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Development and validation of a molecular assay and evaluation of the GeneXpert® MTB/RIF assay for the rapid detection of genital tuberculosis

by

Cebolenkosi Maxwell Sokhela

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Promoter: Doctor D Goedhals, Department of Medical Microbiology and Virology, University of the Free State, Bloemfontein

Co-Promoter: Professor AA Hoosen, Department of Medical Microbiology and Virology, University of the Free State, Bloemfontein

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Declaration

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Presentations and prize

Development and validation of a molecular assay and evaluation of the GeneXpert® MTB/RIF assay for the rapid detection of genital tuberculosis. CM Sokhela, JDP Strydom, AA Hoosen & D Goedhals. Faculty of Health Science's Faculty Research Forum 2016. Bloemfontein 25-26 August 2016 (Oral Presentation)

Development and validation of a molecular assay and evaluation of the GeneXpert® MTB/RIF assay for the rapid detection of genital tuberculosis. CM Sokhela, JDP Strydom, AA Hoosen & D Goedhals. Annual Free State Provincial Health Research Day 2016. Bloemfontein 27-28 October 2016 (Oral Presentation)

Detecting low level rifampicin resistance in *Mycobacterium tuberculosis*. CM Sokhela & A van der Spoel van Dijk. Faculty of Health Science's Faculty Research Forum 2015. Bloemfontein 27-28 August 2016 (Oral Presentation and winner of top 10 Young researcher)

Abstract

Tuberculosis (TB) is a communicable disease which is caused by the bacterium *Mycobacterium tuberculosis* (MTB). According to the World Health Organization, globally in 2015 there were 10.4 million new cases and 1.4 million deaths due to TB. TB is one of the leading causes of death in South Africa resulting in approximately 8.4% of deaths in 2015. The most common manifestation of TB involves the lungs, defined as pulmonary TB (PTB), while TB affecting other organs is defined as extrapulmonary TB (EPTB). EPTB accounts for only 20% of all TB cases in human immunodeficiency virus negative individuals. Approximately 1.8% of all TB cases have a genitourinary site, with the prevalence of genital TB (GTB) in South Africa reported to range from 6.2-21.0%. One of the leading symptoms of GTB in females is infertility, usually resulting from the involvement of the fallopian tubes and endometrium. Approximately 40-80% of women with GTB will become infertile.

The detection of microorganisms through microscopy is the oldest technique for laboratory diagnosis. While microscopy is rapid and inexpensive, it requires a high bacterial load which is not present in paucibacillary EPTB samples. Culture of MTB is widely regarded as the gold standard for TB diagnosis. While culture has a long turnaround time, culture remains important since it is more sensitive than microscopy. In addition, growth is required for species identification, drug susceptibility testing and genotyping of cultured organisms may be useful for epidemiological studies. Little is known regarding which technique is best for the detection of GTB from clinical samples apart from culture. Molecular based techniques hold the promise of a more rapid and accurate diagnosis of EPTB.

The aim of this project was the development and validation of an in-house nested PCR assay and the validation of the GeneXpert® MTB/RIF (GeneXpert) assay for the laboratory diagnosis of GTB. In total 54 samples were submitted for GTB screening from women being investigated for infertility at the Unit for Human Reproduction, Universitas Academic Hospital, Bloemfontein. This included 44 endometrial tissue samples and 10 menstrual fluid samples. All samples underwent testing with the GeneXpert, the in-house nested PCR and culture. The nested PCR was designed targeting the insertion sequence element *6110* (IS*6110)* found in members of the MTB complex. The analytical sensitivity/limit of detection (LOD) for the GeneXpert was determined to be 250pg while the LOD for the nested polymerase chain reaction (PCR) was 62.5fg. Both assays displayed excellent analytical specificity by discriminating TB deoxyribose nucleic acid (DNA) from other bacterial and nontuberculous mycobacterial DNA. The diagnostic sensitivity and specificity was determined using culture as the reference method. Culture was able to detect GTB in 2 of the 54 samples including one menstrual fluid and one endometrial tissue sample, thus indicating a GTB prevalence of 3.7%. The GeneXpert detected 1 of the 54 samples as positive indicating a sensitivity of 50% and a

specificity of 100%. The nested PCR detected both positive samples resulting in a sensitivity and specificity of 100%. The GeneXpert obtained a positive predictive value (PPV) of 100% and a negative predictive value (NPV) of 98.1%, while the nested PCR obtained a PPV and NPV of 100%. The two GTB isolates underwent genotyping using spoligotyping and mycobacterial interspersed repetitive unit – variable number of tandem repeats (MIRU-VNTR). The menstrual fluid isolate was characterised as a Beijing strain and the endometrial tissue isolate as an X3 strain.

