

The evaluation of the Xpert® MTB/RIF in the diagnosis of *Mycobacterium tuberculosis* complex and detection of rifampicin resistance in extrapulmonary (pleural and ascitic) fluid samples received for routine immunophenotypic analysis in a high-burden tuberculosis setting.

Kim Michelle Kilfoil

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 Haematology.

Johannesburg, 2015.

DECLARATION

I, Kim Michelle Kilfoil, declare that this research report comprises my own work. It is being submitted for the degree of Master of Medicine in the branch of Haematology to the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree or examination at any other University.

Kim Michelle Kilfoil

On the _____ day of _____ 2015.

DEDICATION

This work is dedicated to my four beautiful children: Ronan, Kait, Owen and Finn.

RESEARCH OUTPUT

KM Kilfoil, E Mayne, L Scott, W Stevens

A high burden Human Immunodeficiency Virus and Tuberculosis resource limited setting, gains from including Xpert MTB/RIF in the diagnostic algorithm of fluid specimens submitted for exclusion of lymphoma by immunophenotypic analysis.

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ABSTRACT

Introduction: The Gene Xpert MTB/Rif assay (Xpert) is a nucleic acid amplification technique that has been studied in the diagnosis of both pulmonary and, to a lesser extent, extrapulmonary tuberculosis (TB). This study was performed in the National Health Laboratory Services (NHLS) laboratory at Charlotte Maxeke Hospital which services a population with a high prevalence of Mycobacterium Tuberculosis Complex (MTBC) infection. The study aimed to develop a protocol for the processing of pleural and ascitic fluid samples to be run on Xpert for MTBC diagnosis, to evaluate the sensitivity and specificity of the Xpert assay as compared to the gold standard MTBC culture assays and to assess the utility of the Xpert assay as part of the diagnostic algorithm for fluid samples received in high prevalence MTBC laboratories. **Materials and methods:** A total of 392 pleural and ascitic fluid specimens were received for routine immunophenotypic analysis between August 2012 and February 2013 at the NHLS flow cytometry laboratory in Charlotte Maxeke hospital. Of these specimens, 229 had sufficient residual volume (>0.5ml) after routine immunophenotypic analysis to be tested on Xpert. Specimens were processed as per the manufacturer's guidelines for pulmonary specimens and results were compared to the gold standard culture for *Mycobacterium tuberculosis*. **Results:** Xpert positivity was detected in 8.7% (20/229) of the total specimens. Only 43% (99/229) of these specimens were submitted for concurrent MTBC liquid culture (Mycobacterium Growth Indicator tube, MGIT) testing based on the laboratory information system history. Positivity on Xpert was shown in 9% (9/99) of specimens compared to 17% (17/99) on MGIT. One false positive was detected on Xpert. More than half of the specimens, 57% (130/229) were not referred for concurrent MTBC culture. The Xpert detected MTBC in 8.5% (11/130) of these specimens, with 1 Rifampicin resistant case identified. Xpert sensitivity and specificity in this study were 50% (CI:26-75%) and 99% (CI:91-100%) respectively. **Conclusion:** The sensitivity and specificity of Xpert in this study was comparable to that found in other studies performed on fluid samples. Importantly, this study demonstrates that in a high burden HIV/TB setting like South Africa, more than 50% of fluid specimens referred for immunophenotypic analysis to exclude

lymphoma are not referred for concurrent MTBC culture testing. Incorporation of Xpert into the laboratory diagnostic algorithm (LDA) in the immunophenotypic laboratory would, therefore, have a number of benefits, improving overall patient work-up and care. Implementation and policy uptake, however, would require a full costing analysis as Xpert testing would be performed in addition to, and not instead of, routine testing.

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ETHICS CLEARANCE

Ethics clearance was granted by the University of the Witwatersrand Human Research Ethics Committee with the clearance number HREC M121010 (See Appendix 5.4).

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ABBREVIATIONS

BCG	Bacilli Calmette-Guerin
BSL	Biosafety Level
CD	Cluster of differentiation
CI	Confidence Interval
CMJAH	Charlotte Maxeke Johannesburg Academic Hospital
CSF	Cerebrospinal fluid
DNA	Deoxyribose Nucleic Acid
EC	Extracellular
EP	Extrapulmonary
EPTB	Extrapulmonary tuberculosis
HIV	Human Immunodeficiency Virus
HL	Hodgkin Lymphoma
IFN	Interferon
IC	Intracellular
IGRA	Interferon Gamma Release Assay
IL	Interleukin
INH	Isoniazid
LDA	Laboratory Diagnostic Algorithm

LIS	Laboratory Information System
MAPc	Mycolic acid-arabinogalactan-peptidoglycan complex
MC+S	Microscopy, culture and sensitivity
MDRTB	Multi-drug resistant tuberculosis
MGIT	Mycobacterium Growth Indicator Tube
MOTT	Mycobacterium other than tuberculosis
MSMD	Mendelian Susceptibility to Mycobacterial Diseases
MTB	Mycobacterium tuberculosis
MTBC	Mycobacterium tuberculosis complex
NAAT	Nucleic Acid Amplification Technique
NHL	Non-Hodgkin Lymphoma
NHLS	National Health Laboratory Services
PCR	Polymerase Chain Reaction
RIF	Rifampicin
RNA	Ribose Nucleic Acid
rpoB	β -subunit of reverse polymerase gene
RRDR	Rifampicin-resistance determining region
SR	Solvent reagent
STAT	Signal-transducer and activator of transcription
TB	Tuberculosis

TST	Tuberculin Skin Test
UV	Ultraviolet
WHO	World Health Organisation
XDRTB	Extensive drug-resistant tuberculosis

1 INTRODUCTION

In 1882, Robert Koch identified the causative organism that resulted in tuberculosis (TB) and referred to it as the *Tuberkelbazillus* [1]. The binominal term *Mycobacterium tuberculosis* (MTB) which means “fungus bacterium” was only introduced in 1891, after a mould-like pellicle was observed on the surface of fluid media containing members of this genus [1].

Mycobacterium leprae (*M. leprae*) was the only other member of the genus known at that time and was the bacillus identified as the causative organism of leprosy. Subsequent studies identified mycobacterium in other species, which lead to the discovery of zoonotic mycobacterial species *M. bovis* (bovine), *M. avium* (avian), *M. fortuitum* (amphibians) and *M. chelonae* (reptiles). These were subsequently referred to as MOTT (Mycobacterium other than tuberculosis) [1].

1.1 CLASSIFICATION

Mycobacterium tuberculosis belongs to the genus Mycobacteria which is divided into obligate and environmental pathogens. The obligate pathogens include *M. tuberculosis* and *M. leprae*, among others [1].

1.2 CHARACTERISTICS OF MYCOBACTERIA

The mycobacteria are non-sporing, non-flagellate gram positive organisms that are mostly rod like or curved structures. When treated with dilute mineral acid, the bacilli retain the colour of arylmethane dyes. This resulted in the term “acid-fast” bacilli, which is often used as a synonym for mycobacterium [1, 2]. The acid-fast staining technique used today is known as the Ziehl-Neelsen stain. Specimens are stained with hot carbolfuchsin, then decolourised with a dilute mineral acid in water or alcohol and then counterstained with a green or blue dye. The organisms appear as red-coloured bacilli against the counterstained background [1, 2]. See Figure 1.

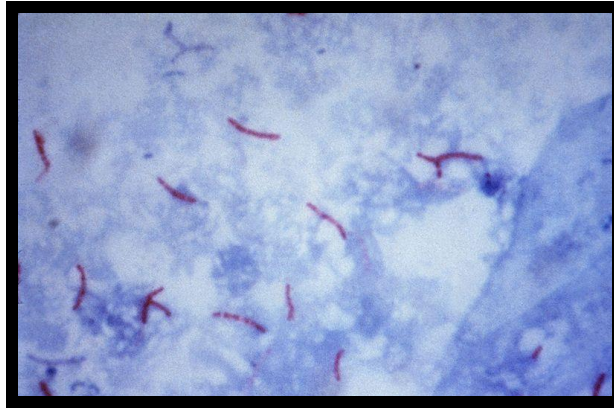


Figure 1 Ziehl-Neelsen stain showing acid-fast bacilli [3].

1.2.1 THE STRUCTURE OF MYCOBACTERIA

Mycobacteria, like other bacteria, have cell membranes comprised of a double phospholipid layer (Figure 2). Characteristically, they have a complex and thick lipid-rich cell wall external to their cell membrane. The cell wall has an inner layer which is a net-like macromolecule, either peptidoglycan or murein, that is comprised of long polysaccharide chains cross-linked by short peptides 4 amino-acids in length. This layer is responsible for the rigidity and the shape of the bacillus. The outer layer of the cell wall is another macromolecule known as arabinogalactan which is a branched polysaccharide comprised of arabinose and galactose. Attached to this layer are long chain fatty acids known as mycolic acids which result in the thickness of the cell wall and its “acid-fast” nature when stained [1, 3].

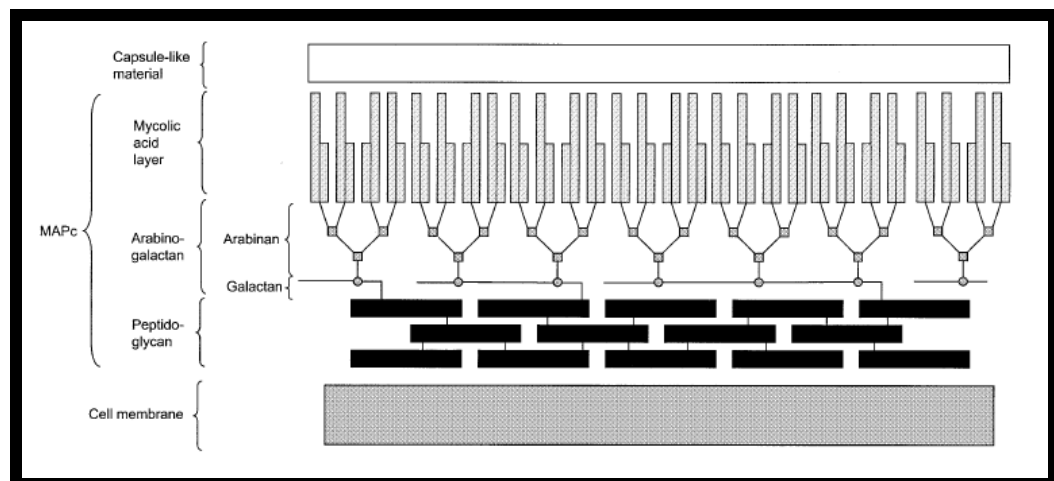


Figure 2 Cross-section of the *M. tuberculosis* envelope [4].

1.2.2 THE GENOME OF M. TUBERCULOSIS

The MTB genome was sequenced in 1998. It comprises 4 411 529 base pairs encoding approximately 4000 genes [1, 2, 4]. The *Mycobacterium tuberculosis* genome encodes for approximately 250 enzymes involved in the synthesis and metabolism of lipids, resulting in the thick, lipid-rich cell wall characteristic of MTB [1, 2, 4].

1.3 TUBERCULOSIS IN SOUTH AFRICA

TB is the second leading cause of death worldwide as a result of an infectious disease, second only to HIV infection [5]. The World Health Organisation (WHO) estimated that, in 2010, there were approximately 8.5–9.2 million cases of *Mycobacterium tuberculosis* complex (MTBC) infection and a resultant 1.2–1.5 million deaths (in HIV negative and positive patients) globally [5]. The figures reported by the WHO for 2012 were similar with approximately 8.6 million new MTBC infection cases with approximately 1.3 million associated deaths [6].

In South Africa, the incidence rate of MTBC infection increased from 2010 to 2012 from an estimated 981/100 000 population to 1000/100 000 population. In 2010, there was an approximate 60-61% HIV prevalence in incident MTBC cases which increased to 62-63% in 2012 [5, 6]. It is, therefore, important to identify MTBC infected patients in South Africa and to institute appropriate treatment timeously.

1.3.1 TB IN THE HIV NEGATIVE POPULATION

Approximately 35% of cases of active MTBC infection occur in Human Immunodeficiency Virus (HIV) negative individuals [5, 7]. The mortality rate of TB in this population is 50 per 100 000 population [5]. These patients may be immunocompetent or immunocompromised for reasons other than HIV infection including uncontrolled diabetes, chemotherapy and extremes of age. The immunocompetent patients develop active disease in ~10% of cases while the remaining majority are able to control the infection effectively [8].

