- Permanent marker
- Heating block, water bath or any isothermal devices
- Centrifuge, Vortex and Timer
- 5 – 10 ml centrifuge tubes
- 1.5 ml centrifuge tubes, with safe-lock feature
- Normal saline

10. Devices, instruments and aids (listing)

- Safety cabinet biological class II
- Vortex
- Timer
- Sterile transfer pipettes, 3.5 ml; SARSTEDT; Catalog no. 86.1172.001 / 840 pcs
- Micro tube 1.5 ml, PP; SARSTEDT; Catalog no. 72.62.105 / 1000 pcs
- Sterile Falcon tubes (16.5x120 mm); 15 ml; BD; Catalog no. 352096 / 500 pcs
- Barcode labels for cartridge identification
- Lockable container with submitted disinfectant
- Autoclave bag
- Refrigerator

- Rack for Falcon tubes of 15 ml
- Disinfectant for surface and skin
- Disposable gloves

11. Procedure

Bring Liquefying Buffer, DNA Extract Solution, Detection Device and specimens to room temperature before use.

Sample preparation and DNA extraction

Steps 1.1 and 1.2 are applicable to sputum samples ONLY. Other sample types such as cerebro-spinal fluid, pleural effusion, peritoneal, pericardial, ascites, synovial fluid and FNA fluid should start from step 1.3 onwards, but pus-like samples should follow sputum-liquefying steps (including pus-like samples FNA in PBS).

1.1. Transfer <5ml sputum specimens into 15ml centrifuge tubes (NOT provided) and add same volume of Liquefying Buffer into the tube. The volume of Sputum Liquefying Solution depends on the condition of the sample, usually a sputum:buffer rate of 1: 1 to 1:2.5, but more buffer may be needed for complete liquefying of some samples.

1.2. Incubate the mixture at room temperature (RT) for 30 minutes; vortex 4 to 5 times during incubation to ensure proper mixing.

Remark: More Liquefying Buffer can be added if the specimen appears to be very dense, but sample to buffer ratio should not exceed 1:2.5

1.3. Transfer 1ml (all of the sample if the volume is less than 1 ml) of the liquefied sputum sample, OR non-liquefied form of other sample types into a 1.5ml centrifuge tube* (NOT provided), centrifuge at >10,000rpm for > 10 minutes. Decant the supernatant and keep the pellet for the next step.

* Use tubes with safe-lock feature to prevent the caps from popping open during heating in subsequent steps.

The liquefied sputum sample should proceed to step 1.4 while the other sample types to step 1.6 directly.

1.4. Wash the pellet two times with 1 ml normal saline (0.9%w/v NaCl), centrifuge at >10,000 rpm for 10 minutes, decant the supernatant and keep the pellet for the next step.

1.5. Thaw the DNA Extract Solution and ensure insoluble particles in the solution are evenly distributed by vortex or shaking.

1.6. Pipette 40 ml of DNA Extract Solution (with the insoluble particles) into the tube containing the pellet (from step 1.3 or 1.4). Vortex to mix well.

1.7. Incubate the mixture at 95 to 100°C for 10 minutes.

1.8. Cool the tube at room temperature for 5 minutes.

1.9. Centrifuge at >10,000 rpm for > 5 minutes and save the supernatant as amplification template.

1.10. The amplification template can be used immediately or can be stored at -20°C for not more than 1 week.

Amplification and hybridization

1. Determine total number of tubes of Reaction Mix needed for the test set, one tube each for every sample and control. Positive and Negative Control are recommended for every test set.
2. Label the tubes for identification.
3. Add 15µl of Resuspension Buffer into each Reaction mix (glassified) tube. Incubate at room temperature for 2-3 minutes to allow complete dissolution.
4. For Negative Control, add 4µl ddH_2O into the tube and mix well using micropipette.
5. For each sample, add 4µl amplification template (samples from step 1.9) into each labeled tube and mix well using micropipette.
6. For the Positive control add 4µl of positive control into the positive control labeled Reaction Mix tube and mix well using micropipette.

