The evaluation of the diagnostic utility and sensitivity of the Xpert® MTB/RIF in the detection of *Mycobacterium tuberculosis* and rifampicin resistance on bone marrow aspirate samples

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in the Branch of Pathology (Haematology).

DECLARATION

I, Nishanti Nadhiya Subramony declare that this thesis is my own work. It is being submitted for the degree of Master of Medicine in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

20 November 2017

DEDICATION

To the Almighty God.

Dedicated to my parents, Sam and Tholsie and to my husband, Nash.

ABSTRACT

In South Africa, the World Health Organisation estimated 454 000 new cases of *Mycobacterium Tuberculosis (M.tb)* infection (MTB) in 2015. Disseminated tuberculosis arises from haematogenous spread of the bacilli and seeding of the bacilli in extrapulmonary sites. The current gold standard for the detection of MTB of the bone marrow is TB culture which has an average turnaround time of 6 weeks. Although shorter, histological examinations of trephine biopsy cores to diagnose MTB also have a time delay owing mainly to the 5-7 day processing period prior to microscopic examination. Adding to the diagnostic delay is the non-specific nature of granulomatous inflammation which is the hallmark of MTB involvement of the bone marrow. A Ziehl-Neelson stain (which highlights acid-fast bacilli) is therefore mandatory to confirm the diagnosis but can take up to 3 days for processing and evaluation. Owing to this delay in diagnosis, many patients are lost to follow up or remain untreated for up to six weeks while the results are awaited, thus encouraging the spread of undiagnosed TB.

The Xpert® MTB/RIF (Cepheid, Sunnyvale, CA) is the molecular test used in the South African national TB program as the initial diagnostic test for pulmonary TB in adults and children. In 2013 the Xpert® MTB/RIF was applied to diagnose extrapulmonary TB and despite being available in 207 testing sites nationwide, it was never investigated for its potential application in diagnosing TB in bone marrow. Therefore, this study investigates the optimisation and performance of Xpert® MTB/RIF on bone marrow aspirate specimens.

BMA specimens received for routine immunophenotypic analysis as part of the investigation into disseminated MTB or in the evaluation of cytopenias in immunocompromised patients were used in this study. Processing BMA on the Xpert® MTB/RIF was optimised to ensure bone marrow in EDTA and heparin did not inhibit the PCR reaction. Inactivated *M.tb* from an Xpert® MTB/RIF external quality assessment program was spiked into the clinical bone marrow specimen and distilled water (as a control). A volume of 500µl and an incubation time of 15 minutes with sample reagent were investigated as the processing protocol.

A total of 135 BMA specimens had sufficient residual volume for Xpert® MTB/RIF testing however 22 specimens (16.3%) were not included in the final statistical analysis as an adequate trephine biopsy and/or TB culture was not available. Xpert® MTB/RIF testing was possible in

the presence of heparin or EDTA, but the overall detection of MTB in BMA was low compared to histology and culture.

The sensitivity of the Xpert® MTB/RIF when compared to both histological and culture findings was 8.7% with a 95% confidence interval (CI) of 1.07-28.04%. The sensitivity of the Xpert® MTB/RIF compared to histological findings only was 11.1% with a 95% CI of 1.38-34.7%. The specificity of the Xpert® MTB/RIF was 98.9% (95% CI: 93.9-99.7%).

Although the Xpert® MTB/RIF has a shorter turnaround time than histology and TB culture and is less expensive than culture and drug susceptibility testing, the low sensitivity of the Xpert® MTB/RIF in this study limits its current use for the diagnosis of MTB in bone marrow aspirate specimens until the diagnostic algorithm or the assay is further defined.

ACKNOWLEDGEMENTS

To the Almighty God without who none of this would be possible.

To my parents for their unwavering love and continuous support through my undergraduate and postgraduate training.

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LIST OF ABBREVIATIONS

AFB: acid fast bacilli

ART: anti-retroviral treatment

BCG: Bacillus Calmette-Guérin

BM: bone marrow

BMA: bone marrow aspirate

CMJAH: Charlotte Maxeke Johannesburg Academic Hospital

Ct: cycle threshold

EDTA: Ethylenediaminetetraacetic acid

ELISA: enzyme linked immunosorbent assay

EPTB: extra-pulmonary TB

HIV: Human Immunodeficiency Virus

IFN-γ: interferon gamma

IGRA: interferon gamma release assays

INH: isoniazid

LAM: lipoaribinomannan

LAMP: loop-mediated isothermal amplification assay

LF-LAM: lateral flow lipoaribinomannan assay

LIS: laboratory information system

LMTBI: latent MTB infection

LPA: line probe assays

MDR-TB: Multi drug resistant-TB

MGIT: Mycobacteria Growth Indicator Tube

MOTT: Mycobacterium Other Than TB

M.tb: Mycobacterium tuberculosis

MTB: *M.tb* infection

NAAT: nucleic acid amplification test

NHLS: National Health Laboratory Services

PCR: polymerase chain reaction

RIF: rifampicin

RRDR: Rifampicin Resistance Determining Region

SOP: standard operating procedure

SPC: sample processing control

SR: solvent-reagent

TAT: turnaround time

TB: Tuberculosis

TNF: Tumour Necrosis Factor

TST: tuberculin skin test

WHO: World Health Organisation

ZN: Ziehl-Neelson

CHAPTER 1: INTRODUCTION

1.1 HISTORY AND CHARACTERISTICS OF MYCOBACTERIA

The genus Mycobacterium is hypothesised to have emerged more than 150 million years ago however Hermann Heinrich Robert Koch was the first to identify the tubercle bacillus (*Mycobacterium tuberculosis*(*M.tb*)) and postulate its infectious nature in humans in 1882 (1, 2). The *M.tb* bacilli are rod-shaped, non-spore forming, aerobic organisms spread by airborne droplets (3). They are classified as acid-fast bacilli owing to their ability to retain the colour of arylmethane dyes when exposed to dilute mineral acid (4). The lipid cell wall of the bacillus is comprised of mycolic acid attached to a polysaccharide arabinogalactan and has many integral roles which include providing resistance to antibiotics and influencing the growth rate of the organism (5). As the organism replicates, it acquires mutations within its genome as a result of random DNA transcriptional errors. This allows for phenotypic variation between infections such as sensitivity to drugs, interaction with the host immune cells and the ability to disseminate to other tissues such as bone marrow (4).

The term MOTT (Mycobacterium Other Than Tuberculosis (TB)) was used when subsequent studies identified the organism in other species. This includes *M. avium* (avian) and *M. bovis* (bovine) (4).

1.2 PATHOPHYSIOLOGY

Once inhaled, most of the bacilli are trapped in the upper airways in close proximity to the goblet cells. These cells serve as the first line of defence owing to their mucus producing properties and are sufficient for the prevention of full-blown infection in healthy individuals (6, 7). Those bacilli that are capable of evading the goblet cells reach the alveoli and are phagocytosed by macrophages. This is enhanced by the binding of complement protein C3 to the bacterial cell wall (opsonisation). Despite being engulfed by alveolar macrophages, the bacilli continue multiplying at a rate of one cell division every 25-32 hours (6). The cytokines released from the macrophages recruit T lymphocytes thus shifting the immune response from an initial innate mediated to a cell mediated response. This process can take 2-12 weeks (8).

In an immunocompetent host, granulomatous inflammation occurs around the bacillus as the next step in containing the infection. Granulomas are predominantly comprised of T lymphocytes and macrophages and are characterised by low pH levels, low oxygen content and limited nutrients. These areas then undergo fibrosis and calcification resulting in a latent bacillus contained within a healed lesion (9). In immunocompromised patients, the inability of the immune system to contain the primary infection leads to symptomatic pulmonary disease. In addition, the formation of a granuloma is usually unsuccessful with early breakdown and extradition of the material into a blood vessel. This allows for haematogenous dissemination of the bacilli to extrapulmonary sites (9).

1.2.1 *M.tb* INFECTION (MTB) IN THE HIV POSITIVE POPULATION

Human Immunodeficiency Virus (HIV) is the biggest risk factor for acquiring *M.tb* infection (MTB) which in turn is the leading cause of death in people with HIV (10). As mentioned above, cell mediated immunity plays a vital role in the initial containment of MTB. This is driven by CD4+ T cells and is therefore compromised in HIV where the hallmark of disease is the steady depletion of CD4+ T cells. Other mechanisms that contribute to increased susceptibility to MTB (both primary and reactivation/post primary disease) include impaired tumour necrosis factor (TNF)-mediated macrophage apoptotic response to the bacillus (11), up-regulation of receptors on macrophages facilitating MTB entry (12) and impaired chemotaxis (13).

The TB-HIV relationship is a mutually beneficial one as *M.tb* increases the expression of CCR5 and CXCR4 receptors on CD4+ T cells which are known entry points for the HIV virus (14). Replication of the HIV virus is increased and has been demonstrated at sites of MTB infection in the lung and within macrophages and lymphocytes of the pleural space (15, 16). This is reflected in vivo by the rising viral loads noted in patients with both infections. Increased viral replication occurs as a bystander effect when TNF is secreted by T lymphocytes with the primary aim to inhibit *M.tb* growth (17). *M.tb* can directly stimulate TNF production through a component of its cell wall, lipoarabinomannan. This occurs via the NF-κB pathway which leads to the transcriptional activation of the long terminal repeat promotor thus enhancing HIV replication (18).

The diagnosis of MTB in a HIV positive person is challenging given the frequency of smear negative cases, the unusual/atypical presentations and the lack of granuloma formation on histological specimens. A high index of suspicion is required as well as novel diagnostic methods to shift the focus away from acid-fast bacilli detection in the diagnosis of active TB. Latent MTB infection (LMTBI) is described as an entity in which there is no manifestation of disease and *M.tb* cannot be cultured however clinical suspicion of infection exists (19). Identifying LMTBI is beneficial as it allows for an early diagnosis in individuals at high risk for transformation to active MTB (such as the immunocompromised). This can be done via two methods - the tuberculin skin test and interferon gamma release assays. Whilst much information is available regarding these tests, LMTBI is not the focus of this study.

1.3 EPIDEMIOLOGY

1.3.1 GLOBAL PERSPECTIVE OF MTB

In 2015, there were approximately 10.4 million new cases of MTB globally with a male to female ratio of 1.5:1. South Africa was one of six countries that accounted for 60% of these new cases (Figure 1). Despite this seemingly large number, the World Health Organisation (WHO) reports a decline in MTB incidence at a rate of 1.5% from 2014 to 2015. Deaths owing to MTB were estimated at 1.4 million in 2015 with MTB retaining its position as one of the top ten causes of death worldwide (20).

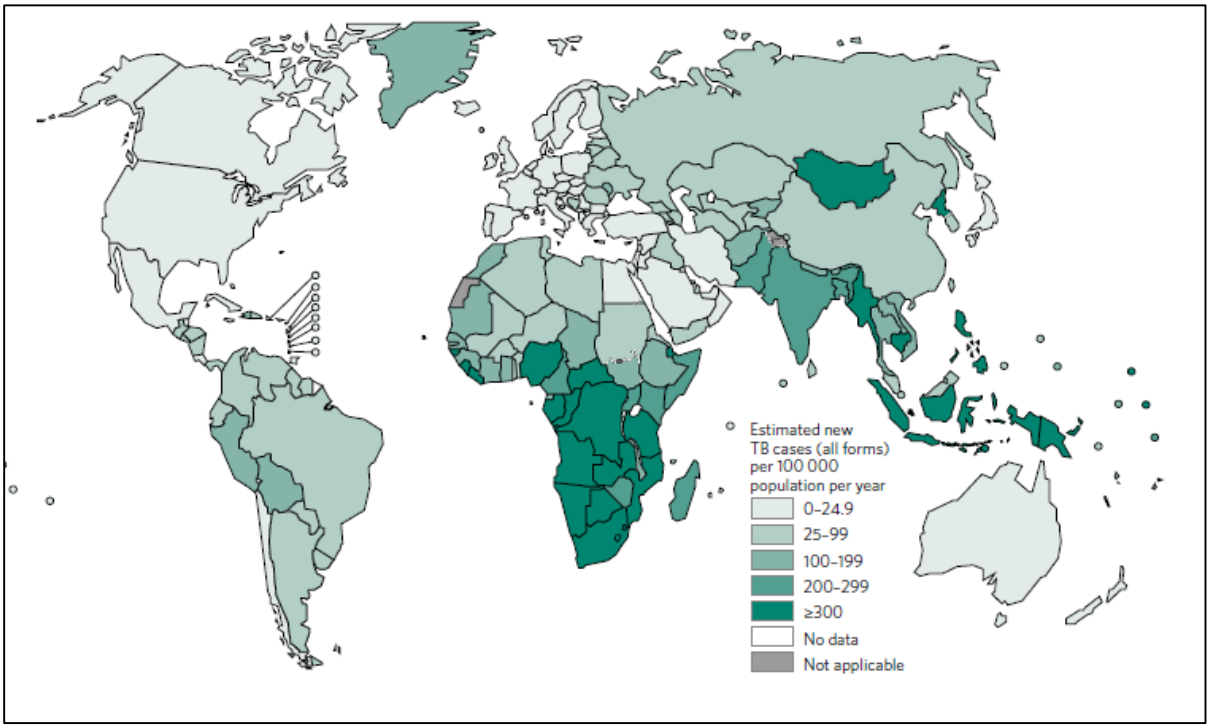


Figure 1: Global incidence of TB for 2015 (20)

New cases of multidrug-resistant TB (MDR-TB; defined as resistance to, at least, rifampicin and isoniazid) were estimated at 480 000 in 2015 with 100 000 new cases of rifampicin resistant TB (20).