Numerous factors contribute to the outcome of MTBC infection including environmental conditions (poverty and malnutrition), bacterial and host genetic factors [8]. Host genetic factors have been implicated in susceptibility to active MTBC [8]. In Mendelian Susceptibility to Mycobacterial Diseases (MSMD) for example, genes encoding various components of the host immune response have been implicated, such as those encoding interleukins (ILs), γ -Interferon (IFN) and STAT-1 (signal-

transducer and activator of transcription) [1, 9]. It is thought that the longer a certain population is exposed to the MTBC bacillus, the lower their genetic susceptibility to MTBC and that there is a strong selection against the genes that make one susceptible to MTBC infection [8]. Furthermore, patients of African descent show a greater susceptibility to MTBC than patients of European descent [10].

1.3.2 TB IN THE HIV POSITIVE POPULATION

MTBC infection can occur at any CD4 T-cell count in HIV positive patients, but it is more common at lower CD4 T-cell counts ($<200 \times 10^6/l$) [11, 12]. Up to 70% of presumptive MTBC cases in South Africa occur in HIV co-infected patients [13]. In 2011, the incidence of MTBC infection was 993/100,000 and 15% (47,285) of the new cases reported were extrapulmonary TB (EPTB) [14, 15].

Certain patient groups are at an increased risk of both HIV and MTBC infection, for example people living in jails and infirmaries. This is attributed to factors like overcrowding, late detection and treatment of infectious diseases, poor infection control measures and malnutrition [16]. The TB epidemic often parallels HIV infection and as such, health programmes will need to target both infections in order to gain control over the spread of these infections.

1.3.3 ESSENTIAL ANTIMICROBIALS FOR THE TREATMENT OF MTBC INFECTION

The South African Department of Health publish annual guidelines for the management of MTBC infection [17]. The following antimicrobial agents are considered essential in the management of MTBC infection: Isoniazid (INH), Rifampicin (Rif), Pyrazinamide, Ethambutol and Streptomycin. These drugs have varying mechanisms of action and properties, targeting the bacilli in various life stages (Table 1) [17].

The standard treatment regimen for all patients is made up 2 phases. The initial intensive phase lasts 2 months and is a combination of 4 drugs, namely INH, Rif, Pyrazinamide and Ethambutol which rapidly kill the MTBC bacilli [17]. The intensive phase is then followed by the continuation phase which lasts a further 4 months, during which a combination of 2 drugs (INH and Rif) is used. The sterilising effects of INH and Rif result in elimination of the remaining bacilli and prevention of subsequent relapse (Table 1) [17].

1.3.4 DRUG RESISTANT TB

MTBC resistance can be defined as multi-drug resistant (MDRTB) or extensive drug-resistant TB (XDRTB). MDRTB is resistant to both INH and Rif. XDRTB is resistant to any fluoroquinolone as well as to key second-line drugs including kanamycin, amikacin or capreomycin (Table 2) [1, 17].

The true extent of MDRTB and XDRTB in South Africa is largely unknown as a number of cases may not be diagnosed or reported. The WHO figures of reported MDRTB cases in South Africa range from 9070 in 2009, 7386 in 2010, 10 085 in 2011 and 15 419 in 2012 [5, 6, 18].

The WHO found that the number of MDRTB cases reported worldwide had tripled since 2009 to 2013 and reached a total of 136 000 cases, making up 45% of the total cases of notified MTBC infection [19]. An average of approximately 9% of these MDRTB cases are XDRTB [19].

Mutations in the *rpoB* (β -subunit of reverse polymerase) gene of MTBC are responsible for the development of Rif resistance and a number of possible single amino-acid mutations in the short region of the *rpoB* gene have been identified [20]. DNA sequencing studies have shown that >95% of the Rif resistant MTBC strains have a mutation within the 81- base pair hot spot region of the *rpoB* gene, known as the Rifampicin resistance determining region (RRDR) [21, 22]. Resistance to Rif alone is rare and more than 90% of Rif resistant cases also show resistance to INH. The detection of Rif resistance is therefore used as a surrogate marker to detect MDRTB [22].

The South African guidelines for the treatment of MDRTB recommend an intensive phase of six months with a combination of five antimicrobial agents, namely Kanamycin/Amikacin, Moxifloxacin, Ethionamide, Terizidone and Pyrazinamide [23]. See Table 2. This is followed by a continuation phase using a combination of four drugs, namely Moxifloxacin, Ethionamide, Terizidone and Pyrazinamide. In areas with confirmed low prevalence of Ethambutol resistance and in patients who did not receive Ethambutol for >1 month prior to the diagnosis of MDRTB, Ethambutol may be used as an additional antimicrobial agent [23]. Treatment is continued for 18-24 months in total [23].

Factors that contribute to the development of drug resistant MTBC include patient non-compliance as a result of high tablet volume, improvement of symptoms and socio-economic circumstances [24]. MTBC has the ability to undergo slow (but constant) and spontaneous mutations in the genome that confer resistance to antimicrobial agents [23]. The probability of spontaneous mutations resulting in resistance to individual first-line MTBC drugs varies between antimicrobial agents and occurs as follows [23]:

INH:	1/every 106 cell divisions
Rif:	1/every 109 cell divisions
Streptomycin:	1/every 106 cell divisions
Ethambutol:	1/every 105 cell divisions
Pyrazinamide:	1/every 105 cell divisions

Spontaneously occurring resistance to more than 1 antimicrobial agent is extremely rare and is the result of selection of resistant mutants in the bacterial population by the eradication of susceptible MTBC bacilli with antimicrobial agents [23]. Suboptimal treatment e.g. monotherapy and insufficient antimicrobial dose, greatly exacerbates the development of resistant strains.

Table 1 Antimicrobial agents used as first-line treatment in MTBC infection [17, 24]

<u>Antimicrobial</u>	<u>Drug property</u>	<u>Target bacilli</u>	<u>Mechanism of action</u>	<u>Site of action</u>
Rifampicin (Rif)	Bactericidal in <1hour. High potency. Most effective sterilising agent.	All populations including non-replicating bacilli.	Inhibition of DNA-dependent RNA polymerase [25]	Intracellular (IC) and extracellular (EC).
Isoniazid (INH)	Bactericidal after 24 hours. High potency. Kills >90% bacilli in first few days of treatment.	Rapid and intermediate growing bacilli	Activated INH inhibits the synthesis of mycolic acids, an essential component of the bacterial cell wall[25]	IC and EC
Pyrazinamide	Bactericidal. Low potency. Sterilising effect occurs within 2-3 months.	Slow growing bacilli.	Inhibits the enzyme fatty acid synthase I, required by the bacterium to synthesise fatty acids. Disrupts membrane potential and interferes with energy production [25]	IC only (within macrophages)
Ethambutol	Bacteriostatic. Low potency. Minimises the emergence of drug resistance.	All bacterial populations.	Inhibits arabinosyltransferases which are involved in cell wall biosynthesis[25]	IC and EC
Streptomycin	Bactericidal. Low potency.	Rapidly growing bacilli.	Protein synthesis inhibitor[25]	EC only

Table 2 Antimicrobial agents used as second-line treatment in MTBC infection [22, 24]

<u>Antimicrobial</u>	<u>Drug category</u>	<u>Mechanism of action</u>
<u>Kanamycin</u>	Aminoglycoside	Protein synthesis inhibitor[25]
<u>Amikacin</u>	Aminoglycoside	Protein synthesis inhibitor[25]
<u>Capreomycin</u>	Polypeptide	Protein synthesis inhibitor[25]
<u>Ethionamide</u>	Thioamide	Nicotinic acid derivative related to INH. Inhibits the synthesis of mycolic acids, essential component of the bacterial cell wall[25]
<u>Terizidone and Cycloserine</u>	Azolidines	Competitively inhibits two enzymes important in cell wall synthesis, L-alanine racemase and D-alanylalanine synthetase[25]
<u>Moxifloxacin</u>	Fluoroquinolone	Inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV[25]

1.3.5 EXTRAPULMONARY TB (EPTB)

EPTB is defined as TB infection outside of the pulmonary system (i.e. outside the lungs). This occurs as a result of seeding of the MTBC bacilli via mucosal, lymphoid or haematogenous routes. EPTB is more common in patients infected with HIV, especially in the advanced stages, who often present with disseminated or miliary TB [1]. This may be as a result of reactivation of latent MTBC, rapid progression of the disease after initial exposure or repeated exposure to the infective organism. The MTBC bacilli disseminate to extrapulmonary (EP) sites as a result of the associated immunosuppression. An estimated 50% of patients with HIV and MTBC infection have EP involvement and the signs and symptoms of EPTB are often masked in the advanced stages of HIV infection [1].

Approximately one third of MTBC-related deaths in African HIV positive persons are from EPTB infection [26-28]. Only half of these cases are diagnosed prior to death [26, 28]. In 2009, of the 340 000 new TB case findings in South Africa, it is estimated that 16% were EPTB [29]. In 2013, 13% of the new TB case findings were EPTB [30].

Pleural effusions and ascites related to MTBC infection are both classified as exudative effusions. This is an accumulation of serous fluid with high protein content. The pleura and peritoneum are directly involved by the inflammatory/infective process of MTBC infection [31].

1.3.5.1 TUBERCULOUS PLEURAL EFFUSIONS

Despite the close relationship between the pleural cavities and the pulmonary system/lungs, tuberculous pleural effusions are classified as EPTB and they account for >20% of all EPTB cases [32]. Pleural effusions are the most common presentation of EPTB and may result from progression of primary MTBC infection or arise as a complication of post-primary (secondary/reactivated) TB [33].

Pathogenesis

There are two main mechanisms associated with the development of tuberculous pleural effusions. The majority are a result of a delayed-type hypersensitivity reaction that develops in response to invasion of the pleural space by MTBC bacilli. The invasion is often a consequence of the rupture of a sub-pleural caseous focus. Lymphocytes and macrophages in the pleural space are exposed to MTBC antigens resulting in a localised cell-mediated immune response. Granulomatous inflammation of the pleura and lymphocyte-rich pleural exudates then develop [1].

The second mechanism of development of tuberculous pleural effusions is the increased capillary permeability associated with inflammation. The effusion accumulates when there is a disturbance in the balance of lymph drainage, capillary permeability and hydrostatic and osmotic forces [1].

1.3.5.2 TUBERCULOUS ASCITES

Abdominal TB accounts for approximately 11-16% of all EPTB cases and is the sixth most frequent site of EPTB [1]. TB can affect the peritoneum, pancreatobiliary system and the entire gastrointestinal tract from the mouth to the anus.

Pathogenesis

The MTBC bacilli are able to reach the gastrointestinal system via four main mechanisms: haematogenous spread from the primary pulmonary focus, ingestion of bacilli from expectorated sputum, direct spread from adjacent organs and via the lymphatic system from nearby infected lymph nodes [34].

The visceral and parietal peritoneum both gradually become studded with tubercles of MTBC bacilli. When active, these tubercles secrete a proteinaceous fluid which accumulates, resulting in the development of ascites [34].

1.3.5.3 INVESTIGATION OF PLEURAL EFFUSIONS AND ASCITES IN THE SOUTH AFRICAN SETTING

Many fluid specimens (pleural and ascitic) are received for testing in multiple laboratories including microbiology (culture), chemistry (biochemical studies) and histopathology (cytology). The haematology laboratory receives these fluid samples for immunophenotypic analysis by flow cytometry, many of which are from HIV positive patients suspected of having lymphoma. In HIV infected patients, the incidence of all subtypes of Non-Hodgkin lymphoma (NHL) is increased by 60-200 times [35, 36] with pleural effusions occurring in 20-30% of these patients [37].

In the immunophenotypic laboratory of the National Health Laboratory Services (NHLS) laboratory at Charlotte Maxeke Hospital, a standard panel for exclusion of B and T-cell lymphoproliferative disorders and non-haemopoietic tumours (using the monoclonal antibodies anti-kappa, anti-lambda, CD19, CD5, CD4, CD3, CD8, and CD45) is routinely performed. During the diagnostic work-up of these patients, investigation for EPTB is important in this high prevalence setting to enable early identification of EPTB, identification of MDRTB, timeous initiation of treatment resulting in an overall improvement in patient care.