   IMPORTANT: MAKE SURE YOU MIX WELL.

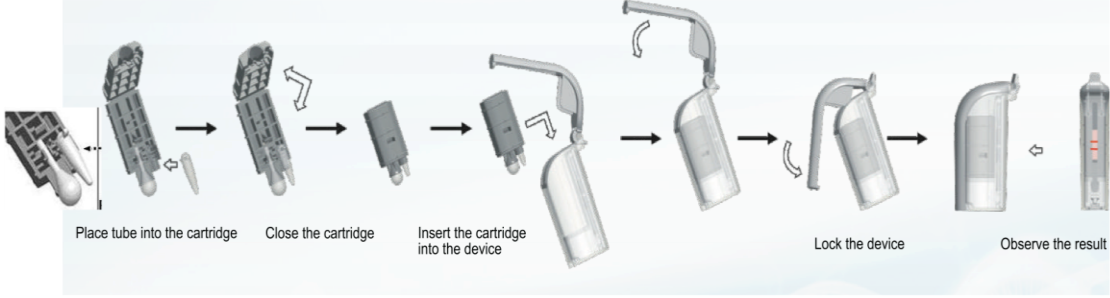
7. Add 20µl of Mineral Oil into each tube (*On top of Reaction Mix. DO NOT MIX)*
8. Centrifuge the Reaction Solution in each tube at >4000rpm for 3 to 5 seconds.
9. Incubate the tubes at 63°C for 60 minutes.

Do not open the tube covers during and after incubation.

Detection

1. Place the tube into the holder of the device cartridge then fold to close the cartridge (if the washing buffer bulb falls off from the holder, reassemble according to figure 1.
2. Insert the cartridge into the Detection Device; push the handle to the locked position.

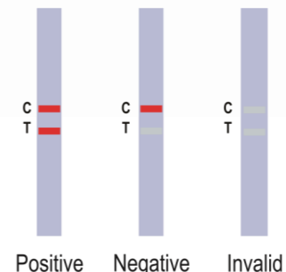
Figure 1:



3. Wait 15 to 30min to read the result.

   Results read after 30min are invalid.

   Figure 2:



12. Data reporting

Write results on USTAR Result Form_v01_03.05.2013

A valid test result MUST fulfill the following requirements (see Figure 2)

1. The Positive Control – two bands should appear; one control line (C) and one test line (T).
2. The Negative Control - ONLY a control line should appear.
3. Every sample tested must produce one control line, with or without a test line.
4. Invalid test results if the control line is absent (even if the test line appears, the result is still invalid)

Interpretation of Test Results

- Specimen with appearance of ONLY Control line indicates absence of *M. tuberculosis* DNA or the DNA copy number is below the detection limit.
- Specimen with appearance of both control line and test line indicates presence of *M. tuberculosis* DNA. Compare the intensity of the test line to the Intensity Scale.
- L4 and above: positive result
- Below L3: negative result
- Between L3 and L4: repeat the test for further confirmation

13. Kit Performance

A NEGATIVE result does not exclude the possibility of exposure to or infection with *M. tuberculosis*

Specificity

Cross-reactivity with positive samples to diseases such as *Pneumococcus, brucellos, Pertussis* vaccine, *M. kansasii, M. marinum, M. Simiae, M. scrofulaceum, M. ranae, M. avium, M. intracellulare, M. Phlei, M. vaccae, M. smegmatis, M. fortuitmn, M. chelonae subs.chelonae* and *M. chelonae subs.abscessus* was found to be insignificant.

Detection limit

Using *M. tuberculosis* H37RV strain, the detection limit was found to be less than ten bacteria.

Reproducibility

Repeat testing on a sample containing one hundred TB bacterial for 10 times, the positive results were reproducible.

