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COMPARISON OF METHODS AND SAMPLES USED IN THE DIAGNOSIS OF CHILDHOOD PTB AND CHARACTERIZATION OF *MYCOBACTERIUM TUBERCULOSIS* ISOLATES

By

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Submitted in fulfilment of the requirements in respect of the Magister in Medical Science, Medical Microbiology degree in the Department of Medical Microbiology and Virology, in the Faculty of Health Sciences at the University of the Free State.

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Declaration

I, **AYODEJI EMMANUEL OGUNBAYO** declare that the master's research dissertation or interrelated, publishable manuscripts / published articles that I herewith submit at the University of the Free State, is my independent work and that I have not previously submitted it for a qualification at another institution of higher education.

I also declare that I am aware that the copyright is vested in the University of the Free State.

I further declare that all royalties as regards intellectual property that was developed during the course of and/or in connection with the study at the University of the Free State, will accrue to the University.

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Ayodeji Emmanuel Ogunbayo

Presentations and Awards

Oral Presentations

Ogunbayo AE, Bulane A, Van Der Spoel van Dijk A. Genotypic and phenotypic characterization of fluoroquinolone-resistant tuberculosis isolates from the Free State province. Free State Provincial Health Research Day 2016.

Ogunbayo AE, Bulane A, Van Der Spoel van Dijk A. Challenges in diagnosing pulmonary tuberculosis in children: A comparative analysis of multiple samples and methods. Health Sciences Faculty Research Forum of the University of the Free State 2017.

Ogunbayo AE. Challenges in diagnosing childhood pulmonary tuberculosis: A better alternative to beat the formidable plague. 3 Minutes Thesis Competition, 2017.

Poster Presentations

Ogunbayo AE, Bulane A, Van Der Spoel van Dijk A. Challenges in diagnosing pulmonary tuberculosis in children: A comparative analysis of multiple samples and methods. Free State Provincial Health Research Day 2017.

Ogunbayo AE, Bulane A, Van Der Spoel van Dijk A. Evaluating the utility of alternative and more easily accessible specimens for the diagnosis of childhood pulmonary tuberculosis Pathological Research Development Congress 2017.

Ogunbayo AE, Bulane A, Van Der Spoel van Dijk A. Challenges in diagnosing pulmonary tuberculosis in children: A comparative analysis of multiple samples and methods. 7th FIDSSA Congress 2017.

Awards

3 Minutes Thesis Competition, 2017: Masters people's choice award.

Free State Provincial Health Research Day 2017: Best poster presentation.

Summary

The diagnosis of childhood pulmonary tuberculosis (PTB) remains an ongoing challenge due to the atypical clinical presentations of the disease. Bacteriological confirmation of PTB and drug susceptibility testing (DST) is imperative in an era of increasing drug resistance, but is seldom achieved in children. This is due to the challenges in obtaining adequate specimens and the low sensitivity of currently available microbiological tests owing to the paucibacillary nature of TB in children as well co-infection with human immunodeficiency virus.

The difficulty in obtaining spontaneously expectorated sputum has necessitated the use of induced sputum or gastric aspirate (GA), which both requires infrastructure and technical expertise. To promote decentralisation and enhance the acceptance of routine specimen collection in children, feasible alternatives (such as stool, urine, and nasopharyngeal specimens) have been proposed. However, operational data on the performance and diagnostic yield of these specimens requires further study.

Furthermore, the occurrence and transmission of *Mycobacterium tuberculosis (M. tuberculosis)* strain families varies by regions and has not yet been documented in children from the Free State. Moreover, since disease progression in children after primary infection mostly occurs within 12 months, genotypic analysis of isolates from children could indicate current transmission patterns of *M. tuberculosis* in a community.

This study aimed to determine and compare the diagnostic yield of various samples [Nasopharyngeal aspirate (NPA), Nasopharyngeal swab (NPS), GA, urine and stool] and methods [smear microscopy, culture and GeneXpert® MTB/RIF (Xpert®)] used in the diagnosis of childhood PTB. Our study further characterise the TB positive isolates with regard to drug resistance using the BACTEC™ MGIT™ 960 System and Genotype® MTBDR*plus,* and strain diversity using spoligotyping and a 24 loci Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats typing.

A total of 126 children with suspected PTB in two hospitals in Mangaung Free State, South Africa, were enrolled in the study. GA, stool, urine and NPA/NPS were collected from each patient. Four children were bacteriologically diagnosed of TB. Two children(1 and 3) were diagnosed only on urine and NPS culture respectively, child 2 on smear microscopy of urine and stool, Xpert® (stool, urine, GA) and culture (stool and urine), and child 4 on Xpert® and culture (GA, urine and stool). Of the remaining children, 18/126 (14.2%) were classified as "unconfirmed TB", whilst 104/126 (82.5%) were classified as 'TB unlikely". DST revealed all the children had a susceptible strain of *M. tuberculosis.* Genotyping showed that child 1 had an X3 strain, child 2 and 4 had a Beijing strain, while child 3 had a T1 strain.

Collectively, our results showed that culture remains the gold standard of diagnosis.. While Xpert® was more sensitive (33%) than smear microscopy (14%), its sensitivity remains suboptimal to culture detecting only 2/4 cases.

The inclusion of alternative specimens was valuable as urine enabled the bacteriological confirmation of TB in 3/4 children compared to GA (2/4). While urine and NPS solely, respectively, allowed the detection of TB in children not detected by routine specimen, stool confirmed the diagnosis obtained by GA. DST result concurred across samples and patients in both assays employed. While the Beijing genotype was a predominant lineage, it was not associated with drug resistance in our study.

Alternative samples outperformed the routine specimen in this study. Although a limitation of this study was the small number of bacteriologically confirmed TB cases, we would suggest at least, the inclusion of urine for routine TB diagnosis in children. However, further studies are required to validate the use of NPS specimen and evaluate other decontamination procedures that can adeqautely prevent the over growth of normal microflora without inhibiting mycobacteria in stool samples. More so, the presence of Beijing strain in 2/4 of the TB positive children raises concern, as Beijing was previously not reported as a predominant strain in the FS population.

Key words: Childhood tuberculosis; Smear microscopy; culture; GeneXpert® MTB/RIF; Gastric aspirate; Stool; Urine; Nasopharyngeal specimens; Drug susceptibility testing; Molecular epidemiology.

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List of Abbreviations

AC	Amplification Control
AFB	Acid-fast bacilli
AM	Amplification Mixes
ART	Antiretroviral therapy
ATL	Animal tissue lysis
BAL	Bronchoalveolar lavage
BCG	Bacille Calmette-Guérin
BSC	Biological safety cabinet
CAS	Central and Middle Eastern Asia
CC	Conjugate Control
CFR	Case fatality ratio
CFU/mL	Colony-forming unit per millilitre
CMI	Cell-mediated immunity
CRISP	Clustered Regularly Interspersed Palindromic Repeats
CT	Computed tomography
Ct	Cycle threshold
DHB	District Health Barometer
DNA	Deoxyribonucleic acid
DR	Direct repeat
DRs	Direct repeats
DR-TB	Drug-resistant TB
DST	Drug susceptibility testing
DS-TB	Drug-susceptible TB
DTH	Delayed-type hypersensitivity
DVR	Direct variable repeats
EAI	East African-Indian
EC	Eastern Cape
ELISA	Enzyme-linked immunosorbent assay
EPTB	Extra-pulmonary TB
EMB	Ethambutol
ETR.Net	Electronic TB register
FDA	Food and drug administration
FDC	Fixed-dose combinations
FFB	Flexible fibreoptic bronchoscope
FQs	Fluoroquinolones
FS	Free State
G+C	Guanine + cytosine
GA	Gastric aspirate
GC	Growth control
GL	Gastric lavage
GP	Gauteng
H	Haarlem
HAZ	Height-for-age z scores
HBCs	High burden countries
HIV	Human immunodeficiency virus
IFN-γ	Interferon-gamma
IGRA	Interferon-gamma release assay
IL	Interleukin
INHMR-TB	INH mono-resistant TB
INH	Isoniazid
INHR-TB	INH resistant TB
IPT	Isoniazid preventive therapy
IS	Induced sputum
IS6110 RFLP	IS6110 restriction fragment length polymorphism

KZN	KwaZulu-Natal
LAM	Latino-American and Mediterranean
LJ	Löwenstein-Jensen
LM	Lipomannan
LP	Limpopo
LPAs	Line probe assays
LSP	Large sequence polymorphism
LTBI	Latent TB infection
M. tuberculosis	*Mycobacterium tuberculosis*
MDR-TB	Multi-drug-resistant TB
MGIT	Mycobacterial Growth Indicator Tubes
MGIT™ 960 System	BACTEC™ MGIT ™960 System
MIC	Minimum inhibitory concentration
MIRU	Mycobacterial intergenic repetition units
MIRU-VNTR	Mycobacterial Interspersed Repetitive Units - Variable Number of Tandem Repeats
MODS	Microscopic observation drug susceptibility assay
MP	Mpumalanga
MTBC	*Mycobacterium tuberculosis* complex
NAAT	Nucleic acid amplification test
NALC-NaOH	N-acetyl-L-cysteine-sodium hydroxide
NaOH	Sodium hydroxide
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NPA	Nasopharyngeal aspirate
NPS	Nasopharyngeal swab
NPV	Negative predictive value
NTM	Non-tuberculosis mycobacteria
NTPs	National TB programmes
OADC™	Oleic acid, albumin, dextrose, catalase
OFX	Ofloxacin
PANTA™	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGL	Phenolic glycolipid
PGRS	Polymorphic GC-rich repetitive sequence
PHCs	Primary health clinics
PIMs	Phosphatidylinositol mannosides
PPD	Purified protein derivative
PPV	Positive predictive value
PTB	Pulmonary TB
PZA	Pyrazinamide
QFT-G	QuantiFERON-TB Gold
qPCR	Real-time polymerase chain reaction
RFLP	Restriction fragment length polymorphism
RIF	Rifampicin
RNA	Ribonucleic acid
RPT	Rifapentine
SA	South Africa
SD	Standard deviation
SI	Sputum induction
SNP	Single nucleotide polymorphism
SOP	Standard operating procedure
ST	String test
T	Default family T
TB	Tuberculosis

TST	Tuberculin skin test
Ultra	Xpert® MTB/RIF Ultra assay
UPGMA	Unweighted pair group method with arithmetic mean
VNTR	Variable number of tandem repetitions
VR	Vital registration
WAZ	Weight-for- age z-scores
WC	Western Cape
WGS	Whole-genome sequencing
WHO	World Health Organization
X	European family
XDR-TB	Extensively drug-resistant TB
Xpert®	GeneXpert® MTB/RIF
ZN	Ziehl-Neelson

List of Symbols

%	Percentage
+	Plus
<	Less than
≤	Less than/Equal to
>	Greater than
≥	Greater than/Equal to
±	Plus/Minus
℃	Degree Celsius
µm	Micrometer
×	Multiply
µL	Microliter
kb	Kilobyte
g	Gram
mL	Millilitres
mm	Millimetres
min	Minutes
nm	Nanometre

Chapter 1: INTRODUCTION

1.1. Background

Tuberculosis (TB) caused by *Mycobacterium tuberculosis (M. tuberculosis)* is a major, but an often underdiagnosed cause of morbidity and mortality in children, predominantly in developing countries (Nachman *et al*., 2015; WHO, 2015a). In 2014, the annual burden of childhood TB was estimated to be 1 000 000 cases with 140 000 deaths, representing 10% and 9% of global TB caseload and mortality, respectively (WHO, 2015a).

Despite the substantial mortality and morbidity rate, childhood TB not until recently has been neglected due to the common discernment that it has no significant contribution to the TB epidemic and that children infrequently develop severe forms of TB (Dodd *et al*., 2014; Seddon and Shingadia, 2014). Also, the global/national TB control program has focused mainly on smear-positive cases, therefore, not on childhood TB, which is mostly paucibacillary and smear-negative (Graham *et al*., 2015b). Consequently, this has led to underdiagnoses and hence underreporting of TB in children (Graham *et al*., 2015b; Nachman *et al*., 2015).

Contrary to previous beliefs and as proven in documented reports and large school community-based outbreaks; children can transmit pulmonary TB (PTB). Besides, they are also at higher risk of disease progression after infection and of developing severe forms of TB disease such as miliary TB and TB meningitis (Batra *et al*., 2012; Elhassan *et al*., 2016; López Ávalos and Montes de Oca, 2012; Nelson and Wells, 2004). Furthermore, childhood TB reflects a recent ongoing transmission in the community and also contributes towards a reservoir from which significant numbers of future adult cases may arise (Batra *et al*., 2012). Childhood TB, therefore requires early reliable diagnosis and adequate prompt treatment (Batra *et al*., 2012; Kalu *et al*., 2015; Tsai *et al*., 2013).

In adults, PTB cases are often easily recognisable by the typical symptoms, radiological features and are mostly bacteriologically confirmed by positive sputum staining (López Ávalos and Montes de Oca, 2012). Childhood PTB is more difficult to diagnose due to varying atypical radiological features, non-specific clinical presentations, inability to expectorate sputum, paucibacillary nature and the presence of human immunodeficiency virus (HIV) co-infection (López Ávalos and Montes de Oca, 2012; Reither *et al*., 2015). As a result, definite and timely diagnosis is seldom achieved (Reither *et al*., 2015; Venturini *et al*., 2014).

In the absence of bacteriological confirmation (the Gold standard for the definitive diagnosis of TB), childhood TB diagnosis relies on a tuberculin skin test (TST) and other non-specific and subjective markers including failure to thrive, chest X-ray suggestive of PTB, with reduced diagnostic accuracy (Berti *et al*., 2014; Reither *et al*., 2015). While bacteriological confirmation is required for definitive diagnosis, additional challenges are often inadequate quality and low quantity of specimen yield

(Jenkins *et al.*, 2014). More so, less than 15% of the TB infected children are sputum acid-fast bacilli (AFB) smear-positive, and mycobacterial culture yield rarely exceeds 30-40% (Gous *et al.*, 2015). Consequently, this not only hampers bacteriological confirmation of childhood PTB but also complicates the diagnosis of drug-resistant TB (DR-TB) in children (Gous *et al.*, 2015; Jenkins *et al.*, 2014).

Furthermore, DR-TB is on the rise globally, and children are as vulnerable as adults but less likely to be counted as cases of DR-TB (Becerra and Swaminathan, 2014). Investigation of multi-drug-resistant TB (MDR-TB) in children is limited, and no routine surveillance data on MDR-TB among children exist globally or in South Africa (SA) (Garcia-Prats *et al.*, 2015; Guo *et al.*, 2016; Velayutham *et al.*, 2015). A study by Yuen and colleagues, involving 189 countries, SA inclusive, reported the absence of publications documenting child MDR-TB and extensively drug-resistant TB (XDR-TB) cases in settings where MDR and XDR-TB in adults were reported. This signals under-detection of children with MDR-TB and also prevents advocating for better childhood TB diagnosis (Yuen *et al.*, 2015b).

Considering these facts, it is imperative to further assess other approaches such as using various specimens and testing methods to ensure early definitive bacteriological diagnosis and/or confirmation of PTB in children (Elhassan *et al.*, 2016; Kalu *et al.*, 2015). More so, the bacteriological confirmation of *M. tuberculosis* in children will facilitate the prompt determination of DR-TB in this population (Moore *et al.*, 2015; Velayutham *et al.*, 2015).

Additionally, successful isolation of the *M. tuberculosis* bacteria would enable the characterisation of the dominant and/or circulating *M. tuberculosis* strain/s in the children population within different geographical regions (Middelkoop *et al.*, 2015; Schaaf *et al.*, 2014). Moreover, since disease progression in children after primary infection mostly occurs within 12 months, the genotypic analysis could further indicate current transmission patterns of *M. tuberculosis* in a community (Marais *et al.*, 2006a; Wootton *et al.*, 2005).

1.2. Problem Statement

The diagnosis of PTB in children is an ongoing challenge due to non-specific characteristics, the paucity of TB disease and HIV co-infection (Dorman, 2015; Reither *et al.*, 2015). A definite diagnosis of PTB is defined as microbiological confirmation of the disease which is still the gold standard of diagnosis (Connell *et al.*, 2011; WHO, 2015a). However, this is rarely achieved in children due to the difficulty in obtaining specimens, the poor performance of smear microscopy/culture and the perception that microbiological yield is low (Chatterjee and Pramanik, 2015). Hence, many children are empirically diagnosed based on unreliable clinical characteristics; consequently, leading to either over-diagnosis or delayed diagnosis (Anderson *et al.*, 2014; Chatterjee and Pramanik, 2015; Perez-Velez

and Marais, 2012). Overdiagnosis contributes to inappropriate treatment of childhood TB and/or a poor outcome, while delayed diagnosis is associated with increased morbidity and mortality rates (Chatterjee and Pramanik, 2015; Pai and Schito, 2015).

However, studies have confirmed that "on collection of adequate specimen", bacteriological confirmation and molecular detection of PTB in children is feasible and possible, even in infants and younger children (Connell *et al.*, 2011). The importance of bacteriological confirmation is the possibility to reach a definitive diagnosis and perform drug susceptibility testing (DST) to diagnose or exclude any form of DR-TB (Dunn *et al.*, 2016).

In an era of increasing DR-TB, SA ranks 10th on the list of high burden countries (HBCs) in terms of absolute number and rates of MDR-TB (WHO, 2015b), yet the magnitude of DR-TB among South African children remains unknown (Moore *et al.*, 2015). A better knowledge of the epidemiology of PTB, DR-TB in children is desired as it can guide appropriate therapy and inform whether programmatic and clinical practices meet the needs of children (Moore *et al.*, 2015).

While the bacteriological confirmation of PTB and DST in children is increasingly important and desirable, difficulty in obtaining good quality specimen is considered the first hindrance (Raizada *et al.*, 2015). The currently recommended standard specimen types for assessing PTB in children are expectorated or induced sputum (IS) and a gastric aspirate (GA) (WHO, 2014a). Usually, expectorated sputum cannot be voluntarily produced by infants and young children but is sometimes attempted in older children (Khan and Starke, 1995; Zar *et al.*, 2000). The collection of GA requires a fair amount of technical expertise and fasting, which often results in overnight hospitalisation and may preclude the use of the method in low resource settings (Marais and Pai, 2006; Zar *et al.*, 2005). Despite multiple reports on the safety and feasibility of sputum induction (SI) even in primary care settings, it is safe only in clinically stable children (Detjen and Walters, 2016). More so, its routine implementation often faces challenges, including the need for equipment, electricity, and a level of expertise in handling possible rare adverse effects (Detjen *et al.*, 2015; Detjen and Walters, 2016).

To enhance the acceptance of routine specimen collection from children in clinical settings, feasible alternatives such as stool, urine, and nasopharyngeal specimens were proposed; as different combinations of specimens have a variable impact on total yield (Detjen and Walters, 2016). Data further suggest that the use of alternative specimens to diagnose PTB have the potential to either replace or complement standard specimen, in both HIV-infected and uninfected children (Detjen and Walters, 2016; Thomas *et al.*, 2014). Consequently, further studies are required to evaluate the feasibility and bacteriological diagnostic yield of these specimens (Detjen and Walters, 2016; Marcy *et al.*, 2016; Thomas *et al.*, 2014).

1.3. Proposed Research Questions

What are the best combinations of existing diagnostic tools and specimens to enhance early diagnosis of PTB and DR-TB in children?

What is the incidence of PTB and DR-TB in children suspected of PTB admitted to selected public hospitals in Mangaung, Free State (FS) Province?

What is the lineage distribution of *M. tuberculosis* among children with TB in the FS, and is there an association between these lineages and DR-TB?

1.4. Research Aim

To determine and compare the diagnostic yield of various samples and methods used in the diagnosis of childhood PTB.

1.5. Research Objectives

To collect GA, nasopharyngeal aspirate (NPA)/nasopharyngeal swab (NPS), stool and urine samples from children (≤13 years) suspected of having PTB from Pelonomi and Botshabelo Hospitals, Mangaung Metropolitan Municipality, FS Province, SA.

To perform *M. tuberculosis* diagnosis on the collected samples using smear microscopy, culture via BACTEC™ MGIT™ 960 System (MGIT™ 960 System) and GeneXpert® MTB/RIF (Xpert®).

To perform DST for rifampicin (RIF) and isoniazid (INH) on *M. tuberculosis* culture-positive isolates using the MGIT™ 960 System and the Genotype® MTBDR*plus* assay to determine the proportion of DR-TB among children with confirmed PTB.

To perform genotypic analysis of *M. tuberculosis* positive isolates using spoligotyping and a 24 loci Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats (MIRU-VNTR) to determine the *M. tuberculosis* strain diversity in the children population.

Chapter 2: LITERATURE REVIEW

2.1. Introduction to Tuberculosis, Epidemiology and Public Health

2.1.1. Historical Perspective

TB is an ancient scourge that has claimed innumerable victims and has been afflicting humanity for centuries (Daniel, 2006). Over the years, various cultures gave the disease names such as consumption, phthisis pulmonalis and the white plague (Shet, 2012). It is believed that *M. tuberculosis* has been around for over 15 000-20 000 years, but the definitive evidence is available from about 10 000 years ago (Shet, 2012).

The theory of TB took a new turn in 1882 when Robert Koch showed that tubercle bacilli are the agents responsible for the disease (Daniel, 2005). Visualisation of the organism was complicated due to its distinctive protein coat until Ziehl-Neelson (ZN) stain was discovered (Hershkovitz *et al*., 2008). In the nineteenth century, sanatoriums were established, where TB patients were treated with rest and improved nutrition (Daniel, 2006; Frith, 2014). Even though this helped many sufferers, it still wasn't effective in curing the disease (Frith, 2014). This led to the development of the now accessible Bacille Calmette-Guérin (BCG) for vaccination in children (Sakula, 1983). This vaccine was created by serial passage of *Mycobacterium bovis* in the laboratory to create an attenuated vaccine strain, which has been widely used since 1921 (Daniel, 2005).

Before discovering the therapeutic effect of antibiotics against *M. tuberculosis*, surgical treatment procedures involving draining pleural effusion from around the lungs were employed (Shet, 2012). Years later, para-aminosalicylic acid was discovered which was disappointing due to only having a bactericidal effect (Daniel, 2006). However, the discovery of more effective drugs like INH, pyrazinamide (PZA), and RIF followed; leading to the new age of TB treatment (Frith, 2014). It led to the dawn of public health care, and TB treatment was gradually extended even to those having latent TB infection (LTBI). Initiating treatment to cure every afflicted person became the aspired objective (Daniel, 2006).

2.1.2. Tuberculosis

TB is an airborne transmitted infectious disease caused by the bacillus *M. tuberculosis*; an intracellular parasite that can affect almost any tissue or organ of the body but mostly affects the lungs (Daniel, 2006). *M. tuberculosis* is primarily a human-adapted pathogen and belongs to the genus *Mycobacterium* which includes a diverse group of organisms with various environmental and animal reservoirs (Glaziou *et al*., 2015; Moore *et al*., 2009). TB is a major global health problem that causes ill-health among millions of people each year and ranks alongside HIV as the second leading cause of death due to infectious disease worldwide (WHO, 2015a). Although research in the past years has

added productive insight into the TB epidemic, much remains to be uncovered in order to effectively reduce the incidence, prevalence, mortality and ultimately eradicate TB (Fogel, 2015).

2.1.3. Classification of *Mycobacterium tuberculosis*

The genus *Mycobacterium* consists of more than 150 species. However, for diagnostic and treatment purposes, these species are classified into *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium leprae* and non-tuberculosis mycobacteria (NTM) (Joao *et al*., 2014; Ng *et al*., 2014).

MTBC comprises of species known to cause human and animal diseases including *M. tuberculosis, Mycobacterium africanum, Mycobacterium canetti, Mycobacterium bovis Bacillus* Calmette–Guérin (culture-adapted strain), *Mycobacterium bovis, Mycobacterium pinnipedii, Mycobacterium caprae, Mycobacterium orygis, Mycobacterium mungi, Mycobacterium suricattae, and Mycobacterium microti* (Clarke *et al*., 2016; Sinha *et al*., 2016). These members share higher than 99% genetic similarity at the nucleotide level with an identical 16s ribonucleic acid (RNA) sequence but differ notably in their host tropisms, geographic distribution and pathogenicity (Brosch *et al*., 2002; Huard *et al*., 2006).

NTM species of medical importance include amongst others: *Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium kansasii, Mycobacterium fortuitum, Mycobacterium chelonae, and Mycobacterium scrofulaceum.* These group of bacteria may cause human disease but do not cause TB (Gopinath and Singh, 2010).

2.1.4. Characteristics and Morphology of *Mycobacterium tuberculosis*

M. tuberculosis is a relatively large obligatory aerobic, nonmotile, rod-shaped bacterium (Pfyffer, 2007). The rods (bacilli) are 2-4 μm in length and 0.2-0.5 μm in width, non-sporulating with no capsules and were previously thought to lack the production of toxin until the discovery of a C- terminal domain of CpnT named tuberculosis necrotising toxin (Hett and Rubin, 2008; Sun *et al*., 2015). *M. tuberculosis* is a facultative intracellular parasite with a slow generation time of 15 to 20 hours, which according to Todar may contribute to its virulence (Todar, 2005).

Mycobacterium species is not classified as either Gram-positive or Gram-negative but is considered more related to Gram-positive bacteria due to the presence of peptidoglycan in its cell walls and may thus stain very weakly Gram-positive (Todar, 2005). It is also referred to as an acid-fast bacterium owing to the presence of a thick mycolic acid structure with the ability to retain the primary stain during the decolourisation step with acid alcohol during ZN staining (Tu *et al*., 2003; Iseman, 2000).

Microscopically, *M. tuberculosis* cells may appear as either straight or curved rods as previously stated and may also appear as distinctive serpentine cords in liquid medium (Tu *et al*., 2003). On solid media,

the *M. tuberculosis* bacterial colonies appear rough with or without pigmentation (Cole, 2002). Pigmented colonies are yellow, orange (rarely pink) due to the carotenoid pigments (Pfyffer, 2007).

The cell wall of *M. tuberculosis* comprises of peptidoglycan and a high content of complex lipids (>60%) composed of mycolic acid, cord factor and wax-D (Alderwick *et al.*, 2007; Brennan, 2003; Todar, 2005). The cell wall is further divided into two layers; The upper layer consisting of free lipids linked to fatty acids, and the lower layer containing peptidoglycan which is covalently linked to arabinogalactan and mycolic acid, resulting in the mycolyl arabinogalactan-peptidoglycan complex (Brennan, 2003; van Soolingen *et al.*, 1997). Also, interspersed in the cell wall are lipoarabinomannan, phthiocerol containing lipids, phosphatidylinositol mannosides (PIMs), lipomannan (LM) and proteins (Brennan, 2003). These distinctive features of the *M. tuberculosis* cell wall which is unique for mycobacterial species call for essential laboratory considerations during specimen staining, culturing in media, and when determining species identification by molecular methods (Caulfield and Wengenack, 2016).

2.1.5. Global Epidemiology of Childhood Tuberculosis

2.1.5.1. Challenges to Estimating Disease Burden in Children

In many TB endemic regions, estimating the burden of childhood TB poses considerable challenges due to diagnostic uncertainties (Graham *et al.*, 2014; Seddon *et al.*, 2015). These uncertainties arise from difficulty in obtaining adequate samples from children for diagnosis, highly variable presentation of the disease and elusive microbiologic confirmation due to paucibacillary nature of TB in children (Graham *et al.*, 2014; Ki and Shingadia, 2017; Seddon *et al.*, 2015). These challenges are however more prominent in infants and young children <5 years of age (Marais *et al.*, 2004b).

Difficulty in obtaining bacteriological confirmation has been noted to further exacerbate the under-diagnosis of DR-TB in children, with many pediatric cases of DR-TB undiagnosed and as such inappropriately treated (Becerra and Swaminathan, 2014; Perez-Velez and Marais, 2012). Clinical and autopsy studies demonstrated that, in TB endemic regions, many cases of childhood TB are misdiagnosed as severe acute pneumonia, further delineating the challenges of accurately estimating the burden of childhood TB (Oliwa *et al.*, 2015). In addition to diagnostic difficulties, a significant challenge to estimating the burden is inadequate routine recording and reporting of children with TB (Ki and Shingadia, 2017). Not until recently, most National TB Programmes (NTPs) were only obligated to report sputum smear-positive TB cases, and children were reported in a broad age group of 0-14 years, leading to the misperception of a low childhood TB burden (Seddon *et al.*, 2015). NTPs are currently required to report all TB cases by two age categories for children (0-4 years and 5-14 years) (WHO, 2006). Notwithstanding, NTPs can report only data for children registered with the NTPs at

the onset of diagnosis, as such a large proportion of children are treated for TB but are not registered with the NTPs (du Preez *et al*., 2011; Lestari *et al*., 2011).

It is further suggested that the lack of TB diagnosis and management in many childhood HIV and malnutrition programs (two conditions known as significant risk factors for childhood TB), inadequate resources for active case finding in most regions, poor case ascertainment and minimal children/pediatric surveillance reporting has contributed to the notable challenges of accurately estimating the burden of childhood TB (Bacha *et al*., 2017; Ballif *et al*., 2015; Bhat *et al*., 2013; Marais and Graham, 2016).

2.1.5.2. Significance of Estimating the Burden of Childhood Tuberculosis

Jenkins defined burden as "a non-specific term measuring the impact of a health problem regarding financial cost, mortality, morbidity or other indicators". He further highlighted that in the absence of an accurate measure of disease burden, it would be impossible to identify gaps in case identification, estimate required resources to reduce the burden or plan and measure the impact of possible interventions (Jenkins, 2016).

Besides the need to advocate for childhood TB, which has been previously neglected (Marais and Schaaf, 2010), reliable estimation of the childhood TB incidence may help identify frail links in the course of symptoms through presentation to diagnosis, treatment and notification. Evaluation of these links may result in interventions that can augment better case detection and reporting (Seddon *et al*., 2015). More so, since TB in children reflects current transmission patterns, and disease progression is mostly within 12 months; childhood TB can provide insight into the strains of *M. tuberculosis* that are currently circulating in a community (including DR-TB strains) (Newton *et al*., 2008). As such, estimation of TB incidence in children can reflect local transmission rates, thus, serves as a general potential indicator for TB control (Shingadia and Novelli, 2003).

The END TB Strategy has the specific target of reducing global TB incidence and mortality by 90% and 95% respectively by the year 2035 (WHO, 2016a). Accurate baseline numbers and trends in incidence and mortality rates over time can enable progress monitoring and assessment of these target goals (Jenkins, 2016; Seddon *et al*., 2015).

2.1.5.3. Incidence of Childhood Tuberculosis

Until recently, the World Health Organization (WHO) did not publish separate childhood TB estimates. This was partly due to difficulties in interpreting notification data for children, unavailability of notifications disaggregated by age from many countries and also owing to official figures for TB incidence being based on smear and culture-positive cases (Seddon *et al*., 2015;

Tebruegge *et al.*, 2015). However, in 2012, the WHO published their first estimate of global annual childhood (<15 years) TB incidence of 490 000, assuming equal ratio of notified to incident cases for adults and children (WHO, 2012).

Subsequently, in 2014, given increasing attention on childhood TB, three estimates of childhood TB incidence were published. Jenkins *et al.* estimated an approximately 1 000 000 incident cases of childhood TB in 2014 (Jenkins *et al.*, 2014). Their estimate was achieved by maximising the age-disaggregated smear-positive cases reported to the WHO to make up for the significant difference that exists between the percentage of adult and children smear-positive cases (Jenkins, 2016). Also, using a mathematical model, Dodd *et al.* estimated that there were 850 000 global incident cases of childhood TB in 2014 (Dodd *et al.*, 2016). Using a combination of both the method of Dodd *et al.* and Jenkins *et al.* (WHO, 2015c), WHO published an estimate of 1 000 000 (range 900 000–1 100 000) incident cases of childhood TB; equivalent to about 10% of the total number of 9 600 000 incident cases that occurred in 2014 (WHO, 2015a).

Of particular concern is that, of these 1 000 000 estimated pediatric incident TB cases, only 359 000 cases were notified to the WHO, suggesting that more than half of the children with active TB disease in 2014 were not notified (WHO, 2015a). It is therefore probable that these children were not diagnosed and as such did not receive treatment (Jenkins, 2016). Estimating the proportion of these invisible children is critical to raising awareness for childhood TB and may influence the numbers of children who are diagnosed, treated and notified to NTPs (Chiang *et al.*, 2015).

The recent edition of the WHO TB report shows that 1 000 000 children (male: female ratio 1.1-0.9) aged 0-14 years fell ill with TB in 2015 (about 10% of the total caseload; similar to that of 2014), with South East Asia and Africa bearing 40% and 31% of the cases respectively (WHO, 2016b, 2016c). Nevertheless, even though there was a 6.35% increase in the global childhood TB case notification (WHO, 2016c), emphasis remains that global statistics still understates the actual burden of TB in children (Seddon and Graham, 2016; WHO, 2016b).

2.1.5.4. Prevalence of Childhood Tuberculosis

An update from the Global TB programme revealed that there is currently no global data available on the prevalence of childhood TB. Considering the current design of national prevalence surveys to estimate PTB, it is considered that including children in a survey would not give a precise prevalence estimate since only a few bacteriologically-confirmed cases would be found. More so, there are ethical considerations regarding mass screening of all children, most of whom are considered otherwise healthy. Considering these factors, and the performance of existing screening and diagnostic tools, including children in the current design of national PTB prevalence surveys is currently not

recommended. Rather, resources are geared towards strengthening surveillance systems to identify incident cases and mortality among children (Sismanidis *et al*., 2015).

2.1.5.5. Mortality in Childhood Tuberculosis

Given the limited number of children bacteriologically diagnosed with TB, quantifying mortality in children with TB presents a unique challenge (Jenkins *et al*., 2017). Previous mortality estimates include the WHO global estimate of 136 000 annual TB deaths among children in 2014 (WHO, 2015a), while an alternative independent approach also estimated about 60 000 deaths among HIV negative children in 2014 (Murray *et al*., 2014). The recent edition of the WHO report estimated 210 000 deaths among children including 40 000 deaths among HIV-infected children (WHO, 2016c).

The WHO estimates is based on data from vital registration (VR) systems and mortality surveys. VR deaths have several limitations. For example, deaths associated with TB and pneumonia may be attributed to only pneumonia as the cause of death, especially if only one cause of death is allowed, despite multiple contributory factors (Graham *et al*., 2014; WHO, 2016b). Measuring TB mortality among HIV-uninfected people is contingent on substantial VR systems coverage and accurate coding of cause of death according to ICD-10. Most high TB burdened countries lack national VR systems, with only a few having conducted mortality surveys. Even with VR systems, quantifying mortality among HIV-infected people is still challenging since death among HIV positive people are coded as HIV deaths and contributory causes such as TB are mostly not recorded (Graham *et al*., 2014; WHO, 2016b). Also, many countries lack the resources to carry out autopsies, hence, many causes of deaths may not be recorded (Vapattanawong and Prasartkul, 2011).