1.4 CURRENT DIAGNOSTIC METHODS FOR MTBC

1.4.1 SPUTUM MICROSCOPY

Sputum smear microscopy is part of the current diagnostic approach to MTBC infection [38]. Serial sputum samples are taken which requires patients to return on repeated occasions to submit their samples to the laboratory. This is problematic as it is an additional expense that these patients incur. The sensitivity of sputum smear microscopy varies from 20-80%, depending on a number of factors. These include the quality of the sputum sample obtained from the patient, the quality and stain of the smear and the experience of the person interpreting the smear [24]. Sputum microscopy is most insensitive in those patients with low bacillary load (including paediatric and HIV positive patients) [24]. In South African rural areas, clinics are understaffed with few laboratory trained personnel available to perform sputum microscopy. There is also a lack of a constant

and reliable supply of water and electricity to these areas, further inhibiting the ability to diagnose MTBC on sputum microscopy [24]. In view of these difficulties, the incorrect diagnosis is made in many cases [1, 39].

As many as 40-50% MTBC culture positive cases are negative on microscopy for acid-fast bacilli. In HIV co-infected patients, only 10-13% of culture positive cases may be smear positive owing to the low bacillary load [38].

Patients with EPTB infection commonly have an unproductive cough and are not able to provide a sputum sample for microscopic analysis. This further limits the ability to diagnose EPTB.

1.4.2 TB CULTURE AND SENSITIVITY TESTING

TB culture and sensitivity testing is the gold standard for the diagnosis of MTBC and the evaluation of drug sensitivity [1]. This process takes up to six weeks during which time patients are often lost to follow-up (See Appendix 5.1 for laboratory standard operating procedure).

Below is a schematic (Figure 3) of the work-flow in the TB referral laboratory of the National Health Laboratory Services (NHLS). This schematic represents only EP fluid samples that are received for MTBC culture. The NHLS uses Mycobacterium Growth Indicator (MGIT) tubes that are placed in the MGIT 960 instrument [38].

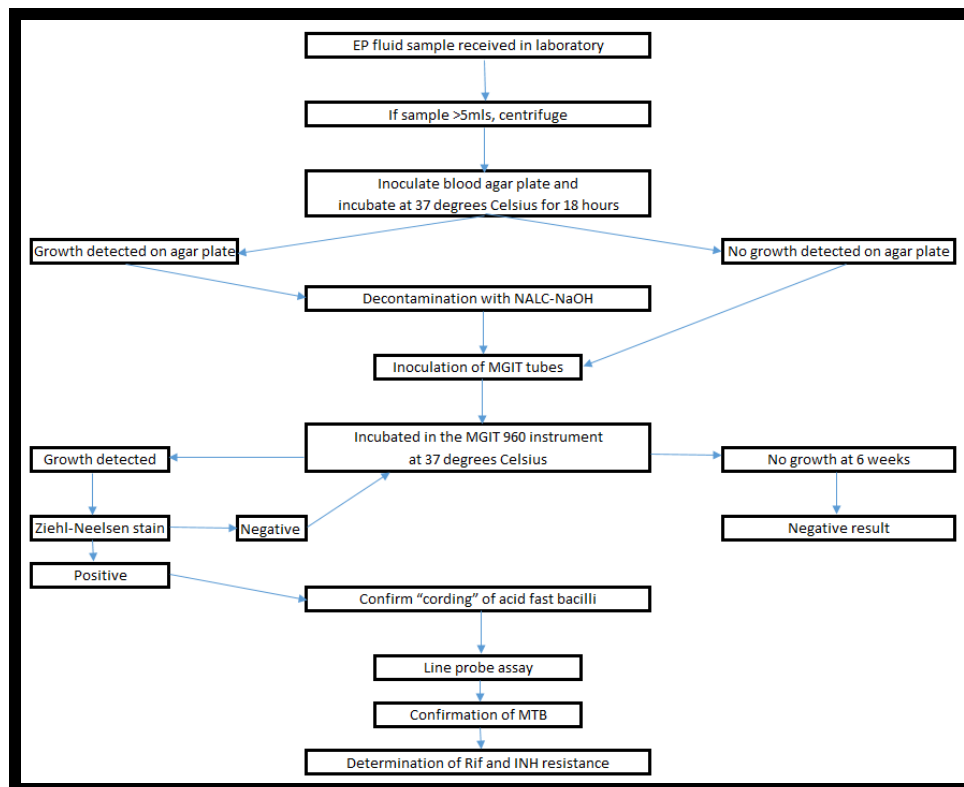


Figure 3 Schematic showing processing of EP fluid samples in the TB reference laboratory of the NHLS [38].

EP fluid samples are extracted from a normally sterile site, however, contamination with other organisms can occur. Sediment from the EP fluid sample is plated on a blood agar plate and cultured. If growth is detected, a decontamination step with N-acetyl-L-cystine sodium hydroxide (NALC-NaOH) is undertaken to destroy the contaminating organisms [38, 40]. These samples are then inoculated into the MGIT and are placed in the MGIT 960 instrument for incubation [38]. If bacterial growth occurs in a specific tube, the free oxygen is utilised by the dividing bacteria and is replaced by carbon dioxide [38, 40]. The oxygen quenched fluorochrome is then no longer inhibited and there is emission of fluorescence within the tube which is detected under ultra-violet (UV) light. The intensity of the fluorescence is proportional to the degree of oxygen depletion and, therefore, bacterial growth.

If no growth is detected after six weeks incubation, the culture is reported as negative. Once a positive signal is obtained from the MGIT 960, the tube is removed and a Ziehl-Neelsen stain is made to confirm the presence of acid-fast bacilli. If other contaminating organisms are found, the sample is reported as contaminated. Characteristically MTBC shows “cording” of the bacilli (see Figure 4). If this is noted, the next step is the line probe assay.

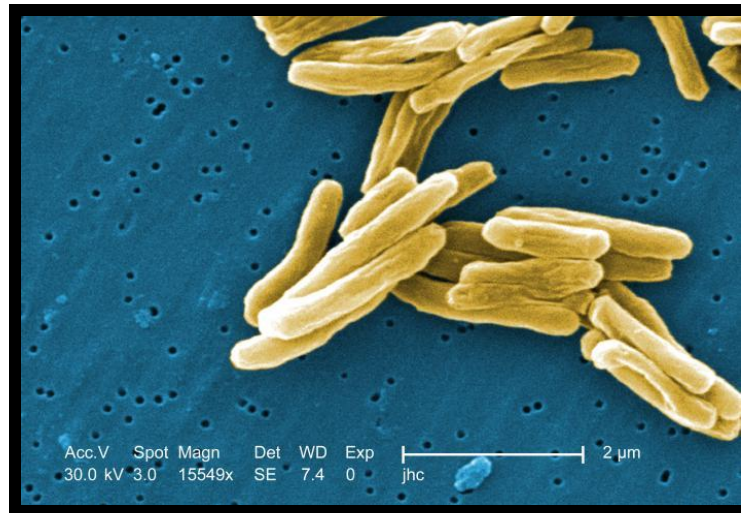


Figure 4 Electron micrograph of MTBC [41].

Molecular line probe assays are based on the application of Polymerase Chain Reaction (PCR) and reverse hybridisation. These assays are highly sensitive (~97%) and specific (~99%) for the detection of Rif resistance alone or in combination with Isoniazid (INH) (sensitivity ~90% and specificity ~99%) [40].

1.4.3 INTERFERON GAMMA RELEASE ASSAY (IGRA)

Interferon gamma release assays (IGRAs) are an alternative to the tuberculin skin test (TST) and have been evaluated for use in screening patients for latent MTBC [42]. The test is based on the principal that T-cells from a patient infected with and sensitised to MTBC will release gamma interferon on re-exposure. A high level of gamma interferon would, therefore, suggest MTBC infection [43].

Unlike IGRAs, the TST is a mixture of a number of antigens from MTBC and non-tuberculous mycobacterium species. As a result, the TST has a low specificity in countries with a high BCG (bacilli Calmette-Guerin) coverage or a high frequency of non-MTBC mycobacterial infection [43]. IGRAs are currently considered to be a cost-effective alternative to the TST in screening for MTBC infection in high risk groups. This would include non-vaccinated patients, healthcare workers, immigrants from MTBC endemic countries and close contacts of infected patients [42]. Used as a screening tool to identify latent MTBC infection, IGRAs can be used to motivate for chemoprophylaxis in latently infected individuals. Less is known about the use of IGRAs in paediatrics patients owing to the limited data available from previous studies [44]. There is a concern that age-related immunologic factors may affect the sensitivity of IGRAs in the paediatric population. As children are more at risk of developing disseminated MTBC disease, a

negative IGRA should not be used to exclude infection and delay the initiation of treatment [44].

1.4.4 NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAAT)

Recently, much attention has been given to the use of nucleic acid amplification techniques (NAAT) in the diagnosis of MTBC. Compared with the gold standard of MTBC culture, NAATs allow rapid identification of MTBC infection and Rif resistance with results available within two hours. This results in timeous identification of patients with active MTBC and allows earlier treatment of the disease and prevention of the spread of drug resistant TB within the community.

The Gene Xpert MTB/Rif assay (Xpert) has been studied in the diagnosis of both pulmonary and, to a lesser extent, extrapulmonary disease. It is currently endorsed by the Scientific and Technical Advisory board of the World Health Organisation (WHO) as a sensitive and quick method of diagnosing TB in paucibacillary pulmonary TB samples [45]. The benefits of this assay include the low rate of cross-contamination, the absence of a required biosafety facility and the assay's ease of use requiring very little training [46].

The Xpert assay uses hemi-nested real time PCR to amplify the *rpoB* gene sequence which is specific to MTBC. The *rpoB* gene is probed with molecular beacons to determine the presence or absence of a mutation associated with Rif resistance within the 81 base pair (bp) RRDR [47, 48]. 5 molecular beacons/probes (Probe A, B, C, D and E) are used which are complementary to overlapping regions of the *rpoB* gene (See Figure 5). These probes, when unbound, are curved in shape, closely approximating the fluorescent dye on one end with the quencher on the other end. When bound to the complementary *rpoB* gene, the probes flatten out and the quencher no longer inhibits the fluorescence. Each probe emits a specific colour which is detected as a positive signal [47].

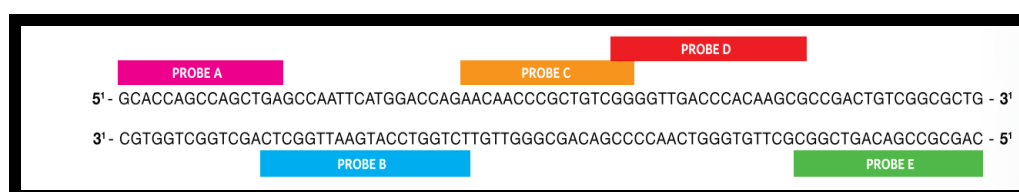


Figure 5 The *rpoB* gene [46]

The data analysis algorithm of the Xpert assay is designed to report MTBC detection when at least two or more *rpoB* specific probes transmit a positive signal within two cycles of each other. In the case of Rif resistance, mutations in the RRDR result in the corresponding complementary probe not binding which in turn results in the absence of a fluorescent signal. A difference in the cycle threshold of 3.5 between the earliest and latest *rpoB*

signals, or no detectable cycle threshold for a particular probe are both interpreted as Rif resistance [47]. Also included within the cartridge is a hemi-nested molecular beacon assay to detect the presence of *Bacillus globigii* DNA which acts as an internal control [47].

The Xpert assay is fully automated. All reagents are in a disposable cartridge, making it easy to use. Results are usually available within two hours. Specimens typically contaminated with other pathogens e.g. sputum and stool have been shown to produce reliable results. The *rpoB* gene is specific to MTBC and, therefore, the assay does not report a positive finding for other mycobacterial species [47]. See Figure 6 for interpretation algorithm.