The nested PCR showed a greater sensitivity than the GeneXpert as a result of the better LOD. Despite this, both techniques could be implemented for GTB screening in combination with culture. Screening of menstrual fluid samples using the GeneXpert assay would be well suited for GTB screening in resource limited areas.

Keywords: extrapulmonary TB; genital TB; nested PCR; IS*6110*; GeneXpert® MTB/RIF; MGIT culture; predictive values; Cohen's Kappa; spoligotyping; mycobacterial interspersed repetitive unit – variable number of tandem repeats.

Opsomming

Tuberkulose (TB) is ‘n aanmeldbare siekte wat veroorsaak word deur die bakterium *Mycobacterium tuberculosis* (MTB). Volgens die Wêreld Gesondheidsorganisasie, was daar wêreldwyd 10.4 miljoen nuwe gevalle en 1.4 miljoen sterftes as gevolg van TB in 2015. TB is een van die hoofoorsake van sterftes in Suid-Afrika en gevolglik verantwoordelik vir ongeveerd 8.4% van sterftes in 2014. Die mees algemene beeld van TB betrek die longe en word gedefinieer as pulmonale TB (PTB), terwyl TB wat ander organe affekteer gedefinieer word as ekstrapulmonale TB (EPTB). EPTB verteenwoordig slegs 20% van TB gevalle in menslike immuungebrekvirus-negatiewe individue. Ongeveer 1.8% van alle TB gevalle affekteer die genito-urinêre area met ‘n voorkoms van genitale TB (GTB) in Suid-Afrika van 6.2-21.0%. Een van die mees algemene simptome van GTB in dames is onvrugbaarheid wat gewoonlik is as gevolg van die betrokkenheid van die fallopiusbuise en endometrium. Ongeveer 40-80% van dames met GTB sal onvrugbaar word.

Die opsporing van mikro-organismes deur mikroskopie is die oudste tegniek vir ‘n laboratorium diagnose. Mikroskopie is vining en goedkoop, maar benodig ‘n groot hoeveelheid bakterieë wat gewoonlik nie teenwoordig is in pauci-basillêre EPTB monsters nie. Kweking van MTB word wyd beskou as die goue standard vir die diagnose van TB. Alhoewel die metode ‘n lang omkeertyd het, bly kweking van die organisme belangrik aangesien dit meer sensitief as mikroskopie is. Kweking van die organisme word vereis vir spesie-identifikasie, middel vatbaarheidstoetsing en genotipering wat van waarde kan wees vir epidemiologiese studies. Huidiglik is daar beperkte kennis met betrekking tot watter tegniek, anders as kweking, die beste is vir die opsporing van GTB in kliniese monsters. Daar is ‘n moontlikheid dat molekulêre tegnieke gebruik kan word vir ‘n vinniger, tog akkurate diagnose van EPTB.

Die doel van hierdie projek was die ontwikkeling en validering van ‘n in-huis geneste polimerase kettingreaksie (PKR) toets, asook die validering van die GeneXpert® MTB/RIF (GeneXpert) toets vir die laboratorium diagnose van GTB. In total was daar 54 monsters ingedien vir GTB toetsing van dames wat huidiglik ondersoek word vir onvrugbaarheid by die Eenheid van Menslike Voortplanting, Universitas Akademiese Hospitaal, Bloemfontein. Hierdie monsters het 44 endometriale weefsel en 10 menstruele vloeistof monsters ingesluit. Alle monsters was getoets met die GeneXpert toets, die in-huis geneste PKR toets en met kweking. ‘n Geneste PKR toets was ontwerp wat die IS*6110* area gevind in die MTB kompleks teiken. Die analitiese sensitiwiteit/limiet van opsporing (LVO) vir die GeneXpert toets was 250pg, terwyl die LVO vir die geneste PKR toets 62,5fg was. Beide toetse het uitstekende analitiese spesifisiteit om tussen TB Deoksiribose nukleïensuur (DNS) en ander bakteriële en nie-tuberkuleuse mikobakteriële DNS te onderskei. Die diagnostiese sensitiwiteit en spesifisiteit was bepaal met kweking as verwysingsmetode. Kweking kon GTB in 2 van die 54 monsters opspoor wat een menstruele vloeistof en een endometriale weefsel monster ingesluit het.