Africa and particularly Southern Africa accounted for the highest percentage of HIV and MTB co-infection however only 81% of patients notified for MTB had a documented HIV result implying that the burden of co-infection may be higher (20) (Figure 2).

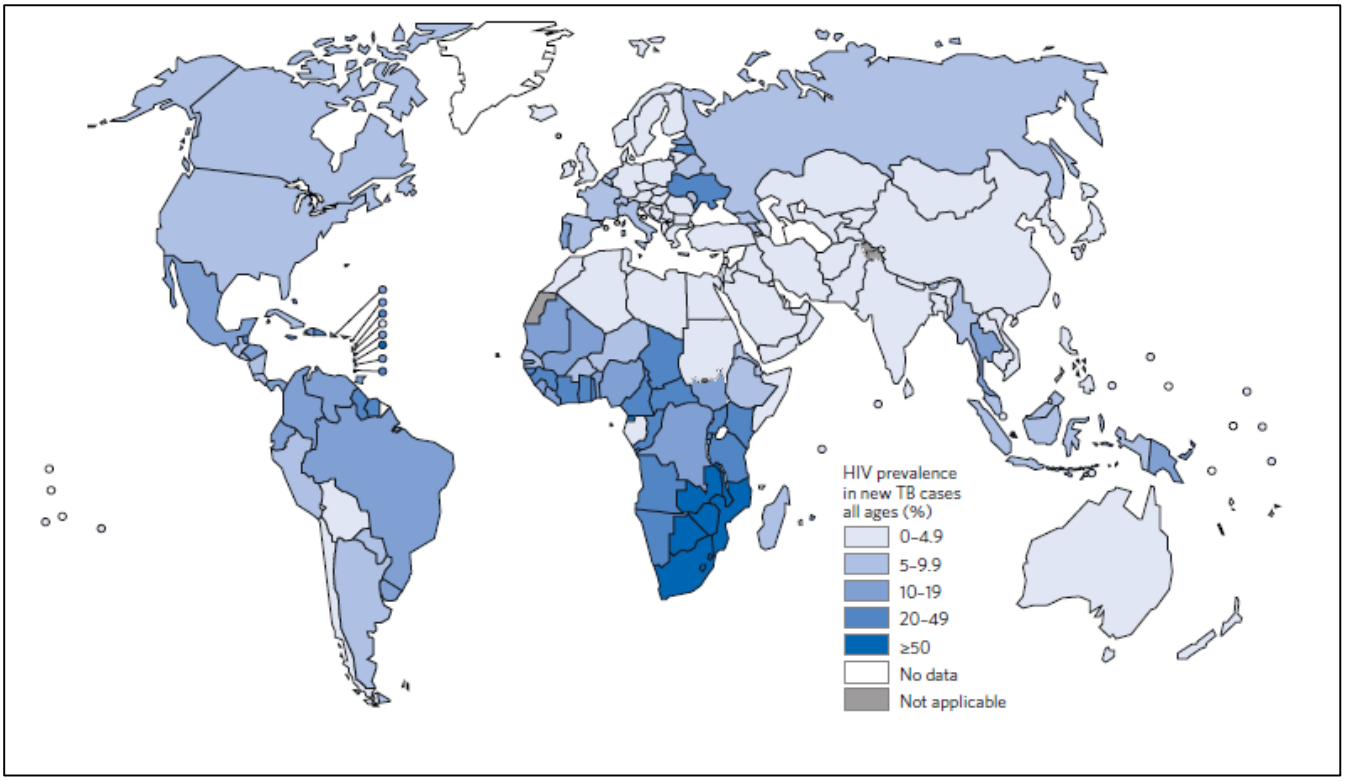


Figure 2: Estimated HIV prevalence of new and relapsed TB cases in 2015 (20)

1.3.2 MTB AND HIV IN SOUTH AFRICA

MTB in South Africa is rife in poorer socio-economic environments making MTB identification, control and reporting a challenge (21).

The WHO Global TB Report 2016 reported 454 000 new cases of MTB in South Africa of which 56% were co-infected with HIV(20). Data regarding incidence (reported as rate per 100 000 population) from 2000 – 2015 is represented graphically in Figure 3 and shows an initial upward growth but a decline after 2005. From 2014 however, the total incidence appears to have stabilised.

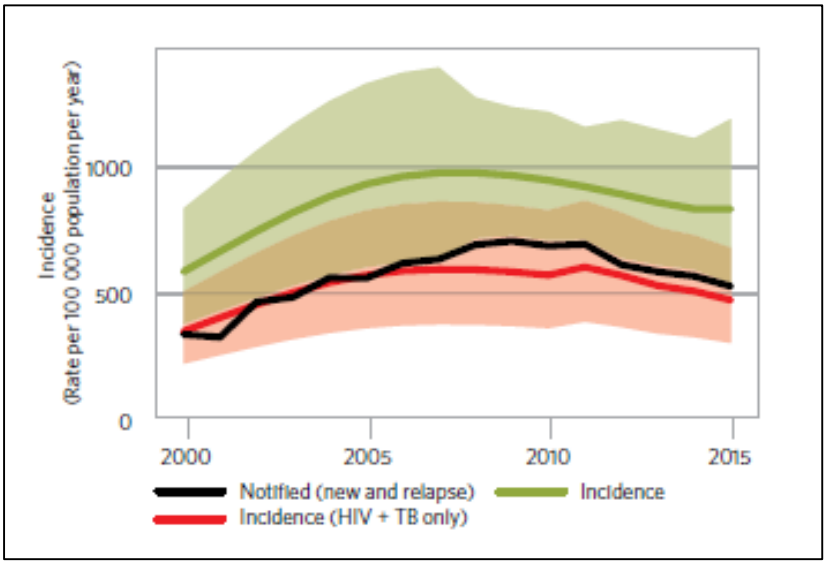


Figure 3: MTB incidence in South Africa from 2000 – 2015 (20)

Overall mortality from MTB amounted to 98 000 cases with a fatality ratio of 0.22 (0.1-0.42). The majority (~74%) occurred in the HIV positive population with a mortality rate of 133 per 100 000. These statistics are similar to the previous year (22).

In 2015, MTB accounted for 0.4 million deaths in the HIV positive population (20). Of the new and relapsed cases of MTB in 2015 in South Africa, 57% were positive for HIV. Of these, 85% were on anti-retroviral treatment (ART). Whilst this appears impressive, it is not in line with the WHO recommendation that all HIV positive people with MTB infection should receive ART (23). This recommendation by the WHO is supported by a study performed in South Africa which showed that by increasing the ART roll-out, the incidence of confirmed pulmonary MTB declined over a period of four years (2008-2012) (24).

1.4 EXTRAPULMONARY MTB

Extrapulmonary MTB infection (EPTB) is defined as MTB outside of the pulmonary system and occurs as a result of seeding of the *M.tb* bacilli via haematogenous, lymphoid or mucosal routes. If two or more non-contiguous sites are involved, the term disseminated TB (synonym: miliary TB) is used. EPTB is more common in those with advanced HIV as the associated immunosuppression allows for rapid progression of the disease following exposure/reactivation of the organism (4).

In South Africa, as per the WHO Global TB report 2016, EPTB cases accounted for 10% (20). Further specification regarding the site involved was not included in this report however a study performed locally (at the National Health Laboratory Services (NHLS) Mycobacteriology Referral Laboratory in Johannesburg) revealed the most common extrapulmonary specimens received for TB culture. These are cerebrospinal fluid, fine needle aspirates (predominantly from lymph nodes) and serous effusions (pleural or ascitic) (Table 1). In this study, the prevalence of EPTB was 23% (277 of 1,175 specimens tested) (25).

Table 1: Extrapulmonary specimens received for TB culture (in order of frequency). Table adapted from (25).

Specimen type	Frequency received in 6 mos, *n* (%)
Cerebrospinal fluid	2,719 (34)
Fine-needle aspriate (mostly lymph node)	2,536 (32)
Fluid (pleural, ascitic, other)	2,008 (25)
Pus	417 (5)
Tissue biopsy	184 (2)
Dialysis fluid or urine	35 (0.4)
Scrapings	7 (0.1)
Bone	5 (0.1)
Stool	3 (0.1)
Catheter tip	2 (0.3)
Total *n*	7,916

1.5 DISSEMINATED MTB INVOLVING THE BONE MARROW

Bone marrow involvement by the *M.tb* bacilli occurs as a result of haematogenous spread and can present with specific haematology findings (such as cytopenias), pyrexia of unknown origin and/or non-specific constitutional symptoms (fever, night sweats and loss of weight) (26). In an 18 month period at Chris Hani Baragwanath Academic Hospital, south of Johannesburg, 15% of the bone marrow aspirates and trephines received were for suspected disseminated TB. Over half of these cases had unexplained cytopenias (27). A study conducted in 2010 in India concluded that bone marrow examinations are highly useful in HIV positive

patients presenting with pyrexia of unknown origin (PUO) as MTB was the commonest cause of PUO, accounting for 60% of all cases in this study (28).

Disseminated TB with bone marrow involvement requires ~9 months of treatment (comparable to other extra pulmonary sites) with the same drugs used for pulmonary TB. There is however a higher mortality rate among patients with bone marrow TB involvement when compared to other sites (29). For these reasons, if clinical suspicion of disseminated TB involving the bone marrow exists (e.g. the presence of cytopenias), a bone marrow aspirate and trephine biopsy with material for TB culture is indicated.

1.5.1 CURRENT DIAGNOSTIC PRACTICES FOR DISSEMINATED TB INVOLVING THE BONE MARROW

When a bone marrow procedure is performed as part of the investigation of disseminated mycobacterial infection, three components are essential. These are outlined in Figure 4 and explained in detail in the text that follows. Of note, three pathology disciplines are involved in the processing and diagnosis of MTB. This includes haematology (analyses bone marrow aspirate smears and trephine biopsies), microbiology (processes TB cultures) and anatomical pathology (processes the trephine biopsies and performs the Ziehl-Neelson (ZN) stain).

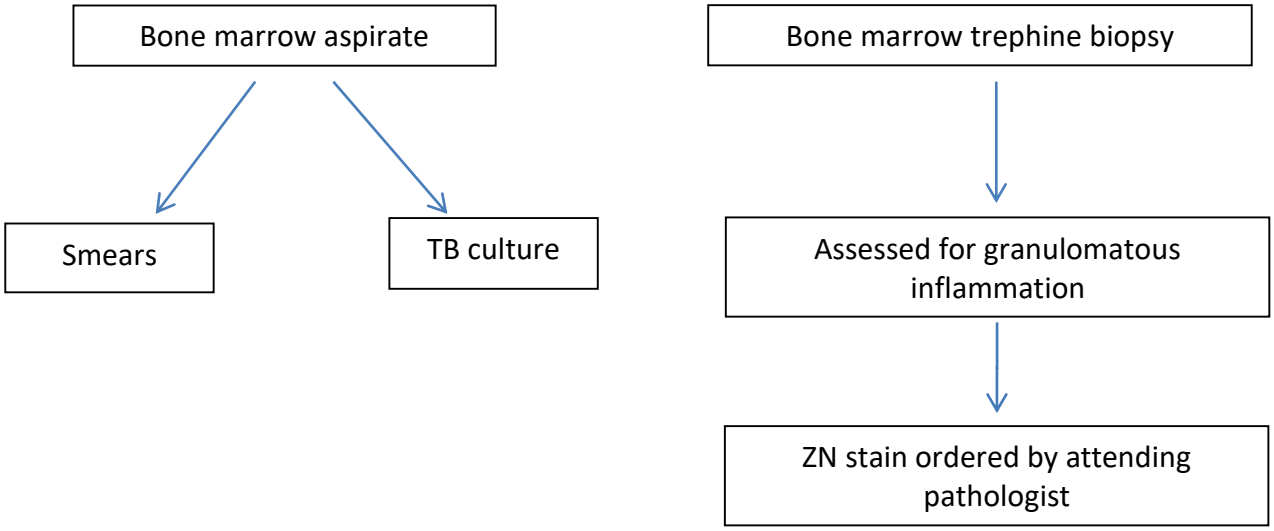


Figure 4: Outline of the components involved in the diagnosis of MTB in bone marrow as followed by the NHLS

1) Bone marrow (BM) aspirate slides are fixed in 10% methanol and stained twice with Giemsa's stain. Examination usually reveals reactive features which are non-diagnostic. The slides can be stained with auramine O for the identification of acid fast bacilli using fluorescent microscopy however this is not commonly done as a study performed locally revealed an overall low yield of positive cases using this method (7 of 123 cases) (27).
2) Bone marrow trephine biopsies undergo 24 hours of formalin fixation followed by a minimum of 48 hours decalcification in ethylenediaminetetraacetic acid (EDTA) disodium salt. The specimen is then embedded into moulds, cut and stained with haematoxylin and phloxine.