In the absence of VR systems or mortality surveys, TB mortality can be estimated as the product of TB incidence and the case fatality ratio (CFR) (WHO, 2016b). As such, a recent systematic review and meta-analysis by Jenkins *et al*. quantified CFR among children with TB. They compared childhood mortality between the pre-treatment era and modern times. Their comparison suggests that in the pre-treatment era, the pooled CFR was 21·9% and was significantly higher in children aged 0–4 years than in those aged 5-14 years (43·6%). By contrast, in studies since 1980, in which most included children that had access to treatment, the pooled CFR was 0·9% and only 2·0% in children aged 0-4 years (Jenkins *et al*., 2017). In view of this study by Jenkins and colleagues, Starke Jefferey highlighted a disturbing fact from the WHO 2016 Global Tuberculosis Report, where the pooled CFR of 22% was reported for children <14 years. This is almost identical to that determined by Jenkins and colleagues in the pre-chemotherapy era (Starke, 2017).

The reality is that a significant proportion of childhood TB cases are not being detected. As a result, many deaths are often misclassified as pneumonia, meningitis, HIV or malnutrition deaths (Graham

et al., 2014; Marais, 2016; Starke, 2017). Therefore, estimating the actual contribution of childhood TB deaths that have been attributed to other causes is a challenge (Graham *et al*., 2014).

2.1.6. Situation in South Africa

2.1.6.1. Epidemiology of Tuberculosis

The recently published lists of high TB burdened countries reveal that SA ranks in the 30 high TB burden countries, the 30 high MDR-TB burden countries and the 30 high HIV/TB co-infection burden countries (WHO, 2016b, 2015b). About 450 000 new TB cases (incidence rate of 834/100 000 population) were estimated in 2014, of which only 318 193 cases were notified and prevalence was estimated at 696/100 000 population (WHO, 2015a). Of concern is that the unreported 131 807 cases constitute the missed cases that may sustain the TB burden, with each infectious case estimated to infect between 11 and 22 people per year (Wood *et al*., 2011).

The WHO 2014 report further shows a mortality rate of approximately 96 000, with 75% of these deaths occurring in TB/HIV co-infected patients (WHO, 2015a). The 2015 report is relatively similar to that of 2014 with an incidence and mortality rate of 834/100 000 population and 98 000 TB deaths respectively (WHO, 2016b). Notably, TB is a significant cause of death among people in the economically active age group and is the leading cause of deaths among youth aged 15-35 years; responsible for 10 962 in 2013 (StatsSA 2015). TB also ranks as part of the ten underlying causes of death in all age groups and was the leading cause of death among people 15-44 years and 45-64 years; constituting 12,4% and 8,9% of deaths respectively in 2015 (StatsSA 2017).

As noted, there has been an increase in the detection rate of MDR-TB which may be linked to improved diagnostics and recording or partly due to increasing direct transmission of resistant strains between individuals; with 19 613 and 1 024 laboratory confirmed cases of MDR-TB and XDR-TB respectively in 2015 (WHO, 2016b).

Furthermore, even though both prevalence and incidence rates have declined in SA since 2009, as seen in figure 2.1; the rate of decline has been slow. The slow trend can be compared to the global trend, with a decline rate of 1.5% per annum between the year 2000 and 2013 (Murray *et al*., 2014).

Previous estimates also gave an insight breakdown into the burden of TB in the different provinces of SA. As seen in Figure 2.2, KwaZulu-Natal (KZN), Eastern Cape (EC) and Western Cape (WC) are the three provinces with the highest incidence of TB with 922; 782 and 730 cases per 100 000 population respectively. Mpumalanga (MP) at 467 cases per 100 000; Gauteng (GP) at 388/100 000 and Limpopo (LP) 354/100 000 are the three lowest ranking provinces in terms of TB incidence (Massyn *et al.,* 2014).

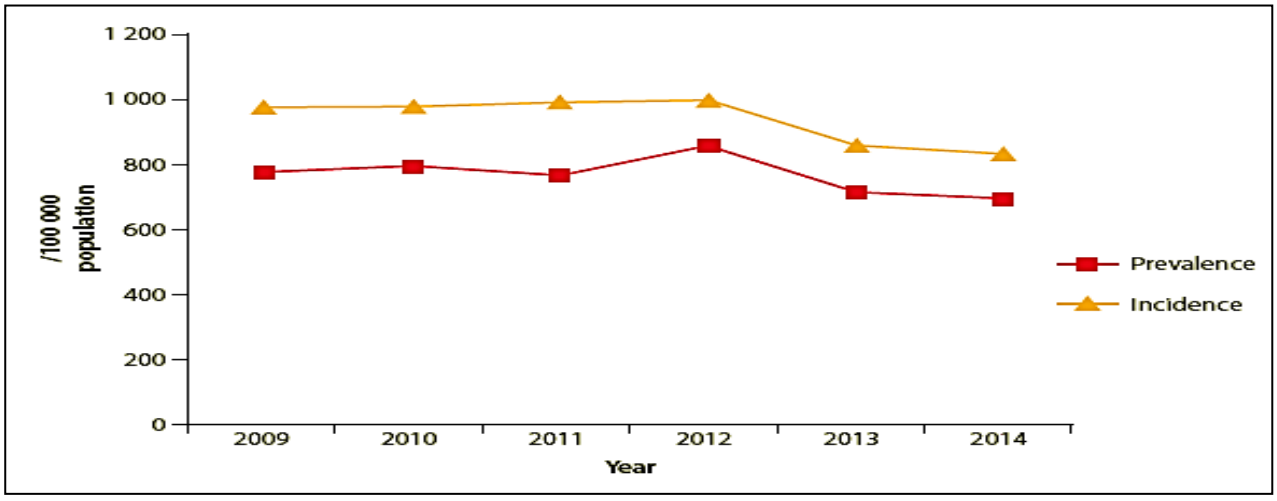

Figure 2.1. Tuberculosis prevalence and incidence estimate in South Africa from the year 2009–2014 Adapted from Moyo and Rehle 2017.

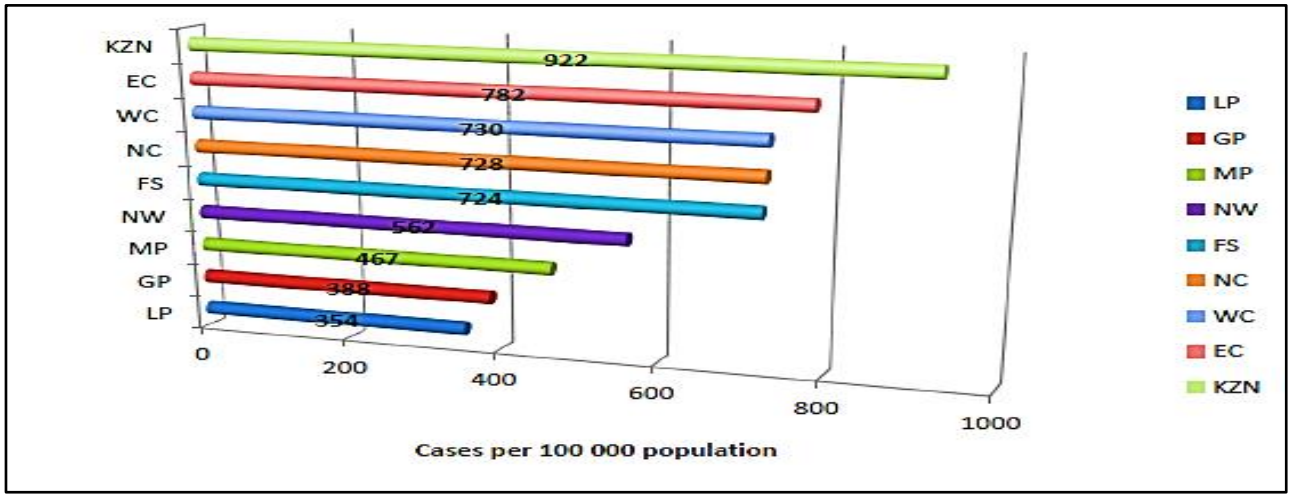

Figure 2.2. The incidence of tuberculosis in South Africa by province. Estimates for the year 2013. Adapted from Massyn *et al.*, 2014.

As evident by the increase in successful treatment outcomes, most importantly cure rate, it is acknowledged that the SA TB programme has made progress in addressing TB over the last years. However, to achieve the Millennium Development Goals set for TB, other challenges to be addressed include the size of the TB burden in the country, high TB/HIV co-infection rates, and several patient-related issues (Massyn *et al.*, 2015).

2.1.6.2. Burden of Childhood Tuberculosis

TB remains a leading cause of premature death in SA; with postmortem studies showing that undetected TB and DR-TB are of significant contribution to hospital deaths (Massyn *et al.*, 2015). Mortality data show that TB is the 4th leading cause of death in all children under 14 years of age, and the 4th leading cause in children 1-4 years of age (StatsSA 2017)

Estimates from the WHO shows that among the 306 166 new and relapse cases of TB notified in 2014, 31 977 (10%) occurred in children <15 years of age (WHO, 2015a). This also correlates with the SA national estimates released in the 2015 district health barometer (DHB) which revealed that children make up the caseload in the following order; 0-4 years (6.6%), 6-9 years (2.2%) and 10-14 years (1.4%) respectively (Massyn *et al*., 2015).

These published statistics has howecer been argued to have underestimated the actual burden of childhood TB in the country as microbiological confirmation is still challenging and a notable proportion of children diagnosed with confirmed TB are often not registered (Brennan *et al*., 2016; du Preez *et al*., 2011). A study at five local primary health clinics (PHCs) in the WC province showed that 54 of 354 (15.3%) children with TB were not recorded in the facility-based TB registers; all of these children were diagnosed at a referral hospital, and a significant percentage had disseminated disease (Marais *et al*., 2006c). In a cohort study by du Preez *et al*., only 166 of 267 (62%) children diagnosed with culture-confirmed TB were registered in the routine provincial electronic TB register (ETR.Net); highlighting a large scale underreporting of hospital-diagnosed TB in children, and consequently absence of these children in provincial, national and, ultimately, international TB reports (du Preez *et al*., 2011).

SA has one of the worst TB epidemics in the world; with high incidence (834/100 000 population) (WHO, 2016b) and prevalence (696/100 000 population) rates (WHO, 2015a), associated with increasing MDR/XDR-TB epidemics and HIV co-infection (Churchyard *et al*., 2014; WHO, 2016b). Such incidence and prevalence rates drive a high force of infection which leads to children being exposed and infected at a younger age. Moreover, a correlation is said to exist between the total TB burden in a community and the proportion of that burden in children; suggesting that the higher the overall burden, the higher the proportion in children (Seddon and Graham, 2016).

Data on the burden of TB in children in SA is inadequate, hence, children aged <14 years are argued to account for 15-20% of the total TB disease burden (Dodd *et al*., 2014; Gous *et al*., 2015; Hiruy *et al*., 2015). This claim may be substantiated by a previous study in an urban community in the WC where 39% of the TB caseload was found in children <14 years old (van Rie *et al*., 1999).

An increase in the number of MDR-TB cases had been reported in children in some provinces of SA which reflects the resistance pattern of prevailing strains circulating in the community (Seddon *et al*., 2012). Studies from academic centres in Johannesburg and the WC gave insight to the description of DR-TB among children in SA. In the WC, a 17-years period survey showed a 15.4% and 8.9% DR-TB and MDR-TB among children with culture-confirmed TB respectively (Schaaf *et al*., 2014, 2013; Seddon *et al*., 2012). A study in Johannesburg in 2008 equally showed that 9% of children with a recorded DST

result had MDR-TB (Fairlie *et al*., 2011). Moore *et al*. also published a cohort review describing DR-TB among children and adolescents in four South African provinces from 2005–2010. During the review period, 774 children and adolescents (median age 11.3 years) were diagnosed with DR-TB at the selected facilities, while 626 patients were diagnosed with MDR-TB (Moore *et al*., 2015).

The relationship between HIV and TB is well documented and cannot be overemphasised as HIV remains a crucial driver of the TB epidemic in SA (Churchyard *et al*., 2014). A study in SA estimated the incidence of TB to be 23 per 100 child-years among children on antiretroviral therapy (ART) (Walters *et al*., 2008). A laboratory-guided surveillance study conducted in SA also found that overall incidences of TB were 1 596 cases per 100 000 population among HIV-infected infants and 65.9 cases per 100 000 population among HIV-uninfected infants (Hesseling *et al*., 2009). Furthermore, the incidence of disseminated TB was 240 cases per 100 000 population among HIV-infected children compared to 14 cases per 100 000 among HIV negative children of the same age (Hesseling *et al*., 2009). Estimates also show that HIV prevalence among children with TB ranges from 10% to 60% in moderate TB burdened countries (WHO, 2011a) while higher estimate was noted in SA at 35–50% (Fairlie *et al*., 2011; Moyo *et al*., 2010). It can be deduced that like globally, the extent of childhood TB in SA is yet to be uncovered. However, better childhood TB surveillance programs, increased diagnostic strategies with interventions such as active case finding could make a considerable impact (Knight *et al*., 2015; Moore *et al*., 2015).

2.1.7. Drug-Resistant Tuberculosis in Children

MDR-TB refers to TB disease caused by strains of *M. tuberculosis* resistant to INH and RIF; the backbone of the current first-line therapy, while XDR-TB refers to strains resistant to first-line therapy MDR-TB, with an additional resistance to any of the fluoroquinolones (FQs) (such as levofloxacin or moxifloxacin) and at least one of the three injectable second-line drugs (amikacin, capreomycin, kanamycin or viomycin) (WHO, 2011b). DR-TB may be primary drug resistance (transmitted resistance) or secondary drug resistance (acquired resistance). Primary drug resistance occurs in patients who are naïve to anti-TB therapy, while acquired drug resistance occurs when a patient develops resistance during or following anti-TB therapy (AL Qurainees and Tufenkeji, 2016). Children however generally have transmitted resistance, since the disease is mostly paucibacillary, acquired resistance rarely occurs (Schaaf *et al*., 2009; Seddon and Schaaf, 2016).

While underdiagnoses of DR-TB exists among all age groups owing to diagnostic costs and resources and the unavailability of testing facilities in many regions, several factors have contributed to making children with DR-TB much more difficult to “find” and “treat” (Becerra and Swaminathan, 2014; McAnaw *et al*., 2017). These factors include the well-acknowledged challenges of bacteriological confirmation from children with TB, lack of good diagnostic tools, and under-representation of

children sick with TB and DR-TB in systematic information sources (Becerra and Swaminathan, 2014). An additional factor is a large-scale failure to implement the one programmatic strategy (contact investigation) that can efficiently identify children with TB (Abubakar *et al*., 2013; McAnaw *et al*., 2017).

A study by Yuen *et al*. captured the notable absence of literature documenting child MDR- and XDR-TB cases in regions where adult MDR- and XDR-TB cases were reported (Yuen *et al*., 2015b). This suggests both exclusion of childhood DR-TB from the public discourse and possible underdetection of children suffering from this form of TB (Becerra and Swaminathan, 2014; Yuen *et al*., 2015b). However, in settings where the rates of DR-TB have been monitored, the proportion of DR-TB among children was observed to be similar and in some cases slightly higher than those among adults (Dodd *et al*., 2014; Moore *et al*., 2015). A representative DR-TB survey by Zignol *et al*. also proposed an association between MDR-TB and being <15 years of age in five countries; SA inclusive. Their result established that children aged <15 years with TB have a considerable likelihood of harbouring MDR-TB strains compared to adult patients aged >15 years (Zignol *et al*., 2013).

Although children <15 years old constitute 26% of the global population (World Bank, 2017), until recently the incidence of MDR-TB in this group was never estimated. However, with increasing awareness, the first estimate of pediatric MDR-TB incidence was published in 2014 by Jenkins *et al*. They estimated that, globally, approximately 32 000 children had MDR-TB disease in 2010 (3.2% of their overall TB incidence estimate) (Jenkins *et al*., 2014).

Furthermore, the importance of other forms of DR-TB such as of INH mono-resistant TB (INHMR-TB) cannot be overemphasised and is progressively acknowledged (Garcia-Prats *et al*., 2016; Stagg *et al*., 2017; van der Heijden *et al*., 2017; Yuen *et al*., 2013). Initially, MDR-TB strains possess acquired resistance to INH and subsequently to RIF. In effect, this makes INHMR-TB a gateway to MDR-TB disease, which is associated with poor clinical and microbiological outcome, post-treatment relapse and death. As such the burden of other forms of DR-TB requires quantification (Dodd *et al*., 2016; Otero *et al*., 2017).

Yuen *et al*. recently estimated that 12.1% of children with TB globally had INH resistant TB (INHR-TB) disease (including INHMR-TB combined with other forms of resistance), representing 121 000 annual cases of the disease. They further proposed that the majority of these children might not have received treatment or standardised empirical first-line therapy (consisting of at most three effective drugs in the intensive treatment phase and only one effective drug in the continuation phase) (Yuen *et al*., 2015a). Hence, it is imperative to identify children at high risk of INHR-TB disease or infection so that other treatment or preventive measures can be incorporated (Garcia-Prats *et al*., 2016; Yuen *et al*., 2015a).

In addition, a recently published extension of Dodd *et al*. mathematical model also quantified different forms of DR-TB in children. They estimated that in 2014, 24 800 had MDR-TB (2.9% of all TB incidence cases), 58 300 had INHMR-TB (6.9% of all TB incidence cases), and 1 160 had XDR-TB (Dodd *et al.*, 2016).

From the estimated burden of DR-TB in children, it is evident that a vast gap exists between incidence and treatment (Dodd *et al*., 2016). Many children are not treated for DR-TB globally, and a large number of the untreated ones are sentinels for future cases of DR-TB (Becerra and Swaminathan, 2014; Eckhoff, 2016). Moreover, since children who are diagnosed and receive adequate early treatment have a higher chance of recovery with better treatment outcomes, improved estimates of the incidence of MDR-TB disease in children will enable improved predictions of the resources needed to find and treat childhood MDR-TB successfully (Jenkins *et al*., 2014; McAnaw *et al*., 2017; Seddon and Schaaf, 2016; WHO, 2016c).

2.1.8. Tuberculosis in HIV-infected Children

In children, the interaction between TB and HIV infection is less characterised compared to the adult population (Nelson and Wells, 2004). The availability of little data on the prevalence of HIV among children living with TB and incidence of TB among HIV-infected children makes it difficult to give an accurate estimate of HIV/TB co-infection in children (Venturini *et al*., 2014). However, evidence from a previous survey of children with TB in Sub-Saharan Africa showed a co-infection rate (with HIV) of 11–64% (Coovadia *et al*., 1998). This concurs with the WHO estimate of 10-60% HIV prevalence among children with TB in countries with moderate to high prevalence (WHO, 2011a). Dodd *et al*. further estimated that 5.0% of TB incidence in the WHO declared 22 HBCs occurs in HIV-infected children; equivalent to 32 500 HIV-infected children developing active TB disease in the HBCs in 2010 (Dodd *et al*., 2014).

In the HIV-uninfected population, only around 10% of people infected with TB will develop the disease compared to HIV positive people, with a 20-30 fold increased relative risk of developing TB disease from the latent state (Lancella *et al*., 2016; Venturini *et al*., 2014). This risk surpasses those of other risk factors such as malnutrition (Venturini *et al*., 2014).

Due to the intricate interaction between HIV and TB, HIV/TB co-infected children exhibit considerable mortality from TB, with mortality as high as 20–35% in resource-limited settings (Edmonds *et al.*, 2009; Walters *et al*., 2008). HIV-infected children also have a heightened risk of rapid TB disease progression, possible poor response to TB therapy, increased risk of TB recurrence and are more prone to developing disseminated TB and other forms of PTB and extra-pulmonary TB (EPTB) disease that reflect poor organism containment (Marais *et al*., 2007; Nwokeukwu *et al*., 2013).

While ART reduces TB incidence in HIV-infected children (Abuogi *et al.*, 2013; Auld *et al.*, 2014), the occurrence of TB in HIV-infected children remains significantly higher compared to the uninfected paediatric population (Anígilájé *et al.*, 2016; Crook *et al.*, 2016). Although clinical disease presentation of TB in HIV-infected children can be compared to that observed in HIV-uninfected children; immunocompromised children exhibit more severe characteristics (Marais *et al.*, 2007). This requires further studies that focus on the complicated interaction between HIV and TB (Nelson and Wells, 2004).

2.2. Tuberculosis Infection and Disease in Children

2.2.1. Transmission, Exposure, and Infection

The transmission of *M. tuberculosis* mostly results from the inhalation of tubercle bacilli in aerosolised respiratory droplets released via sneezing or coughing by an infectious source with pulmonary or laryngeal TB (Newton *et al.*, 2008). In children, after exposure to an infectious source case, the risk of infection depends on the infectiousness of the source case, as well as the proximity and duration of the contact (Marais *et al.*, 2004a). In most cases, the source is an adolescent or adult with bacilleferous cavitary pulmonary disease in whom the estimated transmission rate is about 35% compared to 17% observed among those with non-bacilleferous forms of TB (Erkens *et al.*, 2010; Marais *et al.*, 2004a; Moore *et al.*, 2009).

The infectiousness of bacilleferous cavitary and non-bacilleferous forms of TB was examined in a study by Singh *et al.* where they evaluated the prevalence of TB infection in children with adult household contact with PTB. They highlighted a striking difference in contagiousness between smear-positive patients and smear-negative ones. In their study, 95 out of 281 children had TB (33.8%), of which 65 (68.4%) were contacts of sputum smear-positive adults whereas only 30 (31.6%) were contacts of sputum smear-negative ones (Singh *et al.*, 2005).

TB exposure is defined as a recent close encounter with an adult or adolescent with infectious PTB (presumptive or bacteriologically confirmed), but with no documentation of infection nor clinical or radiological findings suggestive of disease (Roya-Pabon and Perez-Velez, 2016). TB infection is clinically defined as an infection with any species of the MTBC, demonstrated by a positive T-cell-based test [TST and/or Interferon-gamma release assay (IGRA)] result, without clinical manifestations or radiological findings suggestive of active TB (Roya-Pabon and Perez-Velez, 2016).

Although children with TB are considered less/rarely contagious due to the presence of unproductive cough, paucibacillary nature of the disease, and absence of cavitary lesions in lungs in most cases (Cruz and Starke, 2011; Marais *et al.*, 2006a). Nonetheless, some studies reported transmission of *M. tuberculosis* from children to contacts in 2 to 20% of smear-positive children 7-10 years old (Curtis *et*

al., 1999; Lee *et al*., 2005). Molicotti *et al*. also reported a TB transmission from a ten years old child to 21 of 29 (72%) contacts as seen by positive IGRA tests (Molicotti *et al*., 2008).

2.2.2. Role of the Immune System and Disease Progression

Childhood TB is marked by a short interval between infection and development of symptomatic disease (Marais *et al*., 2005a). Subsequent to inhalation of *M. tuberculosis* bacilli, innate immunity controls infection in immunocompetent individuals depending on the variation in equilibrium between the pathogen and the host immune response (Marais *et al*., 2005a; Newton *et al*., 2008). This may lead to the *M. tuberculosis* being destroyed immediately by the innate immune response, in which case no adaptive T-cell response is developed (Lerner *et al*., 2015; Sia *et al*., 2015; van Crevel *et al*., 2002).

Many cases of childhood primary TB infection are asymptomatic, self-healing and may persist without notice or inadvertently discovered at a later stage (Marais *et al*., 2004a). However, when infection is established, a focal nonspecific inflammatory response follows. Initially, TB bacilli lodges in the lungs and immunity prevent dissemination, resulting in an enlarged regional lymph node visible on chest radiography and may be associated with Ghon focus; the hallmark of primary TB (Marais, 2011; van Crevel *et al*., 2002). At this stage, children are often asymptomatic or may present with non-specific respiratory symptoms (Piccini *et al*., 2014).

The progression from primary infection to disease is signified by the development of symptoms and signs of TB (Marais, 2011). Of note, the rate of progression from primary infection to active disease is markedly different between children and adults. The risk is higher in infants (30-40% in those younger than one year of age) and children (24% in 1-5 years of age) compared to adolescents (15%) and adults (5-10%) (Nelson and Wells, 2004). The risk of progression drops beyond five years of age and increase again during adolescence (Newton *et al*., 2008). More so, in children, the majority of cases (>90%) progress to active disease within one year after primary infection, in contrast to an extended time interval in adults (Marais, 2011; Marais and Schaaf, 2014).

While a vigorous immune response in immunocompetent adolescents tends to result in adult type disease, poor cell-mediated immunity (CMI) in young or HIV co-infected children enables unfettered multiplication of bacilli with progressive parenchymal lung damage and dissemination (Marais *et al*., 2005b; Newton *et al*., 2008).

Disease manifestation in children also varies according to the age at which the infection becomes active (Marais, 2011). The influence of age on disease manifestation is illustrated in figure 2.3, where the schematic age-line indicates the age profile of different disease manifestations following primary infection with *M. tuberculosis* (Marais, 2011).

Progressive disease in infants and younger children tend to result in haematogenous dissemination, causing significantly advanced disease such as miliary disease or meningitis (Marais, 2011). About 4% of children infected under the age of 5 years, in fact, develop tubercular meningitis or miliary TB (Carrol *et al.*, 2001). When approaching adolescence, the presenting phenotype mimics adult-type reactivation disease, as characterised by cavitary lung lesions and associated pulmonary changes (Marais *et al.*, 2005a).

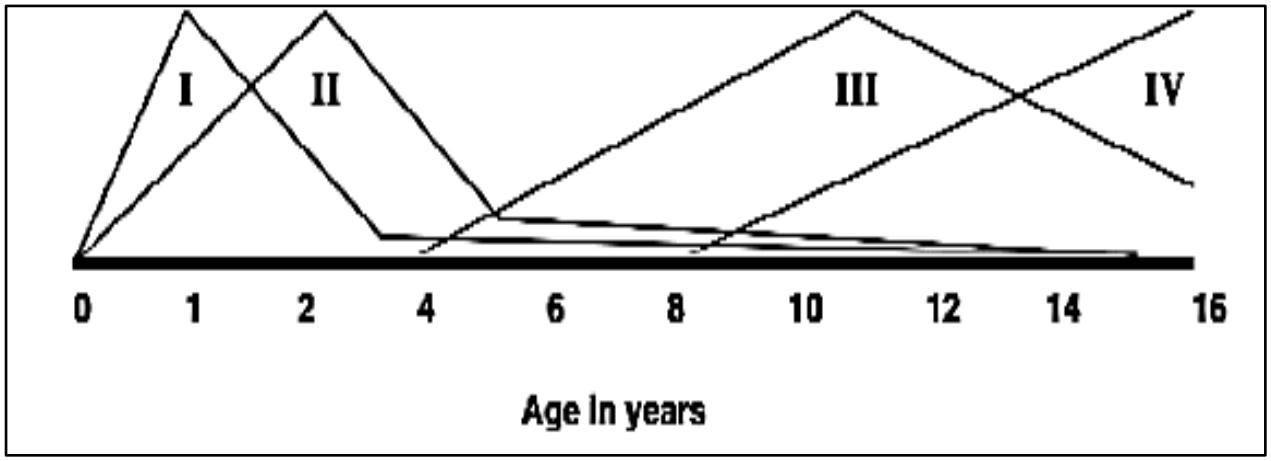

Figure 2.3. Schematic age-line, indicating the age profile of different disease manifestations following primary infection with *Mycobacterium tuberculosis*. I: complicated Ghon focus, disseminated miliary disease and Tubercular meningitis; II: uncomplicated and complicated lymph node disease; III: pleural effusion; IV: adult-type disease. Adapted from Marais, 2011.

2.2.3. Risk Factors for Developing Tuberculosis Disease in Children

2.2.3.1. Malnutrition

Malnutrition (both micro and macro deficiency) increases the risk of TB as it has direct effects on the proper functioning of immune cells that would allow the child to either clear the infection or drive it into a latent state (Abba *et al.*, 2011; Cegielski and McMurray, 2004; Lancella *et al.*, 2015). Furthermore, malnutrition affects genetic expression and immune function, thereby predisposing children to TB progression (Jaganath and Mupere, 2012; Lancella *et al.*, 2015). The resulting disease and inflammatory response worsen the nutritional state by decreasing appetite and changes in metabolic processes (Grobler *et al.*, 2016; Jaganath and Mupere, 2012).

BCG vaccine trials also suggested that malnourished children have a decrease in TB-associated cell-mediated immune responses and are twice as likely to contract TB disease compared to their nourished peers (Comstock and Palmer, 1966; McMurray *et al.*, 1981). A cohort study in SA further found malnutrition to be an independent predictor of unfavourable treatment outcome and mortality among MDR-TB and HIV co-infected children (Hicks *et al.*, 2014).

2.2.3.2. Human Immunodeficiency Virus

HIV alters the pathogenesis of TB. It decreases chemotaxis, causes defective granuloma formation, impairs antigen processing, and results in generalised loss of CD4+ cells (Berti *et al.*, 2014; Hesseling *et al.*, 2009). It further causes selective clonal depletion of *M. tuberculosis*-specific CD4+ lymphocytes, increases immune-mediated TB susceptibility and thereby increasing the risk of developing active TB (Nwokeukwu *et al.*, 2013; Hesseling *et al.*, 2009). More so, HIV co-infection is marked by deficient CMI, as such often result in disseminated TB disease, especially in advanced stages of HIV-infection. This leads to increased mortality in contrast to immunocompetent children (Palme *et al.*, 2002).

2.2.3.3. Young Age

As stated in the natural history of TB in children, young age is one of the predisposing factors to TB disease (Marais *et al.*, 2005a; Marais, 2011). The risk of developing active disease is significantly higher in children than in adults, (being about 15% in adolescents, 24% in children aged 1–5 years, and 40–50% in those aged <2 years) with higher risk of developing the more severe forms such as tubercular meningitis or miliary TB especially in those <2 years (Lancella *et al.*, 2015).

2.2.3.4. Genetic Susceptibility

Individuals susceptibility to TB disease may be heightened by various genetic conditions and acquired defects in host immune response pathways (Kampmann *et al.*, 2005; Levin and Newport, 1999; Newport *et al.*, 1996). Some carriers of mutations in the genes encoding interleukin (IL)-12 beta and beta-1 receptors or interferon-gamma (IFN-γ) receptors 1 and 2 are more susceptible to both TB and NTM (Filipe-Santos *et al.*, 2006; Kampmann *et al.*, 2005; Rossouw *et al.*, 2003). Most of these observations were initially noted in children with reduced ability to activate macrophage antimycobacterial mechanisms through defects in the IFN-γ76, 77, 79 / IL-1280 pathway, resulting in severe mycobacterial infection (Kampmann *et al.*, 2005; Newport *et al.*, 1996).

2.2.4. Latent Tuberculosis Infection

LTBI is defined clinically by a reactive TST (Dutta and Karakousis, 2014). This indicates a delayed-type hypersensitivity (DTH) response to intradermal injection of *M. tuberculosis* or a T-cell response to *M. tuberculosis*-specific antigens (Dutta and Karakousis, 2014). Chest radiography may seldom exhibit evidence of healed infection such as calcified non-enlarged regional lymph nodes or pleural thickening (Ki and Shingadia, 2017). The initial febrile illness may be resolved by children who develop LTBI as such they remain asymptomatic (Powell and Garret Hunt, 2006).

Although LTBI is suggested to be a form of bacterial containment in inactive form, the definition does not consider the duration and activity of the latent focus (Dutta and Karakousis, 2014). It is not a

homogenous entity but somewhat varies from person to person based on timing and on host and pathogen-specific factors (Barry *et al*., 2009; Dutta and Karakousis, 2014; Salgame *et al*., 2015).

2.2.5. Clinical Manifestation of Tuberculosis Disease in Children

In those children who progress to active TB following primary infection, one or more body systems may be involved. PTB account for 60–80% of all cases, while 25-35% children present with extra-pulmonary manifestation (Cruz and Starke, 2007; Jahromi and Sharifi-mood, 2014).

2.2.5.1. Pulmonary Tuberculosis

The most common form of clinical manifestation of PTB in children is the pulmonary parenchymal disease and associated intrathoracic adenopathy (Cruz and Starke, 2007). Pulmonary involvement in children ranges from primary parenchymal, progressive primary to reactivation disease (Cruz and Starke, 2010). Primary parenchymal disease often occurs in infants and adolescents and mostly presents with signs and symptoms of lung disease. Progressive disease largely arises as a result of inadequate organism containment, and reactivation disease usually develops during adolescence (Cruz and Starke, 2007, 2010).

Symptoms associated with PTB in children include prolonged non-productive cough, low-grade fever, weight loss or failure to thrive, and wheezing (Esposito *et al*., 2013; Piccini *et al*., 2014). These symptoms are however non-specific and may overlap with many other chronic diseases, as such are not easily distinguished from other forms of lung disease (Piccini *et al*., 2014).

PTB in children often involves the well-ventilated lobes which include the right upper and middle lobe and left upper lobe (Mandalakas and Starke, 2005). Pleural involvement may occur from the direct spread of caseous material from lymph nodes or as a result of hypersensitivity reaction to mycobacterial antigens (Cruz and Starke, 2007, 2010).

2.2.5.2. Extra-pulmonary Tuberculosis

Tubercular lymphadenitis is the most common form of EPTB in children. It is part of the primary complex that often occurs in response to a pulmonary focus within their drainage area, and it mostly involves cervical, submandibular, supraclavicular, preauricular or submental areas (Carrol *et al*., 2001; Donald, 2010). The most severe manifestations of EPTB in children includes tubercular meningitis (more common in early childhood), tuberculoma (usually found in older children), and skeletal TB (marked by bone and joint lesions and are more frequent in children than in adults) (Carrol *et al*., 2001).

2.3. Diagnostic Challenges in Childhood Pulmonary Tuberculosis

Diagnostic difficulties pose a considerable challenge to childhood PTB management compared to adult PTB on many levels as listed in table 2.1. (Tsai *et al*., 2013). These diagnostic difficulties among others include the absence of a practical reference test or gold standard, the nonspecific clinical presentation, the inability of young children to expectorate sputum and paucibacillary nature of TB in children (Lodha and Kabra, 2004; Marais and Pai, 2007a; Nelson and Wells, 2004).

Table 2.1. Clinical similarities and differences between adult and childhood tuberculosis with relevancy to successful diagnosis. Adapted from López Ávalos and Montes de Oca, 2012.