Die voorkoms van GTB was dus 3.7%. Die GeneXpert toets het 1 van die 54 monsters as positief getoon met ‘n sensitiwiteit 50% en ‘n spesifisiteit van 100%. Die geneste PKR toets kon beide positiewe monsters opspoor met ‘n sensitiwiteit en spesifisiteit van 100%. Die GeneXpert toets het ‘n positiewe voorspellingswaarde (PVW) van 100% en ‘n negatiewe voorspellingswaarde (NVW) van 98.1%, terwyl die geneste PKR toets ‘n PVW en NVW van 100% gehad het. Genotipering in die vorm van spoligotipering en mikobakteriële verspreide herhalende eenheid – veranderlike aantal tandem herhalings (MIRU-VNTR) was bepaal. Die menstruele vloeistof isolaat was gekarakteriseer as ‘n Beijing stam en die endometiale weefsel isolaat as ‘n X3 stam.

Die geneste PKR toets het hoër sensitiwiteit as die GeneXpert toets getoon en gevolglik ook ‘n beter LVO. Ten spyte hiervan, kan beide tegnieke in kombinasie met kweking gebruik word vir GTB opsporing. Toetsing van menstruele vloeistof monsters vir GTB met behulp van die GeneXpert toets sal gepas wees vir die opsporing van GTB in areas met beperkte hulpbronne.

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"A journey of a thousand miles begins with a single step" - Lao Tzu

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β	Beta
δ	Delta
ε	Epsilon
γ	Gamma
µl	Microliter
×g	Times gravity
%	Percentage
°C	Degrees Celsius
(w/v)	Weight/volume
AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BCG	Bacillus Calmette–Guérin
bp	Base pair
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
CTB	Centre for Tuberculosis
Da	Dalton
DNA	Deoxyribonucleic acid
DR	Direct repeat
E	Ethambutol
EPTB	Extra-pulmonary tuberculosis
GACVS	Global Advisory Committee on Vaccine Safety
GeneXpert	GeneXpert® MTB/RIF
GTB	Genital tuberculosis
H	Isoniazid
HE	Haematoxylin and Eosin
HIV	Human immunodeficiency virus
IS*6110*	Insertion Sequence *6110*
ITS	Internal transcribed spacer
IVF	In Vitro Fertilization
LJ	Löwenstein-Jansen
LOD	Limit of detection
MDR-TB	Multidrug-resistant tuberculosis
MOTT	Mycobacteria Other Than Tuberculosis
MGIT	Mycobacteria growth indicator tube
Min	Minutes
MIRU	Mycobacteria interspersed repetitive unit
ml	Millilitre
MRI	Magnetic resonance imaging
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* Complex
NAAT	Nucleic acid amplification test
Na_2EDTA	Disodium ethylenediamineteraacetate
NaOH-NALC	Sodium hydroxide-N-acetyl-L-cysteine
NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Disease
NPV	Negative predictive value

NTM	Nontuberculous mycobacteria
PANTA	Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, Azlocillin
PCR	Polymerase chain reaction
pg	Picogram
PGL	Phenol glycolipid
PPV	Positive predictive value
PTB	Pulmonary tuberculosis
R	Rifampicin
rRNA	Ribosomal ribonucleic acid
RFLP	Restriction fragment length polymorphism
RRDR	Rifampicin Resistance Determining Region
SANAS	South African national accreditation system
Sec	Seconds
SIT	Spoligo international type
SNP	Single nucleotide polymorphism
SOP	Standard Operating Procedure
SR	Sample reagent
Stats SA	Statistics South Africa
TAE	tris-acetate disodiumethylenediaminetetraacetate
TB	Tuberculosis
TMC	Trudeau Mycobacterial Collection
tRNA	Transfer ribonucleic acid
USA	United States of America
UV	Ultra violet
VNTR	Variable number of tandem repeats
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis
Z	Pyrazinamide
ZN	Ziehl-Neelsen