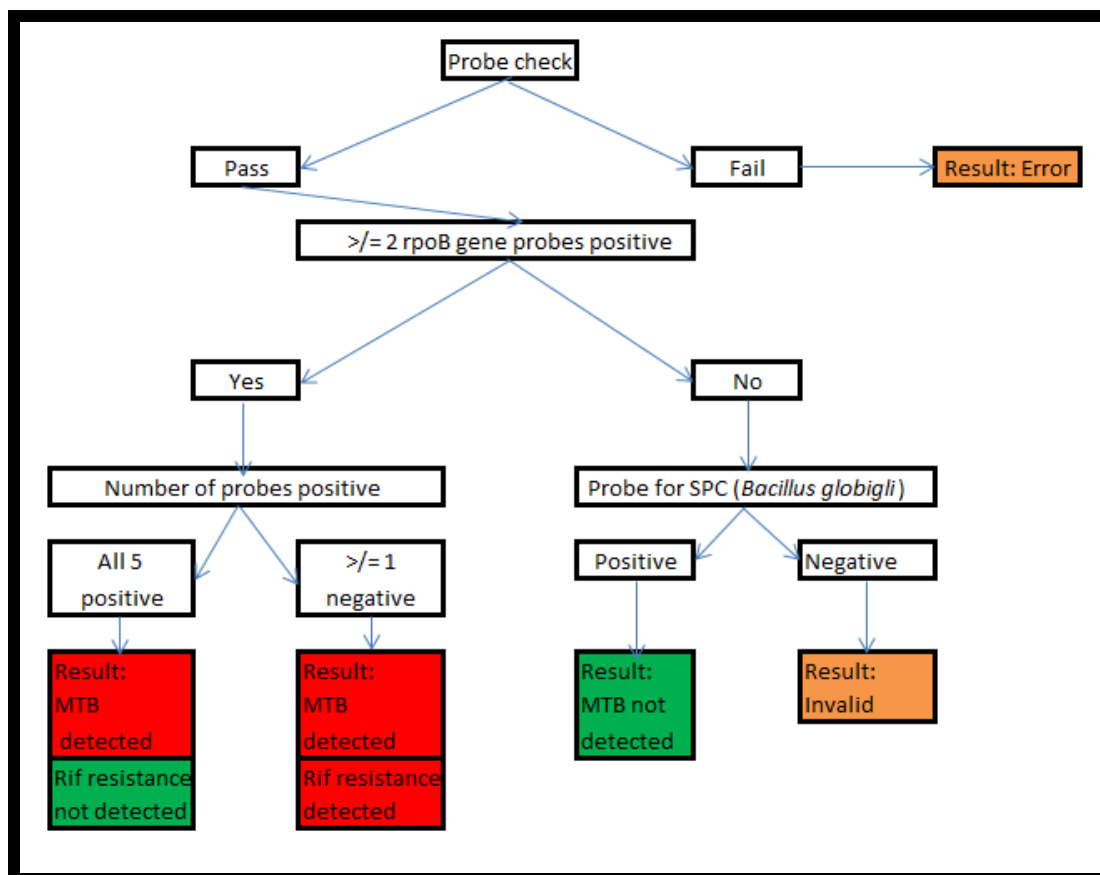


Figure 6 Interpretation algorithm for Cepheid MTB/Rif assay [46]

The Xpert assay is performed in three steps:

Step 1: The reagent is added to the sample and incubated at room temperature for 15 minutes [46].



Step 2: Using a pipette, the diluted sample is inserted into the cartridge containing all the necessary reagents [46].



Step 3: The cartridge is inserted into the Xpert machine for the assay to commence [46].



IGRAs have been proposed as a diagnostic assay in active MTBC infection however they have some disadvantages when compared to NAAT [43, 49, 50]. See Table 3.

Table 3 Advantages of NAATs compared to IGRAs [42, 47, 49]

<u>NAATs</u>	<u>IGRAs</u>
Results available within 2 hours	Incubation time ranges from 1 - 6 days
Identifies Rifampicin resistance	Does not identify drug resistant cases
Use not limited to a laboratory setting	Performed in laboratories only
Self-contained and self-run assay, no laboratory personnel required	Laboratory trained staff necessary
Detects MTBC bacillus directly	Indirect test of patient's immune response to the MTBC bacillus.
Used on a variety of specimens e.g. urine, stool, tissue, fluid	Tested on blood only
Used in paediatric and adult samples	Limited data on use in paediatric samples
Reliable results in immunocompromised patients	T-cell based assay, data limited in patients with T cell immunodeficiency e.g. HIV

1.4.5 DATA FROM PREVIOUS STUDIES EVALUATING THE XPERT ASSAY

Numerous studies have been performed to evaluate the sensitivity and specificity of the Gene Xpert assay. Smear and/or culture positive pulmonary samples show a sensitivity of up to 100% and a specificity of 98.3%. Smear negative pulmonary samples have shown a sensitivity of 74.2% [21, 48]. The sensitivity of the Xpert assay is higher in pulmonary specimens compared with EP specimens (Table 4).

Compared with studies on pulmonary samples, fewer studies have been performed on the use of Xpert for the diagnosis of EPTB owing to variations in study population, sample size, methodology and specimen type. Xpert is, however being investigated for inclusion in national Tuberculosis programs [51, 52]. The findings from some of the studies are summarised in Table 4 below.

Table 4 Sensitivity and specificity data obtained for EP samples processed on Xpert

<u>Authors</u>	<u>Sample type</u>	<u>Diagnosis of MTB</u> <u>Sensitivity</u>	<u>Diagnosis of MTB</u> <u>Specificity</u>
Ligthelm et al. [53]	Fine needle aspirate	96.7%	88.9%
Peter et al.[7]	Urine	48%	98%
Ioannidis et al. [22]	EP (CSF, gastric fluid, lymph node, pericardial fluid, pleural fluid, pus, synovial fluid, tissue, urine)	100%	91.6%
Teo et al.[39]	EP (CSF, gastric aspirate, urine, fluid)	90.9%	89.5%
Tortoli et al. [45]	EP (biopsies, pleural fluids, gastric aspirates, CSF)	79.0%	97.3%
Hilleman et al. [48]	EP (Urine, stool, skin tissue)	79.5%	98.2%
Zeka et al. [21]	EP (pleural fluid, lymph node, ascites, CSF, pericardial fluid, skin biopsy, urine)	100% (smear positive) 63% (smear negative)	100%
Vadwai et al. [54]	EP (lymph nodes, fine needle aspirates, fluids, CSF)	81%	99.6%
Armand et al. [55]	EP(lymph node, pleural fluid, bone, abscess, urine)	100% (smear positive) 37% (smear negative)	-
Causse et al. [56]	EP (CSF, pleural ascitic articular and pericardial fluid, biopsy, gastric aspirates and purulent exudates)	95%	100%
Friedrich et al. [57]	EP (pleural fluid)	25%	100%
Moure et al. [58]	EP (fluid, lymph node, tissue, abscess, stool)	26.9-76.5%	-
Malbruny et al. [59]	EP (lymph node, CSF, pleural joint ascitic fluids, biopsies, urine, bone marrow)	85.7%	97.3%
Chang et al. [60]	Meta-analysis of 18 studies	80.4%	86.1%
Scott et al. [51]	EP (pus, lymph nodes, pleural and ascitic fluid)	59%	92%
WHO meta-analysis [52]	Meta-analysis of 15 studies	43.7%	98.1%

Smear positive EP samples have a higher sensitivity (100%) than smear negative EP samples (63%) [21]. The lower sensitivity may be attributable to the low bacillary load within the fluids and/or the presence of an inhibitor within the fluid [61]. The specificity of the assay remains high throughout all types of specimens but the sensitivity varies between EP sample types. The sensitivity of the assay is higher on peritoneal and ascitic fluids (86-100%) compared with pleural fluids (63-73%) [62].

Data suggest that the Xpert assay should be used to confirm the diagnosis of pulmonary TB but it cannot be used to exclude MTBC infection because of the variable sensitivity of the assay (i.e. if MTBC is not detected by the Xpert assay, this does not exclude MTBC infection in that patient). At present, data do not support the replacement of conventional testing (including MTBC culture) with the Xpert assay [33].

The specificity and sensitivity of the Xpert assay in determining Rif resistance is promising with a specificity of 98-100% and a sensitivity of 98-99.1% recorded in some trials [22, 54].

1.5 OBJECTIVES OF THIS MMED RESEARCH REPORT

This study was performed in the NHLS laboratory at Charlotte Maxeke Hospital which services a population with a high prevalence of MTBC infection. The first aim of the study was to develop a protocol for the processing of pleural and ascitic fluid samples to be run on Xpert for MTBC diagnosis, in any setting, not limited to an immunophenotypic laboratory. The second aim was to evaluate the sensitivity and specificity of the Xpert assay as compared to the gold standard MTBC culture assays and, thirdly, to assess the utility of the Xpert assay as part of the diagnostic algorithm for fluid samples received in high prevalence MTBC laboratories.

Results showing a sensitivity and specificity of the Xpert assay comparable to those seen in other studies on EPTB samples (see Table 4), would encourage the use of a NAAT as part of the routine diagnostic work-up on fluid samples received in the immunophenotypic laboratory.

2 MATERIALS AND METHODS

2.1 SPECIMENS

Pleural and ascitic fluid specimens received for routine immunophenotypic analysis between August 2012 and February 2013 at the NHLS flow cytometry laboratory in Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) were used in this study. Ethical approval was obtained from the University of the Witwatersrand Human Research Ethics committee (HREC M121010).

All specimens were processed for routine immunophenotypic analysis and specimens with a residual volume >0.5ml were then tested on Xpert (Figure 7). Limited information on each specimen was obtained from the laboratory

information system (LIS), see Appendix 5.3, including the MGIT culture results that were performed by TB referral laboratory of the NHLS (NHLS).

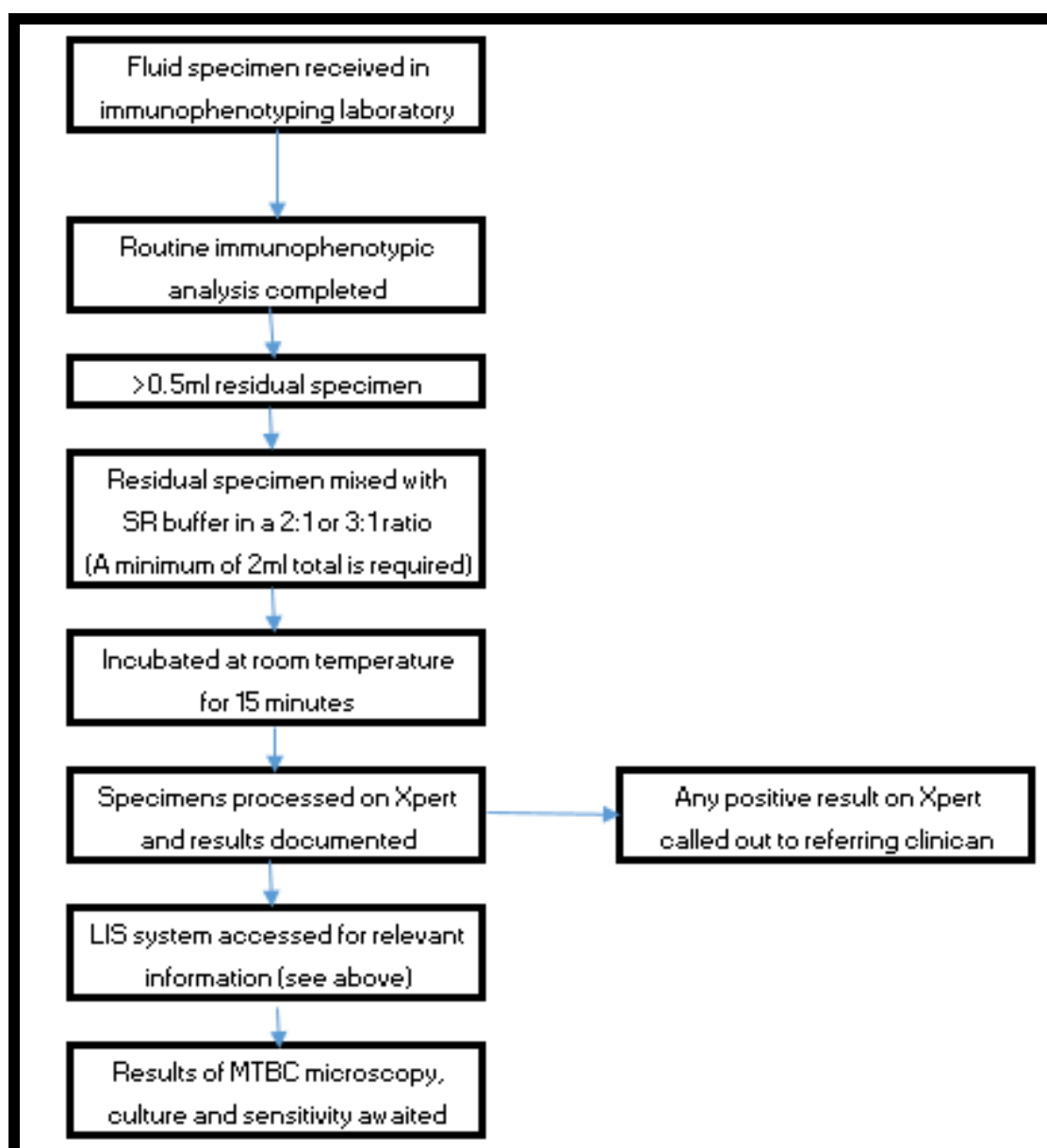


Figure 7 Method algorithm

2.2 EXCLUSION CRITERIA

- I. Insufficient residual specimen (<0.5ml) after routine immunophenotypic analysis.
- II. Samples with diagnosed lymphoma
- III. Samples that were not submitted for the gold standard MTBC culture and sensitivity testing will not be included in the final data analysis to determine sensitivity and specificity of the Xpert assay.