Feature	Adult	Children
Typical signs	Radiological features and a positive sputum smear	TB can mimic many common childhood diseases. The clinical symptoms in older children are cough, fever, wheezing, fatigue, and failure to gain weight. In younger children, symptoms are pulmonary parenchymal disease and intrathoracic adenopathy, lymphadenopathy, and central nervous system involvement
X-rays findings	Classical cavitation in lungs	Enlargement of hilar, mediastinal, or subcarinal lymph nodes and lung parenchymal changes. Hilar lymphadenopathy with or without a focal parenchymal lesion
TST	Cross-reaction with BCG vaccination and exposition with other mycobacteria	Cross-reaction with BCG vaccination and exposition with other mycobacteria
Sampling	Easy sputum and blood sampling	Difficulty to expectorate, blood sampling usually painful in paediatric children
Bacillary load	High bacillary load, easy to find the bacillus when technician is skilful	Lower bacillary load and is usually smear-negative even with fluorescent dyes
Bacillus growth in culture	High yields of 90–100%	Confirmation by culture rarely exceeds 30–40%
Tropism of *M. tuberculosis*	Commonly localized infection in the lungs	Mostly pulmonary but extrapulmonary, disseminated mal also occur

TST:Tuberculin skin test, BCG: Bacille Calmette-Guerin

Bacteriological confirmed diagnosis of PTB is achieved in less than 15% of children using AFB smear staining and 30%–40% using culture (Marais and Pai, 2006; Swaminathan and Rekha, 2010). Further complicating the challenges of bacteriological confirmation is the difficulty in obtaining a good quality specimen for the diagnosis of PTB in children (Raizada *et al*., 2015). Young children rarely produce spontaneously expectorated sputum or may swallow their sputum, necessitating IS or gastric aspiration, which are resource intensive and require a level of expertise (Zar *et al*., 2005).

In the absence of bacteriological confirmation, diagnosis is based on; exposure to an adult index case, non specific chronic respiratory symptoms, documented weight loss or failure to thrive, a positive TST and the presence of suggestive signs on the chest radiography (Graham *et al*., 2004; Nelson *et al*., 2004; Swaminathan and Rekha, 2010). These criteria, however, are of limited applicability in TB endemic countries where TB exposure is often undocumented and TST/IGRA result is common and cannot distinguish between latent infection and active disease (Machingaidze *et al*., 2011; Marais *et al*., 2006b; Marais and Pai, 2007b). More so, chest radiography findings may be normal for a significant proportion of children with confirmed PTB. Moreover, high intra- and interobserver variability and non-availability further limit the use of chest radiography in resource-poor settings (Jahromi and Sharifi-mood, 2014; Swaminathan *et al*., 2008).

Various clinical scoring systems to aid in the diagnosis of childhood TB have been developed but are limited by a lack of standard symptom definitions, varying diagnostic accuracy and adequate validation against the standard of culture-confirmed diagnosis (Anderson *et al*., 2014; Chatterjee and Pramanik, 2015; Koura and Mohammed, 2012). However a recently published editorial by Holm and Wejse suggests an optimism to the use of clinical scoring systems for the diagnosis of TB even in children with an HIV co-infection (Holm and Wejse, 2017), nonetheless, the WHO still does not approve the use of a clinical scoring system (WHO, 2014a).

Serologic tests are currently unable to diagnose childhood TB accurately and as such is of little value (Lodha and Kabra, 2004). Although they offer rapid tests and does not require a sample from the site of disease, its sensitivity and specificity depend on the antigen employed, and its use in TB diagnosis is currently not recommended by the WHO (Lodha and Kabra, 2004; WHO, 2011c). Moreover, several factors can alter the results such as age, exposure to other mycobacteria, BCG vaccination or other causes of immune compromise; all of which are particularly prevalent in TB-endemic regions (Lodha and Kabra, 2004; Marais and Pai, 2007a).

Furthermore, childhood TB diagnostic challenges are more pronounced in HIV-infected children as seen by the relatively poor performance of the current diagnostic algorithm in this group (Venturini *et al.*, 2014). The exacerbated diagnostic difficulty in this group is characterised by the lesser sensitivity of TST, the relative similarity between chronic pulmonary symptoms and other HIV-related conditions and the complicated interpretation of chest radiography among others (Van Rheenen, 2002).

As a result of these challenges, a significant proportion of children are either under-diagnosed or over-diagnosed. Over-diagnosis leads to inappropriate treatment of children without TB. Conversely, under-diagnosis results in poor outcome leading to increased morbidity and mortality rates and often

patients are only identified as having TB at the critical disease stage or during postmortem investigations (Chatterjee and Pramanik, 2015; Pai and Schito, 2015).

2.4. Diagnosis of Childhood Pulmonary Tuberculosis

2.4.1. Samples Used for Diagnosing Childhood Pulmonary Tuberculosis

Good quality samples are one of the significant parameters that determine the performance of a microbiological diagnostic test, a scarce luxury in children (Dunn *et al*., 2016).

As seen in figure 2.4, bacteriological confirmation of intrathoracic TB in children can be achieved using a broad variety of specimen types. These may be examined microscopically, cultured, or tested using gene amplification (Roya-Pabon and Perez-Velez, 2016; Triasih, 2015).

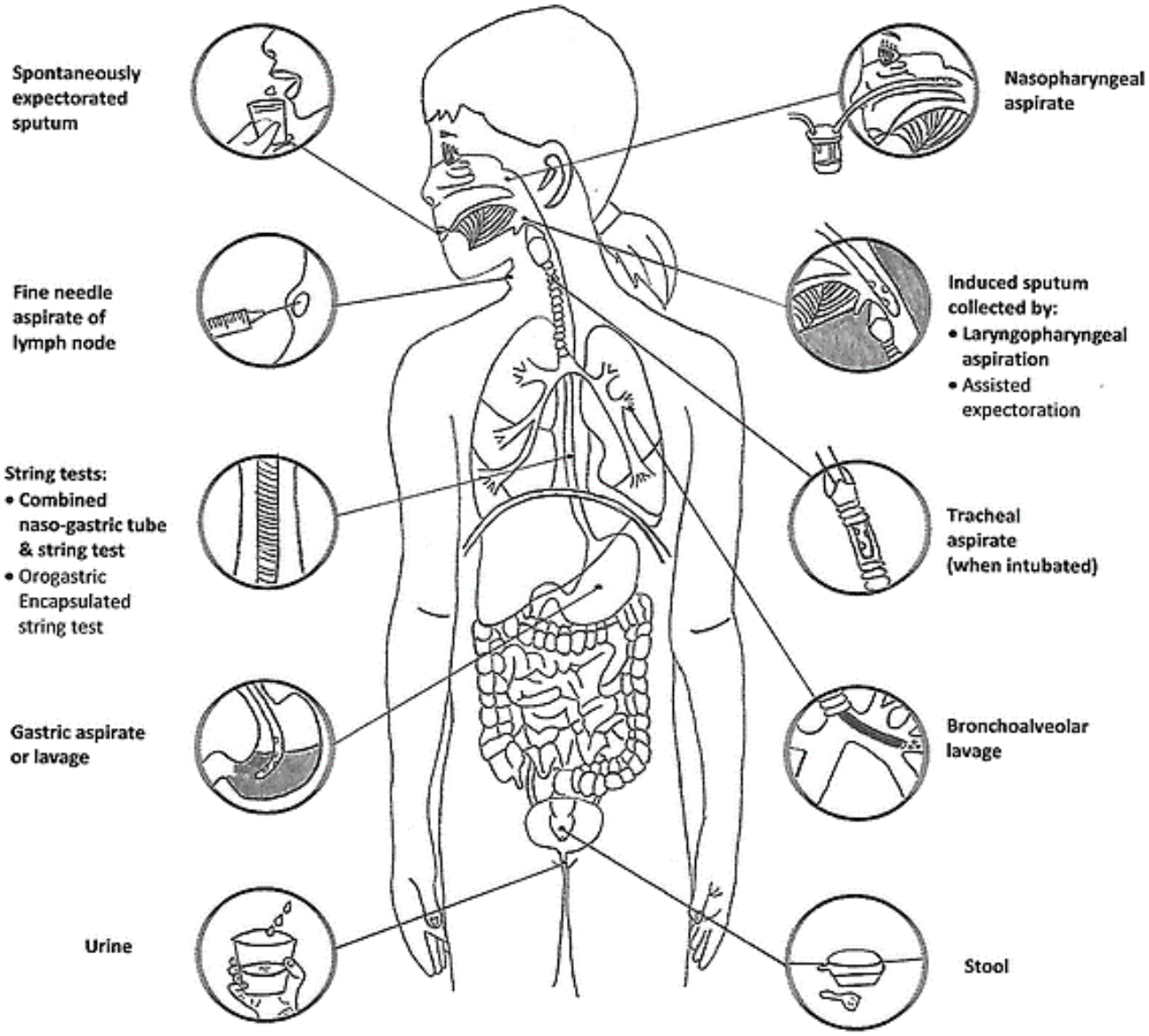

Figure 2.4. Illustrated list of specimens suitable for the bacteriological confirmation of intrathoracic tuberculosis in children. Adapted from Roya-Pabon and Perez-Velez 2016.

2.4.1.1. Gastric Aspirate/Gastric Lavage

The use GA or gastric lavage (GL) for the identification of *M. tuberculosis* is based on the rationale that sputum is moved to the upper airway by the mucociliary system of the respiratory tract where it is swallowed down into the stomach and remains there until gastric emptying occurs (Perez-velez *et al*., 2017). GA/GL is performed after overnight fasting for three consecutive mornings to obtain sputum swallowed whoile sleeping (Dunn *et al*., 2016). Sample volume of about 5 to 10 mL are collected on three consecutive days, and if not processed within four hours of collection, are adjusted to neutral pH with sodium bicarbonate since long-term exposure to stomach acid may decrease the viability of mycobacteria (CLSI, 2008). However, a recent study reported that neutralising the pH of GA before processing results in higher contamination rate without any significant difference in detection rate (Parashar *et al*., 2013).

For many years, GA/GL has been the accepted standard procedure for attempting microbiological confirmation of PTB disease in young children. Culturing of these samples achieved a yield of 30-40% and up to 80% in young infants and in cases with advanced endobronchial disease (Triasih, 2015). However, the low bacteriological yield of *M. tuberculosis*, the requirement of skilled personnel, and the need for overnight hospitalisation preclude the use of this method in low resource areas where the burden of TB may be most significant (Nicol and Zar, 2011).

2.4.1.2. Induced Sputum

Sputum induction is a well-established procedure for obtaining respiratory samples for the diagnosis of PTB in children (Gonzalez-Angulo *et al*., 2012). It involves the administration of an inhaled bronchodilator followed by nebulised hypertonic (3%–5%) saline and then suctioning of the nasopharynx or continuous expectoration of mucus from the lower respiratory tract (Grant *et al*., 2012).

IS has been successfully used for diagnosis of childhood PTB in several regions and has been suggested to give a better yield of microbiological confirmation than GA (Bart *et al*., 2016; Triasih, 2015). In a study of 149 children (median age nine months) admitted with acute pneumonia, 10% had a positive TB culture, and the yield from a single IS sample (10%) was better than that of sequential GL samples (6%) (Zar *et al*., 2000). Better results with IS was corroborated in another more substantial study of children suspected of PTB (250 children, median age 13 months) where a significantly higher cumulative yield was noted for 3 IS samples (87%) compared with 3 GL samples (65%). The yield from one IS sample was equivalent to three GL samples (Zar *et al*., 2005). Other studies also showed similar results using IS compared to GA (Ruiz Jiménez *et al*., 2013; Thomas *et al*., 2014). Nonetheless, regardless of multiple reports on the safety and feasibility of IS even in primary care settings, sputum induction is considered safe only in clinically stable children (Detjen *et al*., 2015; Detjen and Walters, 2016).

2.4.1.3. Nasopharyngeal Aspirate

NPA is an easy to perform, less invasive procedure requiring minimal training and facilities; it is achieved by passing a cannula through the nostril into the nasopharynx which elicits a cough reflex (Connell *et al.*, 2011). Earlier studies of patients with suspected TB found similar results using NPA and GA specimens (30% vs 38%) (Franchi *et al.*, 1998) and between NPA and IS specimens (24% vs 22%; median age, 48 months (Owens *et al.*, 2007). Contrary, in Yemen (n 5213; median age, five years), culture results were positive for 14% of IS samples, 9% of GA samples, 8% of sputum samples and 7% of NPA samples (Al-Aghbari *et al.*, 2009). Similarly, two Peruvian studies involving 165 (Oberhelman *et al.*, 2006) and 218 (Oberhelman *et al.*, 2010) children reported a lower yield from NPA samples compared to GA samples (3.8% vs 6.8% and 10% vs 5.5%, respectively).

Nevertheless, a recent meta-analysis on diagnostic testing of NPA using the Xpert® assay documented both comparable performances to standard specimens 69% and 66%, respectively (Detjen *et al.*, 2015). Similarly, another study reported that NPA had similar sensitivity to IS when tested on Xpert®. Xpert® on two IS samples detected 16 of 28 and on two NPA detected 11 of 28 culture-confirmed cases (Zar *et al.*, 2013).

However, Nicol and Zar speculated that the observed differences in the culture diagnostic yield of NPA from different studies might be related to specimen collection techniques, patient population, and methods employed for culturing *M. tuberculosis* (Nicol and Zar, 2011).

2.4.1.4. Bronchoalveolar Lavage

Bronchoalveolar lavage (BAL) is a technique that samples the alveolar lining fluid and has been used for the diagnosis of several respiratory infections, including TB (Swaminathan *et al.*, 1996). In children beyond the neonatal period, BAL is performed using a paediatric flexible fibreoptic bronchoscope (FFB) with an external diameter of 3.5±3.7 mm, while children >9 years of age mostly tolerate the FFB with a diameter of 4.6±4.9 mm (de Blic *et al.*, 2000). BAL procedures in children are usually performed under sedation in combination with local anaesthesia (de Blic *et al.*, 2000).

Regarding the controversies surrounding the use of BAL in the diagnosis of PTB in children, a study compared the sensitivity of GA with BAL for isolating *M. tuberculosis* from 20 children with suspected PTB. Cultures of BAL on two patients (2 of 20 or 10%) were positive for *M. tuberculosis*, while cultures of the GA from the same two patients were also positive for *M. tuberculosis*. Eight additional patients had positive GA cultures with negative BAL cultures, resulting in a total of 10 of 20 (50%) patients with positive GA cultures. (Abadco and Steiner, 1992). This was further evaluated in a study of 50 children with suspected PTB, mean age of five years (range seven months to 12 years). Of the 50 cases, *M. tuberculosis* was cultured in six BAL samples (12%) and in 16 GL samples (32%) resulting in a total

of 17 culture-confirmed cases (34%). Of the six BAL positive cases, GL was also positive in five cases. (Somu *et al*., 1995). Both studies concluded that GL is better than BAL for bacteriologic confirmation of PTB in children as BAL does not significantly increase bacteriological confirmation.

In contrast, a study by Menon *et al.* assessed the diagnostic yield of BAL compared to GA in 52 children with suspected PTB, AFB on smear was identified in 19 (36.5%); BAL was positive in 16 (30.8%), and GA was positive in 11 (21.15%). GA alone was positive in three (5.67%) while BAL alone was positive in eight (15.38%) (Menon *et al*., 2011). Other studies also showed that the use of BAL with Xpert® resulted in additional diagnostic yield and that the use of both GL and BAL for bacteriological isolation can complement each other to double the diagnostic yield (Singh *et al*., 2000; Walters *et al*., 2014). Nonetheless, it is suggested that BAL should be performed only when less invasive specimens are not possible to collect, as the bacteriological yield is no better than a series of IS or GA specimens (Perez-velez *et al*., 2017).

2.4.1.5. Nasopharyngeal Swab

Trevathan and Philips suggested that when patients with TB coughs, droplets of bacilli laden sputum may be forced into the nasopharynx, nose and sinuses. They further proposed the possibility that a patient with TB bacilli might inhale the infectious droplets expelled during coughing. The mucus coated nasopharyngeal vibrissae are known to possess an efficient filtering action on bacteria suspended in the inspired air and are presumably capable of trapping some of the tubercle bacilli inhaled (Trevathan and Philips, 1959). The nasopharynx is a secure gateway for some mycobacterial species such as *M. bovis* and *M. leprae*, as such *M. tuberculosis* can cross lymphoepithelial barriers in vitro, but its ability to colonies the nasopharyngeal mucosa in vivo is yet to be fully established (Balcells *et al*., 2016).

A previous study has proven the possibility of detecting *M. tuberculosis* from nasal swabs of some adult TB patients (Warndorff *et al*., 1996), while a recent study also detected TB in one of 89 adult patients who had a positive Xpert® on a NPS specimen, with a normal chest X-ray, negative QuantiFERON but a culture-positive sputum (Balcells *et al*., 2016). Disappointingly, data on the performance of NPS in the detection of PTB in the children population is still lacking.

2.4.1.6. String Test

Classically, in a string test (ST), patients are required to swallow a gel capsule consisting of a coiled nylon string (Marais and Pai, 2006). The string unravels as the capsule descends and dissolves in the stomach. The unravelling string gets coated with gastro-intestinal secretions containing any present pathogens. The string passes in the faeces after four hours and may be used for mycobacterial culture (Marais and Pai, 2006). The ST has been shown to be well tolerated and subsequently demonstrated a

comparable level of sensitivity to IS in adult and older children (Chow *et al*., 2006; Nansumba *et al*., 2016; Vargas *et al*., 2005). In the adult population, the culture yield from ST samples (9%) was better than that from IS (5%) (Vargas *et al*., 2005). While this procedure was well tolerated in older children (median age eight years) it has not been well studied in younger children (<4 years of age) who account for a high proportion of TB cases in some settings (Chow *et al*., 2006). Nansumba and colleagues further suggested that the tolerability of ST in young children might be improved by the reduction of the size of the capsule (Nansumba *et al*., 2016).

2.4.1.7. Urine

It was postulated that tubercle bacilli could be excreted through the kidneys and that the organisms could be detected in the urine of active PTB patients with no symptoms pertaining to the urinary tract (Gopinath and Singh, 2009). This hypothesis was evaluated by studies carried out in HIV-negative and HIV-positive cases; suggesting that urine can be used as an additional specimen due to the convenience and non-invasive nature of collection (Aceti *et al*., 1999; Bentz *et al*., 1975; Kafwabulula *et al*., 2002; Sechi *et al*., 1997).

A study by Torrea *et al.* also confirmed the utility of urine specimens for diagnosing PTB by nested polymerase chain reaction (PCR) with a sensitivity of 64.3% in culture-negative PTB cases and 46.3% in EPTB cases (Torrea *et al*., 2005). In another study, of the 81 patients suspected of having PTB, 46 (56.8%) were sputum culture-positive, of these, 12 (26.1%) were also urine culture-positive for *M. tuberculosis*. Of the 35 sputum culture-negative cases, three (8.6%) were urine culture-positive (Gopinath and Singh, 2009). In a study of Thomas *et al*. among 118 children with probable TB, urine provided an additional diagnosis with culture positivity on three patients and MDR-TB was detected by urine culture alone in one child >5 years old (Thomas *et al*., 2014).

2.4.1.8. Stool

The feasibility of using a stool for the diagnosis of PTB was established based on the rationale that children swallow their sputum and *M. tuberculosis* deoxyribonucleic acid (DNA) may survive intestinal transit (Perez-velez *et al*., 2017; Triasih, 2015). As such, stool may contain swallowed *M. tuberculosis* bacilli (Perez-velez *et al*., 2017). Therefore, PCR and mycobacterial culture of stool are expected to be useful for detecting *M. tuberculosis* originating from the lungs (Gopinath and Singh, 2009; Perez-velez *et al*., 2017; Triasih, 2015). However, the challenges associated with the use of stool in the diagnosis of PTB is that other rapidly growing bacteria may overgrow the slowly replicating *M. tuberculosis* bacillus and the need for stringent decontamination procedures to prevent overgrowth of normal bowel flora may also kill or inhibit the growth of mycobacteria (Banada *et al*., 2016).

Over the years, stool culture was proposed to be insensitive (Oberhelman *et al.*, 2006), but new studies have provided insights that despite the low sensitivity, it can still serve as a useful alternative or additional specimen for the diagnosis of PTB in children (Ouédraogo *et al.*, 2016).

The roll-out of Xpert® has enhanced the feasibility of using stool to diagnose childhood PTB (Detjen and Walters, 2016; Shenai *et al.*, 2013), with more studies in recent years indicating stool as a promising test for diagnosis of PTB in both HIV-infected and uninfected children (Banada *et al.*, 2016; DiNardo *et al.*, 2015; Marcy *et al.*, 2016; Nicol *et al.*, 2013; Walters *et al.*, 2012). A recent study with Xpert® on stool of pediatric TB suspects and controls suggested an assay sensitivity of 85% and 84% for 0.6g and 1.2g stool samples, respectively, and a specificity of 100% and 94%, respectively (Banada *et al.*, 2016). Welday and colleagues in their research found that Xpert® testing of stool showed 100% sensitivity and 89.36% specificity without missing any positive from sputum smear microscopy. They further emphasised that stool DNA extraction should be considered for further investigation as this helps to detect cases missed in the direct stool Xpert® test (Welday *et al.*, 2014). Dorman also suggested that more studies are required to clarify the sensitivity of stool specimens with more precision, and newer optimising techniques are needed to achieve a maximal diagnostic yield (Dorman, 2015).

2.4.2. Methods Used in the Diagnosis of Childhood Pulmonary Tuberculosis

2.4.2.1. Clinical Symptoms

With the use of a clinical symptoms approach only, the status can be classified into probable TB, possible TB, TB unlikely and Not TB (Graham *et al.*, 2012).

Probable TB is defined as suspected TB with either (1) a positive Chest X-ray, (2) a positive response to anti-TB treatment, (3) documented exposure to *M. tuberculosis* (4) immunologic evidence of *M. tuberculosis* infection. Possible TB is defined as suspected TB with a positive Chest X-ray, in addition to either (1) a positive response to anti-TB treatment or (2) documented exposure to *M. tuberculosis* or (3) immunologic evidence of *M. tuberculosis* infection. TB unlikely includes children that are symptomatic but not fitting the above definitions and no alternative diagnosis established. Not TB fits the diagnosis for TB unlikely but with an established alternative diagnosis (Graham *et al.*, 2012). A recent expert consensus statement recommendation has regrouped the case definitions into confirmed TB, unconfirmed TB, and unlikely TB (Graham *et al.*, 2015a).

An ongoing challenge in the diagnosis and classification of TB in the paediatric population is in children who are not bacteriologically confirmed to have TB, but in whom diagnosis relies on clinical investigations and parameters with notable limitations (Graham *et al.*, 2015a). In TB endemic countries, symptoms associated with TB often occur in association with other common childhood illnesses, and this lack of specificity may limit their diagnostic usefulness (Marais *et al.*, 2005; Mulenga

et al., 2015). Besides, it is expected that symptom-based diagnostic approaches would have less value in high-risk children (<3 years of age and/or immune compromised) (Marais *et al.*, 2006a). In these cases progression may occur rapidly, emphasising the need for early diagnosis in this group (Khan and Starke, 1995).

In general, scepticism exists regarding the diagnostic value of the symptom-based approach due to its poor clinical validation (López Ávalos and Montes de Oca, 2012). Nonetheless, it is suggested that symptoms may have diagnostic value if appropriate risk stratification is established (Marais *et al.*, 2006a).

2.4.2.2. Immunodiagnostics Tests

2.4.2.2.1. Tuberculin Skin Test

TST or the Mantoux test is based on the intradermal injection of a standard dose of purified protein derivative (PPD) tuberculin which induces a cutaneous DTH reaction (Lalvani and Millington, 2007). The reaction is measured as mm of induration after 48 to 72 hours (Khan and Starke, 1995). A TST according to the WHO guidelines is considered positive when the diameter of the induration is >10 mm in patients with no history of BCG vaccination and induration >15 mm in previously BCG vaccinated patients (WHO, 2014a).

TST, however, has many shortcomings, with the most pronounced being its poor specificity (Chapman and Lauzardo, 2014). Some studies reported that approximately 10%–40% of immunocompetent children with confirmed bacteriologically active TB were initially TST negative, thus a negative TST result does not necessarily exclude the possibility of TB infection and/or disease (Auld *et al.*, 2013; Chiappini *et al.*, 2016; Starke and Taylor-Watts, 1989).

2.4.2.2.2. Interferon-gamma Release Assays

IGRA were developed to address the drawbacks of the TST by adressing the lack of specificity due to antigen overlap with NTM and the BCG vaccine strains (Hertting and Shingadia, 2014; Starke, 2014). Recent versions of IFN-γ assays uses antigens which are encoded within the region of difference of the *M. tuberculosis* genome and are more specific to *M. tuberculosis* considering that they are not shared with any BCG vaccine strains or selected NTM species (Detjen *et al.*, 2007; Ling *et al.*, 2011; Nicol *et al.*, 2005a). Commercially available IGRA includes the QuantiFERON-TB Gold (QFT-G) and T-Spot TB assay (Sudha, 2016; Pai *et al.*, 2014).

One of the advantages of IGRA over TST is the lack of cross-reactivity with previous BCG vaccination, as such it is often possible to distinguish patients with TB infection from those who are BCG vaccinated and those with NTM infections (Pai *et al.*, 2014). Nonetheless, it is suggested that antigens used in

IGRA may still be expressed by some NTM, such as *Mycobacterium kansasii*. Therefore false positive results are still possible (Chiappini *et al*., 2016). Like TST, IGRA cannot differentiate between latent infection and active TB, with a suboptimal performance in children aged <5 years and immunosuppressed patients (Chiappini *et al*., 2016). More so, owing to the lack of unequivocal data of these assays, they are not recommended in children <5 years (Chiappini *et al*., 2016).

2.4.2.3. Radiological Approach

In children with minimal or no symptoms of PTB, imaging is considered a part of the diagnostic process of classifying TB as infection or active disease (Swingler *et al*., 2005). However detection of hilar and mediastinal lymphadenopathy; the two most pronounced cardinal signs of primary PTB is limited in children, with varying observations and non-specific findings (De Villiers *et al*., 2004). Consequently, the decisive role of chest X-ray in PTB diagnosis is of limited value in children with about 74% specificity and 40% sensitivity and is even lower in immunocompromised children (Bélard *et al*., 2014).

2.4.2.4. Bacteriological Diagnosis

Definitive bacteriological confirmation is important for TB diagnosis in children (Connell *et al*., 2011). Each microbiological test has a different threshold for detecting *M. tuberculosis* bacilli. The minimum threshold for detection of *M. tuberculosis* in smear microscopy is 10 000 colony-forming unit per millilitre (CFU/mL), in liquid culture is 10–100 CFU/mL, and in nucleic acid amplification assays is 100–150 CFU/mL (Triasih, 2015).

2.4.2.4.1. Smear Microscopy

Smear microscopy following direct staining of obtained samples provides rapid identification of acid fast bacilli (Gomez-Pastrana, 2013; Triasih, 2015). Smear microscopy conventionally relies on the ZN or cold Kinyoun staining of sputm smears to examine the presence of AFB. The principle of this method is based on the resistance of mycobacteria to decolourisation by acid alcohol post staining with phenol dyes. This resistance is a feature specific to mycobacteria due to the presence of mycolic acid in their cell wall (Caulfield and Wengenack, 2016).

Light microscopy of ZN stained smears is specific in the detection of *M. tuberculosis* bacilli, but the sensitivity of this technique varies between 20–80% in immunocompromised patients, with significantly reduced sensitivity in the pediatric population (Rathod *et al*., 2016). In a recent review, the pooled value of sensitivity of ZN staining was found to be less than 10% on sputum and GA in pediatric patients (Giang *et al*., 2015) and very poor on other specimen types (Singh *et al*., 2015).

Efforts have been made to improve the diagnostic performance of smear microscopy through concentration of specimens by centrifugation, and the use of Auramine staining that can be visualised by fluorescence microscopy (López Ávalos and Montes de Oca, 2012; Steingart *et al*., 2006a, 2006b). This has improved the sensitivity by 10% and reduces the time for screening while specificity is unaffected (Steingart *et al*., 2006a).

Despite the technique optimisation, the paucibacillary nature of TB disease in children has contributed to the very low sensitivity of smear microscopy (below 15%, except older children >5 years with adult type disease) (Gomez-Pastrana, 2013; Marais *et al.*, 2005b). Of importance is that acid-fast stains are not specific for MTBC as they cannot differentiate between mycobacterial species and as such cannot be used as an exclusive mycobacterial test for detecting *M. tuberculosis* (Roya-Pabon and Perez-Velez, 2016).

2.4.2.4.2. Culture Techniques

Culture remains the WHO recommended gold standard for the diagnosis of TB disease. Isolation of the organism is imperative for definitive diagnosis and DST (Dunn *et al*., 2016). Mycobacterial culture can be performed on either a solid or a liquid medium with a documented higher yield of *M. tuberculosis* as well as reduced time to detection from a liquid medium (e.g., Middlebrook 7H9) compared to a solid egg-based LJ medium or a solid agar-based Middlebrook 7H11 medium) (Brittle *et al*., 2009; Cruciani *et al*., 2004). Since normal body flora and fungi may inhibit the growth of *M. tuberculosis*, decontaminating patient samples is essential when isolating mycobacteria (Chatterjee *et al*., 2013). Common decontamination methods include N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH), cetyl pyridinium chloride-sodium chloride, oxalic acid, benzalkonium chloride-trisodium phosphate, or chlorhexidine (Ferroni *et al*., 2006; Yajko *et al*., 1993).

There are three food and drug administration (FDA) approved commercial platforms for the semi-automated broth-based culture of mycobacteria: The MGIT™ 960 System (Becton Dickinson Microbiology Systems), the VersaTREK system (Trek Diagnostic Systems), and the MB/BacT Alert 3D (bioMérieux). The MGIT™ 960 system is named for its use of Mycobacterial Growth Indicator Tubes (MGIT). Each tube contains a modified Middlebrook 7H9 broth and a fluorescent indicator that is quenched by the presence of oxygen within the tube. The growth of mycobacteria in the medium consumes oxygen over time and allows the fluorescent indicator to signal as positive once a certain growth threshold has been met. The instrument continuously monitors tube fluorescence allowing the identification of positive tubes (Caulfield and Wengenack, 2016).

A study by Brittle and colleagues in SA compared mycobacterial yield and time to detection of *M. tuberculosis* in 801 different pediatric specimens and highlighted the advantages of the MGIT™ 960

System over LJ. The yield obtained with the MGIT™ 960 System was significantly higher than that obtained with LJ (11% vs 1.6%), and the time to detection of mycobacteria using the MGIT™ 960 System was 12.4 days compared to 26.8 days using LJ (Brittle *et al.*, 2009). This was also validated in a recent study of Roy and colleagues, where recovery of *M. tuberculosis* using the MGIT™ 960 System and LJ medium was compared in children suspected of tubercular meningitis. The MGIT™ 960 System showed a higher rate of recovery of *M. tuberculosis* (28%) than LJ media (8%) with a shorter mean time to detect *M. tuberculosis* (13.2 days) compared to (32.4 days) with LJ media (Roy *et al.*, 2016).

Bacteriological confirmation of childhood TB disease is mostly achieved in <40% cases, while positivity rate may be as high as 70% in TB endemic regions depending on factors such as age, disease severity, type, quality of the specimen, and culture method used (Chiang *et al.*, 2015; Detjen *et al.*, 2015; Marais *et al.*, 2006b; Zar *et al.*, 2005). It was however suggested that since the process of liquification and decontamination of pediatric specimen before culture follows the same procedure as that for adult specimens (CLSI, 2008), and considering the paucibacillary nature of TB disease in children, stringent decontamination procedures may easily render the small concentration of bacilli present nonviable for culture (Burdz *et al.*, 2003).

A proposed low-cost alternative to MGIT™ culture is the MODS assay. The technique involves the direct inoculation of the decontaminated specimen into wells of a tissue culture plate containing liquid growth media (Nicol and Zar, 2011). Anti-tuberculous drugs like INH and RIF can be incorporated in some wells at critical concentrations, and thus enables the concurrent detection of drug resistance (Moore *et al.*, 2004; Nicol and Zar, 2011). Using "clinical diagnosis" as reference standard, MODS is substantially more sensitive than solid culture in specimens from children (Ha *et al.*, 2009; Oberhelman *et al.*, 2010; Tran *et al.*, 2013), and its sensitivity in children has been found to be similar to that of Xpert® (Nhu *et al.*, 2013). However, MODS is labour intensive and also requires a degree of experience to read the cording in the wells (Nhu *et al.*, 2013).

2.4.2.4.3. Drug Susceptibility Testing in Liquid Media

WHO in 2007 recommended the use of liquid media for DST in low-income countries and middle-income countries for possible early detection of DR-TB (WHO, 2007). DST with liquid media is performed after the preparation of primary culture and its identification (Falzon *et al.*, 2011). One of the most widely used systems for MTBC detection and DST in liquid media is the MGIT™ 960 system (Becton Dickinson, Sparks, MD, USA) using MGIT (Hwang *et al.*, 2014). After the identification of *M. tuberculosis* positive MGIT, purity is checked, and the liquid media in the MGIT are used directly for DST, shortening the time to detection of resistance from >30 days in solid media to 4-16 days in liquid media (Piersimoni *et al.*, 2006).

Although the MGIT™ 960 system is widely used, some investigators suggested that the system may yield a false susceptible or resistant DST results, and may also have a doubtful DST result owing to the difficulty in checking for non-visible contamination and overgrowth with NTM and in assessing the colony's morphology (Van Klingeren *et al*., 2007; Piersimoni *et al*., 2006). This may lead to false MDR-TB results, unnecessary treatment prescription and treatment failure (Piersimoni *et al*., 2006). It is also suggested that these challenges are partly due to the work-flow of liquid media DST, the inoculating methods and the characteristics of some systems (Piersimoni *et al*., 2006).

2.4.2.5. Molecular Diagnosis

2.4.2.5.1. Real-Time Polymerase Chain Reaction

A significant advancement in the diagnostic testing for TB is the development of real-time polymerase chain reaction (qPCR) (Connell *et al*., 2011). The Xpert® assay is an FDA approved cartridge-based nucleic acid amplification test (NAAT) that has also been recommended in the 2013 WHO policy update as the initial diagnostic test for children suspected of having MDR-TB or HIV-associated TB (WHO, 2013).

The Xpert® platform is a self-contained cartridge-based system that utilises microfluidics and automated nucleic acid extraction and amplification to provide the detection of *M. tuberculosis* and simultaneously RIF resistance directly from clinical samples in <2 hours (Dunn *et al*., 2016). The test procedure may be performed on either fresh samples or sediments obtained after decontamination and concentration (WHO, 2014b). Xpert® closed cartridge system also reduces the risk of infection via aerosol production compared to smear preparation by making use of a tuberculocidal sample treatment reagent, which may be used as a point of care test in the absence of a biocontainment facility (Banada *et al*., 2010). The assay is FDA cleared for use on smear-positive and smear-negative respiratory specimens, but with no specific clearance for extrapulmonary specimens (Dunn *et al*., 2016).