Chapter 1 Literature review

1.1 Introduction

Tuberculosis (TB), a communicable disease, is caused by the bacterium *Mycobacterium tuberculosis* (MTB). According to the World Health Organization (WHO), globally in 2015 there were 10.4 million new cases of TB of which 5.9 million were men, 3.5 million were women and 1.0 million were children. WHO also reported that 1.4 million deaths have resulted from the disease. In South Africa the incidence is approximately 834 per 100,000 population, ranging from 539 to 1190 including human immunodeficiency virus (HIV) co-infection (World Health Organization 2016, Schaaf et al. 2009). Statistics South Africa (Stats SA) has reported that TB is still the leading cause of death in South Africa but also that it is in decline according to data from 2011 to 2014. Stats SA reported that in 2014 approximately 8.4% of deaths in South Africa were attributed to TB (Statistics South Africa 2015b).

TB infection is usually the result of inhaling droplets that contain the bacteria, MTB. These droplets usually result from an infected person coughing, sneezing or talking. In the absence of a predisposing condition, only a small percentage of MTB infected individuals develop active TB. In individuals with an intact immune system, macrophages and activated T cells form granulomas that prevent the spread and proliferation of the bacteria. Individuals such as these would test positive with a tuberculin skin test even in the absence of active TB, due to the fact that the skin test can only determine if an individual had been previously infected. Individuals with latent TB are not infectious and cannot transmit the organism. In contrast to those with an intact immune system, individuals with silicosis, diabetes mellitus, using immunosuppressive drugs (corticosteroids) and those immunocompromised due to HIV infection have a greater chance of developing active TB (Bass et al. 2000, Schaaf et al. 2009).

The most common clinical manifestation of TB involves the lungs, this is defined as pulmonary tuberculosis (PTB). Manifestations of disease in sites other than the lungs, defined as extra-pulmonary tuberculosis (EPTB), are less common than PTB accounting for only 20% of all TB cases in HIV negative patients. Sites commonly affected by EPTB include the abdominal cavity, brain, bones, joints and genitalia. EPTB is more common in HIV positive patients, this is likely due to the compromised immune system (Frieden et al. 2003). The symptoms of TB will vary according to the site of the infection. In some cases the symptoms will not be specific to the site of infection and this should be taken into account when considering a diagnosis of TB. The most common symptoms of pulmonary TB include: a persistent cough (longer than two weeks), shortness of breath, chest pain, night sweats, fever and weight loss (Bass et al. 2000, Frieden et al. 2003).

In 2010, the WHO released guidelines for the treatment of uncomplicated drug susceptible TB. This can be separated into the intensive phase treatment (two months) and continuation phase treatment (four months). In the intensive phase, the following are recommended antibiotics: isoniazid (H), rifampicin (R), pyrazinamide (Z), and ethambutol (E). With the continuation phase, the following antibiotics are recommended: H and R. The antibiotics HRZE form the first line drugs against TB (World Health Organization 2010). In some cases TB has acquired resistance to antituberculosis drugs which is usually a result of spontaneous mutation. In patients with active TB that have strains that have undergone spontaneous mutation resulting in resistance, the administration of antituberculosis drugs has led to resistant strains becoming the dominant strains by selective pressure. This situation is further compounded when these strains are then spread to individuals that have not been exposed to antituberculosis drugs (Gandhi et al. 2010). Multidrug-resistant tuberculosis (MDR-TB) is TB that has acquired resistance to at least the first line drugs R and H. Resistance to H is a result of mutations in several genes, these include *inhA, katG, ahpC, kasA* and NDH while R resistance is due to mutations in the *rpoB* gene (Ormerod 2005, Palomino et al. 2014). In 2006 it was agreed by WHO and the Centers for Disease Control and Prevention (CDC), USA, that the definition of extensively drug-resistant tuberculosis (XDR-TB) is the acquisition of additional resistance to any of the fluoroquinolones and one of the second-line injectable drugs, amikacin, kanamycin and capreomycin (Centers for Disease Control and Prevention 2006, Sowajassatakul et al. 2014).