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Appendix 24: EasyNAT Result Form

USTAR Result Form

(EasyNAT™ TB Isothermal Amplification Diagnostic Kit)

Subject ID:	|__|__|__|__|__|-|__|__|-|__|__|__|__|__|	
Sample ID:	|__|__|-|__|__|__|__|__|	
Sample type:	☐ Sputum	☐ Other______________
	☐ FNA sample	
Start of Test Date :	|__|__|-|__|__|-|__|__|__|__|	
Reading Results Date:	|__|__|-|__|__|-|__|__|__|__|	
USTAR Test Result:	☐ MTB Positive	
	☐ MTB Negative	
	☐ Invalid	
Comments		
Technician Initials |__|__|__|	Time |__|__|:|__|__|	
Signature ________________	Date |__|__|-|__|__|-|__|__|__|__|	

Version 1.0 dated 03.05.2013 Page **1** of **1**

Appendix 25: Latent Class Analysis Output

Parameter Estimates

Gamma estimates (class membership probabilities): (**Prevalence**): Class: 1 2

0.2283 0.7717

Rho estimates (item response probabilities):

Response category 1: (**Specificity**)

Class:	1	**2**	Class:	**1**	2
CultPosNeg:	0.2552	**0.9028**	CultPosNeg:	**0.7448**	0.0972
CytoPosNeg:	0.0000	**0.9468**	CytoPosNeg:	**1.0000**	0.0532
UstarPosNeg:	0.7080	**1.0000**	UstarPosNeg:	**0.2920**	0.0000
XpertPosNeg:	0.2929	**0.9421**	XpertPosNeg:	**0.7071**	0.0579
ZNPosNeg:	0.8093	**1.0000**	ZNPosNeg:	**0.1907**	0.0000

Response category 2: (**Sensitivity**)

Binary predictor variable correlation using a common latent random effect as TB score

ZNPosNeg:	**0.42**
XpertPosNeg:	**0.82**
UstarPosNeg:	**0.60**
CytoPosNeg:	**0.87**
CultPosNeg:	**0.82**

Latent Class Analysis (LCA) was performed on all FNA diagnostic outputs using Statistical Analysis Systems (SAS) Software version 9.2 SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). The data were prepared for all cases per diagnostic modality; 1 representing positive for TB and 2 to representing negative for TB. Blank cells indicated inconclusive outcomes or missing data. An algorithm is used to determine the probability of TB-presence or TB-absence per case, based on the data input.

The output displays two 'latent classes' created: class 1 for TB-positive and class 2 for TB-negative, with probabilities of 0.228 and 0.772 respectively. The prevalence of TB lymphadenitis in this study population therefore was estimated as 22.8% (0.228).

On creation of these latent classes (the probability of a case being positive or negative), the system assessed the performance of each diagnostic modality against the latent classes (Rho estimates). In 'response category 2' indicating "TB-present" (the probabilities of having positive values [class 1]) were the sensitivities of each diagnostic modality; Xpert 100%, cytology 74.5%, culture 70.7%, Ustar EasyNAT 29.2% and ZN (AFB microscopy) 19.1%. The negative predictive values ('response category 1' "TB-absent" [indicating the probabilities of having negative results]) were very high for all 5 diagnostic modalities (range: 90.3% - 100%), implying good specificities.

The binary predictor variable correlation, using a common latent random effect as TB score, was interpreted as **concordance** of the diagnostic modalities to the "true TB status" of the patients, as estimated by LCA. Accordingly, cytology had the highest concordance of 87% (p:0.87), followed by culture and Xpert at par (p:0.82 each), then Ustar (p:0.60) and ZN (p:0.42)

Appendix 26: Mhimbira, F.A., Bholla, M., et al. 2015. Detection of Mycobacterium tuberculosis by EasyNAT Diagnostic Kit in Sputum Samples from Tanzania. JCM

Detection of *Mycobacterium tuberculosis* by EasyNAT Diagnostic Kit in Sputum Samples from Tanzania

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The EasyNAT assay was evaluated for the detection of tuberculosis in sputum smears from presumptive pulmonary tuberculosis (TB) patients in an African high-TB and high-HIV setting. The sensitivity of the EasyNAT assay was 66.7%, and the specificity and positive predictive value were 100% for the culture-positive patients. The sensitivity was only 10% in the smear-negative and culture-positive patients.