   As mentioned previously, the hallmark of MTB is granulomatous inflammation. This is however non-specific and seen with several other infections (including cryptococcus), sarcoidosis, Hodgkin and Non-Hodgkin Lymphoma (30). As a result, a ZN stain is mandatory for confirmation of MTB. In our setting, this stain is performed only at the request of the attending pathologist. Due to the large amounts of lipid (mycolic acid) in the cell wall of the mycobacterium, traditional staining methods such as the Gram stain are ineffective. The ZN stain utilises heated carbolfuchsin to break through this lipid capsule. Initially, all viable cells stain with carbolfuchsin however after a process of acid and alcohol decolourisation only cells with the protective lipid layer retain the carbolfuchsin dye. Mycobacteria are thus acid and alcohol fast. The specimen is then stained with methylene blue which is taken up by non-acid fast organisms. Acid-fast bacilli (AFB) retain the pink colour of carbolfuchsin (31).

From the description above, it is easy to see that this method of diagnosing MTB in the bone marrow can be protracted, taking on average 4-7 days to reach the pathologist. In addition, the diagnostic yield of bone marrow trephine examinations is influenced by poor quality specimens (owing to clinician inexperience, degree of bone marrow infiltration or patient factors. The latter may include obesity, pelvic bed sores or fractures that interfere with obtaining a good quality, diagnostic specimen), differential sampling and paucibacillary specimens (that may appear negative for AFB on a ZN stain).

3) TB culture and drug susceptibility analysis is the gold standard for detection of disseminated MTB. Myco-F lytic culture vials are required with a minimum of 5ml of bone marrow sample. This is incubated in the Bactec 9240 automated system with weekly

inspections for six weeks. If growth is detected, a smear is prepared from the TB culture bottle containing the bone marrow aspirate specimen and a ZN stain performed. One of the following scenarios can then occur (outlined in Figure 5 below):

a. Only contaminating bacteria are identified and the ZN stain is negative for AFB. No further testing is performed in this scenario.
b. Both AFB and contaminating bacteria are not identified. This implies a false positive flag by the instrument and the sample is re-incubated.
c. If the ZN stain is positive for AFB regardless of the presence of contaminating bacteria, a rapid antigen test (the TB Ag MPT64 Rapid) is performed. This test identifies *M.tb* therefore if negative, polymerase chain reaction (PCR; specifically the Hain Genotype Mycobacterium CM, Hain Lifescience, Germany) is performed to identify the presence of MOTT. If the rapid antigen test is positive and drug susceptibility has been requested by the clinician, a subculture into a Mycobacteria Growth Indicator Tube (MGIT) is performed which is followed by a line probe assay (the MTBDR*plus*, Hain Lifescience, Germany) to identify susceptibility to the first line anti-TB agents, rifampicin and isoniazid.

The protracted nature of this TB culture leads to delayed diagnosis of the disease with the knock on effect of losing patients to follow up and hindering TB infection control.

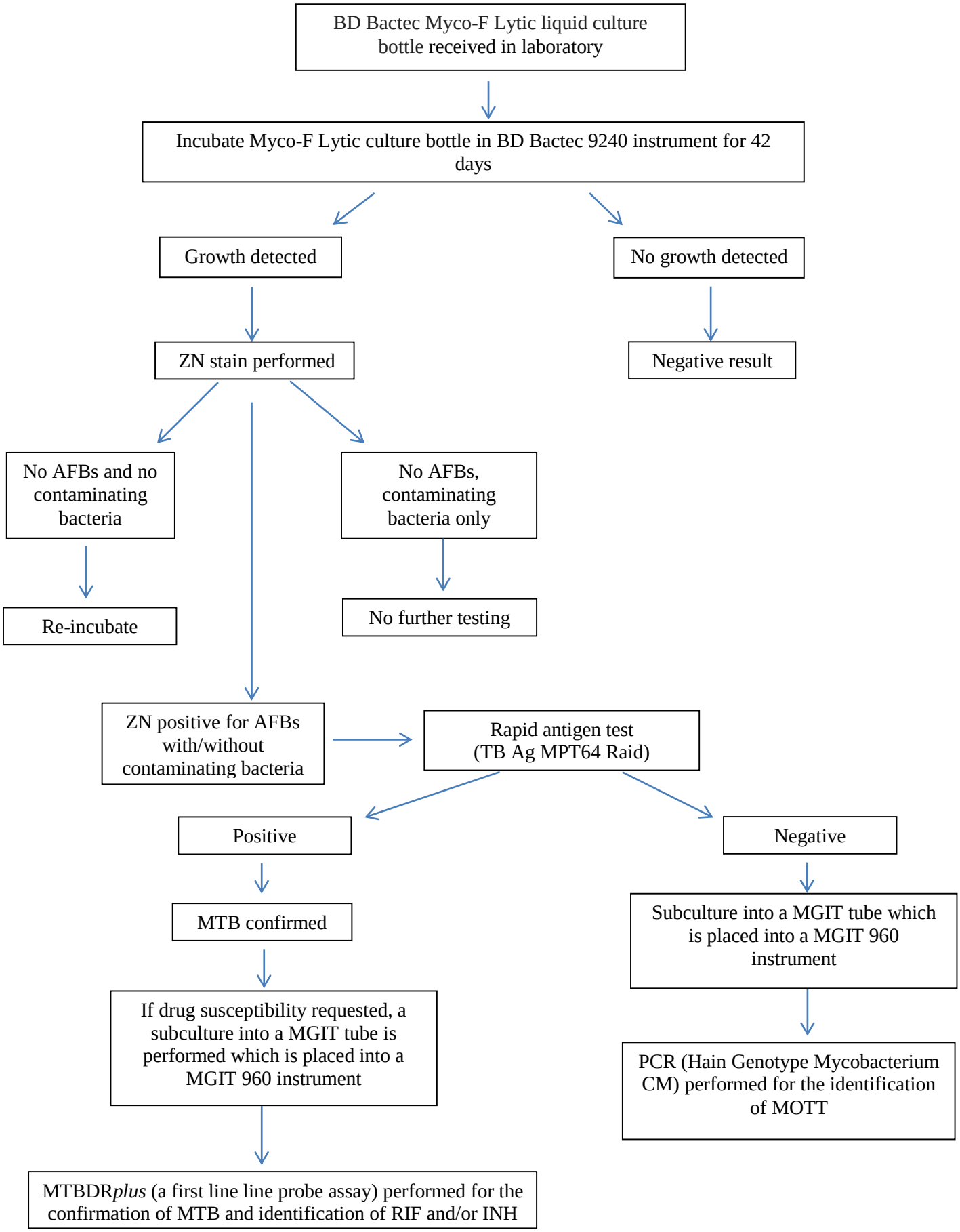


Figure 5: Schematic representation of the processing of bone marrow Bactec Myco-F Lytic culture in the TB reference laboratory of NHLS

1.6 OTHER DIAGNOSTIC METHODS FOR TB

1.6.1 WHO ENDORSED TECHNOLOGIES

1.6.1.1 XPERT® MTB/RIF

The Xpert® MTB/RIF assay from Cepheid (Sunnyvale, California) is a cartridge based assay using hemi-nested real time PCR to amplify the *rpoB* gene which is specific to *M.tb*. In addition, the Xpert® MTB/RIF assay is capable of simultaneously detecting resistance to the most commonly used first line anti-TB drug, rifampicin (RIF).

The assay utilises five probes (A-E; also known as molecular beacons) which are complementary to overlapping regions of the *rpoB* gene (Figure 6) and the 81 base pair RIF Resistance Determining Region (RRDR) which is the site of mutation for more than 95% of all RIF resistant strains (32).

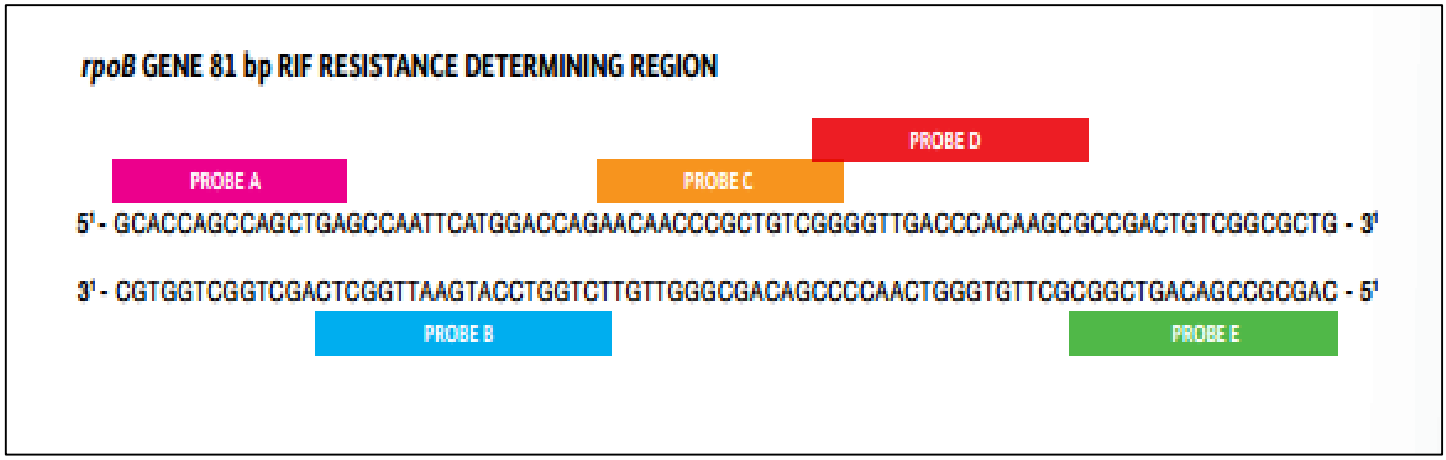


Figure 6: The 81bp RRDR of the *rpoB* gene showing the overlapping molecular probes (33)

Each probe is allocated a specific fluorescent marker. When unhybridised, the probes do not fluoresce as the quencher and the fluorescent dye are in close proximity. This is enabled by the curved conformation of the probes. Once hybridised to the RRDR region, the probe flattens out and the quencher no longer suppresses the fluorescent dye. An increase in fluorescence above the background is reported by the software as a positive result. A positive result for the presence of *M.tb* is deemed if two or more probes emit a signal within two cycles of each other

(34). If there is resistance to RIF, hybridisation of the probe will not occur as there is a change in sequence in the RRDR region of the *rpoB* gene owing to the mutation/s present (34). As a result, a fluorescent signal will not be released. Resistance to RIF is therefore identified by the lack of a detectable cycle threshold for a specific probe or if the difference in cycle threshold between the first and last *rpoB* signals is >4.1. If <4.1, a result of rifampicin sensitive is reported.

Included in the cartridge is a Sample Processing Control (SPC) which is a separate PCR reaction (but occurs simultaneously to the sample PCR reaction) and assesses for the presence of *Bacillus globigii* spores (34). This serves as an internal control and allows for the verification of the integrity and effectiveness of processing.

The Xpert® MTB/RIF is fully automated but does require the addition of a reagent buffer to liquefy and inactivate any *M.tb* bacilli present in the specimen. The limit of detection for the Xpert® MTB/RIF is 150 cfu/mL (34) however with the advent of the Xpert® MTB/RIF Ultra (Cepheid), this may be improved upon (35). This latest assay utilises a new cartridge that allows for double the amount of sample DNA (as compared to the Xpert® MTB/RIF) to be used in the PCR reaction. The five original probes described above are replaced with four probes which detect mutation in the *rpoB* gene as well as real-time probes that target the IS*6110* and IS*1081*genes of *M.tb*. Whilst being as easy to use, this new assay has a 10x lower limit of detection compared to the original Xpert® MTB/RIF and can detect a wider variety of *rpoB* gene mutations (36). As of March 2017, the WHO recommends the use of Xpert® MTB/RIF Ultra as a replacement assay for the current Xpert® MTB/RIF (37).

The steps involved in the Xpert® MTB/RIF assay are shown in Figure 7 (38).