In the Xpert® system, five overlapping nucleic acid hybridisation probes labelled with coloured fluorophores binds to an 81-bp core region of the wild-type *rpoB* gene (Helb *et al*., 2010; Lawn and Nicol, 2011). This region is associated with over 95% of RIF resistant cases (Telenti *et al*., 1993). The molecular beacons hybridise specifically with amplified wild-type *rpoB* gene sequences. The detection of *M. tuberculosis* occurs when more than two fluorophores with *rpoB*-specific molecular beacons signal cycle threshold (Ct) values that are less than 39 and when the Ct values are in the same range (±2) (Blakemore *et al*., 2010).

Multiple studies have been performed to assess the utility of Xpert® in the pediatric population. The WHO 2013 policy update documented a pooled specificity of 98% for Xpert® compared to culture with

a pooled sensitivity of 66% (WHO, 2013). Various studies in the last five years further evaluated the diagnostic potential of Xpert® for diagnosing PTB in the children population. Xpert® was reported to outperform smear microscopy on GA and sputum samples by detecting 47% of smear-negative cases (Bates *et al*., 2013). Nicol and colleagues also reported that Xpert® testing on two IS samples detected twice as many cases (75·9%) compared to smear microscopy (37·9%). Xpert® detected all of 22 smear-positive cases and 22 of 36 smear-negative cases (Nicol *et al*., 2011). In another study, when compared to culture, Xpert® had an incremental yield on testing of the second specimen. The increase in sensitivity from testing a second IS was 17.6% for culture and 25% for Xpert® while sensitivity from testing a second NPA was 26.3% and 36.7 % for culture and Xpert® respectively (Zar *et al*., 2012).

A recent meta-analysis also evaluated the results of 15 studies with 4768 respiratory specimens from 3650 children suspected of PTB. Xpert® detected 11% of the 12% bacteriologically confirmed PTB, with better sensitivity than smear microscopy, but suboptimum when compared with culture. With culture as the gold standard, Xpert® exhibited a sensitivity and specificity of 62% and 98% for IS respectively, and 66% sensitivity and 98 % specificity for GA, with an improved sensitivity and specificity (86 and 98%, respectively) in the detection of RIF resistance (Detjen *et al*., 2015).

Of note, using clinically diagnosed TB as the reference standard, Xpert® sensitivity in culture-negative samples from pediatric patients was reported to be 4% for expectorated or IS and 15% for GA samples (WHO, 2013). This was also corroborated in a study where Xpert® sensitivity in culture-negative children started on anti-tuberculosis therapy was 2% for expectorated or IS (Detjen *et al*., 2015). More so, even though the specificity for detecting RIF resistance rmains higher than 98%, some studies have documented false-positive RIF resistance, which has been suggested to occur due to the detection of *rpoB* mutations that do not affect phenotypic resistance (Ocheretina *et al*., 2016; van Rie *et al*., 2012).

Although Xpert® aids in prompt confirmation of TB disease, with better sensitivity for the diagnosis of PTB in children compared with microscopy, its sensitivity remains suboptimum compared with culture, and as such a negative Xpert® result cannot solely rule out TB disease (Detjen *et al*., 2015; WHO, 2014b). More so, the fact that Xpert® cannot differentiate viable from non-viable mycobacteria precludes its use in treatment monitoring (Friedrich *et al*., 2013; WHO, 2014b). The instrument requirement for steady electricity supply, controlled temperature and a dust free environment also has implications on its point of care capability (Albert *et al*., 2016). Additionally, Xpert® test failures are often not recorded in accuracy studies, since analysis are based on valid index and reference tests. However, reports from routine implementation suggested failure between 7.2%-10.6%, with a common requirement for module replacement (Creswell *et al*., 2014; Raizada *et al*., 2014).

The new Xpert® MTB/RIF Ultra assay (Ultra) has been developed to overcome the known shortcomings of Xpert® (Chakravorty *et al*., 2017; WHO, 2017a). Early demonstration of the Ultra assay's operational characteristics suggests that Ultra will result in remarkable TB case detection rates even in subjects with paucibacillary TB, people with HIV co-infection, and those with EPTB (Chakravorty *et al*., 2017; WHO, 2017a). Ultra was proven to have a similar accuracy with Xpert® in detecting RIF resistance cases (WHO, 2017a). However, Ultra's increased sensitivity has been offset by a decrease in specificity, possibly due to the acknowledged limitations of the reference standard (culture) used as the comparator for both versions of the Xpert® (Dorman *et al*., 2017; WHO, 2017a). In view of the recent evidence, Ultra has also been approved by the WHO as an initial diagnostic test for all adults and children with signs and symptoms of TB, and for the testing of selected extrapulmonary specimens (cerebrospinal fluid, lymph nodes and tissue specimens) (WHO, 2017b).

2.4.2.5.2. Line Probe Assays

As a rapid alternative to phenotypic DST, specialized NAAT known as line probe assays (LPAs) offer *M. tuberculosis* speciation and genotypic DST with results in 1-2 days (WHO, 2008). In 2008, the WHO endorsed the use of Version 1 of the Genotype® MTBDR*plus* assay for rapid detection of INH and RIF resistance on smear-positive or culture-positive samples (WHO, 2008), with the subsequent endorsement of Genotype® MTBDR*plus* Version 2 and the Nipro Corporation NTM+MDR-TB Detection Kit 2 in 2015 (WHO, 2016e).

LPAs such as Genotype® MTBDR*plus* (Hain Lifesciences, Nehren, Germany) identify drug-resistance mutations by detecting the binding of PCR-amplified fragments of *M. tuberculosis* DNA to probes that target mutated sequences in the *rpoB* gene associated with resistance to RIF, and in the *KatG* gene and promoter region of the *inhA* gene associated with INH resistance (Maclean *et al.*, 2017). Two studies concluded that the Genotype MTBDR*plus* assay was highly accurate in detecting MDR-TB when compared to conventional DST method. Both reviews reported sensitivities of 91% and 94% and specificities of 99% and 100%, respectively, for detection of MDR-TB (Abanda *et al*., 2017; Bai *et al*., 2016)

Report by WHO suggest that first-line LPAs offer 98% sensitivity for RIF, but only 85% for INH resistance due to the presence of resistance coding mutations outside the regions of the *inhA* and *KatG* genes detected by the assays (WHO, 2008). The WHO endorsed the use of second-line LPAs (Genotype MTBDR*sl* version 2.0) in 2016, which detects mutations conferring resistance to FQs and second-line injectable drugs (WHO, 2016d).

2.4.2.6. Antigen Detection Tests

The Lipoarabinomannan assay is an enzyme-linked immunosorbent assay (ELISA) based test for detecting mycobacterial antigens (Nicol and Zar, 2011). Lipoarabinomannan is a lipopolysaccharide component of the outer cell wall of mycobacteria that is excreted in the urine after *M. tuberculosis* is lysed by the host immune system (Dorman, 2015; Nicol and Zar, 2011). The sensitivity of Lipoarabinomannan assay in adult studies varied widely (44%-67%), with better sensitivity in patients co-infected with advanced HIV infection, which is due to high bacterial load and increased potential for disseminated forms of TB (Connell *et al.*, 2011; Dorman, 2015).

Nicol *et al.* evaluated the accuracy of the Lipoarabinomannan assay in 532 children (HIV-infected and uninfected) suspected of TB; they reported that Lipoarabinomannan has an insufficient sensitivity (48.3 %) and specificity (60.8 %) for diagnosis of TB compared to culture in this population (Nicol *et al.*, 2014).

2.4.2.7. Immunochromatographic Assays

Immunochromatographic assays, also called lateral flow assays were developed to discriminate between MTBC and NTM (Martin *et al.*, 2011; Park *et al.*, 2009). It is a simple and rapid manual assay with no requirement for sample preparation. Total assay time is <15 min and reactivity is confirmed by the visual colour development of test and control lines (Said *et al.*, 2011). The assay uses a monoclonal antibody to detect the MPB64 protein (Rv1980c; also termed MPT64); a 24 kDa protein, secreted during growth by the MTBC and highly specific for the MTBC, except some variants of *M. bovis* BCG (Andersen *et al.*, 1991; Harboe *et al.*, 1986). There are three commercially available MPT64-based ICT tests; SD BIOLINE TB Ag MPT64 (Standard Diagnostics, Seoul, South Korea), BD MGIT™ TBc Identification Test (Becton Dickinson, Franklin Lakes, USA), and Capilia TB-Neo (Tauns, Izunokuni, Japan).

The MPT64 immunochromatographic assays has been confirmed to be a useful tool for identification of MTBC in liquid cultures of the MGIT™ 960 System and other liquid culture automated systems for mycobacterial detection such as the BacT/Alert system 3D (formerly MB/Bact) (Dinnes *et al.*, 2007; Martin *et al.*, 2011; Yu *et al.*, 2011). A previous study suggested that the test sensitivity, specificity, positive predictive values (PPV), and negative predictive values (NPV) were 98.3%; 97.5%; 99.15%; 95.12%, respectively (Povazan *et al.*, 2012). A study by Said *et al.* suggested a sensitivity, specificity, NPV, and PPV of 100%, 92.4%, 100%, and 92.2%, respectively for the BD MGIT™ TBc Identification Test (Said *et al.*, 2011). Nevertheless, despite the high sensitivity and specificity report of these assays, a recent study by Chew *et al.*, suggests that MPT64 gene mutations may lead to non-reactive rapid immunochromatographic assays; as such, a negative MPT64 assay cannot in itself exclude the diagnosis of TB (Chew *et al.*, 2017).

2.5. Treatment of Childhood Pulmonary Tuberculosis

In the absence of confirmed or suspected DR-TB, the treatment of TB and its accompanying clinical characteristics in children is centred on a four-drug regimen as in adults; INH, RIF, PZA, and ethambutol (EMB) (WHO, 2014a). As per the WHO, the recommended regimen for presumptive drug-susceptible TB (DS-TB) cases in children are a 2-month intensive phase of daily INH, RIF and PZA, with the addition of EMB depending on the region and disease circumstances followed by a 4 month continuation phase of daily INH and RIF (WHO, 2014a).

In SA, three standard treatment regimens are adopted. Regimen 1 is for new and previously treated children >8 years/>30 kg and involves the use of RIF, INH, PZA and EMB for two months at the intensive phase and RIF/INH at the continuation phase for four months. Regimen 3A includes an intensive phase of RIF, INH, and PZA for two months and RIF and INH continuation phase for four months for children <8 years and <30 kg with uncomplicated PTB disease, while regimen 3B comprises of RIF, INH, PZA and EMB intensive phase for two months and four months RIF, INH continuation phase for children <8 years and <30 kg with complicated TB disease (DOH, 2014).

Recommendation by the WHO suggest that children with proven or suspected PTB or tuberculous meningitis caused by MDR-TB bacilli can be treated with any FQs in the context of a well-functioning MDR-TB control programme and within an appropriate MDR-TB regimen, while the decision to treat is taken by a clinician experienced in managing paediatric TB (WHO, 2014a). Conversely, the South African protocol includes, in the majority of MDR-TB cases, the use of high-dose INH as "isolates with an *inh*A promoter region mutation usually have a low minimum inhibitory concentration (MIC)" (Hamzaoui *et al*., 2014).

2.6. Childhood Tuberculosis Preventive Interventions

Childhood TB preventive measures include vaccination, administration of isoniazid preventive therapy (IPT) and treatment of LTBI (Hamzaoui *et al*., 2014; Lancella *et al*., 2015).

In TB prevalent countries, BCG vaccination is recommended in very young children and infants; but has a limited preventive efficacy of 50% against PTB (Britton *et al*., 2013). Nontheless, BCG vaccination reduce the risk of severe disseminated TB disease such as miliary TB and TB meningitis (Getahun *et al*., 2012; Newton *et al*., 2008). Furhermore, it is recommended that following documented exposure to an infectious case of DS-TB, children <5 years of age and/or immunocompromised without active TB should receive IPT daily for six months (WHO, 2014a).

While the use of MDR-TB prophylasis may be suitable in some cases, its use is hampered by the limited evidence available on optimal paediatric chemoprophylasis regimens in child contacts of MDR-TB

cases (Padayatchi and Naidu, 2015; WHO, 2015a). Nonetheless, in the absence of a randomised clinical trial, observational studies suggest that a suitable MDR-TB prophylaxis should be considered in the absence of active TB, when there is a documented exposure to MDR-TB contact and the risk of disease progression is high and outweighs the risk of adverse events (Lange *et al*., 2014; Padayatchi and Naidu, 2015).

2.7. Molecular Epidemiology of *Mycobacterium tuberculosis*

2.7.1. The Genome of *Mycobacterium tuberculosis*

The complete genome sequencing of the H37Rv strain of *M. tuberculosis* was performed by Cole *et al.* in 1998 and generated valuable genetic information of the bacterium. The genome consists of 4 411 529 sequence base pairs; containing about 4 000 genes and high guanine + cytosine (G+C) content of 65.6%. Even though the G+C content is relatively constant throughout the genome, there are regions with higher G+C content which correlate to sequences belonging to a large family of genes which include the polymorphic GC-rich repetitive sequence (PGRS) (Cole *et al*., 1998).

The *M. tuberculosis* genome is rich in repetitive DNA, especially in insertion sequence and multigene families as illustrated in figure 2.5 (Cole *et al.*, 1998; Nicol and Wilkinson, 2008). Of interest are the IS*6110*, a sequence of the IS3 family with different insertion sites and copy numbers and therefore its wide utility for strain typing and molecular epidemiology studies (Cole *et al*., 1998).

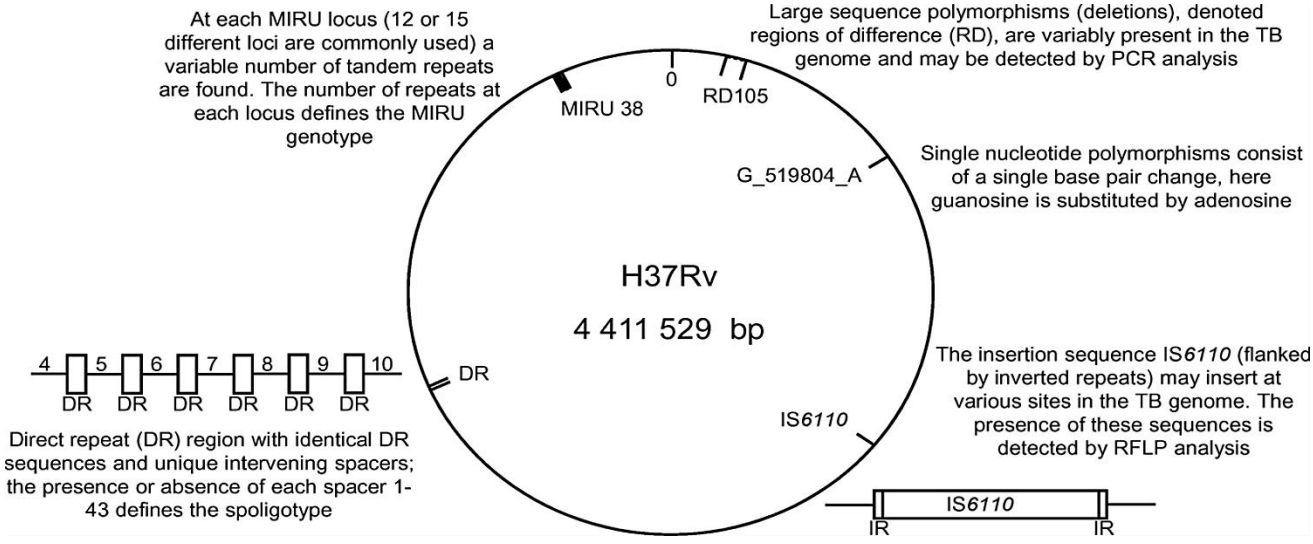

Figure 2.5. Schematic representation of the *Mycobacterium tuberculosis* genome, indicating the genetic basis of genotyping techniques. The circular chromosome of the reference strain H37Rv is shown together with examples of the genetic elements used for strain genotyping. Adapted from Nicol and Wilkinson, 2008.

Another described region is the direct repeat (DR) locus; a member of the Clustered Regularly Interspersed Palindromic Repeats (CRISP) sequences and a highly preserved repetitive region within

the *M. tuberculosis* chromosome (Figure 2.5) (Baker *et al*., 2004; Filliol *et al*., 2006; Supply *et al*., 2000). After the discovery of the DR region, the variable number of tandem repetitions (VNTR) was found followed by the discovery of the mycobacterial intergenic repetition units (MIRU) which is also listed as VNTR multiple locus analysis. After that, the identification of the single nucleotide polymorphism (SNP) and large sequence polymorphism (LSP) was described (Figure 2.5) (Nicol and Wilkinson, 2008).

2.7.2. Molecular Characterisation of *Mycobacterium tuberculosis*

Molecular epidemiology studies use DNA fingerprinting (genotyping) based on polymorphisms in the *M. tuberculosis* genome to characterise the bacteria into different strain families (Mathema *et al*., 2006). The combination of epidemiological data and TB genotyping results provides the tools to investigate the diversity of *M. tuberculosis* strains and the role of reactivation, reinfection and recent transmission in different settings (Filliol *et al*., 2002). Its applicability in children is significant as they usually progress to disease within a year after primary infection; therefore genotypic analysis in children could reflect current transmission patterns within the community (CDC, 2012; Marais *et al*., 2006b; Yates *et al.*, 2016).

Several genotyping methods are currently widely used in the monitoring and study of the dynamics of TB epidemics. These methods include spoligotyping, IS*6110* restriction fragment length polymorphism (IS*6110* RFLP) and MIRU-VNTR (Ifticene e*t al*., 2015; Muwonge *et al*., 2013). The analysis of punctiform mutations using new-generation whole-genome sequencing (WGS) is another recently introduced method (Jagielski *et al*., 2014; Schürch and van Soolingen, 2012).

Of the genotyping assays, IS*6110* RFLP is considered the 'gold standard' (van Embden *et al*., 1993). Spoligotyping and MIRU-VNTR typing have a lower discriminatory power compared to IS*6110* RFLP, however, a combination of these typing assays provides a discriminatory power considered close to that of the IS*6110* RFLP typing method (Blackwood *et al*., 2004).

2.7.2.1. IS6110 Restriction Fragment Length Polymorphism

Insertion sequence elements are small mobile repetitive elements with variable copy numbers located on the chromosome of *M. tuberculosis,* with over 14 types present in the MTBC (Mathema *et al.*, 2006; van Soolingen, 2001). These insertion sequence elements are usually less than 2.5 kb in size and are distributed throughout the chromosome in no particular order (Barnes and Cave, 2003; van Embden *et al*., 1993; van Soolingen *et al*., 1991). The most widely used insertion sequence elements for the genotypic characterisation of *M. tuberculosis* strains is IS*6110*, which is exclusive to the MTBC and ranges from 0 to 25 copies in different *M. tuberculosis* strains (Khosravi and Seghatoleslami, 2009). The IS*6110* element copies vary between *M. tuberculosis* strains in numbers and location, therefore this

method is employed in *M. tuberculosis* strain differentiation (Khosravi and Seghatoleslami, 2009; van Embden *et al*., 1993).

RFLP typing using IS*6110* as a DNA probe is a standardised method against which other molecular typing techniques are evaluated (van Embden *et al*., 1993; van Soolingen, 2001). The IS*6110* RFLP typing assay uses DNA probes to visualise *Pvu*II restriction fragments with repetitive DNA sequences complementary to the specific probe (van Soolingen, 2001). The DNA probe is labelled with peroxidase which enables the chemiluminescence detection of the IS*6110*-containing restriction fragment (Burgos and Pym, 2002).

Previous epidemiological studies suggest that most *M. tuberculosis* strains consist of 8 to 18 copies of IS*6110* insertion elements, a number that enables sufficient discrimination between various *M. tuberculosis* strains (van Soolingen, 2001). Unfortunately, some *M. tuberculosis* isolates have few copies or no IS*6110* elements, and the utility of IS*6110* in such strains is limited due to lesser discriminatory power (van Soolingen *et al*., 1993). Other limitations of this technique include the requirement of high-quality DNA for restriction enzyme digestion, the lengthy execution time, the need for sophisticated and expensive software for analysis as well as experienced technicians (Blackwood *et al*., 2004; Lancella *et al*., 2015).

2.7.2.2. Spoligotyping

Spoligotyping (Spacer Oligonucleotide Typing) is based on identifying polymorphisms in the spacer units of the DR region of the *M. tuberculosis* genome (Groenen *et al*., 1993). The DR region comprises of multiple identical 36 bp direct repeats (DRs) interspersed by unique spacer DNA sequences that are 37 to 41 bp in length with significant strain to strain polymorphisms (Goyal *et al*., 1997; Groenen *et al*., 1993; Kamerbeek *et al.*, 1997). These variations arise as a result of homologous recombination between DRs, or transformation due to the insertion of IS*6110* and the sequential deletion of direct variable repeats (DVR) from the DR region (Groenen *et al*., 1993; van Embden *et al*., 2000). These unidirectional events may occur over time and as such the DR region is believed to be an informative locus for studying the epidemiology of the MTBC (Filliol *et al*., 2006; Warren *et al*., 2004).

The spoligotyping technique uses PCR, reverse hybridisation, and 43 DNA probes covalently bound to a membrane to identify the presence or absence of one or more spacers in the DR region of *M. tuberculosis* strains (Goyal *et al*., 1997; Kamerbeek *et al*., 1997).

The advantages of spoligotyping include its requirement for a relatively small amount of DNA (10 ng) compared to IS*6110* RFLP, which needs about 2-3 µg (Ali, 2014; Kamerbeek *et al*., 1997). It is a simple and robust technique that generates highly reproducible results that can be easily interpreted and

computerised in binary format {present (n)/absent (o)] or an octal code format, which enables intra-laboratory comparisons (Kamerbeek *et al*., 1997; Nicol and Wilkinson, 2008). A further attractive aspect of this method is the possibility to rapidly type strains without the need to subculture isolates for DNA isolation, and the ability to type *M. tuberculosis* organisms from paraffin-embedded blocks or in archaeological samples (Kulkarni *et al*., 2005).

Although spoligotyping is simple and highly reproducible, it has a lower discriminatory capacity compared to IS*6110*-based RFLP analysis, except in strains having low IS*6110* copy numbers (Bauer *et al*., 1999; Goyal *et al*., 1997). More so, spoligotyping targets a single locus, representing less than 0.1% of the *M. tuberculosis* genome compared to IS*6110*-based RFLP analysis, which analyses the distribution of IS6110 throughout the entire genome (Ali, 2014; Jagielski *et al*., 2014). Additionally, spoligotyping is not discriminatory enough for strains having identical spoligotyping patterns, with distinct IS6110 fingerprint profiles. Therefore spoligotyping for the characterisation of the East-Asian/Beijing lineage is of limited use (Bifani *et al*., 2002; Desikan and Narayanan, 2015; van Embden *et al*., 2000).

For rapid global comparisons of various circulating strains, different international spoligotype databases (SpolDB1, SpolDB2, SpolDB3, SpolDB4, SITVIT2 and TB-insight) were created (Brudey *et al.*, 2006; Filliol *et al*., 2002). These databases reveal the global structure of the MTBC population and have played a significant role in the analysis of the global TB epidemiology (Sola *et al*., 2001; Van Embden and van Soolingen, 2000).

2.7.2.3. MIRU-VNTR

MIRU are groups of repetitive units distributed throughout the mycobacterial genome, which was first described by Supply and colleagues (Supply *et al*., 1997). The *M. tuberculosis* genome contains many MIRU (Figure 2.6); some strains possess identical repeat units, while others may contain repeats with slight variation in length and sequence (40 to 100 bp) (Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001; Supply *et al*., 2000).

These MIRU elements are found as tandem repeats and are dispersed in the intergenic region of the *M. tuberculosis* genome (Frothingham and Meeker-O'Connell, 1998; Mazars *et al*., 2001). The VNTR are consecutive base pair repeats located in the non-coding region of the *M. tuberculosis* genome (Supply *et al*., 2000). Between 12 to 24 MIRU loci are found in *M. tuberculosis* and are used to distinguish between *M. tuberculosis* strains (Mathema *et al*., 2006). However, further work by Supply *et al*. identified a total of 41 MIRU loci when analysing the complete genome of the H37Rv strain of *M. tuberculosis* (Supply *et al*., 2000) (Figure 2.6).

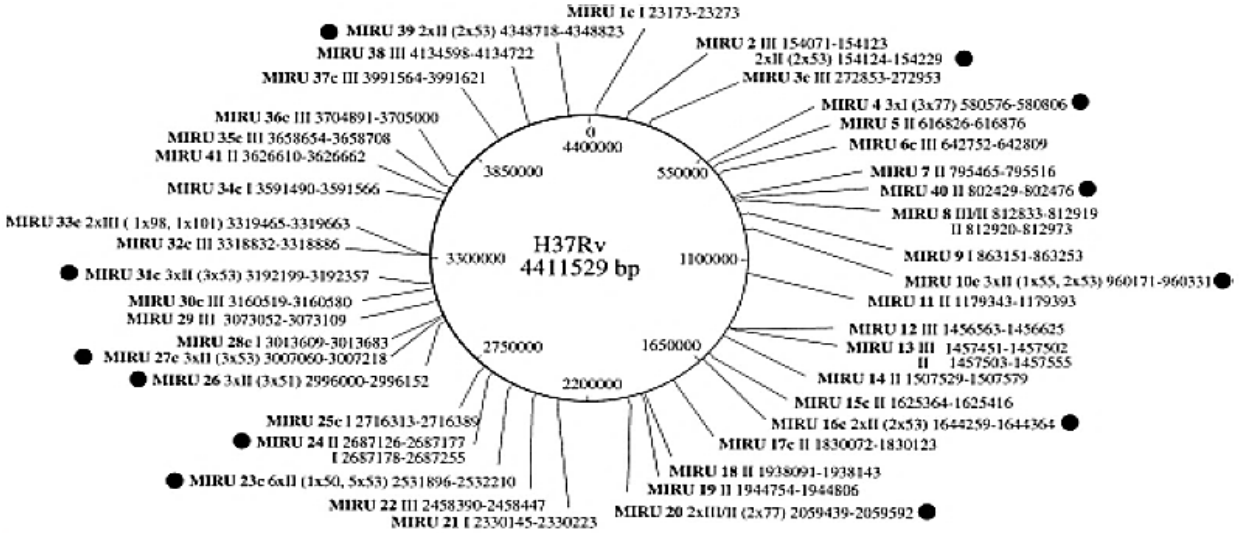

Figure 2.6. The location of MIRU loci in the *Mycobacterium tuberculosis* H37Rv genome. Adapted from Supply *et al*., 2000**.**

The MIRU-VNTR genotyping technique is a PCR based assay that uses specific primers to flank the region of each locus (Supply *et al*., 2001). The number of the targeted MIRU-VNTR marker copies mirrors the discriminatory power of the marker (Mathema *et al*., 2006).

Post PCR amplification, amplicon sizes can be determined using gel electrophoresis, capillary (1, 24 or 28) analysis or nondenaturing high-performance liquid chromatography (Mathema *et al*., 2006). The MIRU-VNTR genotyping method can also be automated using labelled primers and an automated sequencer. Automation enables the use of a quadruplex PCR with each PCR targeting four different MIRU loci (Allix-Beguec *et al*., 2008; Supply *et al*., 2001). MIRU-VNTR results are expressed as numerical strings and can be catalogued on a computer database (MIRU-VNTRplus) for compilation and comparisons of different MIRU patterns (Allix-Beguec *et al*., 2008; Supply *et al*., 2001).

Although MIRU-VNTR typing is an extensively used tool for transmission studies (Bouklata *et al.*, 2015), its discriminatory power is dependent on the number of loci analysed, the more MIRU loci used, the higher the discriminatory capability (Mathema *et al*., 2006; Supply *et al*., 2000). It is further suggested that when more than 12 loci are used, or MIRU analysis is combined with spoligotyping, the discriminatory power is similar to that of IS*6110*-RFLP analysis (Cowan *et al*., 2002; Oelemann *et al*., 2007; Supply *et al*., 2001).

Further exploration of the MIRU loci suggested that optimal discriminatory power can be achieved when a set of 15 MIRU loci is applied for molecular epidemiologic studies and 24 loci for molecular phylogenetic studies (Allix-Beguec *et al*., 2008; van Deutekom *et al*., 2005; Oelemann *et al*., 2007; Supply *et al*., 2006). The advantages of MIRU-VNTR typing includes its reproducibility, generation of

digital results that are readily exchangeable between laboratories and its availability in a global database (Mazars *et al*., 2001; Supply *et al*., 2001; Weniger *et al*., 2010).

2.7.3. Epidemiology and Genetic Diversity of *Mycobacterium tuberculosis* Strains

The MTBC strains are divided into eight major spoligotyping-based families which are further divided into several sub-families (Table 2.2) (Brudey *et al*., 2006; Firdessa *et al*., 2013), and seven lineages (Coll *et al*., 2014b; Gagneux *et al*., 2006b; Yimer *et al*., 2017). The different polymorphic or hypervariable genetic markers are characterised and used to discriminate or sub-speciate clinical isolates of MTBC (Brudey *et al*., 2006; Firdessa *et al*., 2013).

Table 2.2. The eight major *Mycobacterium tuberculosis* spoligotyping based families and subfamilies Compiled from Brudey *et al*., 2006 and Firdessa *et al*., 2013.

Families	Subfamilies
East African-Indian (EAI)	EAI1, EAI2-Nonthaburi. EAI3, EAI4 EAI5, EAI6-Bangladesh/1, EIA7-Bangladesh/2 & EIA8-Madagascar
Haarlem (H)	Haarlem1, Haarlem2, Haarlem3 & Haarlem 4
Central and Middle Eastern Asia (CAS)	CAS1-Delhi, CAS1-Kilimanjaro & CAS2
European Family X	X1, X2 & X3
Default family T	T1-Russia/2,T2-Uganda, T3 -Ethiopia, T3-Oscar, T4-Central Europe/1, T5-Russia/1, T5-Madrid/2,"Tuscany"
W-Beijing	None
Latino-American and Mediterranean (LAM)	LAM1, LAM2, LAM3, LAM4, LAM5, LAM6, LAM7-Turkey, LAM8, LAM9, LAM10-Camreoun, LAM11-ZWE, LAM12-Madrid1
Ethiopian	None

The seven major lineages (figure 2.7) were classified based on large genomic deletions and has been confirmed using WGS (Coll *et al*., 2014b; Gagneux *et al*., 2006b; Yimer *et al*., 2017). Each lineage is strongly associated with a geographic region and as such are known by either numbers or the name denoting the geographical area where they are predominantly isolated (Gagneux and Small, 2007).

Lineage 1 consists of EAI, with strains mostly isolated from East Africa, Southeast Asia and Southern India. Lineage 2 consists of the Beijing strains and are mostly found in East Asia, Russia and SA. Lineage 3 consists of Central and Middle Eastern Asia (CAS) and are mostly found in East Africa and the Indo-Oceanic region. Lineage 4 consists of Haarlem (H), LAM family, Default family (T) and European family (X), and are mostly found in Europe and America. Lineages 5 and 6 consists of the AFRI strains and are predominantly found in West Africa (Firdessa *et al*., 2013; Gagneux and Small, 2007; Yimer *et al*., 2015). A seventh lineage has recently been described from Ethiopia with the name Aethiops vetus proposed for the lineage (Nebenzahl-Guimaraes *et al*., 2016; Yimer *et al*., 2017).

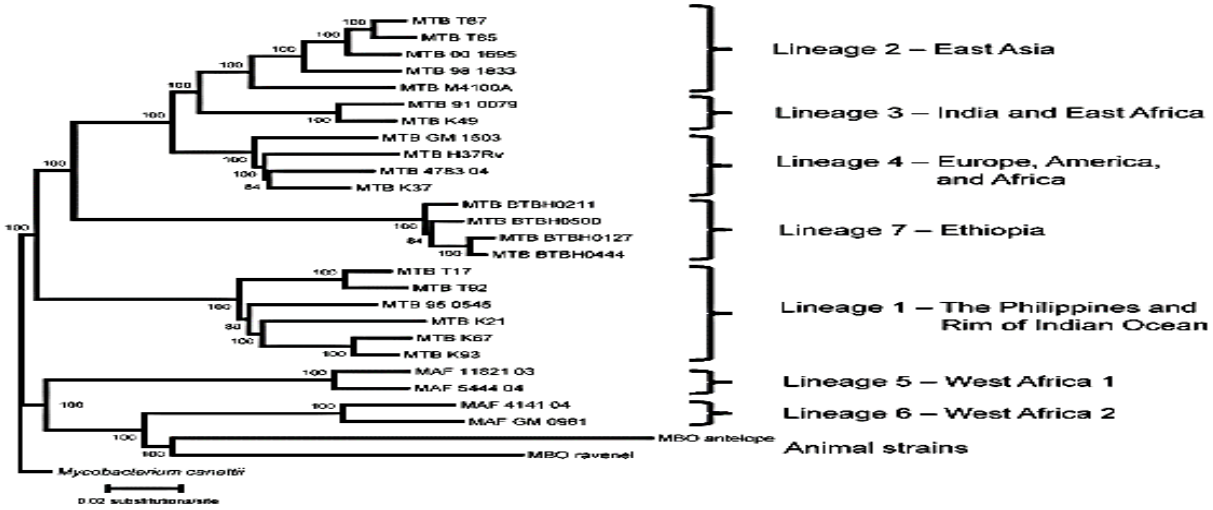

Figure 2.7. Lineages of the MTBC. Adapted from Firdessa *et al.*, 2013.

Lineages of *M. tuberculosis* are important as the strain may play a role in the virulence, clinical presentation, treatment efficacy, transmission of the disease and evolution of DR-TB strains (Coll *et al.*, 2014a). Very little is known in the FS about the genotypes of the strains of TB, especially MDR-TB strains, while no information is available on strains circulating amongst children. According to Van der Spoel van Dijk *et al.*, LAM was the most prevalent lineage in the FS, followed closely by T isolates and X isolates and then Beijing and H isolates (Van der Spoel van Dijk *et al.*, 2016). In contrast, the Beijing lineage was the most dominant lineage reported by other studies in children and adults from other provinces (Hove *et al.*, 2012; Sekati *et al.*, 2015).

Chapter 3: METHODOLOGY

3.1. Study Sites Description

This retrospective study was conducted in the Mangaung Municipality, FS Province. It is a category A municipality located in the central interior of SA covering approximately 6 284km² with 124.7 people per km2 (Massyn *et al*., 2015). The total population of the city is approximately 784 000, while children ≤15 years accounts for 26.9% (Massyn *et al*., 2015). The provincial incidence of TB among all age groups in 2014 was estimated to be 724 cases per 100 000 population with approximately 10% occurring in children ≤15 years (Massyn e*t al*., 2015). Two different public health facilities (Pelonomi regional hospital and Botshabelo district hospital) were involved in this study.