1.2 The genus *Mycobacterium*

At the Berlin Physiology Society meeting, on the 24th of March 1882, German physician and microbiologist Robert Koch made a presentation on the infectious agent responsible for TB. The name Robert Koch gave to this bacteria was Tuberkelbazillus, while the name *Mycobacterium tuberculosis* would be introduced later (Schaaf et al. 2009).

According to Bergey's Manual of Systematic Bacteriology, the genus *Mycobacterium* consists of bacteria that are aerobic to microaerophilic. Their shape can be described as straight or slightly curved rods. Depending on their growth these bacteria can, at some stage of their growth, be strongly acid-alcohol-fast. Other characteristics of the genus are that the bacilli are non-motile and the colonies may appear to be white to cream in colour with some strains being able to produce pigmented colonies that appear yellow to orange in colour. The cells and their cell walls are rich in lipids. The genus consists of opportunistic forms, saprophytes and obligate parasites. The cell growth of mycobacteria can be slow to very slow and the incubation period can range from a few days to eight weeks. Depending on the species, the optimal temperature for growth can range from ambient to 45°C. *Mycobacterium leprae* has not been cultured outside of living cells. One of the characteristics of mycobacteria is resistance to decolourization by the acid-alcohol mix during

staining. This feature is shared with closely related actinomycetes. In general, most mycobacteria are obligate aerobes with some species that show tolerance to reduced oxygen levels. Carbon dioxide (CO_2) is required for growth and the bacteria can obtain it from either the atmosphere or through the growth supplements. Additional growth requirements include potassium, iron salts, sodium, magnesium and sulphur. Numerous species can be cultured in Sauton's agar however mycobacteria that are of clinical importance will be best grown on the egg based Löwenstein–Jensen (LJ) media or Middlebrook media (Goodfellow et al. 2012).

Other bacteria that belong to the *Mycobacterium* genus include the leprosy causing *Mycobacterium leprae* and the cattle borne *Mycobacterium bovis*. The *Mycobacterium* genus can be arranged systematically into two groups; the slow growers and the rapid growers which are related to the genus *Nocardia*. The genus can also be divided between obligate pathogens (MTB and *M. leprae*) and the numerous other species that can be found freely in the environment sometimes termed environmental mycobacteria. The mycobacteria that result in TB in humans and mammals have been grouped into the MTB complex (MTBC) (Schaaf et al. 2009). Table 1.1 describes the species that constitute the MTBC.

Table 1.1 The *Mycobacterium tuberculosis* complex

Species	**Principal host**
MTB	Human
M. bovis	Cattle
M. caprae	Goats
M. africanum	Human
M. microti	Vole
M. canetti	Human
M. pinnipedii	Seal
M. mungi	Mongoose
M. orygis	Oryx
M. suricattae	Meerkats

(Schaaf et al. 2009, Alexander et al. 2010, van Ingen et al. 2012, Parsons et al. 2013)

1.2.1 M. tuberculosis

MTB is a strongly acid-alcohol-fast rod shaped bacterium, which can be straight or slightly curved. When MTB is grown on solid media, the colonies tend to be raised and rough with a wrinkled surface. Growth is usually serpentine in MGIT culture, meaning that the bacilli form cordlike masses which can be observed with the bacilli being in a parallel orientation. Avirulent colonies are in most cases less compact. In liquid media, MTB growth forms a pellicle which in time can become thick and wrinkled (Goodfellow et al. 2012).

MTB optimum growth conditions include: temperature at approximately 37°C (growth can occur at 30-34°C), pH at approximately 6.4-7.0, CO_2 at 5-10% mixed with air has been known to stimulate growth as has the addition of glycerol 0.5% (w/v). Bacilli grown under highly aerobic conditions will

die when the environment rapidly changes to anaerobic however this phenomenon is different if the bacilli are allowed time to settle and grow as they adapt to oxygen deprivation. Members of the MTBC share identical 16s rRNA and internal transcribed spacer (ITS) sequences (Goodfellow et al. 2012).