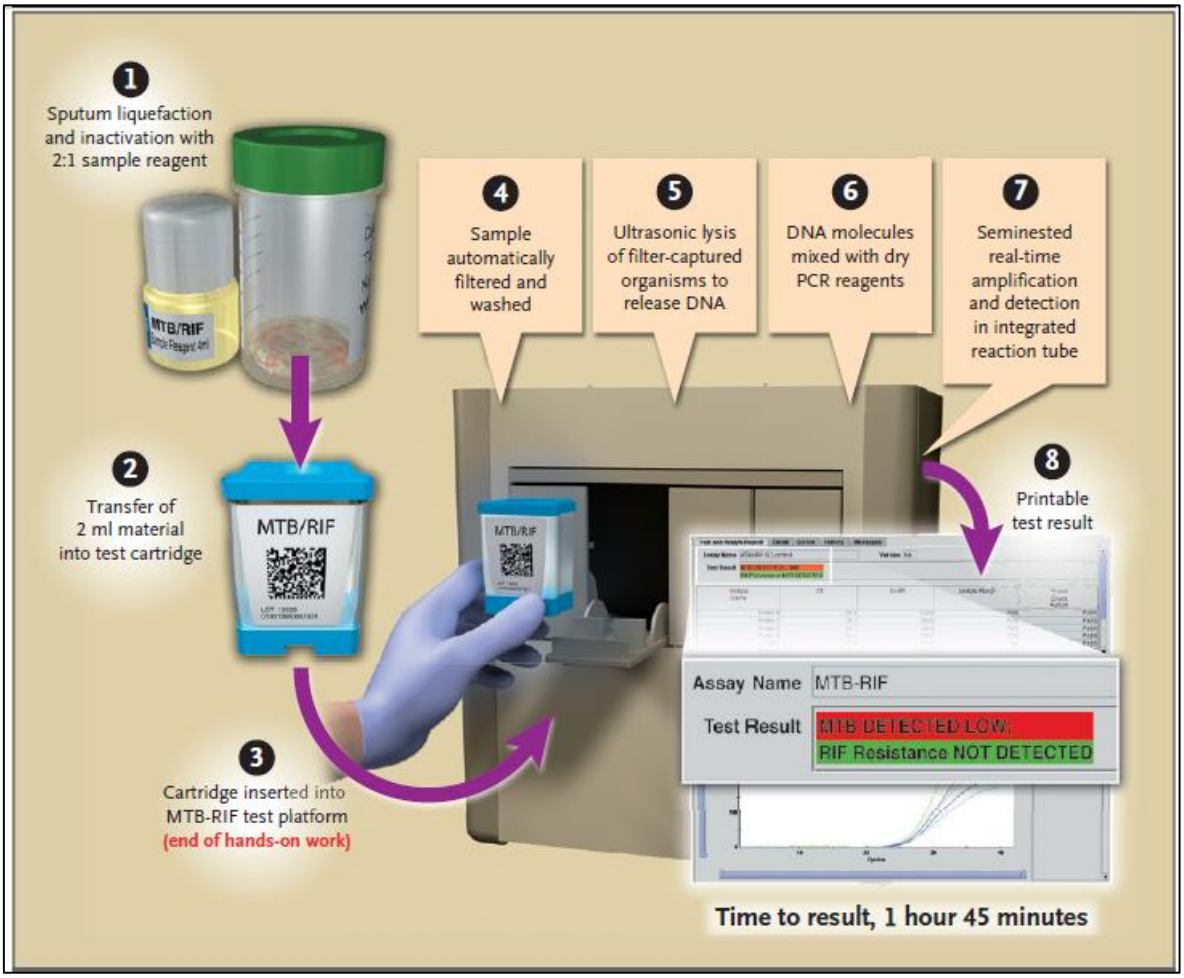


Figure 7: Components of Xpert® MTB/RIF assay. Adapted from (38).

The results are interpreted using the algorithm shown in Figure 8 (33):

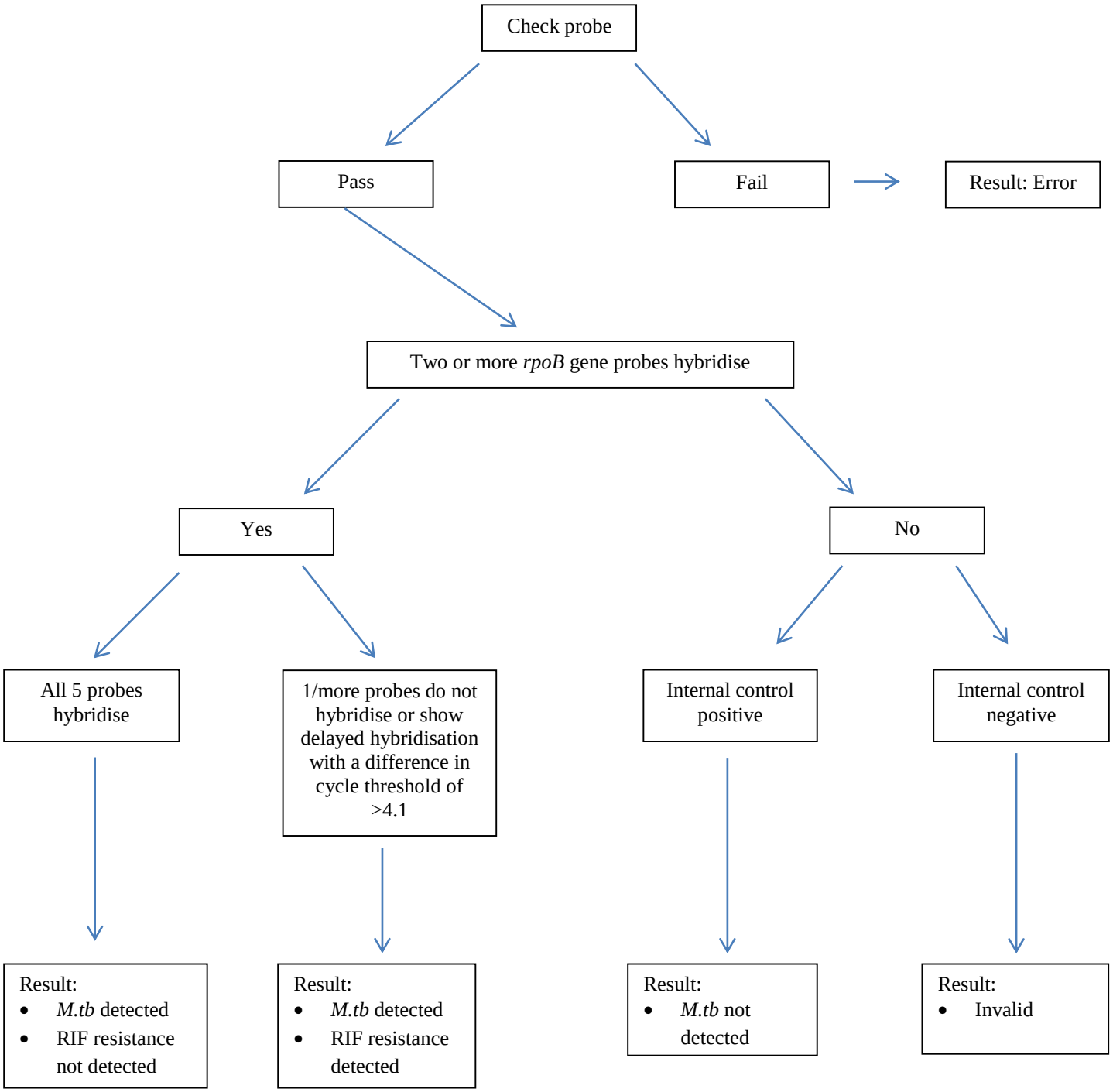


Figure 8: Interpretation of results as reported by the Xpert® MTB/RIF test (33)

In the instances where *M.tb* is detected, the instrument reports levels of *M.tb* DNA in the sample by analysis of the cycle thresholds (Ct). Based on an average of the Ct values of all five probes, a lower Ct denotes a higher DNA concentration and a higher Ct denotes a lower DNA concentration (Table 2) (21, 33).

Table 2: The reported MTB result based on the Ct range

Result	**Ct range**
High	<16
Medium	16-22
Low	22-28
Very low	>28

The 2013 WHO policy update for the use of the Xpert® MTB/RIF assay on pulmonary specimens was based on 27 studies with more than 9000 participants. A meta-analysis of these studies revealed the following (39):

- As an initial diagnostic test used instead of smear microscopy, the Xpert® MTB/RIF assay had a pooled sensitivity of 88% (95% CI: 84–92%) and a pooled specificity of 99% (95% CI: 98– 99%).
- If performed after a negative smear-microscopy result, the Xpert® MTB/RIF had a pooled sensitivity of 68% (95% CI: 61–74%) and a pooled specificity of 99% (95% CI: 98–99%).
- For smear-positive culture-positive MTB, the pooled sensitivity of the Xpert® MTB/RIF was 98% (95% CI: 97–99%).
- In the HIV positive population, the pooled sensitivity of Xpert® MTB/RIF was 79% (95% CI: 70–86%).
- In the HIV negative population, the pooled sensitivity was 86% (95% CI: 76–92%).
- When used to identify RIF resistance, Xpert® MTB/RIF resulted in a pooled sensitivity of 95% (95% CI: 90–97%) and a pooled specificity of 98% (95% CI: 97–99%).

Based on the above findings, the WHO strongly recommends the use of Xpert® MTB/RIF in place of conventional testing (microscopy, culture and drug susceptibility testing) as the initial diagnostic test in both adults and children suspected of having MDR-TB or HIV-associated

TB. A conditional recommendation (acknowledging resource constraints) is also included whereby the Xpert® MTB/RIF can be used instead of the conventional testing mentioned above as the initial diagnostic test in children and adults suspected of having pulmonary TB. It can also be used as a follow-up test to microscopy (especially if smear negative) in adults suspected of having pulmonary TB if they are not at risk of MDR-TB or HIV-associated TB.

The use of Xpert® MTB/RIF assay on pulmonary specimens in South Africa commenced in March 2011 following WHO recommendations and is used in the National TB programme as the initial diagnostic tool for diagnosing MTB. In 2017, evidence 5 years post implementation showed a decline in the TB notification rate in both the HIV positive (19% decrease) and HIV negative (12% decrease) populations since the advent of the rollout. A positive Xpert® MTB/RIF result also surpassed a positive microscopy result as the number one reason for commencing TB treatment. This shows how well clinicians have taken to this relatively new assay in our setting (40).

The results of the meta-analysis performed by the WHO in determining the sensitivity and specificity of the Xpert® MTB/RIF in diagnosing EPTB is summarised in Table 3.

Table 3: A meta-analysis of the sensitivity and specificity of the Xpert® MTB/RIF in diagnosing EPTB at the commonest sites involved (39).

Sample type	**Pooled sensitivity (95% confidence interval)**	**Pooled specificity (95% confidence interval)**
Lymph node	• 84.9% (72-92%)	• 92.5% (80-97%)
Cerebro-spinal fluid	• 79.5% (62-90%) when compared against culture • 55.5% (51-81%) when compared against a composite reference standard	• 98.6% (96-100%) • 98.8% (95-100%)
Pleural fluid	• 43.7% (25-65%) when compared against culture • 17% (8-34%) when compared to a composite reference standard	• 98.1% (95-99%) • 99.9% (94-100%)

Overall, pleural fluid is a poor specimen for the diagnosis of MTB however if Xpert® MTB/RIF is positive, this should be considered evidence of disease and treated as such (39).
A study performed locally demonstrated the sensitivity of the Xpert® MTB/RIF in comparison to liquid culture for various specimen types (including pulmonary and extrapulmonary specimens) (41). This is shown in Figure 9.

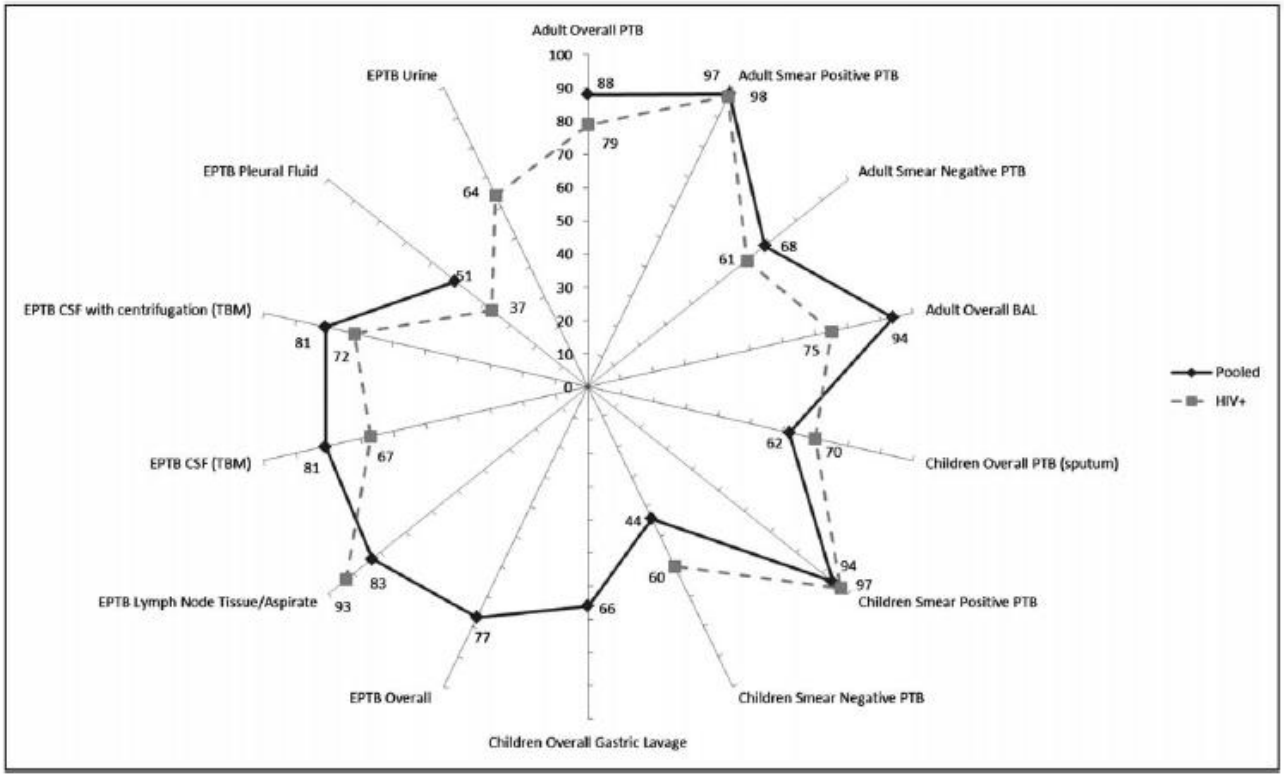


Figure 9: A radar plot of the sensitivity of the Xpert® MTB/RIF compared to liquid culture for various specimen types and including performance among the HIV+ population. Adapted from (41).