Tuberculosis (TB), caused by the *Mycobacterium tuberculosis* complex, was reported in about 8.9 million cases and led to 1.3 million TB-related deaths in 2012 (1). The early and accurate diagnosis of TB and treatment are the mainstays of TB control (2, 3). Smear microscopy is still the sole TB diagnostic tool used in most resource-limited settings, where a high prevalence of HIV disease reduces its sensitivity from 55% to 39% because of paucibacillary disease (3, 4). The use of TB culture is restricted by the slow growth of the pathogen, high operational costs, and comprehensive biosafety requirements. Hence, a point-of-care test (POCT) for the diagnosis of active pulmonary TB is urgently needed (5).

Nucleic acid amplification tests (NAATs) for TB are high-performing tests for detecting TB RNA and DNA in clinical samples (6). The EasyNAT tuberculosis isothermal nucleic acid amplification diagnostic kit by Ustar Biotechnologies Co., Ltd. uses isothermal cross-priming amplification technology for the qualitative detection of *M. tuberculosis*. Six to eight primers target the *gyrB* gene of *M. tuberculosis*, amplifying it at a constant temperature of 63 or 65°C (7). Isothermal amplification is considered a promising platform for the point-of-care molecular detection of TB, because the technology does not need an initial denaturation step or the addition of a nicking enzyme (8), and it has a short hands-on time (9).

The recent clinical evaluation of the EasyNAT TB kit at four country-level TB clinics in China included 2,200 presumptive TB patients. Lowenstein-Jensen (LJ) culture was used as the reference standard. The overall sensitivity of the kit was 84.1%, and the specificity was 97.8%. The positive and negative predictive values (PPV and NPV, respectively) were 83.4% and 97.9%, respectively. In the smear-negative patients, the sensitivity was 59.8% (10).

In this substudy, the diagnostic performance of the EasyNAT assay was evaluated for the first time in an African high-TB-burden setting. We evaluated the detection of *M. tuberculosis* from 1 ml of frozen untreated morning or spot sputum samples from a clinically and microbiologically well-characterized cohort of presumptive pulmonary TB patients in Bagamoyo, Tanzania. Of the 147 participants, four were excluded because information was not available at the 5-month follow-up (Fig. 1). The EasyNAT assay was performed on one sputum sample from each of the 143 eligible participants who were assigned to classification groups, as described in Table 1. In classification groups A and B, the EasyNAT assay was applied only to samples that were also culture positive.

The mycobacteriological reference included the results of smear microscopy for acid-fast bacilli, the Bactec Mycobacterium Growth Indicator Tube (MGIT) 960 system, and LJ culture, as well as subsequent MPT64 antigen or molecular testing (GenoType MTBC or *Mycobacterium* CM/AS assay; Hain Lifescience, Nehren, Germany).

The study was approved by the Ifakara Health Institute (IHI) institutional review board and the Medical Research Coordinating Committee of Tanzania. Informed consent was obtained from all study participants.

The frozen stored sputum samples were thawed, and DNA extraction, amplification, hybridization, and detection using the EasyNAT assay were performed as previously described (10). The appearance of both the control and test lines on the EasyNAT kits indicated the presence of *M. tuberculosis* DNA. A single control line indicated the absence of *M. tuberculosis* DNA or that DNA copies were below the detection limit of the assay (7, 10). All tests were done at the TB research laboratory in Bagamoyo, Tanzania. The laboratory personnel were blinded to the classification of the patients and the microbiological results other than those of the EasyNAT assay. Parallel rapid HIV testing was done, as per the research protocol.