Pelonomi Regional Hospital is a provincial partnership hospital located at 121 Dr Belcher Road, Heidedal in Bloemfontein, FS. Children were enrolled from its three general paediatric wards (3A, 4A, and 4B) that caters for children ≤13 years old. Of note is that access to this hospital is mostly by referral from other health institutions.

Botshabelo District Hospital is a 135-bed government-funded hospital located in Old Police Station Road in Botshabelo, Mangaung Municipality FS. It is situated 57 km East of Bloemfontein on the N8 highway. It offers a full district hospital package of services which includes but are not limited to paediatric, casualty, and an ARV clinic. The hospital receives patients referred from clinics situated in (Botshabelo, Theunissen, and Verkeerdevlei) and refers patients to Pelonomi Hospital.

Laboratory analysis of specimens was performed at the Medical Microbiology Department, University of the FS, Bloemfontein as part of the National Health Laboratory Services (NHLS), Provincial TB Reference Laboratory located in Universitas Bloemfontein.

3.2. Study Design

This was a descriptive observational study conducted among children ≤13 years old from August 2016 to June 2017. Within this period, eligible patients (according to the selection criteria in section 3.4) admitted in the aforementioned health facilities were consecutively enrolled in the study after voluntary consent/ascent and assessment by an attending physician.

3.3. Study Population

The study comprises of patients aged ≤13 years that were suspected of PTB and admitted to the paediatric wards of the mentioned health facilities. These patients exhibited clinical symptoms suggestive of PTB as established by the consensus agreement on Intrathoracic Tuberculosis Definitions for Diagnostic Research in Children as defined in section 3.4.1.

3.4. Selection Criteria

3.4.1. Inclusion Criteria

As defined by the consensus agreement on Intrathoracic Tuberculosis Definitions for Diagnostic Research in Children (Cuevas *et al*., 2012; Graham *et al*., 2012, 2015a), patients were eligible upon suspicion of PTB with a symptom compatible with at least one of the following:

(a) A persistent cough: (>2 weeks), unremitting cough.

(b) Weight loss/failure to thrive: (Unexplained weight loss with 0.5% reduction in weight compared with the highest weight recorded in last three months **OR** failure to thrive **AND** not responding to nutritional rehabilitation or ART if HIV-infected.

(c) Persistent unexplained fever (>38°C): Persistent (>1 week) reported by a guardian or objectively recorded at least once.

(d) Persistent unexplained lethargy or reduced playfulness/activity reported by the parent/caregiver.

(e) For infants <60 days: Any of the criteria listed above (**a** to **d**) plus pneumonia, unexplained hepatosplenomegaly or sepsis-like illness not responding to appropriate treatment "Where other causes are excluded or not excluded".

(f) Chest X-ray suggestive of PTB per attending physician.

(g) History of exposure to *M. tuberculosis*: Reported exposure to a case of TB (household/close contact) within the preceding 12 months.

3.4.2. Exclusion Criteria

Patients were excluded based on the following: children >13 years of age, inability to obtain informed consent from parent/guardian or ascent from children who are old enough to ascent, patients who are on anti-TB treatment and patients having EPTB. Also, children screened for TB at the casualty were excluded; considering they are not admitted, thus collection of other alternative specimens (urine and stool) was not feasible.

3.5. Research Logistics

Logistics involved a continuous liaison via scheduled meetings with doctors, registrars and nursing staff as well as technicians/technologists of NHLS branches in each hospital.

3.6. Study Procedures

The attending physician requested the routine tests for children suspected of PTB. The nursing staff assisted with the supply of study information in the language of choice (Appendices A, B and C). They also obtained informed parental/guardian consent or assent from children (≥10 years) old enough to

assent for inclusion in the study. Nursing staff further assisted with obtaining specimens (GA, stool, urine, and NPA/NPS). Recording of the medical history of participants and the evaluation for PTB were performed per routine practice as per the hospital standard operating procedure (SOP). Research interventions included the provision of sterile specimen containers (100 mL) for specimen collection for the study apart from the routine specimens. Sodium bicarbonate-containing specimen containers were prepared and provided for the collection of GA to enable neutralisation of the gastric acid. The students received and proceed all study samples on arrival at the routine laboratory. Study results were communicated to the clinicians who were in charge or taking care of the patient; however treatment decisions were taken independently of the study.

3.7. Tuberculosis Case Definitions

Clinical case definition categories for TB in children were determined based on standardised case definitions recently published by (Graham *et al*., 2015a).

'**Confirmed TB**' cases were defined as children with microbiologically confirmed TB, defined as at least one positive smear or culture or WHO-endorsed NAAT (Xpert®) in any sample.

'**Unconfirmed TB**' cases were defined as children meeting at least 2 of the following criteria: defined signs or symptoms suggestive of TB; chest radiograph consistent with TB; history of close TB exposure with or without immunologic evidence of *M. tuberculosis* infection, i.e. positive tuberculin testing; and a positive response to TB treatment.

'**Unlikely TB**' cases were defined as children not meeting criteria for 'confirmed TB cases' and 'Unconfirmed TB cases with or without immunological evidence of *M. tuberculosis* infection.

The nutritional status of the children was determined as per hospital procedures by calculating height-for-age z-scores (HAZ) and weight-for-age z-scores (WAZ). Children with a z-score ≤-2 standard deviations (SD) below the mean were defined as being malnourished, and those ≤-3 SD were severely malnourished.

3.8. Specimen Collection

As part of the intensified specimen collection, specimen types (GA, NPA/NPS, urine and stool) were collected per child by the attending physician and/or nursing staff. GA was collected from children and either a NPA/NPS was taking depending on the choice of the attending physician. Urine was obtained as first-morning, clean-catch specimens from children able to spontaneously void, and for younger children via a sterile bag secured to the perineum after skin disinfection with sterile water. The stool was collected directly from the diaper using a scoop. Children who were toilet trained were

given a clean potty into which to pass stool. These specimens were transported together with other routine specimens from the hospital NHLS branch to the TB laboratory of the NHLS central branch Universitas, Bloemfontein. Collected specimens were decontaminated upon receiving, stored at -20°C and processed within 12 to 24 hours.

3.9. Sample Size

The sample size for this study was determined by the formula $\mathbf{N=Z^2p(1-p)/d^2}$ (Thrusfield, 1999), where;

N = Sample size.

Z = the score for a given interval which is 1.96 (S.E) at 95% confidence interval.

P = known or estimated prevalence (10%)

d = (5%) level of precision.

With the estimated prevalence, the minimum calculated sample size (N) required for the study was $1.96^2 \times 0.10 \times 0.90/0.05^2 = 138$.

3.10. Laboratory Procedures

3.10.1. Specimen Liquefication and Decontamination

Each specimen (GA, NPA/NPS, urine and stool) were liquefied and decontaminated using the standard NALC-NaOH-citrate method (Kent & Kubica, 1985).

NALC-NaOH-citrate Procedure: Using a disinfected biological safety cabinet (BSC) class 2A2, a 4g of N-Acetyl-L-Cysteine (Merk KgaA, Germany) was dissolved in a solution containing 400 mL of commercially prepared sodium hydroxide (NaOH) solution (Diagnostic Media Product, NHLS, Johannesburg). An initial concentration of 4% and an equal volume of 2.9% sodium citrate solution (Diagnostic Media Product, NHLS Johannesburg) were used to make a working solution with a NaOH concentration of 2% (Kent & Kubica, 1985).

NOTE: To avoid contamination, daily preparations of processing reagents and buffers were performed, and any leftovers were discarded.

Processing of GA and NPA Specimens: The specimen was transferred into a 50 mL falcon tube and an equal volume of NALC-NaOH-citrate working solution was added resulting in a 1% final concentration of NaOH in the specimen as recommended. The suspension was thoroughly vortexed to ensure proper mixing. The tube was left on a shaker at room temperature for 15 min to achieve a complete liquefication of the specimen. After incubation, the pH was neutralised by adding phosphate buffered saline (PBS) pH 6.8 to a total volume of 50 mL. The resulting solution was lightly vortexed

and centrifuged at 3000 × g for 15 min using aerosol-proof sealed centrifuge cups in a centrifuge (Beckman Coulter, CA USA) at 4°C. The supernatant was discarded in a splash-proof discard container with disinfectant, and the pellet was re-suspended in 2 mL of PBS.

Processing of NPS Specimens: Using sterile forceps, the swab was transferred into a 50 mL falcon tube and broken off to allow the replacement of the tube cap. A 2 mL volume of saline and an equal volume of NALC-NaOH-citrate solution was added to the tube, vortex mixed and incubated on a shaker for 15 min. The swab was removed with forceps while squeezing the liquid out of the swab and discarded. The tube was filled with phosphate buffer up to 50 mL and centrifuged at 3000 × g for 15 min. The supernatant was discarded similar as for GA, and the pellet was re-suspended in 2 mL of PBS.

Processing of Urine Specimens: The specimen was first concentrated by centrifugation at 3000 × g for 20 min using three falcon tubes (3 mL of urine per tube). The sediment in each tube was resuspended in 2 mL saline and then pooled together to a volume of 6 mL. The concentrated specimen was liquefied and decontaminated using an equal volume of a NALC-NaOH-citrate solution similar to GA. The resulting supernatant was discarded similarly as for GA, and the pellet was re-suspended in 2 mL of PBS.

Processing of Stool Specimens: As described by (Nicol *et al.,* 2013), an aliquot of approximately 0.15 g was transferred into a 50 mL falcon tube using a sterile disposable plastic loop. A volume of 2.4 mL PBS was added, and the sample was homogenised by vortex mixing. The mixture was left undisturbed for 15 min at room temperature to allow large particles to settle. The supernatant was collected and centrifuged at 3000 × g for 20 min. The resulting supernatant was discarded, pellets were resuspended in 1 mL PBS and were processed for decontamination in the same manner as GA using an equal volume of NALC-NaOH-citrate solution.

NOTE: Two negative processing controls were included with each batch of specimens processed. This was done by adding 5 mL sterile saline to 5 mL NALC-NaOH-citrate solution in the first (blank 1) and last (blank 2) falcon tubes to be processed. PBS was added up to the 50 mL mark and were processed like other specimens. Blank 1 checks sterility before the processing while blank 2 checks sterility during processing and as such the MGIT of blanks should be negative after six weeks of incubation.

NOTE: The re-suspended specimens (GA, NPA/NPS, urine and stool) in PBS were used for smear preparation, culture with the MGIT™ 960 System, and Xpert® testing.

3.10.2. Smear Microscopy

For smear preparations, a Pasteur pipette was used to deliver one drop of the concentrated decontaminated specimen (GA, NPA/NPS, urine and stool) on a labelled microscopy slide for

Auramine O and cold carbol-fuchsin (Kinyoun staining) techniques. Positive (positive culture of *M. tuberculosis* H37Rv) and negative (blank) quality control slides were included in each batch of stains for both Auramine O and Kinyoun staining to assess the quality of the reagents. It also assisted in determining if the staining was correctly performed and if the microscope was working correctly.

Auramine O Staining Procedure: A drop of Auramine fixative (Diagnostic Media Product, NHLS Johannesburg) was placed on a clean labelled microscope slide with a 1.8 cm diameter circle drawn in the middle with a wax pencil. A drop of the concentrated decontaminated specimen was added and smeared in a circular motion to make the smear as even as possible. The slide was heat dried in a covered stainless-steel container on an electrothermal heater at 80°C for 15 min and transferred to a staining rack over a sink. The slide was flooded with Auramine O stain for 15 min, decolourised with 0.5% acid alcohol for 2 min, and then counterstained with potassium permanganate solution for 2 min. The slide was rinsed with tap water between each step. The smear was air-dried and examined under the 250ox, and 450x objective lenses using a fluorescence microscope for AFB resulting in a final magnification of 200x and 500x. Stained slides were scored as positive for AFB according to the grading in table 3.1.

Table 3.1. Acid-fast smear evaluation and reporting. Adapted from Kent and Kubica, 1985.

Report	No of AFB seen by staining method and magnification		
	Fluorochrome stain		Ziehl-Neelson Stain
	×250	×450	×1000
No AFB seen	0	0	0
Doubtful; repeat	1-2/30 F (1 sweep)	1-2/70 F (1.5 sweeps)	1-2/300 F[b] (3 sweeps)[c]
1+	1-9/10 F	2-18/50 F	1-9/100 F
2+	1-9/ F	4-36/10 F	1-9/10 F
3+	10-90/F	4-36/F	1-9/F
4+	> 90/F	> 36/F	>9/F

AFB, Acid-fact bacilli
[b] F, the microscope field
[c] one full sweep refers to scanning the full length (2 cm) of a smear 1 cm wide by 2 cm long

Kinyoun Staining Procedure: A smear was made using ZN fixative (Diagnostic Media Product, NHLS Johannesburg) and a drop of MGIT culture from MGIT™ 960 System positive specimens as for Auramine O staining. The slide was placed in a covered stainless-steel container and heat dried on an electrothermal heater at 80°C for 15 min and transferred to a staining rack over a sink. The slide was flooded with carbol-fuchsin stain for 5 min, rinsed gently with tap water, and decolourised with 3% acid alcohol for 2 min. This was followed by gentle rinsing with tap water, counterstained with methylene blue for 2 min, washed gently with tap water, air-dried and examined under oil immersion

(10x and 100x lens objective) microscopy. The presence of serpentine corded AFB was an indication of a positive MTBC culture, the absence of other bacteria was used as an indication of a pure culture.

NOTE: Auramine staining was performed to identify the presence of acid-fast bacteria in the decontaminated samples. However, since all acid-fast organisms will be stained by the auramine O, including for example parasites and *Nocardia* spp, Kinyoun staining was used as a convenient method of confirming and differentiating morphology of culture (MGIT™ 960 System) positive tubes. Thus, Kinyoun staining was only done for samples that were MGIT™ 960 System culture-positive.

3.10.3. Culture via MGIT™ 960 System

Culture of the specimens was performed using the automated broth culture via the MGIT™ 960 System as described by the manufacturer (Becton Dickinson, 2004).

Preparation of cultures was done in a BSC class 2A2. In brief, using a sterile pipette, 500 µL of each decontaminated specimen (GA, NPA/NPS, urine and stool) was inoculated into a MGIT (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA). Each tube contained 7 mL liquid medium with 800 µL of enriched reconstituted growth supplement (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) added as per the manufacturer's instructions (Becton Dickinson, 2004). The MGIT growth supplement contains OADC™ (Oleic acid, albumin, dextrose, catalase) and PANTA™ (Polymyxin B, amphotericin B, nalidixic acid, trimethoprim, azlocillin) (Becton Dickinson, Cockeysville, MD, USA).

The inoculated MGIT barcode was scanned and incubated at 37°C in the MGIT™ 960 System. The instrument automatically reads the MGIT hourly until positive results are found or for a maximum of 42 days (approximately six weeks). Cultures were classed as negative when no growth was evident after six weeks of incubation.

Positive MGIT cultures were evaluated using Kinyoun staining and TBc ID antigen testing (BD MGIT™ TBc Identification Test: Becton Dickinson and Company, USA). These tests rule out contamination by bacteria or NTM and/or confirm true positivity. Pure positive MGIT cultures with a positive MPT64 antigen (TBc MTBc ID test) and Kinyoun staining showing the presence of serpentine corded bacilli were further sub-cultured on LJ slants for observation of colony morphology and future use. Heavily turbid contaminated MGIT cultures were reprocessed using 4% NaOH to recover possible mycobacteria, while lightly turbid contaminated MGIT cultures were re-incubated at 37°C till 42 days. After 42 days, Kinyoun staining and TBc ID antigen test (BD MGIT™ TBc Identification Test: Becton Dickinson and Company, USA) were repeated and positivity versus negative growth confirmed.

3.10.4. BD MGIT™ TBc Identification Test

Using sterile filter tips, each TBc ID test device was inoculated with 100 µL of a positive MGIT culture as per the manufacturer's recommendations (BD Diagnostic Systems 2009). Once a TBc ID test device was inoculated, it was incubated for 15 min at room temperature before the results were visually assessed. Positive results are evident by a visible test and reagent function control line.

3.10.5. Sub-culturing on Löwenstein–Jensen Media

Using a cotton-plugged (filtered) tip to prevent cross-contamination, 0.2 mL of confirmed positive MGIT cultures were inoculated on LJ media. The LJ bottle was placed in a slanted position without allowing the pipette to touch the surface of the media while allowing drops to rundown the slant. Inoculated LJ media were incubated in a slanted position at 36°C. Slants were checked twice a week for the first four weeks of incubation and once a week for 5–8 weeks.

3.10.6. Reprocessing/Decontamination of Contaminated MGIT Cultures

The entire contaminated culture from the MGIT was transferred into a 50 mL falcon tube, an equal volume of 4% sterile NaOH solution was added, mixed and incubated for 15 min at room temperature with periodic inverting of the tube. PBS (pH 6.8) was added up to the 40 mL mark, mixed by inverting and centrifuged at 3000 × g for 15 min. The supernatant was discarded, and the sediment was re-suspended in 500 µL of PBS. A 500 µL of this suspension was inoculated into a fresh MGIT containing MGIT™ growth supplement. The tube was scanned for recognition by the MGIT™ 960 System and incubated at 37°C in the MGIT™ 960 System until growth was detected or for a maximum of 42 days (approximately six weeks). Cultures were classified as negative when no growth was detected after six weeks of incubation.

3.10.7. Molecular Diagnosis of *Mycobacterium tuberculosis* and Identification of Rifampicin Resistant-Tuberculosis Using GeneXpert® MTB/RIF

All specimens were processed as per standard Xpert® protocol by addition of sample reagent buffer in a 3:1 or 2:1 ratio to take the final volume to 2 mL (Cepheid, USA).

Decontaminated specimens (GA, NPA/NPS, urine and stool) were used in a 1:2 ratio to the sample reagent. This was done by adding 1 mL of the decontaminated specimen to 2 mL of Xpert® reagent in a sterile BSC class 2A2. The mixture was incubated at room temperature for 15 min with intermittent shaking. A 2 mL of this mixture was added to the Xpert® cartridge and was loaded into the test platforms. The result was available on the machine after 2 hours.

3.10.8. Drug Susceptibility Testing using the BACTEC™ MGIT™ 960 System and Genotype® MTBDR*plus VER 2.0*

First-line DST was performed for RIF and INH using the MGIT™ 960 System AST SIRE kit (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) as described in the MGIT™ 960 System DST package insert (Becton Dickinson, 2004) and the Hain Genotype® MTBDR*plus* assay as per manufacturer's instructions (Hain Lifescience GmbH, Nehren, Germany).

3.10.8.1. Drug Susceptibility Testing using the BACTEC™ MGIT 960™ System

An inoculum was prepared by adding 1 mL of positive culture to 4 mL of sterile saline (1:5). The powered antibiotics; INH and RIF (BACTEC™ MGIT™ SIRE kit; Becton Dickinson, MD) was reconstituted with 4 mL of sterile distilled water to prepare a stock solution. A volume of 0.1 mL of each antibiotic stock solution was added into the 7 mL MGIT tube to achieve a final concentration of 0.1 µg/mL for INH and 1.0 µg/mL for RIF. To this, 0.8 mL of MGIT™ OADC™ and 0.5 mL of the test inoculum was added. The growth control (GC) was prepared by adding 100 µL of inoculum to 10 mL of sterile saline to achieve a final dilution of 1:500. A 500 µL volume of the prepared GC was inoculated into the MGIT™ GC without any antibiotic. All tubes were incubated at 37°C in the MGIT™ 960 System and monitored for growth until the system detected a positive growth result. The automatically printed result was then interpreted according to the MGIT procedure manual (Siddiqi, 2006).

3.10.8.2. Drug Susceptibility Testing using the Genotype® MTBDR*plus* VER 2.0

The procedure is divided into three and was performed as follows;

DNA Extraction: DNA was extracted from cultured samples using a commercial kit called DNeasy® Blood and tissue kit (Qiagen, Valencia, CA) (using the DNA extraction protocol for crude lysates of bacteria) as per the manufacturer's instructions. The manufacturer emphasised that centrifugation must take place at room temperature between 15-25˚C, resulting precipitates must be dissolved in buffer AW1 and AW2. Frozen specimens must be equilibrated to room temperature and buffers should be pre-heated in the incubator to 56˚C (Qiagen, Valencia, CA).

For each specimen, the lysis procedure was done by re-suspending 1-2 colonies of cultured cells from the LJ media into 200 µL of animal tissue lysis (ATL) buffer heated to 56˚C. The bacterial pellet was re-suspended by pulsating vortex for 10 seconds a time.

Following resuspension, the bacteria was heat inactivated at 95-96˚C for 3 × 10 min in a block heater (Bibby Sterlin LTD Stone, Staffordshire UK) and vortexed after each 10 min period. This was followed by incubation in an ultrasonic bath (Ridge Diagnostics, Germany) for 15 min. Afterwards, proteinase

K (20 µL) was added, the suspension was mixed and incubated at 56˚C in the block heater (Bibby Sterilin LTD Stone, Staffordshire UK) overnight for 24 hours or until completely lysed.

A 200 µL volume of buffer AL was added, and the solution was vortexed to mix thoroughly. A 200 µL volume of absolute ethanol (96-100%) was added to the solution, vortexed and transferred to a mini spin column positioned in a 2 mL collection tube. The tube was centrifuged at 6000 × g (8000 rpm) for 1 min, and the resulting flow-through and collection tube was discarded.

Using a new 2 mL collection tube, 500 µL of buffer AW1 was added to the DNeasy® mini spin column and centrifuged at 6000 × g (8000 rpm) for 1 min. The resulting flow-through and collection tube was discarded.

The DNeasy® Mini column was placed in another 2 mL collection tube. A 500 µL buffer AW2 was added to the column and centrifuged for 3 min at 20 000 × g (14 000 rpm). The resulting flow-through and collection tube was discarded.

For DNA elution, a 200 µL of buffer AE was transferred onto the membrane of the DNeasy® Mini spin column positioned in a new 2 mL microcentrifuge tube, incubated at room temperature for 1 min and centrifuged at 6000 × g (8000 rpm) for 1 min.

Extracted DNA was kept in a 4˚C fridge after measuring the DNA concentration and purity at 260 nm and 280 nm respectively, using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific Illinois USA).

PCR Amplification: All reagents needed for amplification such as polymerase and primers are included in the Amplification Mixes A and B [AM-A and AM-B] provided by the manufacturer (Hain Lifescience GmbH, Nehren, Germany). For each sample, a 50 µL reaction was prepared by the addition of AM-A and AM-B and DNA in the following order:

10 µL AM-A, 35 µL AM-B, 5 µL DNA solution.

Using a Life Express Thermal-Cycler (Bioer technology, Tokyo, Japan) the samples were amplified under the following conditions; 15 min at 95°C [1 cycle], 30 secs at 95°C and 2 min at 65°C [10 cycles], 25 secs at 95°C and 40 secs at 55°C [20 cycles], 40 secs at 70°C [20 cycles] and 8 min at 70°C [1 cycle].

Amplification products were stored at 4°C or used at once for hybridisation.

Hybridisation: The hybridisation instrument GT-Blot 48 was used following the manufacturer's instructions (Hain Life Sciences, Gwynedd Wales, UK).

Briefly, all the solutions necessary for the assay (including Conjugate, and Substrate working solutions) were prepared and poured into specific flasks indicated with different coloured flags. These were used to fill all the respective 6 pumps in the machine.

While the assay pre-warms, the Genotype® MTBDR*plus* strips were transferred to a clean tray using forceps and were labelled by sample numbers. Using a clean GT-Blot 48 specific tray, 20 µL of DEN solution was dispensed on the bottom corner of each lane of the tray. To each DEN solution, 20 µL of the PCR product was added, mixed by pipetting and incubated at room temperature for 5 min.

While avoiding touching the DEN-DNA solution, strips were transferred into respective lanes in the GT-Blot 48 specific tray and were placed into the GT-Blot 48 machine.

According to the assay program, the instrument aspirates and add the necessary reagents at the correct time interval and incubate reagents at the proper temperature throughout the test method as indicated in table 3.2.

Table 3.2. Hybridisation instrument GT-Blot 48 assay program.

Step	Colour flag	Time interval and temperature
HYB (hybridisation)	green	30 min at 45°C
STR (stringent wash)	Red	15 min at 45 °C
RIN (rinse solution)		1 min
CON (diluted conjugate)	Orange	30 min at 25 °C
RIN		1 min at 25 °C
Distilled water wash		1 min at 25 °C
SUB (diluted substrate)	Yellow	3 min at 25 °C
Distilled water wash (twice)		1 min each

At the end of the program, forceps were used to transfer strips to the GenoType® MTBDR*plus* sheet provided with the kit and analysed manually as described in the Genotype® MTBDR*plus* VER 2.0 package insert.

NOTE: A known positive (*M. tuberculosis* H37Rv) and negative control (blank) were included in the test, and test validation was confirmed with the appearance of CC (conjugate control) and AC (amplification control) for each sample.

3.10.9. Genotyping of *Mycobacterium tuberculosis* Positive Isolates

Genotyping of *M. tuberculosis* positive isolates was performed by spoligotyping and a 24 loci MIRU-VNTR typing method using the previously extracted DNA from culture-positive isolates.

3.10.9.1. Spoligotyping

Spoligotyping was performed using a commercially available spoligotyping kit (Ocimum BioSolution, India) according to the manufacturer's instructions and as described by Kamerbeek (Kamerbeek *et al*., 1997).

The method is based on PCR amplification of the DRs of *M. tuberculosis* isolates, followed by hybridisation to a membrane having covalently linked oligonucleotides that correspond to the various spacer sequences. The direct-repeat region of the mycobacterial genome was amplified using primers DRa (5'-GGT TTT GGG TCT GAC GAC -3') (biotinylated 5' end) and DRb (5'-CCG AGA GGG GAC GGA AAC-3') (Kamerbeek *et al*., 1997).

Before hybridisation, buffers were prepared from concentrated stock solutions, using de-mineralised water for dilution. (Buffer preparation procedure is added as Appendix D).

Briefly, the PCR reaction was performed in a total volume of 25 µL reaction mix as seen in table 3.3. *M. tuberculosis* H37Rv and *M. bovis* BCG DNA were used as positive controls while deionised water was used as a negative control. A drop of mineral oil was added to prevent evaporation during amplification in the PCR thermal cycler.

Table 3.3. PCR Master mix for spoligotyping.

Master Mix	Per 25µL reaction (µL)
Primer DRa	2
Primer DRb	2
Qiagen Master Mix	12.5
DNA	5
Sterile H2O	3.5

The PCR reaction was performed as follows: Initial denaturation at 96°C for 15 min, denaturation at 96°C for 1 min (30 cycles), annealing at 55°C for 1min (30 cycles), extension at 72°C for 30 secs (30 cycles), a final extension at 72° for 10 min, and a hold at 4°C.

Hybridisation step

A 20 µL volume of the PCR product was added to 150 µL of 2x SSPE/0.1% SDS. To ensure the DNA was single-stranded prior to hybridisation, the diluted PCR product was heat-denatured at 99°C for 10 min in a thermal cycler (Bioer technology, Tokyo, Japan) and immediately cooled on ice.

The spoligotyping membrane (Ocimum BioSolution, India) was washed for 5 min at 60°C in 250 mL 2xSSPE/0.1% SDS (Merck, South Africa) and placed into the miniblotter (Ocimum BioSolution, India) supported by the cushion (Ocimum BioSolution, India). The membrane was placed in the Miniblotter® 45 (Immunetics, Boston, USA) in such a manner that the channels of the miniblotter were perpendicular to the line pattern of the immobilised oligonucleotides. The residual fluid was removed from the slots of the Miniblotter® 45 (Immunetics, Boston, USA) by aspiration. The channels of the miniblotter were filled with approximately 150 µL of denatured PCR products and were left to hybridise at 60°C in a water bath (GFL Germany) for 60 min. After hybridisation, the PCR products were aspirated from the miniblotter, the membrane was carefully removed using sterile forceps and was washed twice in 250 mL 2x SSPE/0.5% SDS at 60°C for 10 min. The membrane was further incubated in a mixture of 2.5 µL (500 U/mL) streptavidin-peroxidase conjugate (Ocimum BioSolution, India) and 20 mL of 2x SSPE/0.5% SDS at 42°C for 60 min in a rolling bottle (Lasec, South Africa). Lastly, the membrane was washed twice in 250 mL of 2x SSPE/0.5% SDS (Ocimum BioSolution, India) at 42°C for 10 min in a shaking incubator (Labcon, US) followed by rinsing twice in 250 mL 2x SSPE at room temperature (25°C) for 5 min.

The detection of the hybridised DNA was performed by incubating the membrane for 1 min in 30 mL of chemiluminescence detection liquid (solution 1 and solution 2) (ECL, Amersham, Sweden). The membrane was then exposed to the light-sensitive X-ray film (Hyperfilm ECL; Amersham) for approximately 1 min. The signal was developed by placing the X-ray film into an X-ray film processing machine (AFP Mini-Medical 90 Film Processor), which automatically develops the X-ray in approximately 5 min.

After generating the spoligotyping results, the spoligotype patterns were entered in an Excel spreadsheet where the pattern of the 43 spacers was represented in a binary form with “n” indicating the presence of a spacer and “0” representing the absence of a spacer.

3.10.9.2. MIRU-VNTR Typing

The MIRU-VNTR typing was performed using the 24 loci MIRU-VNTR typing kit quadruplex versions (GenoScreen, Lille, France). For this study, the MIRU-VNTR PCR analysis was conducted with the Applied Biosystems 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA). Using a sequencer enabled the use of a multiplex PCR with each quadruplex targeting four different MIRU loci

(GenoScreen 2011). Table 3.4 contains the six quadruplex panels, the different MIRU markers as well as their respective aliases.

Table 3.4. 24 Loci MIRU-VNTR Quadruplex panels.

Panel	Marker	Alias
Quadruplex 1	1955	None
	580	None
	2996	MIRU 26
	802	MIRU 40
Quadruplex 2	2163b	QUB11b
	960	MIRU 10
	1644	MIRU 16
	3192	MIRU 31
Quadruplex 3	424	Locus 42
	577	Locus 43/ETRC
	2165	ETRA
	4052	QUB26
Quadruplex 4	154	MIRU 02
	2531	MIRU 23
	4348	MIRU 39
	2401	VNTR 47
Quadruplex 5	2059	MIRU 20
	2687	MIRU 24
	3007	MIRU 27
	4156	VNTR 53
Quadruplex 6	3690	VNTR 52
	2347	VNTR 46
	2461	VNTR 48/ETRB
	3171	VNTR 49

Plate Loading

The PCR reaction was performed in a total volume of 12 µL consisting of 8 µL MIRU-VNTR quadruplex mix and 2 µL of template DNA which was loaded into the 96-well plate based on the PCR spreadsheet in table 3.5. Two positive controls (*M. tuberculosis* H37Rv strain and *M.bovis* BCG strain) and negative control (deionised water) were used.

The 96-well plate was sealed with an adhesive PCR film, spun down and loaded into a thermal cycler (Bioer technology, Tokyo, Japan) using the following amplification cycles: Initial denaturation at 95°C for 15 min, denaturation at 94°C for 1 min (40 cycles), annealing at 59°C for 30 secs (40 cycles), extension at 72°C for 1 min 30 secs, (40 cycles), final extension at 72°C for 10 min and a hold at 4°C.

Post-amplification, the PCR products were stored protected from light at 4°C until capillary electrophoresis was performed.

Table 3.5. MIRU-VNTR PCR loading spreadsheet.

	Quadruplex 1		Quadruplex 2		Quadruplex 3		Quadruplex 4		Quadruplex 5		Quadruplex 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	Ctrl +	ID07	Ctrl +	ID07	Ctrl +	ID07	Ctrl +	ID07	Ctrl +	ID07	Ctrl +	ID07
B	Ctrl -	ID08	Ctrl -	ID08	Ctrl -	ID08	Ctrl -	ID08	Ctrl -	ID08	Ctrl -	ID08
C	ID01	ID09	ID01	ID09	ID01	ID09	ID01	ID09	ID01	ID09	ID01	ID09
D	ID02	ID10	ID02	ID10	ID02	ID10	ID02	ID10	ID02	ID10	ID02	ID10
E	ID03	ID11	ID03	ID11	ID03	ID11	ID03	ID11	ID03	ID11	ID03	ID11
F	ID04	ID12	ID04	ID12	ID04	ID12	ID04	ID12	ID04	ID12	ID04	ID12
G	ID05	ID13	ID05	ID13	ID05	ID13	ID05	ID13	ID05	ID13	ID05	ID13
H	ID06	ID14	ID06	ID14	ID06	ID14	ID06	ID14	ID06	ID14	ID06	ID14

Ctrl + = *M. tuberculosis* H37Rv strain and *M. bovis* BCG strain.
Ctrl - = deionised water
ID= Samples 1 to 14

Fragment Analysis

Fragment analysis was performed on the 3500 Series Genetic Analyzer as per the manufacturer's instructions as well as the guidelines in the GenoScreen MIRU-VNTR Typing Kit.

Briefly, an extemporaneously made master mix of 9.5 µL of HiDi formamide (Applied Biosystems, Foster City, CA) and 0.5 µL of GeneScan™ 1200 LIZ® (Applied Biosystems, Foster City, CA) was prepared per well. A 96-well sequencer plate was loaded with 10 µL of formamide-GeneScan™ 1200 LIZ® mix per well.

The 96-well plate containing the PCR products was centrifuged to prevent cross-contamination when removing the adhesive film. The adhesive film was carefully removed, and 2 µL of the PCR products were added to the 96-well sequencer plate containing the extemporaneously made mix of formamide-GeneScan™ 1200 LIZ® using the same layout as the PCR spreadsheet.

The 96-well sequencer plate was sealed with an adhesive film and centrifuged to remove air bubbles. The DNA was denatured by placing the plate in a thermal cycler at 95°C for 5 min and was immediately placed on ice after denaturation.

The 96-well sequencer plate was loaded on the sequencer, and capillary electrophoresis was performed as described in the ABI manual. Results were available after 4 hours, and sizing of PCR fragments and assignments of the alleles of the 24 loci was performed using the MIRU-VNTR calibration kit (GenoScreen, Lille, France), as well as the GeneMapper® software version 4.0 (Applied Biosystems, USA).

3.11. Genotyping Data Analysis

The spoligopatterns were converted into binary codes where 'n' represents the presence of spacer and 'o' represents the absence of spacers. These binary codes were then converted into octal codes using the SITVIT Database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). The octal codes were used to generate the SIT number for each isolate using SITVIT WEB (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/query). The SIT number was then used to identify the corresponding strain in the SITVIT2 proprietary database (http://www.pasteur-guadeloupe.fr:8081/SpolSimilaritySearch/images/File_S1.pdf). A dendrogram was constructed for the combined result of spoligotyping and MIRU-VNTR typing using the MIRU-VNTRplus web application (http://www.miru-vntrplus.org/MIRU/index.faces).