It is evident that the sensitivity of the Xpert® MTB/RIF is higher with pulmonary samples (and especially so with smear positive specimens) compared to the extrapulmonary samples assessed. Lymph node tissue/aspirate in the HIV positive population is the exception noted in the local study.

Owing to limited availability, data on the use of the assay on bone marrow aspirate (BMA) specimens was omitted from the WHO systematic review and meta-analysis. Often, these specimens are included with other extra-pulmonary specimens and an overall assessment of the performance of the Xpert® MTB/RIF assay is reported. The findings of some of the studies that included BMA samples are shown in Table 4.

Table 4: Studies utilising bone marrow aspirate specimens on the Xpert® MTB/RIF

Authors:	**No. of BMA specimens/total specimens (%)**	**Sensitivity:**	**Specificity:**
Armand et al (42)	6/37 (16.2%)	53%	-
Malbruny et al (43)	2/89 (2.2%)	85.7%	97.3%
Clemente et al (44)	1/72 (1.4%)	73.6%	99.9%
Kim et al (45)	11/1540 (0.7%)	67.7%	98.1%
Moure et al (46)	1/149 (0.7%)	58.3%	100%

The 2013 WHO policy recommendations regarding the use of the Xpert® MTB/RIF assay for extra pulmonary specimens (with the exception of blood, urine and stool) is summarised below (39).

- Strong recommendations:
    - If TB meningitis is suspected, CSF specimens should be processed first on the Xpert® MTB/RIF instead of conventional testing (microscopy, culture and drug sensitivity testing).
- Conditional recommendations (acknowledging resource constraints):
    - For certain non-respiratory specimens, the Xpert® MTB/RIF can be used instead of conventional practices for the diagnosis of EPTB. This includes lymph node specimens.

Although the Xpert® MTB/RIF has a broad global footprint and has been highly successful (especially in the diagnosis of pulmonary TB), there are other technologies endorsed by the WHO which can be used together with or instead of the Xpert® MTB/RIF. The main characteristics of these tests are highlighted in Table 5.

1.6.1.2 LINE PROBE ASSAYS

Line probe assays (LPAs) are strip-based tests that utilise PCR and reverse hybridisation methods to amplify DNA. In addition to identifying the presence of *M.tb*, these assays also detect genotypic susceptibility to RIF and isoniazid (INH) by identifying mutations in three genes - *rpoB, katG* and *inhA* genes (47).

As noted above, the 81 base pair region of the *rpoB* gene is the commonest site for mutations that confer resistance to rifampicin. Similarly, 80-90% of all INH resistant strains can be localised to mutations within the *katG* and *inhA* genes (47).

Figure 10 shows an outline of the steps involved in this assay (48):

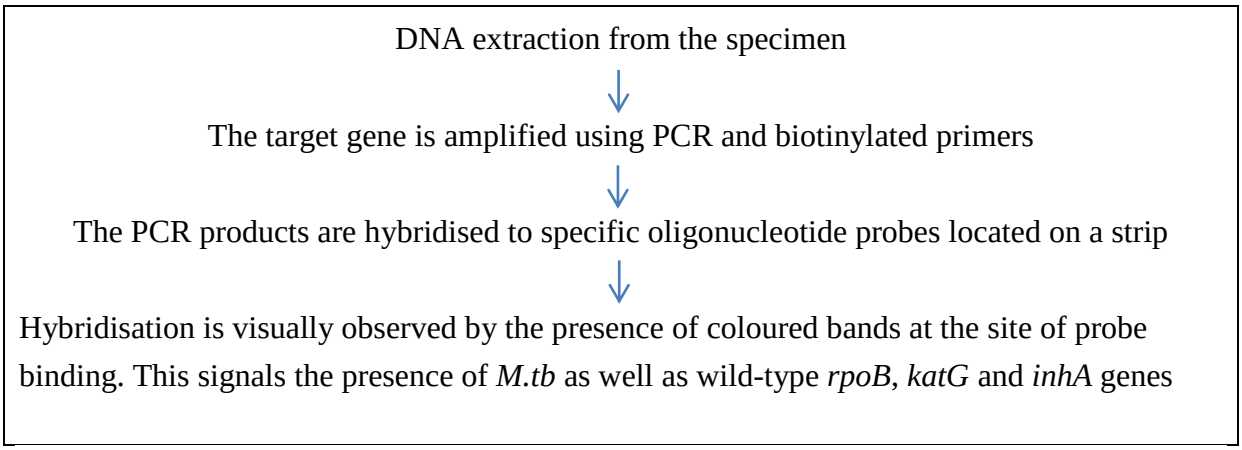


Figure 10: Flow diagram demonstrating the processing of specimens using LPAs

If a genetic mutation is present in one or more of the target genes, the PCR products will not hybridise with the wild type probes but will bind to the specific probes for the commonly occurring mutations.

Advantages of this type of technology include:

- Able to be performed directly on sputum or other specimens
- Detects INH resistance
- Rapid turnaround time (TAT) when compared to culture

Disadvantages include:

- Longer time to result (when compared to Xpert® MTB/RIF)
- Personnel trained in PCR are required
- Regional/centralised laboratory set-up required

Based on the two first-generation assays (INNO-LiPARif.TB assay (Innogenetics, Ghent, Belgium) and Genotype MTBDR assay (HainLifescience GmbH, Nehren, Germany), the WHO approved the use of line probe assays for the diagnosis of MTB and identification of RIF resistance in smear positive cases in 2008 (48). The use of these first generation LPAs for smear negative specimens is not recommended by the WHO, a statement supported by the findings of a recent meta-analysis which showed a 50% difference in sensitivity between smear positive cases (89.4-99.4%) and smear negative cases (20.2-71.7%) (49).

Since 2008, the pioneering LPAs are no longer in use however newer versions have been developed. A meta-analysis commissioned by the WHO evaluated 74 studies which looked at three of these new technologies (Hain Genotype MTBDRplusV1, MTBDRplusV2 and Nipro NTM+MDRTB). This revealed a pooled sensitivity of 96.7% (95% CI: 95.6-97.5%) and pooled specificity of 98.8% (95% CI: 98.2-99.2%) with regards to the detection of RIF resistance. INH resistance had a pooled sensitivity of 90.2% (95% CI: 88.2–91.9%) and a pooled specificity of 99.2% (95% CI: 98.7–99.5%) (49).

LPAs for the detection of resistance to the commonly used second line anti-MTB therapy (termed second-line line probe assays or SL-LPA) utilises probes within the *gyrA, rrs* and *gyrB* genes as well as the eis promoter to identify resistance to fluroquinolones or the injectable agents. These assays are recommended by the WHO for patients with confirmed rifampicin-resistant or multidrug-resistant MTB as the initial test for the identification of resistance to fluroquinolones (50).

1.6.1.3 LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY

Loop-mediated isothermal amplification (LAMP) was described by Notomi *et al* as a DNA amplification method using a DNA polymerase with strand displacement activity, four primers which recognise six sites on the target gene and a constant temperature (~65°C*)* (51).

Subsequently, the technique has been modified to increase the effectiveness and reduce the turnaround time of the test (52, 53).

A meta-analysis performed in 2016 revealed a pooled sensitivity of 93% (95% CI: 92-95%) and a pooled specificity of 94% (95% CI: 92-95%)(54).

The advantages of this assay include (55):

- Temperature independent amplification of DNA
- Rapid TAT (requires less than an hour to perform)
- Simple to perform and requires minimal laboratory equipment – therefore can be used at peripheral healthcare centres
- Safe – similar biosafety requirements as those for sputum smear microscopy
- Easy to read as the endpoint depends on degree of turbidity which is analysed visually under ultraviolet light

Currently, the WHO recommends (on condition) the use of the TB-LAMP assay in adults as a replacement or add-on test for smear microscopy for the diagnosis of pulmonary TB if the signs and symptoms are consistent with TB (56).

As this assay is incapable of detecting rifampicin resistance, the Xpert® MTB/RIF remains the first choice. However, if the Xpert® MTB/RIF cannot be implemented (for example in places with poor electrical supply, poor temperature control, excessive humidity and/or excessive dust), the TB-LAMP assay can be considered as an alternative (56).

1.6.1.4 LIPOARIBINOMANNAN ASSAY

Lipoaribinomannan (LAM) is a 17-19kDa lipopolysaccharide found in the cell wall of *M.tb* microbes and account for 15-18% of the total weight of the bacteria. It is secreted into urine during active MTB infection and is therefore an accessible antigen to assay. Previously, enzyme-linked immunosorbent assays (ELISA) formed the basis of LAM diagnostic testing however these are not feasible in a resource limited setting. Hence, a lateral flow version of the assay (LF-LAM) has been developed as a qualitative point of care test (57).

The commercially available kit is the Determine™ TB LAM Ag (shown in Figure 11).

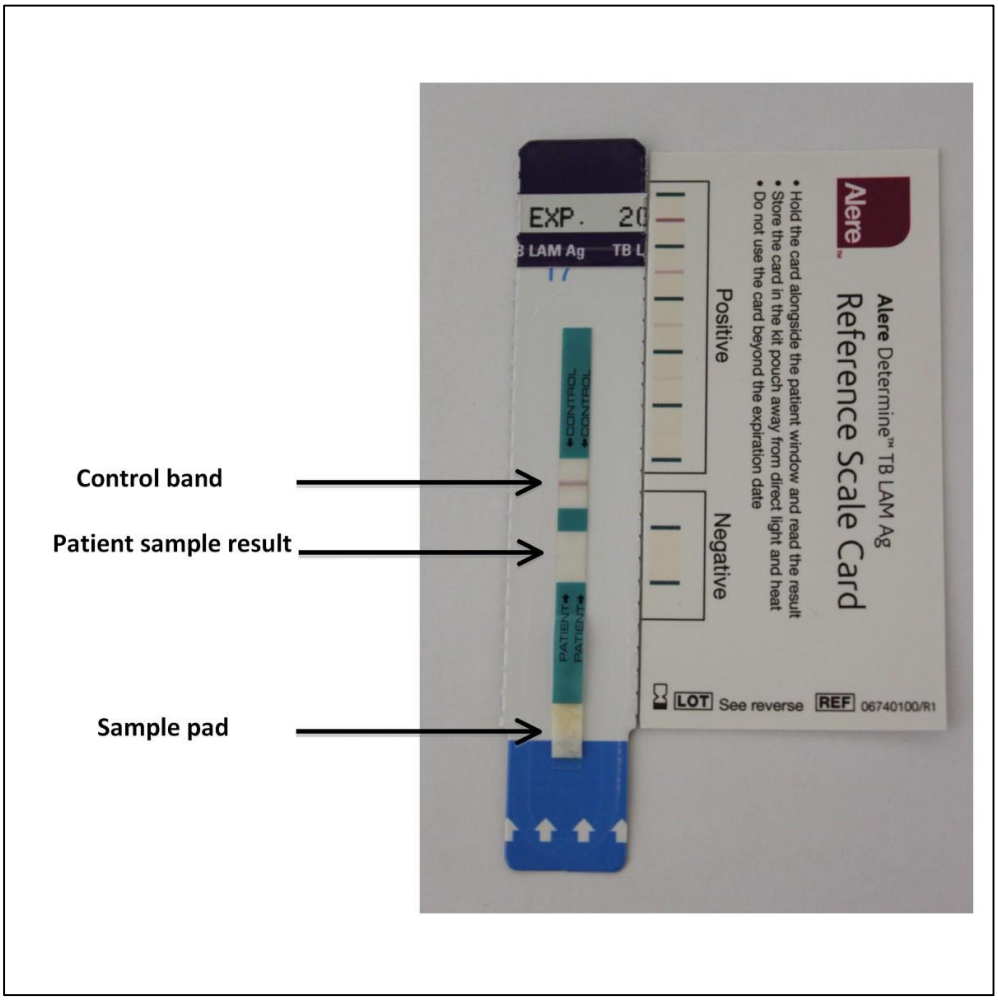


Figure 11: Test strip and reference card of the Determine™ TB LAM Ag kit (57)

A small amount of urine (60µL) is placed onto the sample pad of the test strip and incubated at room temperature for 25-35 minutes. Provided the control band is present, the patient's result can be accepted. This is facilitated by the provision of a reference card to which the band intensity can be compared.

A study performed in South Africa by Peter *et al* evaluated the Determine™ TB LAM among hospital inpatients. Various methods were used to assess the sensitivity which varied between 45% and 71% (58). A meta-analysis performed by the WHO revealed a pooled sensitivity of 44% (95% CI: 31-60%) and a pooled specificity of 92% (95% CI: 83-96%).