The mean patient age was 40.5 years (standard deviation, 15.3 years), and 54.6% of the patients were male. The overall HIV prevalence was 46.2% (95% confidence interval [CI], 37.8% to 54.7%). The HIV prevalences between the culture-confirmed TB patients and controls were 47.9% and 42.7%, respectively (P = 0.568, chi-square test).

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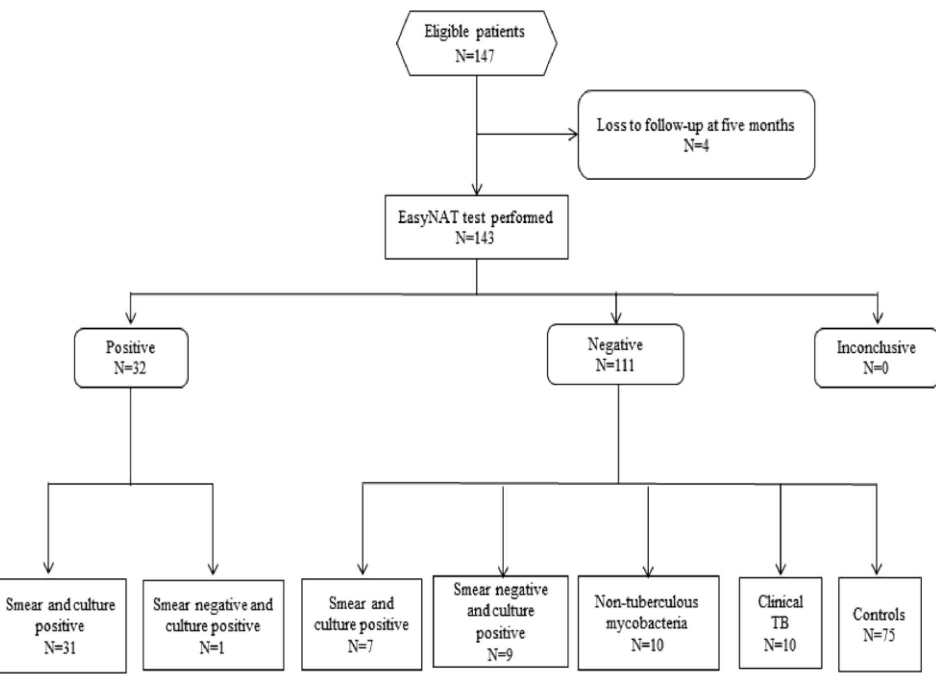

FIG 1 Patient flow and EasyNAT test results by patient classification.

The sensitivity of the EasyNAT assay with culture as the reference standard was 66.7% (95% CI, 51.6% to 79.6%). All patients who were classified as controls were negative by the EasyNAT assay (specificity, 100.0%; 95% CI, 95.2% to 100.0%). The PPV and NPV for the culture-positive patients were 100.0% (95% CI, 89.1% to 100.0%) and 82.4% (95% CI, 73.0% to 89.6%), respectively (Table 2).

The EasyNAT assay identified 31 of 38 smear- and culture-positive patients, respectively (sensitivity, 81.6%; 95% CI, 65.7% to 92.3%). One of the 10 smear-negative and culture-positive TB patients was positive by the EasyNAT assay (sensitivity, 10%; 95% CI, 0.3% to 44.5%). The sensitivity of the EasyNAT assay was 81.6% (95% CI, 65.7% to 92.3%) compared to that with Ziehl-Neelsen (ZN) staining, and 66.7% (95% CI, 51.6% to 79.6) and 69.2% (95% CI, 52.4% to 83.0%), respectively, compared to that with the MGIT system or LJ alone. No *M. tuberculosis* was detected by the EasyNAT assay in 10 patients categorized as having clinically diagnosed TB and in 10 patients who had the following *Mycobacterium* species and strains: *M. fortuitum* strain 1, *M. fortuitum* strain 2/*M. mageritense*, *M. malmoense*/*M. haemophilum*/*M. pasture*, *M. celatum* I+III, *M. simiae*, *M. celatum*, *M. intracellulare*, *M. asiaticum*, *M. scrofulaceum*, or *M. smegmatis*. One patient with both *M. intracellulare* and *M. tuberculosis* had a positive EasyNAT assay result.