2.3 ROUTINE IMMUNOPHENOTYPIC ANALYSIS

A standard panel for exclusion of B and T-cell lymphoproliferative disorders and non-haemopoietic tumours is performed on the majority of fluid specimens submitted to the NHLS laboratory at CMJAH. This work-up includes the following markers: anti-kappa, anti-lambda, CD19, CD5, CD4, CD3, CD8, and CD45.

Additional markers are added to the fluid specimens if the initial panels detect possible pathology.

2.4 PROCESSING ON XPRT

On completion of routine immunophenotypic analysis, specimens with sufficient residual volume were processed for analysis on the Xpert following the manufacturer's guidelines for pulmonary specimens [46]. Specimens were mixed with solvent-reagent (SR) buffer in a 2:1 ratio (if ≥ 1 ml fluid) or a 3:1 ratio (0.5ml fluid), resulting in a minimum of 2ml total volume. The specimen/buffer mixture was incubated with SR buffer for 15 minutes at room temperature. 2-2.5ml of the specimen/buffer mixture was pipetted off and placed into the Xpert cartridge. This was then loaded into the Xpert for analysis (Figure 7).

The ethics clearance received for the project specified that the treating clinician be notified in all cases that tested positive for MTBC on Xpert. Clinical decisions and future treatment were, however, left to the discretion of the clinician.

3 RESULTS

3.1 INTRODUCTION

The study aimed to develop a protocol for the processing of pleural and ascitic fluid specimens to be run on Xpert for MTBC diagnosis, to evaluate the sensitivity and specificity of the Xpert assay as compared to the gold standard MTBC culture assays and to assess the utility of the Xpert assay as part of the diagnostic algorithm for fluid specimens received in high prevalence MTBC immunophenotypic laboratories.

3.2 OVERVIEW OF RESULTS

All specimens were processed according to the manufacturer's guidelines for pulmonary specimens. Figure 8 and Table 4 provide a summary of the results. During the study period, 392 fluid specimens were received for immunophenotypic analysis in the NHLS flow cytometry laboratory at CMJAH. Just over half (229/392) of the specimens had residual volume for Xpert testing of which 74% (169/229) were pleural fluid and 26% (60/229) ascitic fluid. The residual specimen processed on Xpert was raw, unprocessed specimen. Xpert positivity was detected in 8.7% (20/229) of the total specimens.

Only 43% (99/229) of these specimens were submitted for concurrent MTBC liquid culture (MGIT) by the treating clinician at any point during admission of these patients, based on the LIS history. A 3% (3/99) contamination rate on culture was recorded in these specimens and all of these specimens produced a result on Xpert. Positivity on Xpert was shown in 9% (9/99) of specimens compared to 17% (17/99) on MGIT. One false positive was detected on Xpert.

More than half of the specimens, 57% (130/229) were not referred for concurrent MTBC culture. The Xpert detected MTBC in 8.5% (11/130) of these specimens, with 1 Rifampicin resistant case identified.

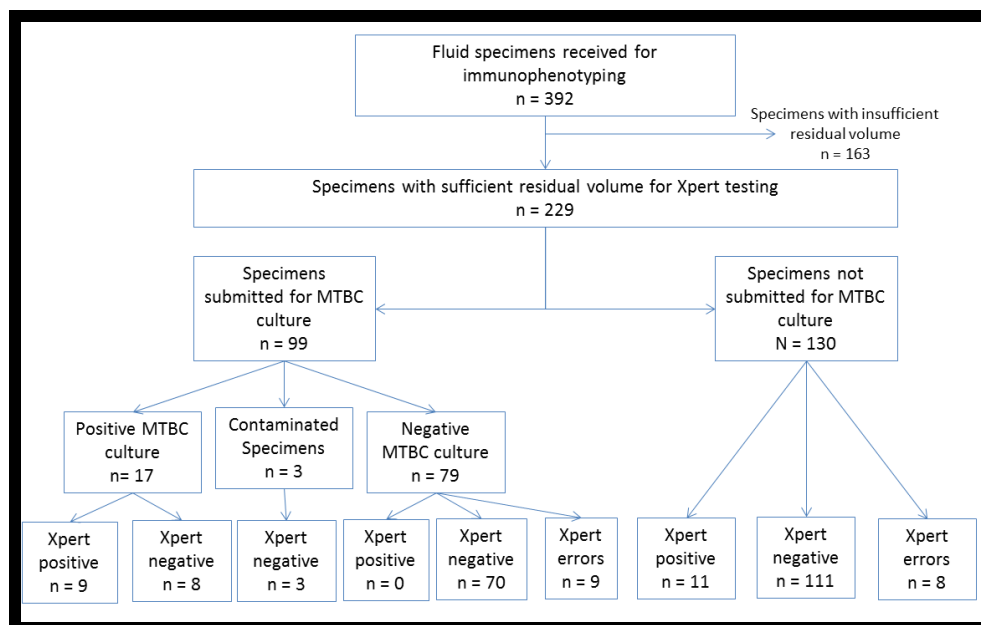


Figure 8 Flow diagram of results obtained

3.3 XPERT ERROR RESULT

The Xpert error rate in this study was 7.4% (17/229) and none of the specimens were repeated on Xpert owing to insufficient sample volume. All of these errors were manufacturer/cartridge error (error code 5006/5007). See Appendix 5.2 for error codes.

3.4 CALCULATION OF THE SENSITIVITY AND SPECIFICITY OF THE XPERT ASSAY

Calculation of the sensitivity and specificity was limited as a result of the low number of specimens submitted for concurrent MTBC culture. See table 5. The sensitivity and specificity of Xpert in this study was 50% (95% Confidence Interval (CI): 26-75%) and 98.5% (CI: 91-100%) respectively. The positive predictive value of Xpert in fluid specimens from patients being investigated for lymphoma and/or TB was found to be 88.9% (CI: 51- 99%).

Table 5 The sensitivity and specificity of the Xpert assay

	<u>Xpert assay (%)</u>	<u>95% Confidence Interval (%)</u>
Sensitivity	50	26-97
Specificity	98.5	91-100
Positive predictive value	88.9	51-99

Table 6 Results table

<u>Xpert assay performance</u>						<u>n(%)</u>
<u>Specimens received</u>						
Fluid specimens received in immunophenotyping laboratory for testing						392
Specimens with insufficient residual for Xpert testing						163(41,5)
Specimens with sufficient residual for Xpert testing						229(58,4)
Pleural fluids						169(74)
Ascitic fluids						60(26)
<u>Specimens submitted for MTBC culture</u>						99(43)
	MGIT Culture	Xpert		Sensitivity (Culture reference)	Specificity (Culture reference)	
Positive n(%)	17(17)	9(9)	}	50% (26,75)	98.5% (91,100)	n = 87
Negative n(%)	79(79)	81(81)				
Contaminated n(%)	3(3)					
Error n(%)		9(9)				
<u>Specimens not submitted for MTBC culture</u>						130(57)
		Xpert				
Positive n(%)		11(8.5)				
Negative n(%)		111(85)				
Error n(%)		8(6)				

4 DISCUSSION

4.1 INTRODUCTION

In 2013, of the estimated 24.7 million people living with HIV in Sub-Saharan Africa, 6.3 million were in South Africa [63, 64]. Furthermore, up to 70% of presumptive MTBC cases in South Africa occur in HIV co-infected patients [13]. The number of cases of MDRTB reported to the WHO has risen steadily in South Africa from 9070 in 2009, to 15 419 in 2012 [5, 6, 18]. In view of the high prevalence of both HIV and TB in South Africa, and the high burden of MDRTB, it is important to identify and initiate the correct treatment timeously.

Current diagnostic methods for the diagnosis of both pulmonary and EPTB present a number of challenges. Contributors to the burden of undiagnosed MTBC in hospitalised HIV positive patients include the patients inability to produce sputum (sputum scarce), smear-negative cases and EPTB [65]. The sensitivity of sputum microscopy ranges from 20-80% as a number of factors (sputum quality and laboratory skill) affect the results [24]. A large percentage of culture positive MTBC cases are negative on sputum microscopy in both HIV negative (40-50%) and HIV positive (87-90%) patients [38]. The gold standard of MTBC culture and drug sensitivity testing is time consuming, resulting in an unnecessary diagnostic delay. Specimens, especially extrapulmonary specimens, are also prone to contamination further affecting the results.

NAATs were therefore introduced into the field of MTBC diagnostics in an attempt to overcome the short comings of the current assays. The Xpert assay targets the *rpoB* gene sequence of the MTBC genome and detects the presence of the MTBC bacilli while simultaneously testing for Rif resistance. The assay is easily run, requiring very little hands-on time, is fully automated and can be used outside of a laboratory setting. Specimen contamination does not impact the assay and the results are available within 2 hours. Xpert is therefore currently endorsed by the WHO as a sensitive and quick method of diagnosing TB in paucibacillary pulmonary TB samples [45]. A WHO meta-analysis of 15 studies on EP samples showed favourable results with the Xpert sensitivity and specificity of 43.7% and 98.1% respectively [52].

Data from previous studies therefore supports the use of Xpert in addition to current diagnostics methods in South Africa to provide a diagnosis in a short amount of time and to enable initiation of the correct treatment, thereby reducing the transmission of MDRTB.

4.2 KEY FINDINGS

This study was performed in the immunophenotyping laboratory of the Charlotte Maxeke Johannesburg Academic Hospital, South Africa. A total of 229 fluid samples from patients in this high prevalence HIV and TB setting were tested in this study.

This first aim of this study was to develop a protocol for the processing of pleural and ascitic fluid specimens to be run on the Xpert. The fluid specimens were processed according to the manufacturer's instructions for pulmonary specimens as no guidelines for the processing of fluid specimens were available at the time of this study. No laboratory preparation was required prior to processing on Xpert and alternative ways of specimen preparation were, therefore, not investigated. As the Xpert instrument is not limited to a Biosafety Level (BSL) 3 laboratory, specimens obtained from a patient suspected to have MTBC infection can also be processed at the bed-side. This will reduce the cost of specimen transportation, the number of specimens misplaced and will allow same-day results. This also allows the use of Xpert to expand into other areas of the laboratory with lower biosafety levels. The possibility of incorporation of routine Xpert testing into diagnostic algorithms in other laboratory tests should be considered.