As of 2015, the WHO recommends the LF-LAM assay in HIV positive hospitalised patients with signs and symptoms of pulmonary TB or EPTB and a CD4 count less than or equal to 100

cells/µL. This assay is also recommended in patients who are unable to produce sputum or in seriously ill HIV positive people regardless of CD4 count (definition of seriously ill: tachypnoea of >30 breaths/minute, temperature >39ºC, tachycardia >120 beats/minute and/or inability to walk unaided). There is a strong recommendation that this assay should not be used as a screening tool (59).

Table 5: Summary of the WHO endorsed technologies

Assay	Description	Specimen type/s	Sensitivity (95% confidence interval)	Specificity (95% confidence interval)	Turn-around time	Capable of detecting resistance (drugs)
Xpert® MTB/RIF	Hemi-nested, fully automated cartridge based assay	• Sputum • CSF • Lymph node • Pleural fluid	• 88% (84-92%) • 55.5-79.5% (51-90%) • 84.9% (72-92%) • 17-43.7% (8-65%)	• 99% (98-99%) • 98.6-98.8 (95-100%) • 92.5% (80-97%) • 98.1-99.9 (94-100%)	2 hours	Yes (rifampicin)
Line probe assays (LPA)	Strip-based tests utilising PCR and reverse hybridisation to detect drug resistance. Visual representation of hybridisation	Smear positive sputum	90.2-96.7% (88.2-97.5%)	98.8-99.2% (98.2-99.5%)	2-3 days	Yes (First line LPA - rifampicin, isoniazid Second line LPA-fluroquinolones)
LAMP	PCR based but temperature independent amplification of DNA	Sputum	93% (92-95%)	94% (92-95%)	<1 hour "hands-on" time	No
LAM assay	Lateral flow("urine-dipstick" format)/ELISA based point-of-care test	Urine	44% (31-60%)	92% (83-96%)	<30 minutes	No

*LAMP,loop-mediated isothermal amplification assay; LAM, lipoaribinomannan; ELISA, enzyme linked immunosorbent assay

1.6.2 TESTS UNDER EVALUATION

1.6.2.1 REAL*TIME* MTB AND REALTIME MTB-INH/RIF ASSAYS

The Real*Time* MTB assay from Abbott Molecular (Des Plaines, Illinois) is a novel nucleic acid amplification test (NAAT) utilising real time PCR to qualitatively detect *M.tb* in pulmonary specimens (60). It is fully automated and has a higher throughput compared to the original Xpert® MTB/RIF (capable of processing 94 specimens per batch). This test amplifies two genes of *M.tb* namely protein antigen B (*paB)* and the multicopy insertion element IS*6110* for the detection of MTB infection. In addition, the Abbott Real*Time* MTB-INH/RIF resistance assay allows for the detection of drug resistance in positive specimens. Whilst this does not occur simultaneously like in the Xpert® MTB/RIF assay, it does allow for the detection of resistance against INH which the Xpert® MTB/RIF assay does not. Similar to the line probe assays, the Real*Time* MTB INH/RIF resistance assay identifies mutations in the *katG* and *inhA* genes for INH resistance and in the *rpoB* gene for resistance to rifampicin. A study conducted by Hofmann-Thiel *et al.* revealed an overall sensitivity of the assay in pulmonary samples of 92.1% (95% CI: 87.9-95.1%) with a lower sensitivity noted in smear negative samples compared to smear-positive cases (76.2% vs 100% respectively). This sensitivity was comparable in extrapulmonary samples. The specificity of this assay was shown to be 99.6% (95% CI: 98.3-99.9%) and with regards to identification of resistance, there was a 99.5% agreement between the Real*Time* MTB-INH/RIF resistance assay and the genotypic and phenotypic manifestations. A local study included HIV positive patients and found a sensitivity of 82.5% (CI: 67.2-92.7%) and specificity of 93.1% (CI: 86.2-97.2) on raw sputum when compared to liquid culture. If the sputum was concentrated prior to processing, the sensitivity declined by 5% whilst the specificity improved by 2%.

Studies are ongoing using this platform in the diagnosis of MTB but so far demonstrate positive findings and certain definite advantages over the Xpert® MTB/RIF assay (60, 61). As it is widely used for HIV viral load testing, it offers the opportunity to integrate multiple tests on a single platform.

1.7 OBJECTIVES OF THIS MMED RESEARCH REPORT

In the current South African landscape, the Xpert® MTB/RIF is improving patient care through the rapid diagnosis of MTB. However, an area where data is lacking is the diagnostic utility of the Xpert® MTB/RIF in bone marrow aspirate specimens. As shown previously, as part of the investigation into disseminated TB involving the bone marrow, three modalities are performed – bone marrow aspirate smear, trephine biopsy and TB culture. The last two tests are associated with a substantial delay in diagnosis. If the Xpert® MTB/RIF is performed at the same time as these other modalities, a reportable result could be obtained within 24 hours allowing timeous initiation of treatment. As all three modalities require processing by different pathology departments, the Xpert® MTB/RIF could assist in integrating these disciplines.

The primary objectives of this study were therefore:

a) To assess the ability of the Xpert® MTB/RIF assay to identify MTB in anticoagulated bone marrow aspirate specimens
b) To evaluate the sensitivity and specificity of the Xpert® MTB/RIF compared to the gold standard histological testing, liquid culture and drug susceptibility assays in BMA specimens.

Secondary objective:

a) To investigate the utility of the Xpert® MTB/RIF using BMA specimens as part of the diagnostic algorithm of MTB in a high prevalence TB and HIV setting.

CHAPTER 2: MATERIALS AND METHODS

2.1 SPECIMEN SELECTION

BMA specimens collected as part of the investigation for disseminated TB or for the investigation of cytopenias in immunocompromised patients and sent for routine immunophenotypic analysis at the NHLS flow cytometry laboratory in Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) between July 2015 and October 2015 were used in this study. BMA specimens for immunophenotypic analysis are routinely collected in EDTA and heparinised tubes. An aliquot of each sample (0.5-1ml) was collected prior to immunophenotypic analysis to prevent possible interference from immunophenotypic processing (e.g. isolating cells by the Ficol method or the addition of phosphate buffer solution). A maximum of 1ml of specimen was utilised as this was found to be the most common volume available for testing that would still allow for adequate cell numbers for immunophenotypic analysis. The longest time delay between collection and processing of the specimens in this study was 3 days and is attributed to the weekend when the flow cytometry laboratory is closed. The same specimen selection criteria were used for the Xpert® MTB/RIF test optimisation and the performance evaluation arms of the study.

Ethical approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (M09-06-88; Appendix 3).

2.2 OPTIMISATION OF THE XPERT® MTB/RIF FOR BONE MARROW ASPIRATE SPECIMENS

2.2.1 ASSESSMENT FOR PCR INHIBITORS IN BMA AND TO ASSESS THE APPROPRIATE SPECIMEN VOLUME

To establish if BMA contains inhibitors to the Xpert® MTB/RIF PCR reaction and to assess test performance using varying volumes of bone marrow, the following were processed (and repeated five times on the same sample):

1) 1ml heparinised BMA specimens with 25µL of inactivated *M.tb.*

2) 500µl heparinised BMA specimens with 25µL of inactivated *M.tb*

3) 500µl heparinised BMA specimens with serial dilutions of inactivated *M.tb* (ranging from 5µl to 25µl).

2.2.2 ASSESSMENT OF THE EFFECT OF ANTICOAGULANTS ON THE XPERT® MTB/RIF PCR REACTION

The following were performed and repeated five times on the same sample:

1) 500µL BMA specimens from EDTA tubes with 25µL of inactivated *M.tb.*

2) From the same patient, 500µL BMA specimens from EDTA tubes and 500µL BMA specimens from heparinised tubes with 25µL of inactivated *M.tb.*

2.2.3 DISTILLED WATER AS A CONTROL:

1) 500µL distilled water with 25µL of inactivated *M.tb.*

2) 500µL distilled water with serial dilutions of inactivated *M.tb* (ranging from 5µl to 25µl).

2.3 SAMPLE PROCESSING

Sample-reagent (SR) buffer was added to the specimens to obtain a 2ml volume of sample/buffer mixture (minimum required for Xpert® MTB/RIF processing (38)). These mixtures were spiked with inactivated rifampicin sensitive *M.tb* that is used as dried culture spots as part of the Xpert® MTB/RIF external quality assurance programme (62) to ensure a known quantity of detectable *M.tb* in the clinical specimen. The mixtures were incubated for a minimum of 15 minutes at room temperature as per the manufacturer's guidelines. The sample/SR buffer mixture was then transferred into the GeneXpert cartridges and inserted into the instrument.

In addition, distilled water was processed in the same way to assess for differences in results that could be attributed to the inhibition of the Xpert® MTB/RIF PCR reaction by BMA and/or the anticoagulant.

The Ct of the sample processing control was documented to ensure validity of the results. In addition, the CT of each probe was documented to assess for possible inhibition to the reaction.

2.4 EVALUATION OF THE XPERT ® MTB/RIF FOR THE DETECTION OF *M.tb* IN PATIENT BMA SPECIMENS

On the basis of the results of the optimization study, the evaluation arm of the study commenced using 500µL of patient specimens from both EDTA and heparinised tubes. This was added to SR buffer in a 1:3 ratio and incubated for 15 minutes at room temperature prior to transfer into the cartridge. The cartridges were immediately inserted into the GeneXpert instrument. The ratio of specimen to buffer differs from the manufacturer's guidelines (Figure 7) and was based on obtaining the total minimum sample/buffer mixture (2ml) required for processing.

2.5 DATA COLLECTION

The Laboratory Information System (LIS) was used to obtain minimal information on each specimen (Appendix 1). This included the HIV status of the patient from which the specimen was obtained as well as the results of the TB culture and trephine biopsy to which the Xpert® MTB/RIF result was compared. The Ct of each probe was documented as this correlates to the amount of *M.tb* DNA present in the specimen and the result reported by the instrument (see Table 2).

2.6 EXCLUSION CRITERIA

- Specimens with no corresponding trephine biopsy or TB culture result were not included in the final statistical analysis.
- Insufficient specimens at the time of receipt (<1ml) were excluded to allow for adequate cell numbers for immunophenotyping.

2.7 STATISTICS

Statistical analysis was performed using the MedCalc Statistical Software programme (Ostend, Belgium) (25). Sensitivity and specificity as well as the positive and negative predictive values were calculated using the number of true positive, true negative, false positive and false negative results. The likelihood ratios were calculated using the sensitivity and specificity:

- Positive likelihood ratio = sensitivity/(100-specificity)
- Negative likelihood ratio = (100-sensitivity)/specificity

CHAPTER 3: RESULTS

3.1 SPECIMENS PROCESSED

BMA specimens obtained for this study:

- Optimisation phase:
  - Five BMA specimens, each of which was analysed five times
  - EDTA and heparinised BMA specimen from the same patient (each analysed in triplicate)
- Evaluation phase:
  - 135 BMA specimens were analysed. Of these:
    - 11 specimens were specifically submitted by the requesting clinican for the exclusion of TB involving the bone marrow
    - The remainder (124 specimens) were chosen as the request form indicated the presence of both HIV and cytopenias in the patient.

3.2 OPTIMISATION OF THE XPERT® MTB/RIF FOR BMA SPECIMENS

3.2.1 ASSESSMENT FOR PCR INHIBITORS IN BMA AND TO ASSESS THE APPROPRIATE SPECIMEN VOLUME

Table 6: Results using 1ml heparinised BMA specimens with 25µL of inactivated *M.tb* in a single sample run in quintuplicate.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
I	Detected – low	Susceptible	Passed	24.5-26.2	26.8
II	Detected – low	Susceptible	Passed	25.0-26.9	30.2
III	Detected – medium	Susceptible	Passed	18.4-20.4	26.7
IV	Detected – medium	Susceptible	Passed	18.3-20.3	29.9
V	Detected – high	Susceptible	Passed	15.0-17.0	25.6

*MTB, Mycobacterium tuberculosis; RIF, rifampicin; QC, quality control; Ct, cycle threshold; SPC, sample processing control

Table 7: Results using 500µL heparinised BMA specimens with 25µL of inactivated *M.tb* in a single sample run in quintuplicate.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
I	Detected – medium	Susceptible	Passed	18.2-20.2	27.0
II	Detected – low	Susceptible	Passed	22.2-23.9	27.7
III	Detected – medium	Susceptible	Passed	19.1-20.8	25.7
IV	Detected – very low	Susceptible	Passed	33.0-35.3	28.2
V	Detected – medium	Susceptible	Passed	20.7-22.5	28.0

These results demonstrate that a minimum of 500µL BMA specimen is sufficient to generate a result using the Xpert® MTB/RIF assay. The results are overall comparable to a higher volume of specimen (1ml) as low and medium results are obtained and the specific Ct values appear to overlap. There is however a single "very low" result seen with the fourth repeat of 500µL of specimen and a "high" result is seen with the fifth repeat of 1ml of specimen, which suggests imperfect reproducibility in Xpert® MTB/RIF results. As the performance between the 2 tested sample volumes was similar, a sample volume of 500µL was opted for the evaluation arm of this study as it allowed for a higher residual volume for immunophenotypic processing. Going forward, using a lower specimen volume is also helpful in the cases of difficult BM aspirations when the amount of specimen obtained can be limited.