In this cohort of presumptive TB patients from sub-Saharan Africa, the diagnostic accuracy for smear- and culture-positive TB patients was lower than that in previously studies on the EasyNAT assay (7, 10) and other isothermal NAATs (11). However, the sensitivity of the EasyNAT assay in the rather small group of smear-negative, culture-positive participants was considerably lower than that in the two evaluation studies from Asia (10% versus 59.8% and 87.5, respectively) (7, 10). Since all patients (n = 10) in this classification group were HIV positive, the low detection rate was most likely caused by a paucibacillary TB disease (12) with DNA copies below the detection limit of the assay. This might also explain why none of the clinically diagnosed TB patients, who presumably also had a low bacillary load, were positive by the EasyNAT assay. The assay did not cross-react with nontuberculous mycobacteria.

The estimated cost per EasyNAT test is $4 to 5 (10), which may be considered low. However, the utility of the EasyNAT assay in primary health care settings might be limited, because the sample preparation techniques are fairly complicated and may require a biological safety cabinet (10). Also, the EasyNAT assay does not

TABLE 1 Classification of study population according to clinical and microbiological data

No. (%) of samples	HIV prevalence (no. [%])	Description
38 (26.6)	13 (34.2)	Smear-positive/culture-positive, *M. tuberculosis*
10 (7.0)	10 (100)	Smear-negative/culture-positive, *M. tuberculosis*
10 (7.0)	7 (70.0)	Smear-negative or smear-positive/culture-positive, NTM[a]
10 (7.0)	4 (40.0)	All cultures negative, chest radiograph and clinical symptoms very suspect for pulmonary TB (clinically diagnosed TB)
75 (52.4)	32 (42.7)	All smears and cultures negative, sustained recovery up to 5 mo after antibiotic therapy (controls)

[a] NTM, nontuberculous mycobacteria.

TABLE 2 Performance of EasyNAT kit with MGIT and LJ combined, smear microscopy, MGIT, and LJ alone versus controls as reference standards

Performance parameter	Estimate ([95% CI]) for reference standards in:			
	Main analysis	Subanalysis		
	Culture MGIT and LJ vs controls	Smear microscopy vs controls	Culture MGIT alone vs controls	Culture LJ alone vs controls
Sensitivity (%)	66.7 (51.6–79.6)	81.6 (65.7–92.3)	66.7 (51.6–79.6)	69.2 (52.4–83.0)
Specificity (%)	100 (95.2–100.0)	100 (95.2–100.0)	100 (95.2–100.0)	100 (95.2–100.0)
Positive predictive value (%)	100.0 (89.1–100.0)	100 (88.8–100.0)	100.0 (89.1–100.0)	100.0 (87.2–100.0)
Negative predictive value (%)	82.4 (73.0–89.6)	91.50 (83.2–96.5)	82.40 (73.0–89.6)	86.2 (77.1–92.7)
Positive likelihood ratio[a]	NC	NC	NC	NC
Negative likelihood ratio	0.3 (0.2–0.5)	0.19 (0.1–0.4)	0.3 (0.2–0.5)	0.3 (0.2–0.5)

[a] The positive likelihood ratio could not be computed, since it is given by sensitivity/(1 − specificity). In all cases, the specificity was 1 (or 100%). NC, not calculated.

concurrently detect drug resistance in TB, which is a disadvantage in high-burden settings.

In conclusion, the EasyNAT assay detected *M. tuberculosis* with an excellent specificity and positive predictive value. The sensitivity was acceptable in the smear-positive patients. However, the low detection rate for the smear-negative, culture-positive sputum samples might be a limitation for wider clinical use and requires further evaluation in larger study populations from different regions that are endemic for TB.

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