The second aim of this study was to evaluate the sensitivity and specificity of the Xpert assay on EP fluid specimens as compared with MTBC culture. This was limited by the large number of specimens without corresponding MTBC culture results, an unexpected finding at the start of this study. This study, nonetheless showed that the sensitivity of Xpert compared to MGIT is 50% (CI: 26-75%) and the specificity is 98.5% (CI: 91-100%). This is comparable to other studies performed on fluid specimens showing overlapping CIs and those reported in the WHO meta-analysis [7, 21, 22, 39, 45, 48, 51-60]. The positive predictive value of Xpert in fluid specimens from patients being investigated for lymphoma and/or TB was found to be 88.9% (51, 99 [66]). Furthermore, our study demonstrated an Xpert error rate of 7.4% which is comparable to that seen in previous studies [15, 47].

The third aim of this study was to evaluate the utility of the Xpert assay as part of the laboratory diagnostic algorithm (LDA) for fluid specimens received in the immunophenotypic laboratory. Importantly, this study demonstrates that in a high burden HIV/TB setting like South Africa, more than 50% of fluid specimens referred for immunophenotypic analysis to exclude lymphoma are not referred for concurrent MTBC culture testing by the treating clinician. These patients would have remained undiagnosed for EPTB without Xpert testing in the LDA. Specimens that are referred for concurrent MTBC culture can take up to 6 weeks for a result and are prone to contamination. Xpert testing in these specimens would also be of importance as it would eliminate an unnecessary diagnostic delay and produce a result, despite possible contamination.

4.3 LIMITATIONS OF THE STUDY

Only a limited number of specimens were tested in this study. In addition, of the total 229 specimens tested on Xpert, more than half had not been referred for concurrent MTBC culture. While this was a major finding in itself, it impacted the calculation of the sensitivity and specificity of the assay.

4.4 RECOMMENDATIONS

It would be of value to expand this study to determine if any laboratory based pre-processing of samples, e.g. centrifugation, would result in an improved sensitivity and specificity of Xpert in fluid samples.

4.5 CONCLUSION

This study shows that incorporation of Xpert into the LDA in the immunophenotypic laboratory would have a number of benefits, improving overall patient work-up and care. For implementation and policy uptake to occur, a full costing analysis would be required taking the cost of delayed diagnosis as well as the number of specimens expected into account. Importantly, Xpert testing would be performed in addition to, and not instead of, routine testing.

As a valuable test to be performed in addition to MTBC culture, the Xpert assay has a number of benefits. The lack of specimen preparation and requirements of a Biosafety level 3 laboratory make it ideal for use in both laboratory and hospital/clinic settings.

In South Africa specifically, the current diagnostic work-up of pleural effusions and ascites seems somewhat lacking and there is potential for Xpert to be included in the immunophenotypic LDA. Routine use in other areas of the laboratory, in particular microbiology, should be investigated. The identification of other specimen types referred to the laboratory for analysis, that would benefit from incorporation of Xpert into the LDA, would be of great value as a continuation of this work.

5 APPENDIX

5.1 STANDARD OPERATING PROCEDURE FOR MTBC CULTURE [38]

STANDARD OPERATING PROCEDURE

Title:	Processing of specimens for primary isolation by culture of mycobacteria using NALC-NaOH digestion & decontamination
Document number:	MIC0462
Version number:	2
Written by:	N. Moloi
Checked by:	Laboratory Working Group
Approved by:	N. Beylis

Written: 15-09-2009

INTRODUCTION & PRINCIPLE

Demonstration of acid-fast bacilli (AFB) in a smear made from a clinical specimen provides a preliminary diagnosis of mycobacterial disease, while the isolation of mycobacteria on culture provides a definite diagnosis of tuberculosis or disease due to Mycobacteria Other Than *M.tuberculosis* (MOTT bacilli) [also known as Non-Tuberculous Mycobacteria (NTM)].

As much as 50-60% of AFB culture-positive clinical specimens may fail to reveal AFB on a smear made from the specimen. In addition, in the setting of a high HIV-prevalence such as South Africa, where there is a high rate of HIV-TB coinfection, smears may be positive in only 10-13% of specimens. As a consequence, culture techniques play a key role in the diagnosis of mycobacterial disease.

Egg-based media, such as Löwenstein-Jensen (LJ) or Ogawa have been used for cultivation of mycobacteria for several decades. In 1958, Middlebrook and Cohn described an agar-based medium to permit more rapid detection of mycobacterial

growth. However, it still required an average of 3-4 weeks to recover mycobacteria from clinical specimens.

In 1969, a technique was developed from semi-automated detection of the metabolism of mycobacteria by measuring the $^{14}\text{CO}_2$ liberated during growth and decarboxylation of ^{14}C -labelled substrate incorporated into the growth medium. This radiometric technique was used widely for blood cultures using the BACTEC 460 instrument.

In 1980, this technique was introduced commercially for the mycobacterial recovery from clinical specimens and for susceptibility testing.

The radiometric BACTEC 460 was evaluated in numerous studies compared to solid media for primary isolation and drug susceptibility testing. It demonstrated excellent results with significant time savings especially for smear negative specimens.

The BACTEC 460 system has been reported to yield 15-20% increased culture positivity of clinical specimens compared to conventional solid culture medium such as LJ medium, with an average time to detection of positive growth from 8-14 days as compared to 3-5 weeks on solid media.

The high efficiency of the BACTEC TB System is due to the use of liquid medium. A growth enhancing substance is added to the medium to further reduce the detection time. Since the introduction of BACTEC 460 TB System, it has been established that liquid medium is far superior to solid media for recovery, time-to-detection and drug susceptibility testing. Certain mycobacteria are reported to grow only in liquid media, failing to be detected on solid media.

Liquid media is more prone to contamination with bacteria that are commonly present as normal flora in certain types of clinical specimens and sometimes survive the decontamination process. Thus, the addition of antimicrobials is needed to suppress contamination in liquid media.

An antimicrobial mixture called PANTA (Polymixin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin) is used for this purpose and reduces the contamination rate to that generally experienced with solid media, when used with BACTEC 460 system.

One of the disadvantages of the BACTEC 460 TB System is the use of ^{14}C -labelled radioactive substrate. Because of strict regulations of handling and waste disposal of radioactive material, it became necessary to develop a non-radiometric technique for mycobacterial culture and susceptibility testing. A new system called Mycobacterial Growth Indicator Tube (MGIT), which is non-radiometric and offers the same rapid, sensitive and reliable methods of testing as the BACTEC 460 TB System was developed. BBL MGIT System is the manual system while BACTEC MGIT 960 (MGIT960) is the fully automated system for the detection of mycobacterial growth and drug susceptibility testing of *M.tuberculosis*. Numerous studies have compared the MGIT system with the LJ and Ogawa media and BACTEC 460 liquid system for primary isolation of mycobacteria. Similarly, the MGIT system for drug susceptibility testing has been thoroughly evaluated.

Mycobacteria Growth Indicator Tube (MGIT) contains modified Middlebrook 7H9 broth base. When supplemented with MGIT Growth Supplement and PANTA, it provides an optimum medium for growth of majority of mycobacterial species. All types of specimens, pulmonary as well as extra-pulmonary (except blood), can be inoculated into MGIT for primary isolation of mycobacteria.

Mucoid (e.g. sputum) specimens are expected to contain contaminating bacteria that are part of the normal flora. Thus these specimens must be digested (liquefaction) and decontaminated before inoculation into the MGIT to optimize the yield of mycobacteria.

Aseptically collected body fluids or tissue biopsies, on the other hand, do not need to be decontaminated. However, since it may be difficult to maintain sterile conditions throughout the collection of certain specimens that would be expected to be sterile, these specimens are inoculated onto a blood agar plate and incubated at 37°C before they are inoculated into a MGIT medium. If the bacteria grow on the blood agar plate, that specimen will be decontaminated before inoculation into the MGIT.

Large volume specimens

Clinical specimens collected in large volumes (of more than 10mls) require centrifugation before decontamination to reduce the overall volume and to concentrate mycobacteria present in the specimen into a smaller volume. After decontamination the specimen should be centrifuged again and the sediment used for preparation of the smear and inoculation for culture.

Numerous procedures for digestion and decontamination have been in use throughout the world. Some procedures are known to be compatible with egg-based media only and may not be used with any other medium not containing egg yolk. The N-Acetyl L-Cysteine (NALC) – NaOH combination is the procedure recommended for the MGIT medium. It is extremely important to follow the standard operating procedure for decontamination recommended for MGIT in order to obtain optimal results. Detection of growth in the MGIT is based on an oxygen sensor system, and a high concentration of N- Acetyl L-Cysteine (NALC) or sodium hydroxide (NaOH) may result in false fluorescence.

Principle of digestion and decontamination

NALC-NaOH procedure: this is the standard recommended procedure to be used with MGIT, which is also recommended by CDC. In this procedure, the initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH-NALC-Sodium Citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1%.

Principle of MGIT medium supplement & PANTA

The MGIT (Mycobacteria Growth Indicator Tube) consists of liquid broth medium that is known to yield better recovery and faster growth of mycobacteria. The MGIT contains 7.0ml of modified Middlebrook broth base. This medium is terminally sterilized by autoclaving. An enrichment called MGIT OADC (Oleic acid, Albumin, Dextrose and Catalase) or MGIT 960 Growth Supplement, is added to make the medium complete. The Growth Supplement is essential for growth of many mycobacteria, especially those belonging to *M.tuberculosis* complex. Addition of MGIT PANTA is necessary to suppress contamination.

Principle of detection

In addition to Middlebrook 7H9 liquid medium, the MGIT tube contains an oxygen-quenched fluorochrome, tris(4,7-diphenyl-1, 10-phenanthroline) ruthenium chloride pentahydrate, embedded in silicone at the bottom of the tube. During bacterial growth within the tube, the free oxygen is utilized and is replaced with carbon dioxide. With depletion of free oxygen, the fluorochrome is no longer inhibited, resulting in

fluorescence within the MGIT tube when visualized under UV light, the intensity of the fluorescence is proportional to the extent of oxygen depletion.

MGIT tubes may be incubated at 37°C and can be read manually under UV light (or using a luminometer) or entered into a MGIT 960 instrument where they are incubated and monitored for increasing fluorescence every 60 minutes. Growth of bacteria as well as mycobacteria increases the fluorescence. In case of *M.tuberculosis* growth, at the time of positivity, there are approximately $10^5 - 10^6$ colony forming units (CFU) per ml of the medium. The instrument declares a tube negative if it remains negative for growth for six weeks (42 days). The detection of growth can also be visually observed by the presence of non-homogenous light turbidity or small granular / flaky appearance in the medium. Growth of some NTM (most commonly rapid growers) results in light turbidity, while contaminating bacteria generally produce heavy turbidity.

AIM

The process consists of liquefaction by NALC which frees bacteria and mycobacteria from mucoid specimens followed by decontamination by NaOH which kills the normal bacterial and/or fungal flora allowing the mycobacteria to survive in order to optimize isolation of mycobacteria from those specimens.

MATERIALS

Disposable 50ml plastic (conical) tubes
Sterile NaOH-NALC-sodium citrate solution Sterile Phosphate buffer pH6.8 (0.067M)
Refrigerated centrifuge with a minimum 3000-3500xg force and safety shield
Weighing balance
Vortex mixer, shaker
3ml sterile disposable pipettes
Timer
5lt bin containing appropriate mycobactericidal disinfectant
MGIT medium
MGIT Media Growth Supplement (enrichment) (refer to MGIT procedure manual page 25)
MGIT PANTA (refer to MGIT procedure manual page 25)
Dry, labelled slides that have been cleaned in alcohol
2lt – 5lt dispenser bottles
70% alcohol in a spray bottle
Fixative – DMP Precipitating fluid Alcohol for slides

SAFETY PRECAUTIONS

Processing of specimens must be performed in a class II biological safety cabinet (BSC). A protective gown, gloves and an N95 respirator must be worn at all times when handling or processing specimens. Use an appropriate mycobactericidal disinfectant for cleaning the work area and for discarding leftover specimen.

Prior to use, examine all MGIT tubes for evidence of damage. Do not use any tube that is cracked or has other defects. Do not use a tube if the medium is discoloured, cloudy or appears to be contaminated.