3.2.2 ASSESSMENT OF THE EFFECT OF ANTICOAGULANTS ON THE XPERT® MTB/RIF PCR REACTION

Table 8: Results using 500µL BMA specimens obtained from ETDA tubes with 25µL of inactivated *M.tb* added in a single sample run in quintuplicate.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
I	Detected – high	Susceptible	Passed	13.7-15.6	25.2
II	Detected – medium	Susceptible	Passed	16.3-17.9	25.5
III	Detected – high	Susceptible	Passed	15.9-17.6	26.0
IV	Detected – medium	Susceptible	Passed	21.9-23.8	29.0
V	Detected – medium	Susceptible	Passed	17.2-19.2	26.5

Table 9: Results of 500µL BMA specimens obtained from the same patient and from EDTA and heparinised tubes run in triplicate for each anticoagulant. 25µL of inactivated *M.tb* added.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
EDTA	Detected – medium	Susceptible	Passed	21.5-23.3	24.9
EDTA	Detected – medium	Susceptible	Passed	21.9-23.7	24.4
EDTA	Detected – low	Susceptible	Passed	24.2-25.8	26.2
HEPARIN	Detected – medium	Susceptible	Passed	19.2-20.8	25.6
HEPARIN	Detected – medium	Susceptible	Passed	21.5-23.2	24.1
HEPARIN	Detected – medium	Susceptible	Passed	21.2-23.0	27.0

The analysis performed with the same BMA specimen showed comparable results for both of the common anticoagulant tubes (EDTA and heparin). This is helpful as these tubes are widely available and should the Xpert® MTB/RIF test be implemented for BMA specimens, it would be easy for the clinicians to submit an extra tube for processing.

3.2.3. DISTILLED WATER AS A CONTROL:

Table 10: Results using 500µl heparinised BMA specimens with serial dilutions of inactivated *M.tb* (ranging from 5µl to 25µl).

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
25µL	Detected – low	Susceptible	Passed	26.3-28.1	25.2
20µL	Detected – low	Susceptible	Passed	25.6-27.6	27.0
15µL	Detected- medium	Susceptible	Passed	20.4-22.2	25.2
10µL	Detected – medium	Susceptible	Passed	21.5-23.5	27.1
5µL	Detected – low	Susceptible	Passed	25.2-27.1	25.2

Table 11: Results using 500µL distilled water with 25µL of inactivated *M.tb* in a single sample run in quintuplicate.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
I	Detected – high	Susceptible	Passed	15.7-17.7	26.8
II	Detected – high	Susceptible	Passed	14.3-16.2	28.1
III	Detected – high	Susceptible	Passed	14.5-16.5	27.0
IV	Detected – high	Susceptible	Passed	13.9-16.2	26.5
V	Detected – high	Susceptible	Passed	14.5-16.6	25.7

Table 12: Results using 500µL distilled water and serial dilutions of inactivated *M.tb*.

Test	MTB status	RIF status	Internal QC status	Ct of probes	SPC Ct
25µL	Detected – high	Sensitive	Passed	14.4-16.4	25.7
20µL	Detected – high	Sensitive	Passed	15.6-17.3	27.2
15µL	Detected – high	Sensitive	Passed	15.5-17.3	25.7
10µL	Detected – medium	Sensitive	Passed	16.6-18	24.6
5µL	Detected – medium	Sensitive	Passed	19.3-20.8	27.2

As expected, the spiked specimens generated a positive result for the detection of *M.tb* however when the Ct values were analysed to provide a semi-quantitative assessment of the *M.tb* concentration in the specimen, the "high" MTB status was seen predominantly with distilled water. The BMA specimens ranged from low to high with a prominence of low and medium results. This implies that BMA or heparin partially inhibits the PCR reaction but not enough to prevent the generation of a result. When serial dilutions of inactivated *M.*tb were utilised, distilled water demonstrated the expected response with a decline in MTB status with the lower concentrations. Interestingly, when BMA specimens were used under the same conditions, a "medium" MTB status was reported with 10µL and 15µL of inactivated *M.tb* yet a "low" result was seen with higher concentrations. The reason for this is unclear but it would be of value to assess if the viscosity of the specimens influenced the result. As the presence of *M.tb* was not excluded in the specimen prior to processing, it is possible that the "medium" MTB status noted with the lower concentrations could be attributed to the *M.tb* bacilli already present in the specimen.

In summary, the results demonstrated the following:

- 500µL of BMA material (clinical specimen) incubated with SR buffer in a 1:3 ratio for 15 minutes does not meaningfully inhibit the PCR reaction of the Xpert® MTB/RIF assay.
- Results are comparable between heparin and EDTA anticoagulated specimens however the higher Ct value seen with the anticoagulated specimens (compared to distilled water) may indicate partial PCR interference.

3.3 EVALUATING THE XPERT® MTB/RIF ON BMA SPECIMENS

Figures 12 and 13 outline the results obtained in this study. During the study period at the CMJAH NHLS flow cytometry laboratory, 135 bone marrow aspirate specimens had sufficient residual volume for Xpert® MTB/RIF testing. Of these, 85.2% (115/135) had a corresponding adequate trephine biopsy and/or had a TB culture submitted. Two (1.5%) had an inadequate trephine biopsy and although a TB culture was submitted, this was reported as contaminated. Therefore, in total, 16.3% (22/135) of the specimens were not included in the final statistical analysis. The prevalence of HIV seropositivity in this study was 85.9% (116/135).

Granulomatous inflammation was seen on the trephine biopsy in 15.9% (18/113) of the specimens, half of which had a positive Ziehl-Neelson (ZN) stain. Five of these 18 specimens had a confirmatory positive result on TB culture (four of which already had a positive ZN stain) and six were negative on TB culture. Seven of the 18 positive results could not be confirmed as the TB culture was either not done or contaminated.

A positive culture result was shown in 8.8% (10/113) with *M.tb* identified in 8 (species identification was not possible in two specimens). All 10 of these positive cases were negative on the Xpert® MTB/RIF and only half showed concordance with histology. Time to positivity of these cases ranged from 1-27 days with a median of 18 days.

Xpert® MTB/RIF positivity was detected in four specimens, one of which had an inadequate trephine biopsy and a contaminated TB culture therefore this specimen was excluded from the final statistical analysis. Positivity on the Xpert® MTB/RIF was therefore shown in 2.7% (3/113) of the specimens. Two of these positive results concurred with the histological finding of AFB positive granulomas however in both instances the TB culture results were negative. One positive result failed to correlate with either the TB culture or the trephine biopsy. Two of the four cases resulted in a "very low *M.tb* detection" whilst the other two had a "low *M.tb* detection" result (Table 13). None of the four positive cases on the Xpert® MTB/RIF showed RIF resistance.

3.3 INCONCLUSIVE RESULTS

The Xpert® MTB/RIF error rate was 1.5% (2/135) and these specimens were not repeated. In both instances, a cartridge error (error code 5007) was reported as the probe check value was below the minimum. This can be linked to sample viscosity, incorrect volume, improperly filled reaction tube or if the probe integrity has been compromised (see Appendix 2).
There was a 12.4% (14/113) contamination rate on culture and all of these specimens produced a result on the Xpert® MTB/RIF (one positive result and 13 negative results). Twelve of the 14 specimens had a corresponding adequate trephine biopsy, two did not.

Figures 12 and 13 summarises the results obtained in this study. Of note, histology and TB culture shared five positive results however the total positive cases of each modality is included.

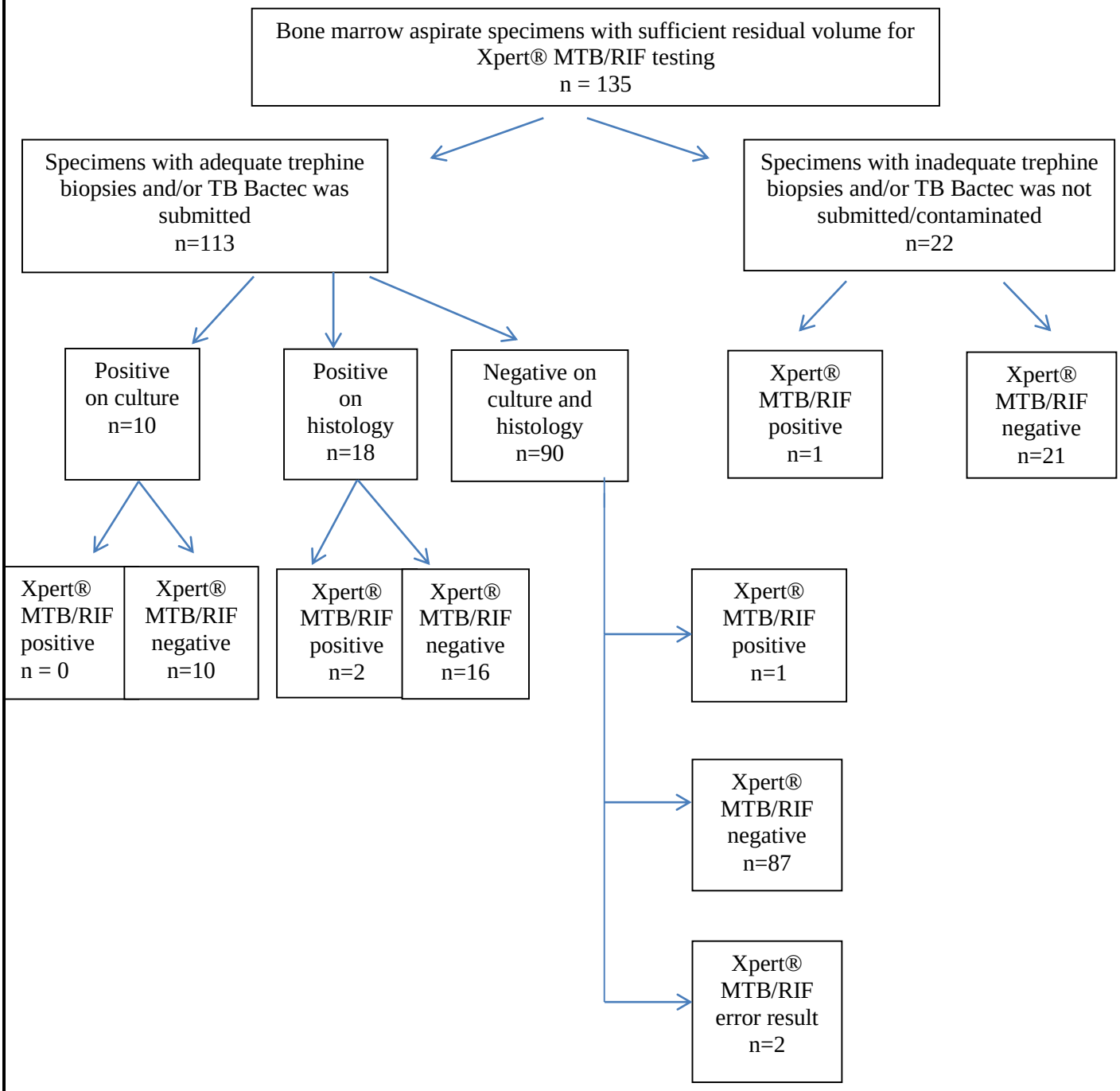


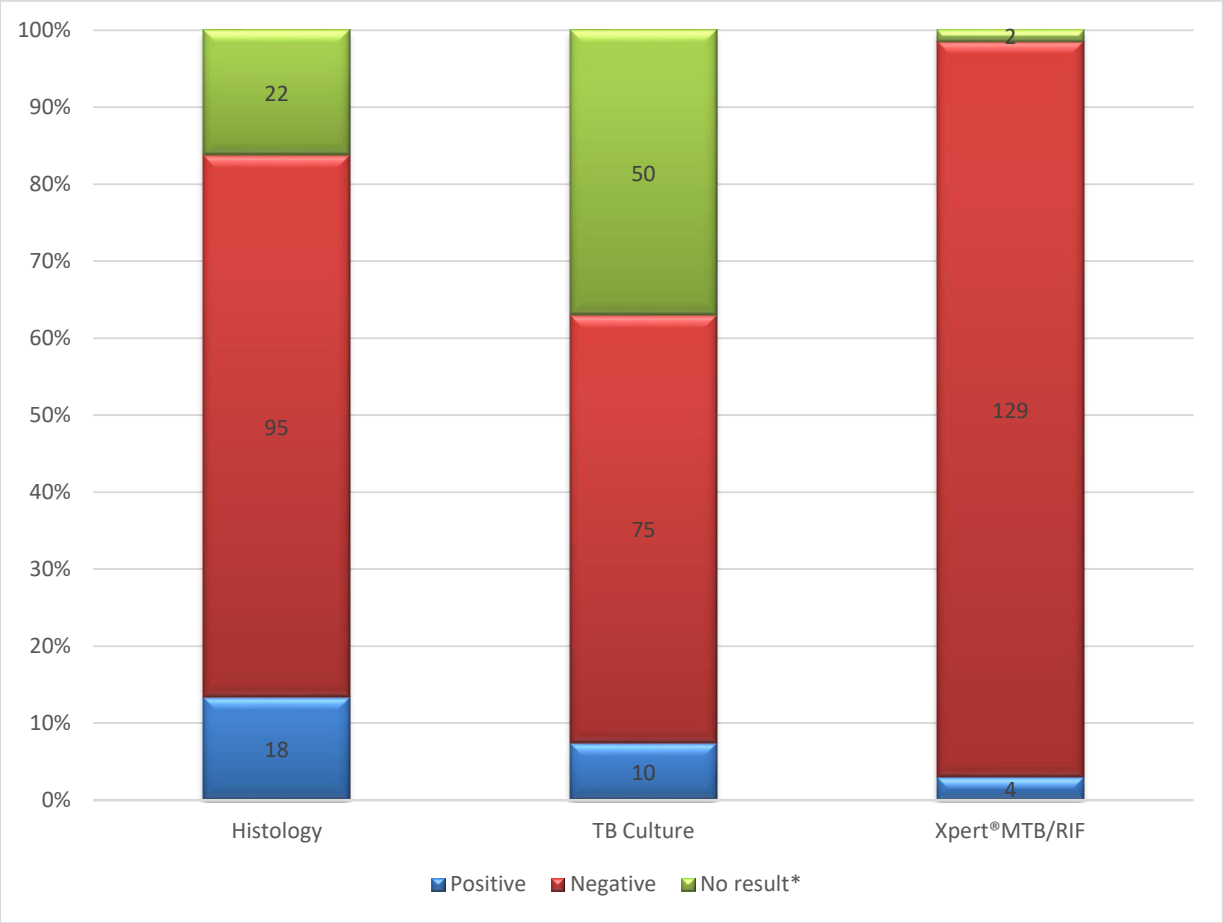
Figure 12: Flow diagram of the results obtained