3.12. Statistical Analysis

The Xpert® assay and microscopy accuracy measures (sensitivity, specificity, PPV, and NPV) against MGIT™ 960 System culture results as the gold standard were estimated using simple percentages. Analyses of diagnostic accuracy were conducted per patient and per specimen.

3.12.1. Diagnostic Sensitivity and Specificity

Diagnostic sensitivity is defined as the ability of a test to accurately diagnose people with a disease as having the disease. In this study, using MGIT™ 960 System culture result as the reference standard, diagnostic sensitivity was measured by determining if a test (Xpert® or smear microscopy) could detect TB in cases that MGIT™ 960 System culture was able to detect. The formula was applied as follows:

$$Sensitivity = \frac{True\ positive}{True\ positive + False\ negative}$$

Diagnostic specificity is defined as the ability of a test to accurately identify people who do not have the disease as being disease free. In this study, similar to diagnostic sensitivity, culture was used as the reference standard. Diagnostic specificity was measured by determining if a test (Xpert® or smear microscopy) could successfully identify negative cases as negative cases by not detecting TB in cases determined as negative by culture. The formula was applied as below. Calculations are attached as Appendix E.

$$Specificity = \frac{True\ negative}{True\ negative + False\ positive}$$

3.12.2. Predictive Values

The PPV is defined as the percentage of patients who tested positive that have the disease. The closer the PPV is to 100%, the closer it is to the gold standard. Using culture as the reference standard The PPV in this study was measured for both smear microscopy and Xpert® using the formula as follows:

$$PPV = \frac{True\ positive}{True\ positive + False\ positive}$$

NPV is defined as the percentage of patients who tested negative and does not truly have the disease. The closer the NPV is to 100%, the closer it is to the gold standard. The NPV in this study, like PPV, was measured for Xpert® and smear microscopy using the formula below: Calculations are attached as Appendix E.

$$NPV = \frac{True\ negative}{True\ negative + False\ negative}$$

3.12.3 Sample Sensitivity

In this study, sample sensitivity was defined as the ability of any of the sample to offer diagnostic confirmation of TB in the patients using on diagnostic method. The formula was applied as shown below: Calculations are attached as Appendix E.

$$Sample\ sensitivity = \frac{Number\ of\ positive\ patient\ (on\ sample)}{Total\ number\ of\ positive\ patients + False\ negative\ patients\ (on\ sample)}$$

3.13. Ethical Considerations

The study was approved by the ethics committee of the University of the FS, (ECUFS 08/2015) (Appendix F). Permission was also granted by the FS Department of Health (Appendix G), Pelonomi Regional Hospital (Appendix H), and Botshabelo District Hospital (Appendix I). Written permission (via consent form) from the parent/guardian (Appendix J) for children to participate in the study was included, and verbal or signed child assent when applicable in the language of choice (Appendix K, L and M) was obtained from the participants. Additional approval for the M.Med.Sc study was obtained from the appointed evaluation committee.

CHAPTER 4: RESULTS

4.1. Diagnosis of Childhood Pulmonary Tuberculosis and Drug Susceptibility Testing of *Mycobacterium tuberculosis* Positive Isolates

4.1.1. Introduction

Definitive diagnosis of TB depends on detection of *M. tuberculosis* from an appropriate clinical sample, a process which is difficult in children and more heightened in HIV-infected children (Brent *et al.*, 2017). These complications occur in the children population owing to their inability to expectorate sputum and lack of TB bacilli in respiratory secretions, as TB bacilli are typically confined to perihilar nodes, preventing it from breaking into the bronchus and thus difficult to detect (Oberhelman *et al.*, 2010). Further exacerbating the diagnostic challenges is the non-specificity of clinical and radiological findings of TB in paediatric patients compared to adults (Thomas *et al.*, 2014). The inability to retrieve bacilli is a key factor that also contribute to the difficulty in establishing bacteriological diagnosis of TB in these population (Zar *et al.*, 2005). The low bacillary load further add to the already poor sensitivity of smear microscopy and culture yield ranging from 10-25% (Moore *et al.*, 2011).

The need for a confirmed diagnosis of TB is becoming increasingly important in the era of DR-TB (Fairlie *et al.*, 2011; Schaaf *et al.*, 2009), as knowledge of a patient's drug-susceptibility pattern is paramount to provide optimal treatment (Gandhi *et al.*, 2006; Thomas *et al.*, 2010). Resistance to TB drugs can be determined using mycobacterial culture DST, LPAs or other NAAT, however, their optimal performance still relies on adequate sample collection and the presence of detectable bacilli (Marcy *et al.*, 2016). Standard samples recommended by the WHO include expectorated sputum, which cannot be produced by younger children; GA, which requires fasting and, most often, hospitalization; and IS, which is only safe in clinically stable children (Marais and Pai, 2006; WHO, 2014a; Zar *et al.*, 2005).

The feasibility of using alternative specimens such as urine, stool, and nasopharyngeal specimens for microbiological diagnosis of PTB in children have not been thoroughly evaluated (Detjen and Walters, 2016; Marcy *et al.*, 2016; Thomas *et al.*, 2014). These samples are easily obtainable than gastric contents and IS and may serve as useful alternative samples for detecting TB in children (Detjen and Walters, 2016; Nicol *et al.*, 2013; Oberhelman *et al.*, 2010). Such specimens may be an option even in children with HIV co-infection, who are more prone to developing disseminated forms of TB (Detjen and Walters, 2016; Nicol *et al.*, 2013; Oberhelman *et al.*, 2010). This study compared the diagnostic yield of alternative specimens (urine, stool, and nasopharyngeal specimens) to that of the standard specimen (GA) obtained from pediatric PTB suspects from two public hospitals in Mangaung SA, while simultaneously assessing the drug susceptibility profile of *M. tuberculosis* positive isolates.

4.1.2. Results

From August 2016 to June 2017, a total of 160 pediatric PTB suspects were referred for this study and 126 met the enrolment criteria (Figure 4.1). These children were evaluated using smear microscopy, culture via MGIT™ 960 System, GenoType® MTBDR*plus* and Xpert® testing.

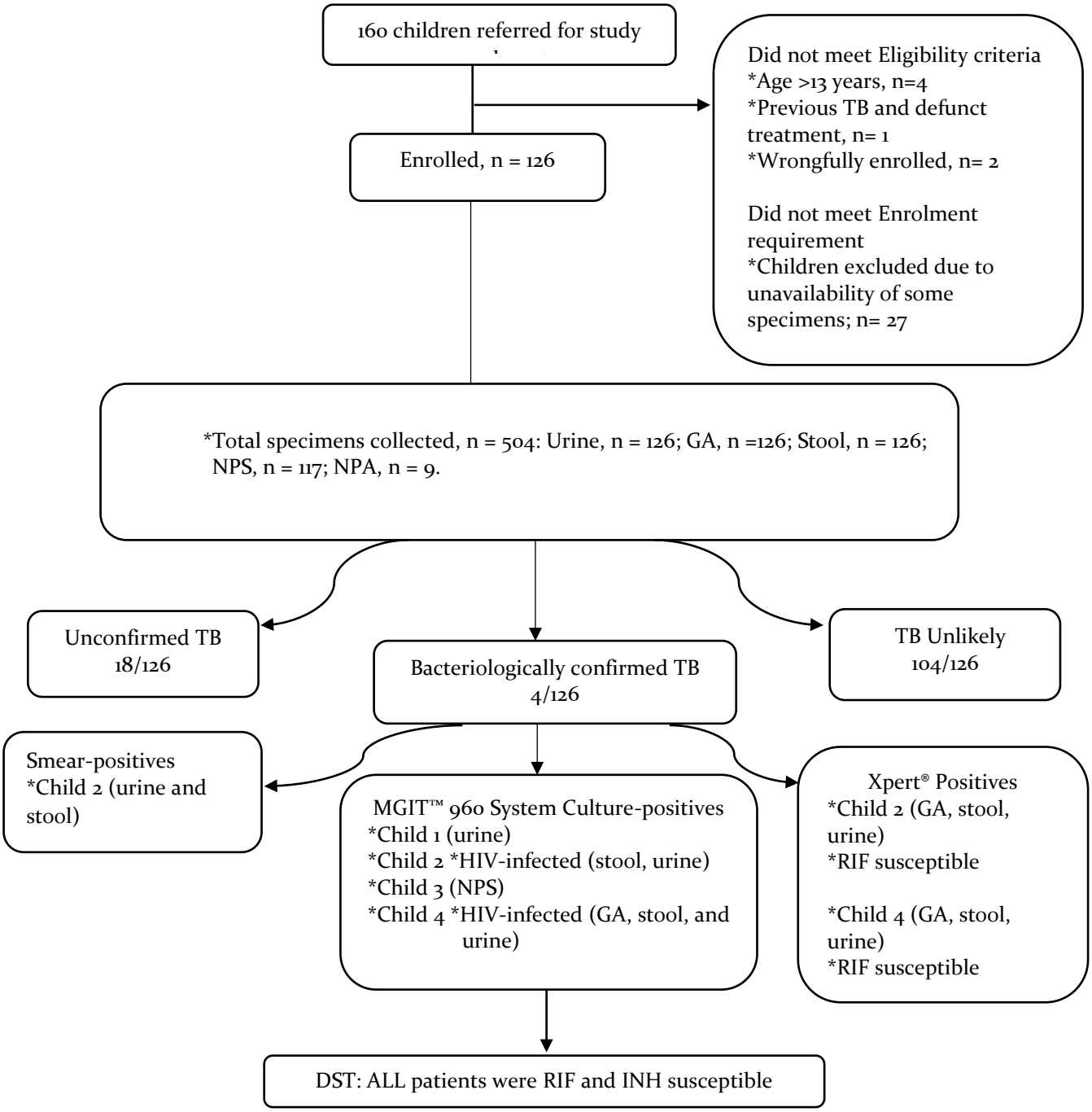

Figure 4.1. A flow diagram depicting the enrolment of children with suspected tuberculosis (TB) in a TB diagnostic and drug resistance study. Gastric aspirate(GA) supplemented with stool, urine and nasopharyngeal aspirate or nasopharyngeal swab were collected per child. Samples were tested using microscopy of auramine-stained smears, culture via MGIT™ 960 System, and GeneXpert® MTB/RIF (Xpert®). Drug susceptibility profile to rifampicin and isoniazid was established using Xpert®, and GenoType MTBDR*plus*.

4.1.2.1. Demographic and Clinical Characteristics

Clinical symptoms of TB in this study population (Table 4.1) included a persistent cough (29.3%), history of TB exposure (5.5%), chest X-ray suggestive of TB (10.3%), persistent lethargy (19%) and persistent fever (15%). Weight loss was the most reported clinical characteristic occurring in (60.3%) of the PTB suspects with a higher proportion in the unlikely TB group. The unlikely TB group also had a higher proportion of malnutrition (43.2%). The overall prevalence of malnutrition was high (39.6%); with severe malnutrition found in 27% of the children. In this cohort, 11% of the children were HIV positive. However, the percentage of those on anti-retroviral therapy are unknown.

Table 4.1. General clinical characteristics of the enrolled patients suspected of pulmonary tuberculosis.

Characteristics	Total population n=126, (%)	Confirmed n=4, (%)	Unconfirmed n=18, (%)	Unlikely n=104, (%)
Persistent cough	37 (29.3%)	0 (0%)	7 (38.8%)	30 (28.8%)
Weight loss	76 (60.3%)	2 (50%)	7 (38.8%)	67 (64.4%)
History of TB exposure	7 (5.5%)	0 (0%)	7 (38.8%)	0 (0%)
Chest X-ray suggestive of TB	13 (10.3%)	0 (0%)	13 (72.2%)	0 (0%)
Severe Malnutrition	34 (27%)	1 (25%)	3 (16.6%)	30 (28.8%)
Persistent lethargy	24 (19%)	4 (100%)	6 (33.3%)	14 (13.4%)
Moderate Malnutrition	16 (12.6%)	0 (0%)	1 (5.5%)	15 (14.4%)
Persistent Fever	19 (15%)	3 (75%)	4 (22.2%)	12 (11.5%)
HIV status Positive	14 (11%)	2 (50%)	5 (27.7%)	7 (6.7%)
Negative	112 (88.8%)	2 (50%)	13 (72.2%)	97 (93.2%)

General demographic characteristics of the study population are presented in table 4.2. Briefly, the male to female ratio was (1:1.37), and the median age was (17.5 months). Of the total population of recruited PTB suspects, children ≤5 years (93%) were more than those >5 years (7%).

Table 4.2. General demographic characteristics of the enrolled patients suspected of pulmonary tuberculosis.

Category	Subcategory	Total population n=126, (%)	Confirmed n=4, (%)	Unconfirmed n=18, (%)	Unlikely n=104, (%)
Age	Median age in months	17.5 months	27 months	8.5 months	18 months
	≤5 years	117 (93%)	4 (100%)	18 (100%)	95 (91.3%)
	>5 years	9 (7%)	0 (0%)	0 (0%)	9 (8.6%)
Sex	Male	73 (57.9%)	2 (50%)	14 (78%)	57 (55%)
	Female	53 (42%)	2 (50%)	4 (22%)	47 (45%)

Demographic and clinical information specific to the four confirmed TB cases are presented in table 4.3. Persistent lethargy and/or fever were reported by each of the confirmed cases. Notably, none of the confirmed TB cases had clinical characteristics of TB exposure, chest X-ray nor a persistent cough.

Table 4.3. Demographic and clinical characteristics of the four patients with bacteriologically confirmed pulmonary tuberculosis.

Patients	Clinical information	Sex	Age	HIV status
Child 1	Persistent unexplained lethargy unexplained weight loss Severe malnutrition Persistent fever	Male	4 years	Negative
Child 2	Persistent unexplained lethargy Persistent fever	Female	3 years	Positive
Child 3	Persistent unexplained lethargy Persistent fever	Female	1 year	Negative
Child 4	Weight loss Persistent unexplained lethargy	Male	1 year 6months	Positive

HIV: Human immunodeficiency virus

4.1.2.2. Diagnostic Results and Accuracy

The clinical reference standard was defined as patients who satisfied characteristics of "confirmed TB" by culture method. Four patients (3.2%) were diagnosed with TB by microbiological confirmation. Using the consensus agreement on Intrathoracic Tuberculosis Definitions for Diagnostic Research in Children (Graham *et al.*, 2015a), 18 patients (14.2%) were classified as having "unconfirmed TB", while 104 patients (82.5%) were classified as 'TB unlikely".

The results of the four confirmed TB cases are summarized in table 4.4. Both child 1 and 3 were HIV-uninfected) and while child 1 was positive by urine culture only, child 3 was positive by NPS culture only. Child 2, (HIV-infected) had a positive auramine staining with a grading of 1+ (1-9 bacilli per field) on the stool and urine samples. The stool and urine samples of this child were also positive on Xpert® and culture, the GA was positive with Xpert®, but the culture became heavily contaminated before positivity. Child 4 (HIV-infected) was positive by Xpert® and culture on GA, stool and urine samples.

Table 4.4. Comparison of results for different diagnostic methods and samples of patient's positive for *Mycobacterium. tuberculosis*.

Pts	GA sm	GA cul	GA Xpt	U sm	U cul	U Xpt	Stool sm	Stool Cul	Stool Xpt	NPS sm	NPS cul	NPS Xpt
Child 1	-	-	-	-	+	-	-	-	-	-	-	-
Child 2	-	CON	+ Low	+	+	+ High	+	+	+ High	-	-	-
Child 3	-	-	-	-	-	-	-	-	-	-	+	-
Child 4	-	+	+ Low	-	+	+ Low	-	+	+ Low	-	-	-

*Pts: Patients, GA: Gastric aspirate, U: Urine, NPS: Nasopharyngeal swab, sm: Smear, cul: culture, Xpt: Xpert™, CON: contaminated, **+:** Positive, -: Negative.

Culture successfully detected *M. tuberculosis* in 4 of the confirmed TB cases, Xpert™ offered diagnosis in 2 children and smear microscopy in 1 child with variable samples positive for different sample types (Table 4.5).

Table 4.5. Diagnostic yield of smear, culture and Xpert® in all positive cases by patient and sample analysis.

	Sensitivity, n (%)		
	Smear	**Culture**	**Xpert®**
By patient (n =4)	**1 (57%)**	4 (100%)	2 (66%)
By ALL Samples (n =16)	**2 (10%)**	7 (44%)	6 (35%)
By sample Types			
GA (n =4)	**0**	1	2
Urine (n =4)	**1**	3	2
Stool (n =4)	**1**	2	2
NPS (n =4)	**0**	1	0

GA: Gastric aspirate, NPS:Nasopharyngeal swab.

Using culture as the gold standard method for diagnosis of TB, on patient analysis; Smear microscopy had a diagnostic sensitivity of 14%, with a 100% diagnostic specificity, a PPV of 100% and a NPV of 97.6%, while Xpert® had a diagnostic sensitivity of 33%, specificity of 100%, a PPV value of 100%, and a NPV value of 98.3%.

The sample-based analysis suggests that GA, urine, stool and NPS had a diagnostic sensitivity of (33%,), (60%), (33%), and (14.2%) respectively.

Drug susceptibility results agree in the two DST assays employed (MGIT™ 960 System culture and Genotype® MTBDR*plus*). Both assays suggest that each child had a RIF and INH susceptible strain of *M. tuberculosis*. The DST result was in concordance with all specimen types in the case of children having more than one culture-positive sample, and with the Xpert® RIF result.

4.1.2.3. Time to Detection

Time to detection was defined as the number of days from sample processing (day 1) to when results were available. Smear microscopy and Xpert® offered a same-day result after processing. The culture mean time to positivity in alternative samples (urine, stool and NPS) were shorter compared to GA (Table 4.6).

Table 4.6. Culture time to detection rate on various obtained samples.

Patients	Sample types	Time to detection rate (days)
Child 1	Urine	19 days
Child 2	GA	Contaminated
	Stool	18 days
	Urine	21 days
Child 3	NPS	26 days
Child 4	GA	28 days
	Stool	22 days
	Urine	23 days

GA: Gastric aspirate, NPS:Nasopharyngeal swab

4.1.2.4. Comparisons Between Diagnostic Results from this Study and Routine NHLS Results

All the pediatric patients recruited for this study also had their samples (2 early morning GA) collected for PTB diagnosis via the NHLS as per normal hospital protocol. Of the 126 suspected PTB cases, bacteriological diagnosis was established by NHLS in two (child 2 and child 4). However, contrary to the result of this study, the NHLS result of "child 2" had a non-contaminated positive culture result for GA. .

4.1.2.5. Contamination Rates

Cultures were contaminated in 60/126 (47.6%) stool, 18/126 (14.2%) GA, 20/126 (15.8%) urine, 5/117 (4.2%) NPS, and 1/9 (11.1%) NPA specimens, which were all classified as "negatives" in this study. Bacteriological identification of MTBC in contaminated samples yielded no positives. Although not subjected to a microbiological identification, contaminating organisms were visually compatible with fungal and bacterial overgrowth.

4.1.3. Summary

A total of 126 children suspected of PTB were enrolled in this study with a male to female ratio of 1:1.37and a median age of 17.5 months. Of the recruited children 93% were <5 years of age. From the clinical data, weight loss, persistent cough and malnutrition were the most prevalent clinical symptoms of children recruited in this cohort. Samples collected included 126 each of GA, stool and urine, while NPS (n =117), and NPA (n = 9) were collected.

Confirmed PTB detected in four children indicated an incidence rate of (3.2%). Of the remaining children without "confirmed TB", 18/126 (14.2%) were classified as "unconfirmed TB" while 104/126 (82.5%) were classified as 'TB unlikely. However, clinically the incidence rate was 17.4% (22/126).

Culture as the gold standard for microbiological confirmation detected 4/4 positive cases, Xpert® and smear microscopy detected 2/4 and 1/4 positive cases respectively. Urine enabled the detection of 3/4 cases with a 60% diagnostic sensitivity. GA and stool attained a similar sensitivity of 33% enabling diagnosis of 2/4 children respectively, while NPS detected 1/4 cases.

Although alternative samples had a better culture turnaround time, a high contamination rate was noted for stool specimens 60/126 (47%), with a comparable contamination rate between GA and urine; 18/126 (14.2%) and 20/126 (15.8%) respectively, while lesser contamination rates were noted for NPS 5/117 (4.2%) and NPA 1/9 (11.1%) specimens.

DST profile to first-line drugs RIF and INH revealed all the positive cases had susceptible strains which correspond across all samples and agrees with the Xpert™ RIF result for child 2 and 4 (the only Xpert™ positive cases in this study).

4.2. Genotypic Characterisation of *Mycobacterium tuberculosis* Positive Isolates using Spoligotyping and MIRU-VNTR

4.2.1. Introduction

Advance developments in molecular epidemiology have resulted in techniques that enable prompt identification and tracking of circulating *M. tuberculosis* strains in the population (Sola *et al*., 2003). Although previous methods such as comparative growth rates, phage typing, colony morphology and susceptibility to antibiotics were beneficial, but they failed to provide adequate information regarding TB epidemiology (Ali, 2014). Through genotypic studies, new infection can be differentiated from relapses (Guernier *et al*., 2008), more so, transmission can be monitored as patients with identical *M. tuberculosis* strains can be identified as clusters indicating recent transmission (Ribeiro *et al*., 2015). Genotyping of strains from children could be significant for population studies as children usually progress to disease within a year of primary infection (Marais *et al*., 2006a). Therefore, genotypic analysis of isolates collected from children could reflect current transmission patterns within the community (Wootton *et al*., 2005).

Genotypic studies further help establish the existence and diversity of specific strain families in different geographical regions (Wang *et al*., 2012). Previous studies suggest that global TB epidemiology is caused by different strains; occurring at frequencies that differ between district, cities, countries and continents (Filliol *et al*., 2003; Mazars *et al*., 2001; Sola *et al*., 1999; Yang *et al*., 1998). Thus, molecular epidemiological studies in a high TB incidence country may provide adequate information on dissemination dynamics and virulence of the pathogen (Ali, 2014).

In this way, molecular typing of MTBC facilitated our understanding of TB epidemiology (van Soolingen, 2001). Initial epidemiological studies were performed using IS6110 RFLP analysis. Although it is still considered the gold standard of genotyping methods, its complexity along with the absence of the IS6110 element in some strains of *M. tuberculosis* led to the development of PCR-based methods (Moström *et al*., 2002; Schürch and van Soolingen, 2012). Recently, widely used methods are spoligotyping based on the variability of the DR locus (Kamerbeek *et al*., 1997), and MIRU-VNTR (Supply *et al*., 2001, 2000, 2006). However, since the lone use of spoligotyping may overestimate the proportion of clustered strains, it is suggested that spoligotyping should be performed with MIRU-VNTR to achieve an optimum discriminatory power (Sola *et al*., 2003).

While some studies have described the local distribution of circulating strains in the FS (Van der Spoel van Dijk *et al*., 2016) and other provinces of SA (Kamudumuli *et al*., 2015; Sekati *et al*., 2015), to date, reports that detail the frequency and distribution of *M. tuberculosis* genotypes, specifically in children

across the different provinces, are limited. The purpose of this study was to describe the molecular characterisation of *M. tuberculosis* isolates from South African children in the FS province.

4.2.2. Results

4.2.2.1. Spoligotyping

The four TB positive isolates identified in this study were assigned to specific genotype families using the SITVIT WEB database (http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/). The four isolates were divided into respective genotype families (Table 4.7).

Table 4.7. Spoligopatterns, binary codes, octal codes and SITVIT WEB lineage identification of the four *M. tuberculosis* positive isolates. and positive controls.

Sample	Spoligopatterns and binary codes	Octal codes	Lineage
Child 1	□■■□□□□□□□□□■■■■■□■■■■■■■■■■■■■■□□□□■■■■■■■ **onnooooooooonnnnnonnnnnnnnnnnnnnnooooonnnnnnn**	300076777760771	X3
Child 2	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■ **nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnooooooooo**	000000000003771	Beijing
Child 3	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■ **nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnoooonnnnnnn**	777777777760771	T1
Child 4	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■ **nnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnnooooooooo**	000000000003771	Beijing
BCG	■■□■■■■■■□■■■■■■■□■■■■■■■■■■■■■■■■■■■■■■□□□□□ **nnonnnnonnnnnnonnnnnnnnnnnnnnnnnnnnnnnooooo**	676773777777600	BOVIS_BCG
H37Rv	■■■■■■■■■■■■■■■■■■■□□■■■■■■■■■■■□□□□■■■■■■■ **nnnnnnnnnnnnnnnnnnnnoonnnnnnnnnnnnnoooonnnnnnn**	777777477760771	H37Rv (451)

X3: X Family, T1: T Family, BCG Bacille Calmette-Guérin (positive control 1), H37Rv (positive control 2)

The Beijing family was observed in two of the isolates, while the T1 and X3 families were found in the remaining two isolates respectively. BCG and H37Rv strains were included as controls. The X-ray film copy of the spoligotyping result is attached as (Appendix N).

4.2.2.2. MIRU-VNTR

The 24 loci MIRU-VNTR typing analysis showed a total of four distinct unique MIRU patterns for the four genotyped isolates. These results, as well as the BCG and H37Rv positive controls were exported into an excel spreadsheet as seen in table 4.8.

Table 4.8. MIRU-VNTR 24 loci copy numbers.

Name/MIRU	154	424	577	580	802	960	1644	1955	2059	2163b	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348
child 1	2	4	3	2	4	4	3	3	2	3	2	4	4	2	5	1	5	3	3	3	4		3	2
child 2	2	3	4	3	4	3	2	1	2	3	4	4		2		1	4		2	3	3	4	2	
child 3	2	3	5	2	1	3	3	4	2	8	4	4	4	2	5	1	6	3	3	5	3	1	3	3
child 4	2	4	4	2	3	3	3	5	2	2	4	2	4	2	5	1	7	3	3	5	3	8	2	4
BCG	2	0	6	2	2	2	3	1	2	3	5	2	2	5	4	2	5	3	3	3	2	5	0	1
H37Rv	2	2	4	3	1	3	2	2	2	5	3	4	2	3	6	1	3	3	3	3	5	5	2	2

4.2.2.3. Combined Results of Spoligotyping and MIRU-VNTR Typing Methods

Combining the spoligotyping (octal code) and MIRU-VNTR (24-digit allelic profile) typing results using the MIRU-VNTRplus database, resulted in 4 distinct genotypes. Other reference strains (*M. bovis*, LAM, Beijing, X1, Haarlem, T and EAI) obtained from the MIRU-VNTRplus web application and SITVIT web database were also included in the analysis for comparison.

While the SITVIT WEB database was able to identify child 1 strain as an X3 family with the SIT no 2286, the MIRU-VNTR plus database could not categorise the strain as seen in figure 4.9. This is evident by a lack of lineage identification in the lane titled VNTRplus.

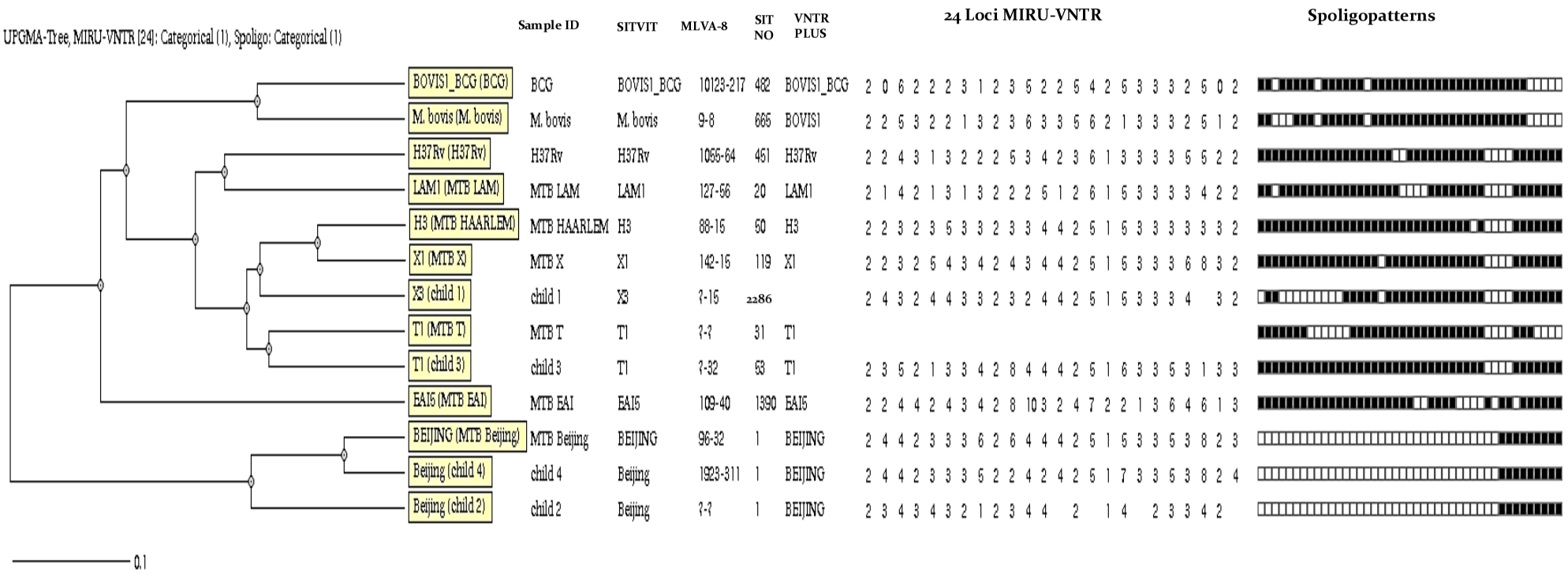

Figure 4.2. The combined results of spoligotyping and MIRU-VNTR typing. The UPGMA dendrogram (Unweighted Pair Group Method with arithmetic mean) was constructed from the strain genotypes using the online MIRU-VNTRplus database (http://www.miru-vntrplus.org/MIRU/index.faces). The result consists of the four isolates obtained from children suspected of tuberculosis (child 1 to 4) and two positive controls (H37Rv and BCG). Other *M. tuberculosis* reference strains (*M. bovis*, Haarlem, T1, X1, Latino-American and Mediterranean, EAI and Beijing) were included in the tree for comparison purposes. The figure contains (from left to right) a phylogenetic tree, the sample ID, the SITVIT lineage identification, the MIRU-VNTR MLVA 8 code, isolates SIT number, MIRU-VNTRplus lineage identification, the 24 loci MIRU-VNTR copy numbers and the spoligopatterns.

4.2.3. Summary

The four *M. tuberculosis* positive isolates in this study were successfully genotypically characterised using spoligotyping and MIRU-VNTR assays. Three different strain families were identified; the Beijing family, the T family and an X3 family.

Respectively, child 1 had an X3 strain, genotyped from DNA extracted from a urine culture, child 2 and 4 both had a Beijing strain genotyped from DNA extracted from stool and GA cultures respectively, while child 3 had a T1 strain genotyped from DNA extracted from a NPS culture.

CHAPTER 5: GENERAL DISCUSSIONS AND CONCLUSIONS

5.1. General Discussions

The challenges associated with establishing a bacteriological diagnosis of TB in children cannot be overemphasised. While a clinical diagnosis of TB can be established in some older children using well-defined symptoms and contact history, it is often more difficult and complicated in young children and HIV-infected children who often present with non-specific symptoms and with varying differential diagnosis (Marais *et al*., 2005). The limitations of the current tests, the paucibacillary nature of TB in children and the inability to obtain good quality and quantity of standard specimens has necessitated the research into the use of more readily accessible specimens (Detjen and Walters, 2016; Zar *et al*., 2000).

This study examined the diagnostic yield of multiple specimen types (GA, urine, stool, NPA/NPS) using various diagnostic methods (smear, culture and Xpert®) in a rural-urban setting. We found that, even though alternative specimen did provide additive culture yield with a shorter mean time to detection compared to the standard specimen (GA), overall, culture confirmation remained low and Xpert® had an even lower yield.

Of the 126 pediatric PTB suspects enrolled, four patients (3.2%, n = 4/126) had microbiological confirmed TB. Of the remaining 122 children, using the consensus agreement on Intrathoracic Tuberculosis Definitions for Diagnostic Research in Children (Graham *et al*., 2015a), 18 (14.2%, n = 18/126) patients were classified as having 'unconfirmed TB', while 104 (82.5%, n= 104/126) were classified as 'TB unlikely'. Therefore, clinically 22 children of a cohort of 126 had TB. This is an incidence rate of 17.4% of the study population. Reflective of the high prevalence of HIV in the community, 2 of 4 of the children with bacteriological confirmed PTB were HIV positive, although information regarding their CD4 cell count was not requested during enrolment.

Of all participants, malnutrition and weight loss were the most significant clinical characteristics reported. Persistent fever and lethargy were more significant in the confirmed TB group, with none of them having reported a history of TB exposure nor chest X-ray suggestive of TB. This suggests that TB exposure is not only limited to household contacts as undocumented community exposure may have occurred. This is in agreement with studies done by other researchers (Marais *et al*., 2009; Middelkoop *et al*., 2008; Wood *et al*., 2010).

Often, clinicians depend on the clinical method of diagnosing pediatric TB. However, the previous classification layout for 'possible' and 'probable' TB (Graham *et al*., 2012) would not have accurately categorised our participants with culture-confirmed disease. In this cohort, the four culture-confirmed TB patients only met symptom criteria for "possible TB", emphasising the limited sensitivity of this classification.

Culture detected TB in all four patients with positivity on 7 out of a possible 16 samples (4 different specimens per child) in both HIV-infected and uninfected children, reiterating its use as the gold standard of diagnosis. Although culture had a slow turnaround time, the mean time to positivity on alternative samples (urine, stool and NPS) was shorter compared to GA (refer to table 4.6, pg 72)

The use of culture on urine for the diagnosis of pediatric PTB is being considered more regularly and increasingly studied (Thomas *et al.*, 2014). However, most of the studies focused on detection of PTB using Lipoarabinomannan (Kroidl *et al.*, 2015; Nicol *et al.*, 2014). In this study, culture successfully detected TB in the urine of three children (one HIV-infected and two HIV-uninfected), offering confirmed diagnosis solely for one of the cases. Similarly, a study by Thomas *et al.* and Gopinath and Singh reported an increased diagnostic yield of culture on urine from pediatric and adult PTB suspects respectively (Gopinath and Singh, 2009; Thomas *et al.*, 2014).

A number of studies have reported the suitability of culture on a stool for the diagnosis of PTB in HIV-infected and uninfected pediatric suspects (Oberhelman *et al.*, 2010; Ouédraogo *et al.*, 2016; Walters *et al.*, 2017). In this study, culture on stool enabled the additional confirmation of TB in 2 of 4 children, and unlike previous studies, diagnosis by culture on stool was established only in the HIV-infected patients. Nonetheless, it was suggested that regardless of the low sensitivity of culture on stool samples it can still serve as an alternative method of diagnosis especially in the HIV-infected population (Ouédraogo *et al.*, 2016).