PROCEDURE

1. Preparation of NaOH-NALC-sodium citrate solution:

- a. This is prepared fresh daily. The solution is poured into sterilized glassware (see SOP for cleaning of glassware).
- b. Prepare equal quantities of 4% sodium hydroxide and 2,9% sodium citrate (Prepare only as much volume as can be used in a day)
 - i. Add NALC powder to achieve a final concentration of 0.5% (0,5gNALC powder into 100ml solution)
- c. Example of the working solution: mix equal amounts of sodium hydroxide (1,000ml) and sodium citrate (1,000ml) together and add 10g of NALC to the mixture
- d. At the end of the working day discard the residual

2. Pouring of specimens

- a. If the specimen is not collected in a 50ml centrifuge tube, it must be transferred to a 50ml centrifuge (conical) tube with a screw cap to enable centrifugation of the decontaminated specimen.
- b. General for all specimens
 - i. Pouring of specimen into the 50ml conical tube is always performed in a class II BSC
 - ii. Soak the paper on the floor of the BSC with mycobactericidal disinfectant
 - iii. For each specimen, always check that the PUIN (TTB number) on the universal specimen container is the same as that on the 50 ml conical tube before pouring
 - iv. The 50ml tube should be single use, easily degradable and have easily labelled walls
 - v. Liquid specimens: spin down the specimen in the conical tube before processing. Decant the supernatant and retain the concentrated specimen for processing with NALC-NaOH
 - vi. Thick specimens: extra NALC powder may be added
- c. Pouring according to specimen type
 - i. **Pulmonary/Respiratory specimens** (e.g. sputum, gastric aspirates, bronchoalveolar lavage/washings) and urine, stool (these are considered non- sterile and require decontamination)
 1. Follow the procedure as above in point b.
 2. Take note of the consistency of the specimen. If the specimen is sticking to the sides of the universal container, add 0.5ml normal saline with a sterile pipette to loosen it from the sides.
 3. Remove the cap from the 50ml tube and grip with small finger
 4. Pick up the specimen container and pour into the centrifuge tube. Transfer the entire volume, up to a maximum of 10mls. If thick or mucopurulent, divide the specimen on the rim of 50ml tube
 5. After the specimen has been poured into the conical tube, replace the screw-cap of the 50ml tube and return the conical tube to the rack

6. Replace the cap of the universal specimen container and discard this into the discard plastic bag in the BSC

ii. Extra-pulmonary specimens (“specials”: these are expected to be sterile) e.g.

CSF, FNA, fluids (pleural/peritoneal/pericardial)

1. These are kept in the refrigerator immediately after reception, until they are processed
2. Requirements: Specimen, blood agar plate (B/A) divided into 6 areas, labelled with 6 PUINs
3. Stick the laboratory number in the designated book to register purity results
4. Ensure that the PUIN on the specimen is the same as that on the B/A plate
5. Centrifuge all specimens that are more than 10ml in volume. Use the sediment to inoculate on the blood agar
6. Aseptically remove a sterile plastic loop from the plastic bag
7. Open the specimen container with small finger and hold the lid
8. Return the open specimen to the rack
9. Put a loop into the specimen and mix the specimen
10. Remove a loopful of specimen and inoculate the corresponding blood agar plate
11. Close the blood agar plate
12. Incubate the blood agar plate for 18 hours at 37°C.
13. Read the blood agar after incubation to check for purity
14. Keep the specimens in the fridge for the duration of incubation of the blood agar plate
15. After reading of the blood agar plates
 - a. transfer each specimen for which there is bacterial growth into a labelled 50ml conical tube
 - i. These specimens will be decontaminated following the procedure for primary isolation of mycobacteria for non-sterile specimens
 - b. For those with no bacterial growth, inoculate directly without decontamination

iii. Other specimens

1. Pus / laryngeal / ear swabs
 - a. Transfer the swab into a labelled sterile 50ml conical test tube
 - b. Break off the swab stick so that the centrifuge tube cap can be placed onto it and tightened
 - c. Add 2ml NaOH-NALC solution
 - d. Replace the cap and mix well in a vortex mixer.
 - e. Let stand for 15 minutes.
 - f. Remove the swab by with forceps, squeezing the liquid out of the swab and discarding it.
 - g. Fill the tube with phosphate buffer.
 - h. Mix and centrifuge at about 3000x to 3500 g for 15-20 minutes.

- i. Discard the supernatant fluid and resuspend the sediment in 1-2 ml sterile buffer.
- j. Use this suspension for smear and culture

2. Tissues

- a. Homogenize the tissue in a tissue grinder with a small quantity of sterile water (2-4ml) and sterile sea sand (tip of the spatula).
- b. Large pieces of tissue can be placed in a sterile Petri dish and torn apart with the help of two sterile needles or cut with a sterile blade and transfer into the tissue grinder.
- c. Transfer the homogenized specimen into the sterile 50ml conical test tube
- d. All this work must be done in a biohazard safety cabinet using sterile instruments.
- e. Inoculate the homogenized specimen onto the blood agar to check

3. Milk

- a. Pour the milk specimen into a 50ml conical tube
- b. Concentrate the milk by centrifugation in the 50ml conical tube for 20 minutes at 3000g force
- c. Remove the cream at the top of the tube and place the cream into another conical tube, using a sterile pipette.
- d. Discard the supernatant, leaving the sediment in the original conical tube
- e. Process each conical tube (one with the cream and the other with the sediment) as per decontamination procedure for specimens

3. Decontamination of specimens

a. Non-sterile respiratory specimens

- i. To the conical tube, add NaOH-NALC-sodium citrate solution in a volume equal to the quantity of specimen.
- ii. Tighten the cap of the tube.
- iii. Vortex lightly or hand mix for about 15-30 seconds.
- iv. Invert the tube so that the whole tube is exposed to the NaOH-NALC solution
- v. Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30-35 grams) directly to the specimen tube.
- vi. Mix well by vortexing
- vii. Put the conical tubes on a shaker for 20 minutes (up to 25 minutes maximum)

1. Alternatively, if there is no shaker, vortex or hand mix/invert every 5-10 minutes
- viii. At the end of the 20 minutes (maximum 25 minutes), add phosphate buffer pH6.8 up to the 50ml mark on the 50ml disposable conical tube
- ix. Mix well (lightly vortex or invert several times). (Addition of sterile water is not a suitable alternative for phosphate buffer).
- x. Centrifuge the specimen in a refrigerated centrifuge at a minimum speed of 3000g force for 20 minutes. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria.
- xi. After centrifugation, carefully decant the supernatant into a suitable container containing an appropriate mycobactericidal disinfectant. Ensure there is no delay between centrifugation and decanting as the bacilli will float up into the supernatant and may be discarded with decanting. Make sure the sediment is not lost during decanting of the supernatant fluid.
- xii. Add a small quantity (1-2 ml) of phosphate buffer (pH6.8) and resuspend the sediment with the help of a pipette or a vortex mixer.
- xiii. Use the resuspended pellet for making smears and for inoculation of MGIT tubes

b. Non-sterile non-respiratory specimens

Follow the same procedure to decontaminate extra-pulmonary specimens that are contaminated (few bacteria on the blood agar).

1. Pus and other mucopurulent specimens
 - a. If the specimen is thick or mucoid and less than 10ml in volume, digest and decontaminate with NaOH-NALC method similar to the procedure used for sputum specimens
 - b. If the specimen is not thick, it may be treated with 2-4% NaOH
 - c. The concentration of NaOH depends upon the contaminating bacteria expected to be present in the specimen
 - d. If the volume is over 10-12ml, process only 10ml or first concentrate by centrifugation at 3000x g for 15-20 minutes
 - e. In such a situation, if the specimen is thick, liquefy the specimen by adding a small quantity of NALC only (50-100 mg powder) and mix well
 - f. After the concentration step, resuspend the sediment in 5 ml sterile water, decontaminate with NaOH and concentrate again by centrifugation
 - g. Always resuspend the sediment (pellet) in buffer to reduce the pH
2. Gastric aspirates
 - a. Concentrate by centrifugation before decontaminating

- b. Resuspend the sediment in about 5ml of sterile water and decontaminate with NaOH- NALC or 2-4% of NaOH as recommended for sputum
 - c. After decontamination, concentrate again prior to inoculation of the sediment into culture media.
 - d. Due to the low pH, gastric aspirates should be processed as soon as possible (within 4 hours of collection). If the specimen cannot be processed quickly, it should be neutralized with NaOH before transportation and storage
- 3. Bronchial washings
 - a. All other pulmonary specimens, such as bronchial washings (BAL) may be treated as sputum.
 - b. If the specimen is up to 10ml in volume, process the whole specimen
 - c. For larger volumes, concentrate the specimen by centrifugation (3000x g, 15-20 mins)
 - d. If the specimen is thick or mucoid, liquefy by adding a small quantity of NALC powder (50-100 mg).
 - e. After centrifugation, resuspend the sediment in 5 ml sterile water and decontaminate like sputum
- 4. Laryngeal swabs
 - a. Transfer the swab into a sterile centrifuge tube and add 2ml sterile water (normal saline we use)
 - b. If necessary, break off the swab stick so the cap of the centrifuge tube can be placed on it and tightened.
 - c. Add 2ml of NaOH-NALC solution replace the cap and mix well in a vortex mixer
 - d. Let stand for 15 mins.
 - e. Remove the swab with forceps, squeezing the liquid out of the swab and discarding it
 - f. Fill the tube with phosphate buffer
 - g. Mix and centrifuge at about 3000x to 3500x g for 15-20 minutes.
 - h. Discard the supernatant fluid and resuspend the sediment in 1-2ml of sterile buffer h. Use this suspension for smear and culture
- 5. Tissue
 - a. Tissue biopsies are generally collected aseptically and therefore decontamination procedures are not required.
 - b. In our laboratory, we check the specimens for sterility by plating onto blood agar before processing them
 - c. Homogenize the tissue in a tissue grinder with a small quantity of sterile water (1-2ml)
 - d. All steps must be performed in a BSC and lab equipment must be sterile
 - e. Decontaminate the homogenised specimen following the same NaOH-NALC procedure as sputum.

- f. After resuspension of the sediment with phosphate buffer, inoculate 0.5ml MGIT tube
- g. If the tissue grinder is not available, use a mortar and pestle
- h. Tissue may also be placed in a Petri dish with sterile water (2-4ml) and be torn apart with the help of two sterile needles
- i. Work under the hood and use sterilised materials

6. Urine

- a. Isolation of mycobacteria from urine has not been validated due to a very small number of specimens in BD trials
- b. As a routine isolation method, a totally voided, early morning urine specimen is used for mycobacterial culture
- c. Pooled or midstream urine specimens are not recommended
- d. The specimen is concentrated by centrifugation using several 50ml conical tubes (with screw caps) for at least 20-25 minutes
- e. Resuspend the concentrated specimens with 4% NaOH for 15-20 minutes
- f. After decontamination, proceed in a manner similar to sputum

7. Other body fluids

- a. Body fluids, such as cerebrospinal fluid (CSF), synovial fluid and pleural fluid, are collected aseptically and thus can be inoculated into MGIT medium without decontamination (with the addition of PANTA)
- b. However, since sterility cannot be guaranteed, it is recommended that these specimens be tested for bacterial growth by inoculation onto blood agar media before processing. If they are contaminated, they should be (lightly??) decontaminated
- c. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000-3500x g for 15-20 minutes
- d. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100mg)
- e. After centrifugation, resuspend the sediment in about 5ml of saline and then decontaminate following the procedure similar to that for sputum

8. Blood

- a. Isolation of mycobacteria from blood using MGIT has not been evaluated thoroughly. BACTEC Myco/F Lytic medium is recommended for isolation of mycobacteria (and fungi) from blood samples.