Table 13: Positive Xpert® MTB/RIF results in this study

Case	**Correlating with histology/culture**	**MTB Result**	**RIF Result**	**Ct* of probes**	**Ct of SPC***
1	Yes (histology)	Low	Not detected	Probe A – 24.7 Probe B – 25.5 Probe C – 25.2 Probe D – 25.7 Probe E – 26.5	25.7
2	Yes (histology)	Very low	Not detected	Probe A – 29.2 Probe B – 29.3 Probe C – 28.8 Probe D – 29.7 Probe E – 30.9	25.3
3	No	Low	Not detected	Probe A – 26.9 Probe B – 27.6 Probe C – 27.0 Probe D – 28.1 Probe E – 28.8	24.6
4	Unknown	Very low	Not detected	Probe A – 31.7 Probe B – 30.9 Probe C – 31.6 Probe D – 31.7 Probe E – 33.6	25.1

*Ct, cycle threshold; SPC, sample processing control.

Figure 13 describes the separate performance of histology, TB culture and the Xpert® MTB/RIF in this study. The latter confers the shortest time to reportable result but had the fewest positive results. Overall, histology reported the most number of positive cases. TB culture has the highest number of "no results" as it was either not performed by the clinician or was contaminated.



*No result owing to inadequate/contaminated specimens, test not done or error code reported by Xpert® MTB/RIF instrument

Figure 13: 100% stacked bar graph depicting MTB results for each diagnostic modality used in this study

3.4 CALCULATION OF SENSITIVITY AND SPECIFICITY

The overall prevalence of MTB in this study based on the positive TB culture and histology results was 20.4% (23/113). Table 14 summaries the findings that follow. The sensitivity of the Xpert® MTB/RIF when compared to both histological and culture findings was 8.7% with a 95% confidence interval (CI) of 1.07-28.04%. The sensitivity improves when the Xpert® MTB/RIF is compared to histological findings only (11.1% with a 95% CI of 1.38-34.7%). The specificity of the Xpert® MTB/RIF was 98.9% (95% CI: 93.9-99.7%). The positive predictive value of the Xpert® MTB/RIF assay in bone marrow aspirate specimens was found to be 66.7% (95% CI: 15.93-95.48) with a positive likelihood ratio of 7.7. The negative predictive value of the Xpert® MTB/RIF assay for bone marrow aspirate specimens was 80.7% (95% CI: 78.66-82.65%) with a negative likelihood ratio of 0.92 (25).

Table 14: Summary of statistical analysis of Xpert® MTB/RIF assay on bone marrow aspirate specimens

	Xpert® MTB/RIF assay (%)	**95% Confidence Interval**
Sensitivity • Histology and culture • Histology only	 8.7% 11.1%	 1.07-28.04% 1.38-34.7%
Specificity	98.9%	93.9-99.7%
Positive predictive value	66.7%	15.93-95.48%
Positive likelihood ratio	7.7%	
Negative predictive value	80.7%	78.66-82.65%
Negative likelihood ratio	0.92	

CHAPTER 4: DISCUSSION

This study evaluated the performance of the Xpert® MTB/RIF in bone marrow specimens submitted to the CMJAH immunophenotyping laboratory in Johannesburg, South Africa as part of the investigation of disseminated TB or to assess the cause/s of cytopenias in immunocompromised patients. The initial optimisation stage of the study showed that:

- 500µL of BMA material (clinical specimen) incubated with SR buffer in a 1:3 ratio for 15 minutes does not meaningfully inhibit the PCR reaction of the Xpert® MTB/RIF assay.
- Results are comparable between heparin and EDTA anticoagulated specimens however the higher Ct value seen with the anticoagulated specimens (compared to distilled water) may indicate partial PCR interference.

These findings support the feasibility of Xpert® MTB/RIF testing on bone marrow aspirate specimens.

The second arm of this study assessed the sensitivity and specificity of the Xpert® MTB/RIF assay in comparison to the current diagnostic practices (i.e. TB culture (employing Myco-F Lytic culture vials) and histological assessment of the trephine biopsy) in 113 bone marrow specimens. This showed the sensitivity of the Xpert® MTB/RIF assay was 8.7% (95% CI: 1.07-28.04%). The two specimens in which the TB culture failed to identify a species may have been non-tuberculous mycobacteria which the Xpert® MTB/RIF would not have detected. The sensitivity improved when the Xpert® MTB/RIF was compared to histological findings only (11.1% with a 95% CI of 1.38-34.7%). When compared to other studies utilising extrapulmonary specimens, the sensitivity obtained in this study is lower. Possible reasons for this include paucibacillary specimens (this is supported by the high Ct values demonstrated in the positive specimens) and may be compounded by the low volume of specimen used. In addition, bone marrow as a specimen type and/or the presence of anticoagulants may interfere with the Xpert® MTB/RIF PCR reaction and result in a higher Ct. This may hamper detection of the bacilli particularly in instances where the bacterial load is already low. Studies performed previously to evaluate the sensitivity and specificity of the Xpert® MTB/RIF assay on extrapulmonary specimens did not often include bone marrow aspirate specimens. When they did, an overall assessment of the sensitivity and specificity was reported with no information

pertaining to bone marrow aspirate specimens specifically (42, 43, 45). In these cases, the sensitivity of the Xpert® MTB/RIF assay may be skewed especially if certain specimens (such as CSF and lymph node) are included in the study as the assay has already been shown to have a higher sensitivity with these specimens (39). A meaningful comparison between studies regarding the sensitivity of the Xpert® MTB/RIF assay on bone marrow aspirate specimens is therefore not possible owing to the limited data available.

The specificity of the Xpert® MTB/RIF assay in this study was shown to be 98.9% (95% CI: 93.9-99.7%). This correlates well with other studies performed on extrapulmonary specimens.

The last objective of this study was to develop a protocol to include the Xpert® MTB/RIF assay in the processing of bone marrow aspirate specimens to diagnose or exclude MTB infection. As mentioned above, the specimens were collected at the flow cytometry laboratory of CMJAH. Whilst this is a quaternary hospital, the Xpert® MTB/RIF instrument is compact and does not require a Biosafety Level 3 laboratory. It can therefore be used at the peripheral hospital performing the bone marrow investigation. This will reduce the need for specimen transportation, reduce the number of lost specimens and will allow same-day results. When performing a bone marrow investigation, material is aspirated for immunophenotypic analysis. Similarly, a small amount of material can be aspirated and placed into a separate heparin or EDTA tube for Xpert® MTB/RIF testing. The specimen will then be processed as per the manufacturer's guidelines and as determined in this study.

This study shows that a separate bone marrow aspirate specimen could be submitted for processing on the Xpert® MTB/RIF as it will generate a faster result than histology and TB culture. The assay is less expensive than culture and drug sensitivity testing (63) however the low sensitivity of the Xpert® MTB/RIF in this study warrants alternative methods (see recommendations in section 4.2) to improve the sensitivity before introducing the assay into the diagnostic algorithm for the identification of MTB in bone marrow. Negative Xpert® MTB/RIF results do not necessarily exclude disease but may be related to a low bacterial burden in the specimen. This observation can be compared to the lower sensitivity of the Xpert® MTB/RIF assay noted in smear negative pulmonary cases.

Limitations to this study are:

- Only a limited number of specimens were tested with the final number included in the analysis further reduced as an adequate corresponding trephine biopsy and/or TB culture was not available.
- Owing to the small volume of specimen used, paucibacillary specimens may have resulted in false negative results.

It would be of benefit to expand on this study to assess if a higher volume of BMA specimen may have improved the outcome and resulted in a better detection of MTB using the Xpert® MTB/RIF.

This study used 15 minutes as the incubation time for the sample/SR buffer mixture as per the manufacturer's guidelines. It would however be of interest to assess if an increased exposure to the SR buffer would improve the sensitivity of the assay. It is postulated that this longer time prior to processing may allow macrophages to release the ingested bacilli.

With the recent recommendation by the WHO (37), assessment of the sensitivity of the Xpert® MTB/RIF Ultra assay to diagnose MTB in bone marrow would be a valuable study considering the improved sensitivity of this assay particularly in paucibacillary and extrapulmonary specimens.

4.1 CONCLUSION

Although the Xpert® MTB/RIF has a shorter turnaround time than histology and TB culture and is less expensive than culture and drug susceptibility testing, the low sensitivity of the Xpert® MTB/RIF in this study limits its current use for the diagnosis of MTB in bone marrow aspirate specimens until the diagnostic algorithm or the assay is further defined.
Alternative methods (such as the Xpert® MTB/RIF Ultra assay, longer incubation times and/or higher volume of BM specimens) are warranted to further optimise the assay. Negative Xpert® MTB/RIF results do not necessarily exclude disease but may be related to a low bacterial burden in the specimen. More sensitive TB diagnostic tools such as the Xpert® MTB/RIF Ultra assay warrant re-evaluation of the clinical and diagnostic algorithms for patient care. Although TB culture remains the gold standard for the detection of *M.tb* in bone marrow aspirate specimens, it is limiting in its time to diagnosis and clinical relevance.

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APPENDIX

APPENDIX 1: PATIENT INFORMATION OBTAINED FROM LIS (LABORATORY INFORMATION SYSTEM)

- Laboratory number
- Referral hospital
- Hospital number
- Date of collection
- History as per the bone marrow request form
- Latest Full Blood Count and Differential Count
- Bone marrow aspirate findings
- Bone marrow trephine biopsy findings
- Ziehl-Neelson result
- TB Bactec result
- Other Xpert® MTB/RIF assays performed – results and type of sample

APPENDIX 2: XPERT® MTB/RIF ERROR CODES

Invalid	Internal control has failed. PCR reaction was inhibited.
5006/5007/5008	Probe check control has failed. Can be linked to sample viscosity, incorrect volume, reaction tube is improperly filled or probe integrity is compromised
2008	Pressure exceeds the maximum pressure allowed or Xpert® MTB/RIF module failure. Usually a random error linked to sample viscosity
No result	Insufficient data was collected as the test has failed to run to completion.

APPENDIX 3: ETHICS CLEARANCE

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Tobias Health Sciences Building 2nd floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265

Ref: W-CJ-141008-3 08/10/2014

TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

Investigator: Dr N H Subramony. (Student number 0500378R).

Project title: The evaluation of the diagnostic utility and sensitivity of the Xpert® MTB/RIF in the detection of *Mycobacterium tuberculosis* complex and rifampicin resistance on bone marrow aspirate samples.

Reason: This is an *in vitro* laboratory study to compare GeneXpert® MTB/RIF results to the current gold standard diagnostic methods for bone marrow TB namely histological testing, culture and drug sensitivity assays. The research falls within the and already approved investigation M090688. Specimens to be used will be those submitted for routine testing, no specimen specifically for the research will be collected from human participants.

UNIVERSITY OF THE WITWATERSRAND
PROF PE CLEATON - JONES
HREC (MEDICAL)
2014 -10- 08
JOHANNESBURG

Professor Peter Cleaton-Jones

Chair: Human Research Ethics Committee (Medical)

Copy - HREC(Medical) Secretariat : Zanele Ndlovu.

APPENDIX 4: SUPERVISOR LETTER & TURN-IT-IN REPORT

Chris Hani Baragwanath Hospital
Johannesburg
25/07/17

To whom it may concern,

Re: TURN-IT-IN report for the MMed research report entitled: The evaluation of the diagnostic utility and sensitivity of the Xpert® MTB/RIF in the detection of *Mycobacterium tuberculosis* and rifampicin resistance on bone marrow aspirate samples.

As per standard university protocol, this MMed thesis was analysed with TURN-IT-IN software. This has yielded a similarity index of 19%. However, this is largely due to the use of technical jargon which cannot be expressed in any other way. As the supervisor of this project, I have approved submission of this thesis.

Yours sincerely,

Dr Jenifer Vaughan
Supervisor of Dr Nadhiya Subramony

MMEDfinalLSJV.docx

ORIGINALITY REPORT

19%	14%	15%	6%
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