Whilst some studies have reported culture yields between 30% and 38% for GA and NPA (Franchi *et al.*, 1998), some reported a lower yield of 3.8% vs 6.8% and 10% vs 5.5%, for GA and NPA specimen respectively (Oberhelman *et al.*, 2006, 2010). In this study, culture was positive only on one NPS, with no positivity on NPA specimens. Generally, the sensitivity of culture on NPA and NPS in this study was low. However, due to the few numbers of NPA samples collected in this study, no definite conclusions can be drawn regarding the sensitivity of culture on NPA samples.

Contrary to culture, in this study Xpert® had a diagnostic sensitivity of 33%, specificity of 100%, a PPV value of 100%, and a NPV value of 98.3%. Xpert® detected only 2/4 confirmed TB cases (mainly HIV-infected) with positivity on 6/16 samples. There was a concordance between Xpert® testing of GA, and stool, which was similar to a study of Hasan *et al.*, where Xpert® PTB testing of stool detected 21% (10 cases) and GA 22% (11 cases) of TB cases (Hasan *et al.*, 2016). Currently, there is insufficient data on the use of Xpert® on urine for the diagnosis of PTB in pediatric suspects. However, in this study, Xpert® urine performance was comparable to that of Xpert® stool and GA in the HIV-infected children. Since the completion of this study, the Ultra, an update on Xpert® was introduced into the market (WHO, 2017b). This method is reported to have a better sensitivity in paucibacillary TB cases and in

extrapulmonary specimens (Chakravorty *et al*., 2017; Dorman *et al*., 2017) and could have improved the diagnostic yield.

Several studies have reported the suitability of Xpert® testing on stool specimen in children, especially in the HIV-infected population. In a study by Nicol *et al*. stool Xpert® testing from 115 children with suspected PTB detected 8/17 (47%) culture-confirmed tuberculosis cases, including 4/5 (80%) HIV–infected and 4/12 (33%) HIV-uninfected children (Nicol *et al*., 2013). In another study by Welday *et al*. using a stool for Xpert® testing showed 100% sensitivity and 89.36% specificity without missing any positives compared to sputum ZN smear microscopy (Welday *et al*., 2014). Marcy *et al*. in their PAANTHER study, also reported that Xpert® performance on 1 stool sample had intention-to-diagnose and per-protocol sensitivities of 62.1% and 68.8%, respectively (Marcy *et al*., 2016). A more recent study by LaCourse *et al*. also reported a higher sensitivity of 63% for stool Xpert™ which significantly increased to 80% among HIV-infected children compared to 60% sensitivity on sputum/GA using Xpert® (LaCourse *et al*., 2018). In our study, stool Xpert® result mirrors the report of larger studies as seen by its sensitivity in the HIV-infected children detecting 2/2 cases. Using Ultra as the testing platform, this number could even increase.

Compared with other studies, Xpert® on NPA in our study yielded no positive results, while other studies reported excellent feasibility and good accuracy on NPA with Xpert® (Marcy *et al*., 2016; Zar *et al*., 2013; Zar *et al*., 2012). The lack of positive Xpert® nasopharyngeal specimens in our study may be due to the paucibacillary nature of childhood TB or as suggested by Nicol and Zar, could be as a result of specimen collection techniques or the patient population (Nicol and Zar, 2011).

Additionally, Xpert® showed a satisfactory result in this study with 100% Xpert® positive and negative cases getting valid results. Invalid or false negative results in several PCR based tests are often due to the presence of inhibitors, substandard assay conditions or possible omission of key steps (Raizada *et al*., 2015). However, these challenges are of less concern on Xpert®, as the assay is automated and self-contained with minimal hands-on specimen manipulation which could have added to its low PCR inhibition rates (Raizada *et al*., 2015).

While sputum smear microscopy can be positive in the majority of adults with PTB, fewer than 20% of children with TB are sputum or GA smear-positive (Al-Aghbari *et al*., 2009). Undeniably, in our study neither GA nor alternative specimens resulted in more than 25% positives. Compared to Xpert® and culture, smear microscopy had a comparatively lower sensitivity (14%) but equal specificity (100%) by detecting TB in 1 patient (HIV-infected) with positivity on 2/16 samples. This is relative to a study by Nicol *et al*., among children with definite tuberculosis, 3/5 (60%) HIV-infected children were smear-positive compared with 1/12 (8%) HIV-uninfected children (Nicol *et al*., 2013). Of importance,

the confirmation of a smear-positive culture-confirmed TB in child 2 (HIV-infected) in this study may suggest more severe forms of TB and higher bacterial loads (Marcy *et al*., 2016). As evident with the high Xpert® positive stool and urine result for child 2 compared to smear-negative, low Xpert® positive stool and urine result for child 4 (Table 4.4 in chapter 4).

The importance of microbiological confirmation of TB in children was emphasised in the recent WHO guidelines for the diagnosis of TB in children. The guideline indicated that GA, expectorated or IS are the most frequently used methods to obtain specimens in children. However, it did not specify whether to use these specimens singly or in combination with other possible specimens (WHO, 2014a).

Few studies have investigated whether the collection of several samples from different sites could be synergistic and increase the recovery of *M. tuberculosis* among symptomatic children. In a study, GA yielded positive TB detection in 22 cases, NPA in 12 cases, and stool in 4 cases (Oberhelman *et al*., 2010). In another study of pediatric TB suspects aged ≥6 months and ≤12 years, among those with positive cultures, 7/8 (88%) were from IS, 5/6 (83%) from GA, 3/8 (38%) from blood, and 3/7 (43%) from urine (Thomas *et al*., 2014). A more recent study reported a comparative performance for stool and GA (LaCourse *et al*., 2018). In this study, regardless of the diagnostic method employed, GA, considered minimally invasive did not give a higher diagnostic yield compared to the alternative samples. GA enabled the detection of TB in 2 children that were detected on alternative samples as well. However, alternative samples (urine and NPS) enabled the detection of TB in two children with no positive result on GA. More so, with a shorter mean time to detection on culture compared to GA, which can enable early treatment decision and exclusion of DR-TB especially in cases where drug resistance is suspected.

As proven by previous studies, stool; an easily accessible specimen is an appropriate sample for the diagnosis of *M. tuberculosis* in children. In this study, stool enabled the detection of *M. tuberculosis* and performance of DST in two HIV-infected children. The sensitivity of stool (culture and Xpert™) in our study, although low, can still be compared to those reported in other studies. From a study by Nicol *et al*. stool on Xpert™ detected 8/17 (47.1%) confirmed TB cases (Nicol *et al*., 2013). A study by Oberhelman *et al*. reported stool culture detected 4/22 (18.2%) confirmed TB cases (Oberhelman *et al*., 2010), while another study of Oberhelman *et al*. reported that stool culture detected 3/15 (20%) confirmed TB cases (Oberhelman *et al*., 2006) and Walters *et al*. reported a stool culture positivity in 6/37 (16.2%) confirmed cases (Walters *et al*., 2017). Similarly, in our study, stool on Xpert™ and culture enabled the detection of 2/4 confirmed TB cases. Although some studies like (Alvarez-Uria *et al*., 2012) and (Hillemann *et al*., 2011) reported unacceptable large numbers of errors (13–25%) generated by stool on Xpert®, probably from sample clogging the Xpert® assay cartridge or due to PCR inhibition from

stool components (Banada *et al.*, 2016; Nicol *et al.*, 2013). Contrary to this, none of the 126 stool specimen tested with Xpert® in this study yielded any error result.

It was further suggested that the sensitivity of <50% reported in some Xpert® stool studies, as seen by negative stool Xpert® in 2/4 confirmed cases in our study could have been due to the low amounts of the samples tested (<0.2g). A study by Banada *et al.* evaluated the diagnostic utility of different stool volumes of TB infected macaques. A 0.6 g stool sample gave significantly more positive results than the 0.2 g stool samples. However, they further reported that 1.2 g of a stool sample did not perform better than the 0.6 g stool sample in their human participants, although tests on one 1.2 g of stool did suggest that a TB negative control participant had TB and was treated clinically for TB (Banada *et al.*, 2016). It was also suggested that developing optimised protocols for stool Xpert® testing which permits the use of larger volumes of the stool are likely to enhance sensitivity and may permit the easier diagnosis of paucibacillary TB (Banada *et al.*, 2016; Nicol *et al.*, 2013). Additionally, Welday *et al.* proposed that stool DNA extraction should be investigated in future as this helps to detect cases missed in the direct stool Xpert® test (Welday *et al.*, 2014). Although not yet evaluated, Walters *et al.* recommend that similar to respiratory specimens, increasing the number of stool specimen collected for Xpert® testing may increase the diagnostic yield (Walters *et al.*, 2017).

As previously stated, stool on culture enabled the supportive diagnosis of TB in 2/4 bacteriologically confirmed TB cases. However, in as much as an inter-study comparison of diagnostic results are essential for a feasibility report, it may be difficult as different studies employ the use of different protocols for stool preparation with varying culture techniques (Walters *et al.*, 2017). Nicol *et al.* used a 0.15 g of thawed stool diluted in 2.4 mL PBS (Nicol *et al.*, 2013), Oberhelman *et al.* used a 0.1 g stool mass diluted in 6 mL PBS (Oberhelman *et al.*, 2006, 2010), Donald *et al.* combined 2 stool specimens (final mass not specified) (Donald *et al.*, 1996), while Walters *et al.* homogenized between 2-3 g of stool with 20 mL PBS (Walters *et al.*, 2017). Excluding Walters *et al.*, the other four studies used a 1% final concentration NaOH for decontamination, as used in this study; while Walters *et al.* used a 1.25% final concentration NaOH. For culture, Donald *et al.* used the BACTEC radiometric culture, while Oberhelman *et al.* used both LJ and MODS assays. Similar to Walters *et al.*, this study used the MGIT™ 960 System (Becton 221 Dickinson, Sparks, MD, USA) culture with PANTA™, which is more sensitive than solid culture (Siddiqi, 2006) and is the method used by many diagnostic laboratories.

As observed in this study, MGIT™ 960 System culture is more prone to contamination by commensal microorganisms (Siddiqi, 2006). The excess microflora present in stool overgrows in culture and impede the detection of the slow-growing *M. tuberculosis* bacilli (Walters *et al.*, 2017). Beside Walters *et al.* who reported 41.5% stool culture contamination (Walters *et al.*, 2017), other published paediatric stool culture studies did not report on contamination rates. However, studies in adults using the

MGIT™ 960 System testing, reported 14-38% stool contamination (Hillemann *et al*., 2011; Oramasionwu *et al*., 2013). Comparatively, our study reported 47.6% stool contamination rates.

Various techniques to reduce stool culture contamination in the laboratory have been evaluated. In a study of Allen, NaOH yielded a better *M. tuberculosis* recovery rates and equal contamination rates compared to benzalkonium chloride-1-hexadecylpyridinium chloride and Portaels solution (Allen, 1991). In a similar but separate study, Allen compared different decontaminating agents and concluded that NaOH was better than sulphuric acid and alkali precipitation for recovery of *M. tuberculosis* and decontamination (Allen, 1989). El Khéchine *et al.* reported the use of 0.25% chlorhexidine in place of NaOH in the processing of stool specimens (El Khéchine *et al*., 2009; El Khéchine and Drancourt, 2011), stating an improved recovery vs contamination, compared to NaOH (El Khéchine *et al*., 2009). Allen further suggested that dilution of stool samples after the 1% NaOH decontamination procedure, before inoculation into the culture medium may inhibit contaminating organisms; citing a significantly reduced contamination rate with the use of a 1:10 dilution with no effect on *M. tuberculosis* recovery rate (Allen, 1989).

Many studies are yet to evaluate the use of higher NaOH concentrations and extended exposure times for sample decontamination and the effect of sample dilution on *M. tuberculosis* detection in the pediatric population. However, it is suggested that considering the paucity nature of PTB in children, such modification may actually reduce mycobacterial yield rather than inhibit bacterial and fungal overgrowth on the already paucibacillary specimens (Walters *et al*., 2017).

Urine is one of the most accessible clinical samples. However, only a few studies have demonstrated its utility in diagnosing TB in both HIV-infected and uninfected patients and mostly in the adult population. Urinary excretion of *M. tuberculosis* was reported as early as in 1923 by Hobbs (Hobbs, 1923). In 1975, a study by Bentz *et al*. in an adults population, reported 4.7% positive urine cultures among patients with clinical diagnosis of active PTB (Bentz *et al*., 1975). Torrea *et al*. evaluated the utility of urine specimens using a nested PCR assay in suspected adult PTB and EPTB cases, their PCR detected *M. tuberculosis* in urine samples from 88 of 217 (40.6%) patients with microbiological-positive PTB, 20 of 30 (66.7%) patients with microbiological-negative PTB and 48 of 84 (57.1%) patients with EPTB. However, the major limitation of their study was a lack of bacteriological confirmation of the urine specimens (Torrea *et al*., 2005). A recent study by Thomas *et al*. reported the diagnostic utility of urine among pediatric TB suspects, with a positive urine culture in 3/7 (43%) bacteriologically confirmed cases (Thomas *et al*., 2014). Another study by Gopinath and Singh reported a high culture positivity rate among 81 adults suspected of PTB. Of the 81 patients suspected of having PTB, 46 (56.8%) were sputum culture-positive. Of these, 12 (26.1%) were also urine culture-positive for *M.*

tuberculosis. Of the 35 sputum culture-negative cases, three (8.6%) were urine culture-positive (Gopinath and Singh, 2009).

In this study, we evaluated the feasibility of using urine for the diagnosis of PTB in pediatric suspects. As seen in our results,3/4 of the smear- and/or culture-positive PTB cases also excreted *M. tuberculosis* in their urine specimens, with positive urine culture and Xpert® results in two cases (HIV-infected), and a urine culture-positive result alone in one case (HIV-uninfected). This is similar to a case from the study of Thomas *et al*., where a child was positive on urine culture only despite presenting with pulmonary symptoms (Thomas *et al*., 2014). Hobbs also reported 6% positive urine cultures in 100 patients with PTB with no clinical manifestation of renal involvement nor urinary tract associated symptoms (Hobbs, 1923). Previous investigators also emphasised that bacilluria due to *M. tuberculosis* could occur in the absence of renal lesions and without any association with genitourinary TB (Bentz *et al*., 1975; Hobbs, 1923).

Although in this study, urine samples were centrifuged at 3000 × g and pooled together, Gopinath and Singh suggest that a centrifugation speed of 10 000 rpm should be employed for urine samples, as centrifugation plays a vital role in the retrieval of living mycobacteria (Gopinath and Singh, 2009). More so, the specimen pooling technique employed in their study could have contributed to the increased sensitivity of the urine samples.

As previously suggested, bacilluria is not a continuous process (Gopinath and Singh, 2009). This may be attributed to the case of child 3 with a positive NPS culture only, suggesting that not all PTB patients will have mycobacteria filtered out through the urine. It is also possible that *M. tuberculosis* or its fragments may not have been excreted during sample collection. However, there is not enough available data on the successful diagnosis of paediatric PTB using urine in Xpert® and culture and. therefore, inter-laboratory comparison of results is limited.

This study reports a confirmed culture-positive case of PTB diagnosed using a NPS. NPS is an easy to collect sample, requiring seconds and no specialised equipment or invasive techniques. NPS in this study enabled the detection of 1/4 confirmed TB cases and subsequently enabled us to perform DST of the isolate. A report by Trevathan and Philips highlighted the result of two studies, one from Japan and the other was from Czechoslovakian. In one, 174 cultures of nasopharyngeal washings obtained on 115 persons exposed to TB yielded one culture-positive for *M. tuberculosis*. In the other study, of the 114 patients with PTB examined for tubercle bacilli using a nasopharyngeal wash, 22.8% yielded a positive result on solid egg-based media (Trevathan and Philips, 1959).

The use of a NPS as a clinical specimen for TB diagnosis may be considered a relatively new technique, but its concept was based on reports by other studies such as the ones highlighted by Trevathan and Philips (Trevathan and Philips, 1959); as such its inclusion in our study was to evaluate/proof the concept of detecting TB in NPSs. Although we were able to establish a confirmed TB diagnosis from an HIV-uninfected child, none of the other three children with confirmed TB had a positive NPS, necessitating additional studies with more extensive and diverse patient populations.

To the best of our knowledge, this is the first study in SA to compare the use of GA, stool, urine and nasopharyngeal specimens (NPA/NPS) for the diagnosis of PTB in pediatric suspects using smear microscopy, culture and Xpert®. Although the overall *M. tuberculosis* positive yield in our study was lower (3.2%) than expected (10%) from this TB endemic region, the low yield may be explained by the rural district setting compared to urban referral centres. The latter may see a higher proportion of TB suspects with later-stage presentations and higher bacillary burden. Additionally, our yield may have been affected by the limited specificity of clinical parameters and the possible presence of other pulmonary infections that could have mimicked TB in this cohort with a high prevalence of malnutrition. The broad eligibility criteria may have played a role in the low culture rate as evident by only (22%) of subjects who met criteria for unconfirmed TB.

Furthermore, in an era of increasing DR-TB, bacteriological confirmation is imperative for DST, and the rapid detection of DR-TB is essential to the effective treatment of patients (Siddiqi *et al*., 2012), and essential to prevent transmission of MDR-TB (Espasa *et al*., 2012). Although Xpert® enables the simultaneous identification of RIF resistance, the increasing number of INH monoresistance TB (a gateway to MDR-TB) necessitates the need for phenotypic DST using the MGIT™ 960 System and molecular assays such as Genotype® MTBDR*plus*. More so, following several analyses of hundred thousand samples on Xpert®, controversies regarding its false-negative and false-positive RIF resistance results emerged (Hillemann *et al*., 2011; Marlowe *et al*., 2011; Somoskovi *et al*., 2013).

The Xpert® version G4 assay was developed by the manufacturers to increase the assay's robustness and lessen the amount of reported false RIF-resistant results while improving the detection of probe E mutants that were difficult to detect with the G3 version (FIND, 2011). While an analytical study from SA reported that the G4 version had lessened the false RIF resistant results (Stevens, 2012), another study by Rufai *et al*. did not find the newer version better, particularly for India. Their study shows that only 64.4% of RIF-monoresistant TB cases were correctly diagnosed by Xpert®. The remaining 35.6% were detected as falsely RIF susceptible cases (Rufai *et al*., 2014).

In this study, culture on two children (child 1 and 3) enabled the exclusion of DR-TB and for child 2 and 4 (Xpert® positive, RIF susceptible) by confirming RIF and INH susceptibility using the MGIT™

960 System and Genotype® MTBDR*plus*. Different studies have already demonstrated the feasibility of the Genotype® MTBDR*plus* assay as an effective tool in early detection of MDR-TB with good concordance with phenotypic DST (Hillemann *et al*., 2007; Lacoma *et al*., 2008; Miotto *et al*., 2006). In our study, both assays agree on DST results across all patient and all various samples, correlating with other studies.

The automated liquid culture systems (MGIT™ 960 System) DST is accurate with reported turnaround times from inoculation to drug susceptibility results ranging from 6 to 18 days with a median time of 12 days (Kobayashi *et al*., 2006). Nonetheless, it is still less efficient compared to Genotype® MTBDR*plus* which is less expensive, and easy to perform with time to detection of fewer than 24 hours. Notwithstanding it is suggested that phenotypic DST may still be required in cases of *M. tuberculosis* clinical isolate, as the molecular assay does not detect 100% of drug resistance, more importantly, INH resistance (Gitti *et al*., 2011).

The genotypic profile of *M. tuberculosis* strains from our study has been described in Chapter 4. In this study, three genotype families were identified The Beijing family characterised by the absence of spacer 1 to 36 was identified in two of the cases, the T family (T1 strain), and the X3 family in the remaining two cases respectively.

In a South African adult study by Stavrum *et al*. in which isolates from eight provinces were included, T1 was found to be predominant in GP (40%), followed by the FS (32%) (Stavrum *et al*., 2009). Contrary, a study by van der Spoel van Dijk *et al*. suggest that the LAM (33%) and T (31%) strain families were predominant in the FS (Van der Spoel van Dijk *et al*., 2016). In another pediatric study by Sekati *et al*., Beijing, T1 and X families were found in all four provinces studied (Sekati *et al*., 2015). Similar to our study, the high prevalence of the Beijing family has been reported in other South African studies (Hove *et al*., 2012; Marais *et al*., 2006; Marais *et al*., 2013; Pillay and Sturm, 2007; Sekati *et al*., 2015). Since previous reports suggest that the genotype analysis of isolates obtained from children usually reflects current transmission patterns within the community (Marais *et al*., 2006a; Wootton *et al*., 2005), the fact that the Van der Spoel van Dijk *et al.* study was done on strains from 2001–2003, may suggest a shift in the strain burden in the FS. This may indicate an urgent need for larger genotypic studies of TB strains in children in the FS.

Previous studies have indicated that there is an interaction between the strain's genetic background and drug resistance (Gagneux *et al*., 2006a; Tsolaki *et al*., 2005). The Beijing strain has often been associated with drug resistance, both in SA (Johnson *et al*., 2010; Klopper *et al*., 2013; Marais *et al*., 2006; Streicher *et al*., 2007) and elsewhere (Drobniewski *et al*., 2005; Kubica *et al*., 2005). In our study, none of the Beijing strains was MDR-TB nor RIF or INH monoresistance. A South African study by Gandhi

et al. also reported identifying the Beijing family in only 3% of MDR TB isolates (Gandhi *et al.*, 2014). Only five percent of isolates belonging to the Beijing family were MDR TB in a study by Hove *et al.* (Hove *et al.*, 2012). The prevalence of the Beijing genotype in our study is of note and may require further investigation.

A previous study hypothesised that the lower susceptibility of Beijing strain to anti TB drugs has contributed to its global spread (Bifani *et al.*, 2002). Additionally, another study in mice found that the Beijing strain was more virulent compared to other strains, while another study in rabbits reported a severe form of TB meningitis occurring as a result of infection by Beijing strain compared to other strains (Dormans *et al.*, 2004; Tsenova *et al.*, 2005). It was further suggested that the hypervirulence of the Beijing spoligotype observed in animal studies could be as a result of the phenolic glycolipid (PGL) (Tsenova *et al.*, 2005). It is believed that the synthesis of PGL has allowed the Beijing strains to evade early immune responses resulting in greater success in inducing disseminated disease compared to strains that cannot synthesise PGL (Caws *et al.*, 2008; Hanekom *et al.*, 2011). This could have also contributed to the lower protection of BCG vaccine against Beijing strains (Hanekom *et al.*, 2011); further reiterating the need for extensive analysis of strains circulating amongst children in the FS.

While spoligotyping only identified child 2 and 4 as Beijing strains, the additional discriminatory power of MIRU-VNTR enabled the differentiation of the two isolates into two different subfamilies and as such is not as a result of community transmission nor laboratory contamination.

Limitations of the Study

There were limitations to our study. The unavailability of some specimens (stool, urine and NPS) in some of the children lowered our final sample number, as the children with missing samples had to be excluded. More so, the collection of limited NPAs also hindered our ability to conclude on the performance of NPA.

Furthermore, our study did not initiate a follow up on the enrolled patients. Although follow up of patients was not a part of the research objective, it could, however, have assisted in determining how many of the unconfirmed and unlikely TB cases had TB treatment initiated based on clinical symptoms regardless of bacteriological results. Additionally, the small number of bacteriologically confirmed TB cases in our study did not permit extensive research into the drug susceptibility pattern and strain distribution of *M. tuberculosis* in the children population of the FS.

5.2. General Conclusions

Bacteriological confirmation by culture is the gold standard of diagnosis of TB disease. In children, however culture confirmation is an endpoint in a series of events clouded with true and perceived obstacles and seldom achieved. The barrier of adequate sample collection limits diagnostic investigation owing to the poor performance of the recommended sample types for evaluating PTB in children. Many high TB burdened countries are faced with the daunting challenges of access to diagnostic tools and the capacity to obtain appropriate clinical specimens in children.

While children are remarkably more vulnerable to infection, disease progression, and death from misdiagnosis and diagnostic delays, compared to adults, they also have a better prognosis in the face of timely and adequate treatment. The challenges of collection of adequate specimens is not a justifiable reason to defend the lower percentage of bacteriologically confirmed cases. Investigation on the most appropriate specimen collection and/or combination and methods that could enhance early confirmatory diagnosis is was evaluated for GA/sputum, urine, stool and NGA/NPA specimens and Xpert, GenoType and culture as the main objective of this study.

Performance of Different Diagnostic Methods

The study found that culture remains the gold standard, with a sensitivity of 100% on per patient analysis and 43% per sample analysis. On patient analysis, Xpert® had a sensitivity of 66% and smear microscopy only 57%. When analysed by sample types, culture detected at least one positive sample from each of the four confirmed positive cases.

Xpert® testing had comparable sensitivity to culture on stool and urine samples in the HIV-infected cases in our study. Although Xpert® only allows partial DR determination, combining the two methods (Xpert® and culture) could improve the sensitivity for both standard (GA) and alternatives (stool, urine and nasopharyngeal specimens), while simultaneously full DST if clinically relevant. The recent development of Xpert® Ultra with an expected lower limit of detection could further impact significantly on the capacity to reach diagnostic confirmation.

Additionally, Xpert® with advantages of quick turnaround time and good interpretable results demonstrated equivalent specificity to culture. Reiterating the evidence base for inclusion of Xpert® in the reference standard for bacteriologically confirmed childhood TB in both WHO clinical guidelines and research.

In concordance with previously reported studies, smear microscopy exhibited a sub-optimal performance compared to Xpert® and MGIT™ 960 culture. Its poor performance in our study may be related to its requirement for a high bacillary load which is often not available in pediatric samples

and as such has little value in pediatric diagnostics. Nonetheless, smear microscopy can still serve as an addon test to newer diagnostic methods like Xpert®.

Comparisons of Various Samples for Diagnosing PTB in Children: Additional Value of Alternative Samples

Alternative samples (urine, stool and NPS) provided a better diagnosis than GA in this study. Urine and NPS providing a definitive diagnosis in children (1 each) that would have been otherwise termed negative using GA samples.

Urine outperforms other samples in this study, providing a diagnosis in 3/4 culture confirmed TB cases. Although only 4 children was diagnosed as positive in this study it suggested that urine compared to GA, may be considered a less invasive, more successful sample for childhood TB suspects.

While stool can easily be collected, the relatively complex and laborious stool sample preparation and processing hinder its acceptance in routine use and laboratory protocols for stool sample processing are yet to be optimised. However, given the limited options for detecting TB in children, a stool culture may still be used as an adjunct specimen in clinical scenarios where confirmation of TB disease and DST results are critical. However. this study, did not found increased value of stool samples compared to GA.

However the study do indicate that increasing the number of samples tested increases the diagnostic yield of any diagnostic test, although this strategy might not always be feasible in routine settings with limited time and resources.

The possibility of using NPS for the diagnosis of childhood TB is a proven concept from this study, nonetheless one positive case from NPS may be too insignificant to draw a definitive conclusion

Our finding that the bacillary burden in stool and urine correlates with that of GA cultures suggests that bacteria survive gastrointestinal transit and can be grown in culture while its DNA can be detected by Xpert®.

While our study found a significant relationship with PTB and culture/Xpert® positivity for *M. tuberculosis* on alternative samples (urine, stool, NPS), we cannot comment on the relationship between EPTB and stool/urine Xpert®/culture positivity. Although each participant was evaluated for EPTB, we may have missed cases of EPTB, as other sites were not cultured for TB. Nonetheless, we believe that the positive alternative samples in our study indicate bacilli of pulmonary origin. However, further studies are required to validate our results.

The second objective of the study was to determine the bacteriologically confirmed incidence of PTB and DR-TB in children suspected of PTB admitted to public hospitals in Mangaung, FS Province. In the study four (3.2%) children <5 year out of 126 were microbiologically confirmed as having TB. A further 18 (14.2%) children were classified as having "unconfirmed TB". However, the population covered by the hospitals could not be confirmed and no definity conclusion on incidence can be drawn. It was also not confirmed if the "unconfirmed" cases were treated and if they recovered. Therefore the incidence can only be estimated as between 3 and 14% of children suspected of TB according to the consensus agreement on Intrathoracic Tuberculosis Definitions for Diagnostic Research in Children.

Finally, to better understand the epidemiology of TB in children in Mangaung FS, positive cases were further characterised phenotypically (for DR-TB) and genotypically (for strain identification).

Drug Susceptibility testing: Value of Molecular Assays

The MGIT™ 960 System and Genotype® MTBDR*plus* assay exhibited a 100% concordance on DST result in this study. Both assays found isolates in this study susceptible to RIF and INH. The GenoType® MTBDR*plus* test is reliable, rapid and easy to perform for the simultaneous detection of RIF and INH resistance in *M. tuberculosis.* Although the low number of positive samples in this study do not allow extensive conclusions, in our sample base GenoType® MTBDR*plus* facilitate adequate identification of susceptible and detection of resistant strains, long before the results of liquid culture DST are available. However, Genotype® MTBDR*plus* does not detect all INH resistant mutations, which has been reported to be a concern in children with TB, it is recommended that the GenoType® MTBDR*plus* test should be used as an early guidance of therapy, which should be followed by a phenotypic DST confirmation of INH susceptibility in all TB positive patients children. More so, we conclude that the incorporation of the molecular test in the SA TB programme has been a significant step forward in the rapid identification of both susceptible and resistant *M. tuberculosis* among suspected patients in the country.

Characterisation of *Mycobacterium tuberculosis* Positive Isolates

Epidemiological studies have shown that the distribution of *M. tuberculosis* strains varies between different geographical regions, however no data on genotypic studies in the children population in the Free State is available.

The spoligotyping and automated 24 loci MIRU-VNTR typing methods applied in this study identified three strain families (Beijing, T1 and X3), with the Beijing strain occurring in 2/4 isolates. The Beijing strain family was also reported in other provinces of SA and the presence in our study is a cause for concern since it was present in limited numbers until 2003. Although only two isolates belonging to the Beijing family were found in our study, it represented 50% of the identified cases. and wWhile not

representative of all paediatric TB in the FS population,These findings indicate a need to conduct larger genotypic studies in the children population, as this may reveal an increased transmission of Beijing strains in the FS population.

Recommendations for Future Research

Regarding stool specimen, further laboratory research should be conducted. Importantly, promising laboratory protocols with proven better sensitivity and low contamination rates, such as those using chlorhexidine, should be evaluated and compared to current protocols. This optimised laboratory protocols may then be applied to specific high-risk paediatric groups such as children at risk of DR- TB and those with HIV-infection, where the diagnosis is most critical.

Although our study proposes a proof of concept to the use of NPS in the detection of *M. tuberculosis*, larger studies is needed to validate these findings.

Whilst the recent development of fast and minimally invasive TB diagnostics has been promising, drawbacks include lack of validation in diverse populations, such as children. Refinement of old tools and testing of new tools in children may improve diagnosis and treatment of TB in these special population.

Furthermore, considering the absence of data on the epidemiology (DR and strain distribution) and limited data on incidence and prevalence rates of TB in children, immediate routine surveillance program on childhood TB are needed as it may help inform public health policy by providing information on the rate of infection, current strain transmission and/or DR patterns, and on the effectiveness of control strategies which may limit the future propagation of TB epidemic.

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Appendix A: Study Information in English

INFORMATION DOCUMENT

COMPARISON OF METHODS AND SAMPLES USED IN THE DIAGNOSIS OF CHILDHOOD PULMONARY TUBERCULOSIS AND CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS ISOLATES

We come from the University of the Free State (Department of Medical Microbiology) and we are doing a research study on comparing how fast we can detect TB in fluids from the tummy, back of the nose (throat), poop (stool) and pee (urine) of children who their doctor think might have TB. Research is just the process to learn the answer to a question.

We are asking/inviting you to be part of this research study or ask for your permission to include your child in this research study. The study includes taking the fluids from the tummy and throat (back of the nose) or sputum, but also collecting poop and pee from the children. Your doctor will in any case need the first two samples to make sure that you do not have TB and we are only asking for an extra stool and urine. Total number of children who will take part in this study is 200 and they will all be under the age of 13yrs. To look for the bacteria that causes TB, the collected fluids, poop and pee will be looked at under the Microscope (this is an instrument used to make very small things look larger so that it is easier to see). It will then be put into a machine called GeneXpert and put into special bottles with nutrients (like vitamins) to encourage the bacteria to grow. Bacteria grown will further be tested with antibiotics to check if it will kill the bacteria. The bacteria that grow in the presence of the antibiotics are said to be resistant. The bacteria that grew will then be used in further studies to see if they are related (the same) and to learn more about them (characterise).

There is no risk in taking part in this study since the specimens that will be obtained are non-invasive (no needles or internal procedures). You can say 'No' or you can say 'Yes' and nobody will be upset if you say 'No'. If you say 'Yes', you can always say 'No' later. We will try to make sure that no bad things happen. You can say 'no' to what we ask you to do for the research at any time and we will stop. Efforts will be made to keep personal information like your name confidential. Absolute confidentiality cannot be guaranteed.

Contact details of researcher(s) – for further information/reporting of study-related matters

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Appendix B: Study Information in Afrikaans

INLIGTINGSDOKUMENT

VERGELYKING VAN METODES EN MONSTERS VIR GEBRUIK IN DIE DIAGNOSE VAN PULMONÊRE TUBERCULOSIS IN KINDERS EN KARAKTERISERING VAN *MYCOBACTERIUM TUBERCULOSIS* ISOLATE.

Ons werk by die Universiteit van die Vrystaat (Department Mediese Mikrobiologie) waar ons verskillende nuwe dinge navors (probeer uitvind). Tans doen ons ‘n studie waar ons wil kyk hoe vining ons TB kan opspoor deur die gebruik van verskillende liggaams-vloeistowwe. Ons vergelyk vloeistof uit die maag, keel (die agterkant van die neus), poep (stoelgange) en piepie (uriene) van kinders wat die dokters dink het TB. Navorsing is ‘n proses waardeur ons antwoorde op vrae kry.

Ons wil u/jou vra/uitnooi om deel te neem aan hierdie navorsings studie of om u toestemming te gee om u kind in te sluit in die navorsing studie. Die studie sluit in die neem van vloeistof uit die maag (gastriese aspirate) en agterkant van die neus (keel) of nasofaringeale aspirate, sputum asook die versameling van poep (stoelgange) en piepie (uriene) van die kinders. U/jou dokter moet in elk geval die eerste twee neem om te toets of jy dalk TB het en ons vra slegs vir stoelgange en uriene ekstra. ‘n Totaal van 500 kinders gaan aan die studie deelneem en hulle sal almal jonger as 13 jaar wees en afkomstig uit die Vrystaat Provinsie van Suid Afrika. Om die bakterie op te spoor wat TB veroorsaak, gaan ons die vloeistof, poep en piepie bekyk onder ‘n Mikroskoop (dit is ‘n instrument wat gebruik word om baie klein voorwerpe groter te laat lyk), daarna word dit in ‘n masjien gesit wat ‘n GeneXpert genoem word en ook in spesiale voedingstowwe om die bakterie aan te moedig om te vermeerder. Die bakterie wat groei sal dan verder met antibiotika getoets word om te kyk of dit die bakterie sal doodmaak. Die bakterie wat steeds kan groei met antibiotika word weerstandig genoem. Die bakterie wat op al die media gegroei het sal dan verder getoets word om te kyk of dit van dieselfde oorsprong af kom en om al hul kenmerke te bepaal.