4. Reconstituting PANTA

Enrichment supplement (ODC) with reconstituted PANTA must be added to the MGIT medium prior to inoculation of specimen in MGIT tube

- a. Reconstitute MGIT PANTA with 15ml MGIT growth supplement by adding the contents of the growth supplement into the PANTA bottle: be cautious when reconstituting to avoid contaminating the lid of the PANTA bottle; place the lid onto the towel that is soaked in mycobactericidal disinfectant. Replace the lid of the PANTA bottle.
- b. Mix the PANTA bottle until completely dissolved
- c. Using an Eppendorf pipette, aseptically add 0.8ml of this enrichment into each labelled MGIT tube
- d. Do not add PANTA/enrichment solution to MGIT tubes after the inoculation of specimen
- e. Do not store MGIT tube after the addition of PANT/enrichment solution

5. Inoculation of MGIT medium and preparing smears

- a. Work under the BSC
 - i. With a 2ml syringe drop a drop of fixative onto each microscope slide with sterile technique
- b. Label MGIT tubes with specimen number
- c. Always check that the PUIN on the specimen conical tube is the same as that on the MGIT
to be inoculated and on the slide
- d. Vortex the sediment of the specimen lightly
- e. Use a separate pipette or pipette tip for each specimen
- f. For each specimen, inoculate the medium first: using the 3ml sterile disposable pipette add up to 0.5ml of the concentrated specimen to the appropriately labelled MGIT tube,
- g. Place the pipette into the conical tube
- h. Immediately recap the MGIT tube tightly and mix by inverting the tube several times. Make sure that the tube is tightly closed
- i. Then place about one drop of the pellet from the conical tube on the matching clean
microscope slide
 - i. Spread the smear about 1½ cm x 1cm
 - ii. Allow the smear to air dry completely inside the cabinet
 - iii. Handle the smear carefully since mycobacteria may still be viable
- j. Wipe tubes and caps with a mycobactericidal disinfectant

6. Incubation of the MGIT tubes

- a. Once the batch has been inoculated, remove the batch from the cabinet
- b. Record the batch in the log sheet on the bench
- c. Immediately after inoculation, all inoculated MGIT tubes should be incubated in the
BACTEC MGIT 960 instrument after scanning each tube (refer to the BACTEC MGIT 960
Instrument Manual for details)

- d. It is important to keep the cap tightly closed and not to shake the tube during incubation.
This helps in maintaining the oxygen gradient in the medium
- e. The instrument maintains the 37°C +/- 10°C temperature. Since the optimum temperature for growth of *M.tuberculosis* is 37°C, make sure the temperature is 37°C
- f. If the specimen is suspected of containing mycobacteria which require an optimum temperature other than 37°C [e.g. *M.ulcerans*, *M.marinum*, *M.chelonae*, *M.haemophilum* require 30°C], then 2 sets of media should be inoculated, one in the instrument at 37°C and the other in an outside incubator at 30°C. these tubes can be monitored using a UV light source (Woods lamp) and can also be checked visually. Specimens from skin and open wounds should always be inoculated into suppiculate MGIT tubes, one for 37°C and the other for 30°C
- g. Length of incubation: MGIT tubes should be incubated until the instrument flags them positive. After a maximum period of 6 weeks, the instrument flags the tubes negative if there is no growth. Some species like *M.ulcerans* and *M.genavense* require an extended incubation time. If such species are expected, incubate them for an additional 2-3 weeks

7. Smears

- a. Remove the smears from the cabinet once dry
- b. The smears are to be stained with auramine (refer to SOP MIC0315)

PRECAUTIONS TO BE TAKEN DURING THE PROCEDURE:

1. One of the major sources of contamination in MGIT medium is environmental contaminants introduced during addition of growth supplement.
2. Make all additions inside a biosafety cabinet.
3. Keep the caps of the MGIT tubes closed until ready to make any addition to the medium
4. Do not open more than one MGIT tube at a time.
5. Open the MGIT tube for as short a period as possible
6. The MGIT growth supplement is a sterile product. Handle it aseptically. Do not use it if it appears turbid or if it appears to be contaminated
7. Do not leave MGIT tube caps open after adding OADC supplement
8. Add the growth supplement in the BSC to avoid contaminating the medium
9. A repeat pipettor is very helpful when adding the growth supplement
10. Always recap the tube tightly. If the cap is left loose, it may affect the detection of fluorescence
11. Volumes greater than 0.5ml of decontaminated specimens may disturb the pH of the medium and may cause false fluorescence. This may also increase contamination or otherwise adversely affect the performance of the MGIT medium
12. Specimens stored at temperatures higher than refrigerator temperature for prolonged periods result in an increased number of contaminants – Keep specimens in the refrigerator if they will not be processed within 24 hours
13. Make sure not to use expired reagents.
14. Always use sterile pipettes, sterile and fresh reagents
15. Keep work areas clean, sterile and tidy

16. Always adhere to good laboratory practice

MEASUREMENT OF UNCERTAINTY

a. False positives

1. Cross contamination

- a) formation during handling, vortexing and pipetting of specimens can result in cross contamination which causes false positivity
 - b) open more than one specimen or MGIT tube at a time
 - c) Always use a new sterile and clean pipette for each sample
 - d) Avoid touching the mouth of the MGIT tube with the pipette.
 - e) Use of long pipette tips will help in avoiding this problem
 - f) Always disinfect the cabinet with the appropriate mycobactericidal disinfectant before and after working with each batch of specimens
2. High pH (e.g. due to excess NaOH) may cause transient false fluorescence
 3. Excess NAOH can also delay or inhibit growth. This may cause false fluorescence and even discolour the media (becomes black in colour).
 4. Residual NALC may cause fluorescence. This will usually disappear in 1-2 days

b. False negatives

1. NaOH is bactericidal for contaminating bacteria. It is also harmful for mycobacteria but to a much less extent. Over-decontamination, due to either over-exposure of sample to the decontamination reagents or addition of excessive NAOH will cause false negatives
2. High pH will lower the positivity rate and increase the time to detection of positive cultures
3. The instrument will not read anonymous tubes that are left unattended.
4. Mycobacteria, being hydrophobic, are hard to centrifuge down.
Lower centrifugation speed (g-force) would not sediment mycobacteria very well and some bacteria would be lost during decanting the supernatant, which will affect the positivity rate. Higher centrifugation speeds and longer time (maximum 25 minutes) result in a better concentration of mycobacteria, while positivity affects smear and culture positivity
5. Temperature increases during centrifugation increases the killing effect in mycobacteria which will decrease the positivity rate and increase time-to-detection. A refrigerated centrifuge with at least 3000g force is ideal.
6. Other reagents during the digestion/decontamination step should not be refrigerated but kept at room temperature. Lower temperatures reduce the digestion decontamination process of NaOH-NALC.

QUALITY CONTROL

1. Use of internal controls

- a. These are used to control test the MGIT medium as well as the procedure and

MGIT instrument

- b. Process negative and positive controls along with clinical specimens, using the same digestion, decontamination and concentration methods
- c. Inoculate into fresh MGIT tubes and incubate similarly to other specimens.
- d. The positive control should show positive growth and time-to-detection should be within a specified time with each testing (this is established by data collected from the positive control after several tests).
- e. The negative control should show no growth within the incubation protocol period. If negative control shows positive fluorescence, check for the presence of bacteria/mycobacteria. If positive for growth, investigate procedures and all the reagents for possible source of contamination
 - i. Negative controls
 - a. Always include negative controls with each batch of specimens
 - b. For the negative control, use 5ml of NALC-NaOH-sodium citrate and phosphate buffer
 - c. For the first batch per operator, there are 2 negative controls (reagent only, no clinical specimen), one as the first tube per batch, the other as the last
 - d. For all other batches per operator, per day, include one negative control at the end of the batch
 - ii. Positive controls
 - a. For the positive control use 5 ml of *M. tuberculosis* suspension (McFarland #0.5 turbidity) diluted 1:500 (see SOP for Internal Controls).
 - b. Every new lot of MGIT medium and every new lot of enrichment should be quality control tested by the user upon receipt and before it is used routinely.
 - c. QC strains to be used: The following three mycobacterial cultures are recommended for quality control testing.

<u>ATCC strain</u>	<u>Expected results</u>
<i>M.tuberculosis</i> H37Rv ATCC27294	Tube fluorescence positive in 6 to 10 days
<i>M.kansasii</i> ATCC12478	Tube fluorescence positive in 7 to 11 days
<i>M.fortuitum</i> ATCC 6841	Tube fluorescence positive in 1 to 3 days

- d. If the ATCC reference strains of *M. kansasii* or *M. fortuitum* cannot be obtained, then a laboratory isolate that is well characterized may be used as a quality control strain.
- e. The control suspension is prepared as specified in SOP for Internal controls
- f. If the above criteria are not met, repeat the test. If QC test still does not give satisfactory results, check the viability of the inoculum, age of the culture if stored frozen and other procedures. If everything meets the established specifications, contact Technical Services at
BD Diagnostic Systems
- g. Frequency of positive controls:
 - i. Include a positive ATCC control with one batch per day; vary the operator and document on the tube who the operator is and the date
- iii. Both negative and positive control results are documented at the culture bench when positive and negative MGITs are unloaded.

2. Use good laboratory practice / procedures

- a. With NALC-NaOH digestion, do not agitate the tube vigorously. Extensive aeration causes oxidation of NALC and makes it ineffective
- b. If the specimen has some blood in it, do not use NaOH-NALC method because
NALC does not work in the presence of blood. Use the NaOH method instead

3. Daily cleaning/ housekeeping

- a. Always clean the work area with the recommended mycobactericidal disinfectant before and after working on the surfaces and in cabinets
- b. In the case of spills, notify the bench supervisor immediately and clean all the spills
immediately using recommended mycobactericidal disinfectant according to the spills procedure (see SOPs for disinfectants and for spills)

4. Cross contamination

- a. Cross-contamination of mycobacteria from specimen to specimen is also known in mycobacteriology laboratories.
- b. Usually it happens during the processing of specimens, especially at the time when
a NaOH-NALC solution is added to the specimen or when a buffer is added to the tubes.
- c. Aerosol generation or splashing during the addition causes cross-contamination by
contaminating the next tube or by contaminating the reagent stock solution.
- d. Touching the lip of the specimen tube with the reagent container during pouring or adding of the reagent may also lead to high contamination.
- e. Sometimes stock solution of a reagent gets contaminated with mycobacteria commonly found in water (*M. gordonae*, *M. xenopi*).

- f. Aliquoting small quantities reduce the chances of cross-contamination.
- g. In the event of a cross-contamination episode, all reagents, equipment and biosafety cabinets must be thoroughly checked.

5. Record keeping

- a. Record the lot numbers for MGIT tubes, MGIT OADC, or MGIT Growth Supplement, MGIT PANTA and other reagents
- b. Keep a record of the batch of specimens processed at one time, date of inoculation, person who did the work, time-to-positivity by fluorescence, smear results from positive tube, contamination, etc.

REFERENCES

Adapted from MGIT Procedure Manual, Prepared for the Foundation for Innovative New Diagnostics, Salman H. Saddiqi, Ph.D. July 2006.

5.2 GENE XPRT MTB/Rif ERROR CODES =

INVALID:	Result indicates that the SPC (Internal Control) failed. The PCR was inhibited due to PCR inhibitors (pus, blood or food particles presence).
ERROR 5006/5007/5008:	Result indicates that the Probe Check control failed: This is mainly linked to the sputum viscosity and/or volume; the reaction tube being filled improperly, or probe integrity problem detected.
ERROR 2008:	Pressure exceeds the maximum pressure allowed or GeneXpert module failure. If this happens randomly, this is mostly linked to the sample viscosity.
NO RESULT:	Indicates that insufficient data were collected. For example the test in progress has been stopped voluntarily or due to electrical failure.

5.3 INFORMATION OBTAINED FROM LIS

- Laboratory number (TJG number)
- Specimen type (i.e. pleural or ascitic fluid)
- Name of patient
- Hospital the sample was referred from
- Age of patient
- Gender of patient
- Date of sample collection
- Result of the Xpert®MTB/RIF assay for MTBC detection
- Result of the Xpert®MTB/RIF assay for drug resistance
- Result of the gold-standard MTBC culture and sensitivity testing from the NHLS DISA system (performed at the regional TB referral laboratory)

5.4 ETHICS CLEARANCE CERTIFICATE



UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Dr Kim Kilfoil

CLEARANCE CERTIFICATE

PROJECT

M121010

The Evaluation of the Xpert MTB/RIF in the
Diagnosis of Mycobacterium tuberculosis
Complex and Detection of Rifampicin Resistance

in Extrapulmonary (Pleural and Ascitic) Fluid
Samples Received for Routine Immuno.....

INVESTIGATORS

Dr Kim Kilfoil

DEPARTMENT

Molecular Medicine & Haematology

DATE CONSIDERED

26/10/2012

DECISION OF THE COMMITTEE*

Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE

26/10/2012

CHAIRPERSON


(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor: Dr Elizabeth Prentice

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.

I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. I agree to a completion of a yearly progress report.

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

6 REFERENCES

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