Daar is geen gevaar (risiko) verbonde daaraan om deel te neem aan die studie nie aangesien net vloeistowwe geneem word wat geen indringende (naalde of inwendige) prosedures behels nie. U/jy kan “Nee” sê of jy kan “Ja” sê om aan die studie deel te neem en niemand sal kwaad wees as jy “Nee” sê nie. Jy mag ook eers “Ja” sê en later besluit om liewers “Nee” te sê. Ons sal ons bes probeer om te sorg dat geen slegte dinge gebeur nie. Jy kan “Nee” sê vir wat ons vir jou vra om te doen vir die navorsing op enige stadium en ons sal stop met wat ons besig is. Ons gaan probeer om alle persoonlike inligting soos jou naam privaat te hou. Ons kan dit egter nie absoluut waarborg nie.

Kontak besonderhede van navorser(s) – vir verdere inligting/aanmelding van studie-verwante sake.

A van der Spoel van Dijk (researcher) – 051 4053462

Prof AA Hoosen (Head Department of Medical Microbiology) – 051 4053076

Appendix C: Study Information in Sesotho

TOKOMANE YA TSEBISO

Tshwantshiso ya mekgwa ya phuphutso e sebediswang ho batlisisa lefuba la matshwafu baneng

Rele bo ramahlale hotswa University ya Freistata, re etsa diphaphatso tsa ho fumana lefuba ho tswa marong a ka mpeng, kgokgothong, bohareng ba nko, mantleng le morotong wa bana, bao dingaka dinahanang hore banale lefuba. Dipatlisiso tsena redietsetsa ho araba dipotso tse renang le tsona, ele hore re fumane mokgwa o potlakileng ho fumana lefuba baneng.

Rea lemema hoba karolo ya dipatlisiso tsena, mme re kopa tumello ya ho kenya ngwana hao papatsong tsena. Dipatlisiso tsena dikenyeleditse ho nka lero ho tswa mpeng, kgokgothong kapa sekgohlela leho nka mantle le moroto baneng. Ka tlwaelo ngaka etlo kopa tse pedi tsa ntho tse bolotsweng hodimo, mme rona re kopa fela ho kenyelletsa mantle le moroto. Diphuphutso tsena di tla etswa ho bana ba katlase ho dilemo tse leshome le metso e meraro (13).

Ho batlisisa kokwanahloko ya lefuba, merong, mantleng le morotong, re tla sebedisa Microscope (mochini o sebeliswang ho hodisa tjhebahalo ya dikokwana hloko, hore dibonahale habonolo). Ka mora moo re tla sebedisa mochini o batlisisang lefuba baneng o bitswang GeneXpert le dibotlolo tsa dijo tse potlakisang ho hola ha kokwanahloko. Diantibiotic (kapa moriana/dipidisi tse boloyang dikokwanahloko) di tla sebediswa kgahlanong le kokwanahloko tse hodisitsweng. Kokwanahloko e sa bolawang ke meriana ena, e tla sebediswa bakeng sa dipatlisiso tse kenelletseng ho ithuta haholo ka twantsho ya tsona.

Ha hona kotsi e amanang leho kena thutong ena kapa dipatlisisong tsena, ka ha ho sebediswe dinale. Onale tokelo ya ho hana kapa ho dumela ho nka karolo dipatlisisong tsena. Le ha o ka dumela, o dumelletswe ho hana ha o ka fetola mehopolo. Re tla e tsa bonnete ba hore haho letho le lebe le tla etsahala. Re tla nka matsapa a ho boloka ditaba tsa hao le lebitso la hao lekunutu. Ho fumana lesedinyana kapa tsebiso bakeng sa diphaphatso tsena etihanye le mmabohlale wa rona Mrs A van der Spoel van Dijk (researcher) – 051 4053462 le Prof AA Hoosen (Head Department of Medical Microbiology) – 051 4053076

Appendix D: Buffer Preparations

20xSSPE:	Stock solution obtained from (Life Technologies™, USA).
2xSSPE/0.1% SDS:	Prepared by adding 100 mL 20xSSPE and 10 mL 10% SDS to 890 mL de-mineralised water.
2xSSPE/0.5% SDS:	Prepared by adding 100 mL 20xSSPE and 50 mL 10% SDS to 850 mL de-mineralised water.
2xSSPE:	Prepared by diluting 20xSSPE ten times (1:10) with de-mineralised water.
10% SDS:	Prepared by dissolving 10 g SDS in 100 mL de-mineralised water.

Appendix E: Calculations

Diagnostic sensitivity- Auramine Smear Micrsocopy

$$Sensitivity = \frac{True\ positive\ (Smear)}{Culture\ True\ positive + False\ negative\ smear}$$

$$Sensitivity = \frac{1}{4 + 3}$$

$$Sensitivity =\approx \mathbf{14\%}$$

Diagnostic specificty -Auramine smear microscopy

$$Specificity = \frac{True\ negative\ (Total)}{True\ negative\ (Total) + False\ positive\ (Smear)}$$

$$Sensitivity = \frac{122}{122 + 0}$$

$$Sensitivity = 1.0 \approx \mathbf{100\%}$$

Positive predictive value -Auramine Smear micrscopy

$$PPV = \frac{True\ positive}{True\ positive + False\ positive}$$

$$PPV= \frac{1}{1 + 0}$$

$$PPV= 1 \approx \mathbf{100\%}$$

Negative predictive value -Auramine Smear micrscopy

$$NPV = \frac{True\ negative}{True\ negative + False\ negative}$$

$$NPV= \frac{122}{122+ 3}$$

$$NPV= 0.976 \approx \mathbf{97.6\%}$$

Diagnostic sensitivity- Xpert®

$$Sensitivity = \frac{True\ positive\ (Xpert)}{True\ positive\ (Culture) + False\ negative}$$

$$Sensitivity = \frac{2}{4 + 2}$$

$$Sensitivity \approx \mathbf{33\%}$$

Diagnostic specificty- Xpert

$$Specificity = \frac{True\ negative}{True\ Negative + False\ positive}$$

$$Sensitivity = \frac{122}{122 + 0}$$

$$Sensitivity = 1.0 \approx \mathbf{100\%}$$

Positive predictive value-Xpert

$$PPV = \frac{True\ positive}{True\ positive + False\ positive}$$

$$PPV = \frac{2}{2 + 0}$$

$$PPV = 1 \approx \mathbf{100\%}$$

Negative predictive value-Xpert

$$NPV = \frac{True\ negative}{True\ negative + False\ negative}$$

$$NPV = \frac{122}{122 + 2}$$

$$NPV = 0.983 \approx \mathbf{98.3\%}$$

Sample sensitivities

$$Sample\ sensitivity = \frac{Number\ of\ positive\ patient\ (on\ sample)}{Total\ number\ of\ positive\ patients + False\ negative\ patients\ (on\ sample)}$$

$$GA = \frac{2}{4 + 2}$$

$$= 0.33 \approx \mathbf{33\%}$$

$$Urine = \frac{3}{4 + 1}$$

$$= 0.6 \approx \mathbf{60\%}$$

$$Stool = \frac{2}{4+2}$$

$$= 0.33 \approx \mathbf{33\%}$$

$$NPS = \frac{1}{4+3}$$

$$= 0.142 \approx \mathbf{14.2\%}$$

Sensitivity of methods on different sample types

Smear microscopy

$$\frac{2}{7+14}$$

$$= 10\%$$

Culture

$$\frac{7}{7+9}$$

$$= 44\%$$

Xpert

$$\frac{6}{7+10}$$

$$= 35\%$$

Appendix F: Approval Letter from the University of the Free State Ethics Committee

UNIVERSITY OF THE FREE STATE
UNIVERSITEIT VAN DIE VRYSTAAT
YUNIVESITHI YA FREISTATA

UFS·UV
HEALTH SCIENCES
GESONDHEIDSWETENSKAPPE

IRB nr 00006240
REC Reference nr 230408-011
IORG0005187
FWA00012784

24 August 2016

MR AE OGUNBAYO
DEPT OF MEDICAL MICROBIOLOGY AND VIROLOGY
FACULTY OF HEALTH SCIENCES
UFS

Dear Mr AE Ogunbayo

ECUFS 08/2015
PROJECT TITLE: COMPARISON OF METHODS AND SAMPLES USED IN THE DIAGNOSIS OF CHILDHOOD PULMONARY TUBERCULOSIS AND CHARACTERIZATION OF MYCOBACTERIUM TUBERCULOSIS ISOLATES

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) took note of and approved the following at the meeting held on 23 August 2016:

   - *This project will now be done in fulfilment of an M.Med (Sci) degree (Medical Microbiology)*
   - *Title change from "Determination of diagnostic yield of multiple non-respiratory samples from children suspected with pulmonary TB in the Free-State province of South Africa and characterisation of the mycobacterium tuberculosis isolates" to " Comparison of methods and samples used in the diagnosis of childhood pulmonary tuberculosis and characterization of mycobacterium tuberculosis isolates"*
   - *Three separate study aims combined into one "To determine and compare the diagnostic yield of various samples (sputum, NPA, GA, urine and stool) obtained from childhood suspected of PTB using smear microscopy, culture and GeneXpert MTB/RIF"*
   - *First objective rephrased with inclusion of sputum as one of the samples and third objective also changed*
     - *First objective= to collect sputum, GA or NPA, stool and urine samples from children (<13years) suspected of having pulmonary TB from the Universitas-, Pelonomi-, National and Botshabelo Hospitals), Mangaung Metropolitan Municipality, Free State Province.*
     - *Third objective= To perform first-line drug susceptibility testing on MTB culture positive samples using BACTEC MGIT 960 system and Genotype MTBDRplus assay to determine the proportion of DR-TB among children with confirmed PTB*
   - *Inclusion criteria and study procedures changed from children <15years to children <13years*
     - *An observational descriptive study was added to study design*
   - *Last sentence of procedure changed to "However, laboratory study results will be communicated to Dr Hallbauer or a seconded specialist in the TB field for consideration when deciding on treatment"*

2. Kindly use the **ECUFS NR** as reference in correspondence to HSREC Administration.

3. The HSREC functions in compliance with, but not limited to, the following documents and guidelines: The SA National Health Act. No. 61 of 2003; Ethics in Health Research: Principles, Structures and Processes (2015); SA GCP(2006); Declaration of Helsinki; The Belmont Report; The US Office of Human Research Protections 45 CFR 461 (for non-exempt research with human participants conducted or supported by the US Department of Health and Human Services-(HHS), 21 CFR 50, 21 CFR 56; CIOMS; ICH-GCP-E6 Sections 1-4; The International Conference on Harmonization and Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH Tripartite), Guidelines of the SA Medicines Control Council as well as Laws and Regulations with regard to the Control of Medicines, Constitution of the Ethics Committee of the Faculty of Health Sciences.

Yours faithfully

.....
DR SM LE GRANGE
CHAIR: HEALTH SCIENCES RESEARCH ETHICS COMMITTEE

Health Sciences Research Ethics Committee
Office of the Dean: Health Sciences
T: +27 (0)51 401 7795/7794 | F: +27 (0)51 444 4359 | E: ethicsfhs@ufs.ac.za
Block D, Dean's Division, Room D104 | P.O. Box/Posbus 339 (Internal Post Box G40) | Bloemfontein 9300 | South Africa
www.ufs.ac.za

Appendix G: Approval Letter from the Free State Department of Health

health
Department of Health
FREE STATE PROVINCE

02 July 2015

Mrs A Van Dijk
Dept. of Medical Microbiology and virology
Faculty of Health Sciences
UFS

Dear Mrs. A Van Dijk

Subject: Determination of diagnostic yield of multiple non-respiratory samples from children suspected with pulmonary TB in Free State & Northern Cape provinces of South Africa and characterization of the mycobacterium tuberculosis isolation.

- Permission is hereby granted for the above – mentioned research on the following conditions:
- Participation in the study must be voluntary.
- A written consent by each participants must be obtained.
- Serious adverse events to be reported and/or termination of the study.
- Ascertain that your data collection exercise neither interferes with the day to day running of Pelonomi Hospitals nor the performance of duties by the respondents or health care workers.
- Confidentiality of information will be ensured and no names will be used.
- Research results and a complete report should be made available to the Free State Department of Health on completion of the study (a hard copy plus a soft copy).
- Progress report must be presented not later than one year after approval of the project to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- Any amendments, extension or other modifications to the protocol or investigators must be submitted to the Ethics Committee of the University of the Free State and to Free State Department of Health.
- Conditions stated in your Ethical Approval letter should be adhered to and a final copy of the Ethics Clearance Certificate should be submitted to khusemj@fshealth.gov.za or sebeelats@fshealth.gov.za before you commence with the study
- No financial liability will be placed on the Free State Department of Health
- Please discuss your study with the institution managers/CEOs on commencement for logistical arrangements
- Department of Health to be fully indemnified from any harm that participants and staff experiences in the study
- Researchers will be required to enter in to a formal agreement with the Free State department of health regulating and formalizing the research relationship (document will follow)
- You are encouraged to present your study findings/results at the Free State Provincial health research day
- Future research will only be granted permission if correct procedures are followed see http://nhrd.hst.org.za

Trust you find the above in order.

Kind Regards

Dr D Motau
HEAD: HEALTH
Date: 15/07/2015

Appendix H: Approval Letter from Pelonomi Regional Hospital

pelonomi hospital
Department of Health
Pelonomi Tertiary Hospital
FREE STATE PROVINCE

INTERNAL MEMO

DATE:	21 SEPTEMBER 2015		ENQUIRIES
TO:	A. BULANE DEPARTMENT OF MEDICAL MICRO BIOLOGY AND VIROLOGY UNIVERSITY OF FREE STATE BLOEMFONTEIN 9300 TEL: 082 357 8278	FROM:	Dr B.A Benganga Head: Clinical Services Bengangaba@fshealth.gov.za 051 405 1936/1942 Bloemfontein

RESEARCH:
A request to conduct a survey on Determination of diagnostic yield of multiple non-respiratory samples from children suspected with pulmonary TB in Free State Province of South Africa and characterisation of the Mycobacterium tuberculosis isolates.

Pelonomi Tertiary Hospital **grants you permission** to conduct researches/studies and the following criteria must be met.

- [X] That you obtain ethical clearance from the human research ethics committee of the relevant university.
- [X] That the Hospital incurs no cost in the course of your research.
- [X] That access to the staff and patients at the Pelonomi Hospital will not interrupt the daily provision of services.
- [X] That prior to conducting the research you will liaise with the supervisors of the relevant sections and introduce yourself with permission letter and to make arrangements with them in a manner that is convenient to the sections.

Yours Sincerely

PELONOMI HOSPITAL
21-09- 2015
DEPARTMENT OF HEALTH

Dr Benganga B.A
`Director: Clinical Services

Appendix I: Approval Lettert from Botshabelo District Hospital

health
Department of Health
FREE STATE PROVINCE

11 February 2016

Ms A. Bulane
Department of Medical Microbiology and Virology
Faculty of Health Science

Dear Ms. Bulane

RESEARCH PROJECT: DETERMINATION OF DIAGNOSTIC YIELD OF MULTIPLE NON – RESPIRATORY SAMPLE FROM CHILDREN SUSPECTED WITH PULMONARY TB IN FREE STATE & NORTHERN CAPE PROVINCES OF SOUTH AFRICA AND CHARACTERIZATION OF THE MYCOBACTERIUM TUBERCULOSIS ISOLATES

Herewith permission for the mentioned project to be done at Botshabelo District Hospital on the following condition:

1. The researcher/s should comply with all the conditions referred to in the approval letter obtained from the HOD's Office: Dr. D. Motau on 15 July 2015.

The Chief Executive Officer must be notified of the findings of the project. A research report needs to be sent to the Head Clinical Services as soon as the study is completed.

Yours sincerely

Dr. K.K. Moeng
Clinical Head
Botshabelo District Hospital

DR. KEREDITSE MOENG
MBCHB MP0541591
0734214826

Botshabelo District Hospital P/ Bag X 527, Botshabelo, 9781
Admin Block, Ground Floor, Botshabelo. Tel: (051) 5330234
Fax: (051) 534 1096 E-mail Address: MoengKK@fshealth.gov.za

Appendix J: Consent Form for the Parent/Guardian

TB NAVORSINGSPROJEK OVS
DEPT. MEDIESE MIKROBIOLOGIE
FAKULTEIT GENEESKUNDE
OVS

TB RESEARCH PROJECT UFS
DEPT. MEDICAL MICROBIOLOGY
FACULTY OF MEDICINE
UFS

BOITHUTO BA LEFU LA LEFUBA (TB)
LEFAPHA LA MICROBIOLOGY
UNIVERSITHI EA FOREISETATA

TOESTEMMING OM AAN PROEF DEEL TE NEEM
PERMISSION TO TAKE PART IN THE PROJECT
TUMELLO YA HO NKA KAROLO BOITHUTONG

Ek gee hiermee
I ---------- hereby give
Nna ke fana

toestemming dat my liggaamsstowwe naamlik slym uit die keel (agterkant van die neus en die mond) en maag, ook genoem nasofaringeale en gastriese aspirate, stoelgang (poep) en uriene (piepie) geneem en in bogenoemde navorsingsproef gebruik kan word. Hierdie studie poog om uit te vind watter stowwe uit die liggaam die beste gebruik kan word vir die opsporing van TB in kinders.

Permission that my samples namely poop (stool), pie (urine) and fluid from the back of my nose (throat) and tummy also called Nasopharyngeal and Gastric aspirates may be collected and used in the above mentioned research study. In this study we will determine which of the different samples will be the best for detecting of TB in children.

ka tumello hore lero le tswang ka mpeng, le le fumanwa karolong e ka morao ya dinko tsa ka, moroto le setuloana tsa ka di sebediswe boithutong bo bolelwang.

Ek het die inligtingstuk oor die proef gelees.
I have read the information brochure concerning the project.
Ke badile tlhaloso mabapi le boithuto bona mme ke utloisisa se bolelwang.

Handtekening: Datum:
Signature: _____Date: _____/_____/____
Tekeno: Letsatsi:

Pasiënt naam:
Pasient name:
Lebitso la mokodi:

_____

***Micro use only**:*
Date sample received:

_____

Kontakperson:
Contact person: AA Hoosen / A van der Spoel van Dijk
Phone number: 051 4053076/ 051 4053462

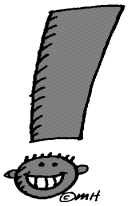
Appendix K: Child Assent Form in English

CHILD ASSENT FORM

What is a research study?

Research studies help us learn new things. We can test new ideas. First, we ask a question. Then we try to find the answer.

Important things to know…



- You get to decide if you want to take part.
- You can say 'No' or you can say 'Yes'.
- No one will be upset if you say 'No'.
- If you say 'Yes', you can always say 'No' later.
- You can say 'No' at any time.

We would still take good care of you no matter what you decide

Why are we doing this research?

We are doing this research to find out more about the illness called TB, which is a bug that can cause you to cough and feel very tired and sick. We are going to look for it from your fluid from your lungs (mucus or sputum), fluid from your throat at the area between the back of your nose to the back of your mouth (called the nasopharynx), pee (urine) and poop (stool).

What would happen if I join this research?

If you decide to be in the research, we would ask you to do the following:

- Mucus comes out of your lungs when you cough and then you swallow it and it lands up in your tummy: We may need to get mucus out of your tummy with a small tube through your nose. The tube is thinner than a straw.
- Fluid from back of nose: Some of the mucus stay at the back of your throat. The same tube used on your tummy will be used to get fluid from the back of your nose. To put the tube through your nose feels ticklish, but is not at all sore.

- Pee and Poop: You will be asked to go on a potty

- Medical records: Your doctor always write down what is causing you to feel ill and which medication he/she gave you to become better when you visit him/her when you are not feeling well. We will look at your past doctor visits and use notes that the doctor wrote down about your illness.
- These will only take about 15 minutes

Could bad things happen if I join this research?

When we put the small tube in your nose it will feel a little funny, but it will not hurt. We will try to make sure that no bad things happen. You can say 'no' to what we ask you to do for the research at any time and we will stop.

Could the research help me?

This study may help you feel better or get well. The researchers might also find out something that will help other children who are coughing just like you later

Who will know what I did in the study?

No one will know except the people who will be working with you, your family and your doctor. We will make sure that your name is not on any study papers

What else should I know about this research?

We have already asked mum and dad/ caregiver if we may get all these from you and they said yes, but we want you to say yes or no too. You would not be paid to be in the study. You can ask questions any time. Ask us any questions you have. Take the time you need to make your choice.

Is there anything else?

If you want to be in the research after we talk, please write your name below or just print your right hand on the paper. We will write our name too. This shows we talked about the research and that you want to take part.

Assent

I want to take part in this study. I know I can change my mind at any time.

_____ **Verbal assent given Yes □**

Print name of child

Written assent if the child chooses to sign the assent.

_____ _____ _____

Signature of Child **Age** **Date**

I confirm that I have explained the study to the participant to the extent compatible with the participants understanding, and that the participant has agreed to be in the study.

_____ _____ _____

Printed name of Person obtaining assent **Signature of Person obtaining assent** **Date**

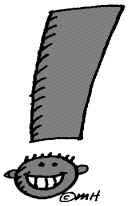
Appendix L: Child Assent Form in Afrikaans

KINDER TOESTEMMINGSVORM

Wat is ‘n navorsings studie?

Navorsings studies help ons om nuwe dinge te leer. Ons kan nuwe idees uittoets. Eers vra ons ‘n vraag. Daarna probeer ons om ‘n antwoord te kry.

Belangrike dinge om te weet…



- Jy kan besluit of jy deel wil wees van die studie.
- Jy kan of “Nee” of “Ja” sê.
- Niemand sal kwaad wees as jy “Nee” sê nie.
- As jy “Ja” sê, kan jy nog altyd later weer “Nee” sê.
- Jy kan enige tyd “Nee” sê.

Ons gaan goed na jou kyk, maak nie saak wat jou antwoord is nie.

Hoekom doen ons die navorsing?

Ons doen die navorsing omdat ons meer wil uitvind van die siekte wat TB genoem word. Dit is ‘n bakterie of baie klein selletjie wat jou kan laat hoes, moeg en siek voel. Ons gaan kyk of ons die bakterie in van die vloeistof (slym) uit jou longe en in jou keel aan die agterkant van jou neus tot by jou mond (dit word die nasofarinks genoem), jou piepie (uriene) en poep (stoelgang) kan vind.

Wat gaan gebeur as ek deel word van die navorsing?

As jy besluit om aan die navorsing deel te neem, sal ons jou vra om die volgende dinge vir ons te doen:

- Slym kom uit ‘n mens se longe uit wanneer jy hoes en dan sluk jy dit in en dit kom in jou maag. Ons kan jou vra of ons ‘n klein buisie in jou neus tot in jou maag kan sit om die slym uit jou maag te kry (die buisie is dunner as ‘n koeldrank strooitjie).
- Van die slym kan ook agter in jou keel bly vassit: Ons kan vra of ons dieselfde buisie wat ons vir jou maag gebruik het ook kan gebruik om die slym agter uit jou keel te kry.

Om die buisie deur jou neus te sit klink grillerig, maar dit is glad nie seer nie, dit kielie net bietjie.

- Piepie en poep: Ons sal jou vra om ‘n potjie te gebruik om te pieps en poef.

- Mediese inligting: Jou dokter skryf altyd alles oor jou siekte en die medisyne wat hy/sy vir jou gee neer as jy vir hom/haar gaan kuier as jy siek is. Ons sal vra om na jou dokter se notas te kyk om te sien wat hy oor jou siekte neergeskryf het.
- Om alles te doen sal net omtrent 15 minute neem.

Kan daar slegte goed met jou gebeur as jy aan die navorsing deelneem?

Wanneer ons die klein buisie in jou neus sit, sal dit bietjie snaaks voel, maar dit sal nie seer wees nie. Ons sal ons bes doen om seker te maak dat niks slegs met jou gebeur nie. Jy mag enige tyd “Nee” sê as ons jou vra of ons iets mag doen en ons sal dan stop.

Kan die navorsing my help?

Hierdie studie kan jou help om beter te voel of beter te word. Die navorsers kan dalk iets uitvind wat later ook vir ander kinders kan help wat net soos jy voel

Wie sal weet wat ek as deel van die studie gedoen het?

Niemand sal weet nie behalwe die mense wat met jou gaan werk, jou familie en jou dokter. Ons sal seker maak dat jou naam nie op enige van die studie papiere (dokumente) is nie.

Wat moet ek nog weet van die navorsing?

Ons het klaar jou ma en pa/ versorger gevra of ons die slym en dinge van jou mag kry en hulle het “Ja” gesê, maar ons wil hê dat jy self besluit en of “Ja” of “Nee” sê. Jy word nie betaal vir die studie

nie. Jy mag enige tyd vrae vra. Vra gerus enige vra wat jy het. Jy mag ook so lank as wat jy wil neem om te besluit of jy wil deelneem.

Is daar enige iets anders?

As jy wil deelneem aan die navorsing nadat ons gepraat het, sal ons bly wees as jy jou naam hieronder sal skryf of druk jou regterhand op die papier. Ons sal ook ons naam skryf. Dit sal wys dat ons oor die navorsing gepraat het en dat jy daaraan wil deelneem.

Toestemming

I wil aan die studie deelneem. I weet dat ek enige tyd anders kan besluit.

_____ **Mondelinge toestemming gegee Ja □**

Skryf die naam van die kind

Geskrewe toestemming as die kind besluit om die toestemming te teken.

_____ _____ _____

Handtekening van kind **Ouderdom** **Datum**

Ek bevestig date k die studie aan die deelnemer verduidelik het op ‘n manier ooreenstemmend aan die deelnemer se begrip end at die deelnemer ingestem het om aan die studied eel te neem.

_____ _____ _____

Naam van person wat toestemming verkry het

Handtekening van person wat toestemming verkry het

Datum

Appendix M: Child Assent Form in Sesotho

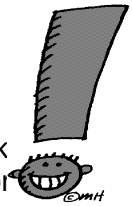
FOROMO YA TUMELO YA NGWANA

Boithuto boo ke ba eng?

Boithuto bona bo re thusa ho beha tekong ntho tse ncha tseo re ka ithutang tsona ka lefuba. Pele re tla o botsa potso mme re tla labella karabo ho tswa ho wena.

Dinhla tsa bohlokwa

- Ke boikhethelo ba hao ho nka karolo boithutong bona
- O ka dumela kapa wa hana
- Boikhethelo ba hao bo ka se kwatisi motho
- Ha o dumetse ho nka karolo o ke so hannelwe ho chencha maik



Ho se nke karolo hwa hao boithutong bona, ho ke ke hwa bolela hor wa fumantswa melemo e o lokelang o le mokuli

Hobaneng re etsa boithuto boo?

Re etsa boithuto bona e le teko ya ho ithuta ka botebo ka lefu la lefuba, le bakwang ke kokwana e bitswang *M.tuberculosis. Kokwana ena e ka etsa hore mokudi a ikutlwe a khathetse, mme a hohlola habohloko. Kokwana ena re tlo batlana le yona lerong le fumanwa ka matshwafong leo e reng ha mokudi a khohlola a le ntshwa a be a le koenya, lero le fumanwang karolong e ka morao ya dinko tsa mokudi, moroto le setuloana sa mokudi.*

Ho ka etsahalang ka nna ha ke ba karolo ya boithuto boo?

Ha o ka dumela ho ba karolo ya boithuto bona, re tla o kopa ho etsa tse latelang:

- Ho hula lero ka hare ho mpa ya hao: ho etsa sena re tla sebedisa letopo le lenyane, mme le tla kena ka hare ho nko ho fihlela le fihla ka hare ho mpa.
- Lero le fumanehang karolong e ka morao ya nko: lethopo le sebelisitsweng ho ntshwa lero ka hare ho mpa le tla boela le sebediswa ho hula lero karolong e kamorao ya nko. Ho etsa sena ha ho bohloko empa ho ka ba botsikinyane

- Moroto le setuloana: o tla kopuwa ho sebedisa bitsa ho ithusa e le hore re tle re fumane moroto le setuloana sa hao

- Bukana tsa ngaka: Re tla bala dinotse tsa ngaka ka bokudi ba hao tseo a di ngolang ka hare ho bukana ya hao.
- Tsena ka o fela di tla nka metsotso e leshome le metso e mehlano

Ke kotsi dife tse ka etsahalang ha ke nka karolo boithutong boo?

Re tla le ka ka hohle hore ho sebe le kotsi e ka etsahang. Ha re kenya lethopo ka nko o ka utlwa e ka ho tla ba bohloko empa ho kekebe hwa ba jwalo.

Boithuto boo bo ka nthusa kae?

Boithuto bona bo ka o thusa ka hore mohlomong re ka fumana kokwana ena ka pele ho wena mme wa fumantswa dipidisi tse nepahetseng ka nako. Bo ithuto bona bo ka re thusa le ho tseba maro ao re ka fumanang kokwana ena kapele, mme ya ba thuso ho bana ba bang batlang ngakeng ka moso bana le lefu lena.

ke mang a ka tsebang ke nkile karolo boithutong boo?

Ha ho motho a ka tsebang ntle le batho ba tsa bophelo bao o tla be o sebetsa le bona, ngaka ya hao le ba lelapa leno. Re tla leka ka hohle hore re se ngole lebitso la hao dipampiring tsa rona empa re ngole nomoro e tla emela lebitso la hao.

keng hape yoo o ka njwetsang yona ka boithuto boo?

Re kopile dumello ho mme le ntate ba hao hore o be karolo ya boithuto bona, mme ba dumetse, empa re batla wena o dumele kapa o hane ho ba karolo. Ha hona ditsiane tse fumanwa ha o le karolo boithutong bona. O ka potsa dipotsa tsohle tseo o nang le tsona, e ka ba hona jwale kapa nakong e tlang.

Hona le ho hong hape?

Ha o dumela ho ba karolo ya boithuto bona, re tla kopa o ngole lebitso la hao karolong e ka tlase ya foromo kapa o hatise letsoho la hao le le tona. Re tla ngola mabitso a rona e le bopaki ba hore re buile le wena mme re dumellane hore o tla ba karolo ya boithuto bona.

Foromo ya ananelo

Ke tla nka karolo boithutong bona. Kea tseba hore nka chencha maikutlo a ka nako yohle.

_____ dumelo ka molomo □

Ngola lebitso la ngwana

Karolo e tlatswa ha ngwana a sa ka khone ho ngola.

_____ _____ _____

Tekeno ya ngwana dilemo Letsatsi la tekeno

Mona ke i kana hore ke hlalosedise mokodi ka boithuto bona mme ke sebedisise mekhwa yohle eo ke neng ke dumella hore mokodi o tla utloisisa boithuto bona ha ke e sebedisa. Mme mokodi o dumetse ho nka karolo boithutong bona.

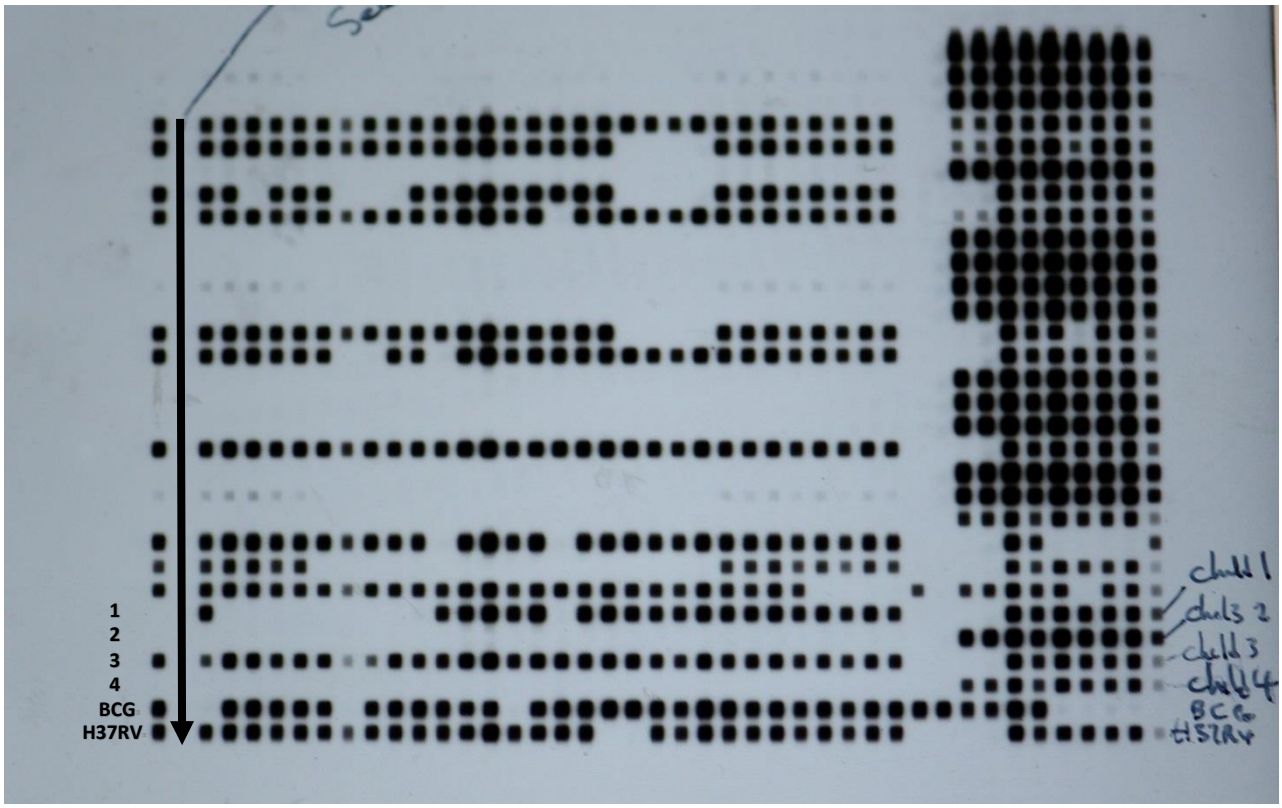
_____ _____ _____

Lebitso la motho a neng a hlalosetsa mokodi

Tekeno ya motho a neng a hlalosetsa mokodi

Letsatsi la tekeno

Appendix N: Spoligotyping X-ray film



1: Child 1

2: Child 2

3: Child 3

4: Child 4

BCG: Positive control 1

H37Rv: Positive control 2

Note: The absence of second spacer as indicated by the drawn downward arrow was as a result of the faulty membrane which was a manufacturers error. However, this did not affect our result analysis, as only the Beijing strain mostly lacks the second spacers.