TB can be thought of as an Old World Disease since it has plagued mankind throughout known human history. It is believed that MTB has killed more people than any other infectious pathogen. Currently there is a hypothesis that the genus could have originated more than 150 million years ago. It is generally believed that members of the MTBC originated from a common ancestor approximately 15,000 to 35,000 years ago in East Africa. While it is difficult to pin point the first human infected with TB, Egypt has documented TB dating back to 5,000 years ago. This was seen with Egyptian mummies displaying characteristics of Pott's disease, a skeletal abnormality of the spine as well as their depiction in ancient Egyptian art (Daniel 2006).

Ancient TB infections have also been recorded outside of the African continent. Prominent examples include ancient written texts uncovered in India and China dating back approximately 2000 years. The Americas, similar to ancient Egypt, contain ancient archaeological evidence of Pott's disease, as this was observed in Peruvian mummies. While Europe was going through the Middle Ages, written records of TB were sparse but this should not be confused with believing that the disease was not present. Archaeological evidence of TB can be found throughout Europe. It was not until the renaissance that there was new found knowledge of the disease. It was only with the publication of Robert Koch in 1882 identifying the causative agent of TB, that our understanding of TB changed (Daniel 2006, Koch 1882).

1.2.2 M. bovis

Zoonosis is defined as an infectious disease in which animals are the principal host but which can be transmitted to humans. The most common zoonotic mycobacteria are the cattle borne *M. bovis* and the goat borne *M. caprae*. While *M. bovis* is 99% genetically identical to MTB, it differs enough to allow the bacteria to infect a wide range of mammals (examples include pigs, horses, foxes, cats and dogs). Determining an epidemiological pattern for *M. bovis* has proven difficult due to the fact that transmission of the disease can occur between farm animals or with wildlife populations. In most cases humans acquire *M. bovis* through three possible routes: inhalation, ingesting derivative products (unpasteurized milk) and traumatic inoculation (Schaaf et al. 2009, Reilly et al. 1995).

1.2.3 BCG

The Bacille Calmette–Guérin vaccine, more commonly known as the BCG vaccine, is considered to be one of the most widely used vaccines in the world with approximately 3 billion doses administered. The BCG vaccine was obtained by performing 231 serial passages (between 1908 and 1920) using

bile salts, that resulted in *M. bovis* becoming attenuated. The attenuation of *M. bovis* was the result of various gene complexes being lost during the attenuation process. Currently the BCG vaccine is the only vaccine available for TB (Dietrich et al. 2003).

In 1921, the first humans were vaccinated with the BCG vaccine in France. Since then laboratories worldwide had performed repeated subculture of the BCG strain which resulted in the emergence of different BCG vaccine strains. With the rise of molecular techniques it became possible to further study and determine the genomic diversity of the different strains. There is a notion that differences in BCG strains result in different levels of efficacy, protection and susceptibility to anti-tuberculosis drugs. This is further complicated by the fact that there are different routes of administering the BCG vaccine which can also result in different levels of protection. Until further information is obtained there is currently no indication as to which BCG is considered “best” or “worst”. Most countries use of a particular vaccine strain is dependent on numerous factors such as cost, historical precedence and logistics (Ritz et al. 2009).

Currently the most widely used BCG strains include Tokyo, Glaxo, Connaught, Moreau, Danish and Pasteur, all of which have shown differences in biochemistry, morphology and immunological effects. The Danish BCG is the strain currently used for vaccination in South Africa (Oettinger et al. 1999, Ritz et al. 2009). Table 1.2 shows the different BCG vaccine strains, their synonyms and their genetic variants such as the copy number of the *IS6110* gene and the presence or absence of the antigenic protein MPT64 (Behr 2002).

Table 1.2 Bacillus Calmette–Guérin vaccine strains

Name	**Synonym**	**Year obtained**	**Copies of *IS6110***	**MPT64**
Russia	Moscow	1924	2	Present
Moreau	Brazil	1925	2	Present
Tokyo	Japan	1925	2	Present
Sweden	Gothenburg	1926	1	Present
Birkhaug		1927	1	Present
Danish*	Copenhagen	1931	1	Absent
Prague	Czechoslovakian	1947 (from Danish)	1	Absent
Glaxo		1954 (from Danish)	1	Absent
Tice	Chicago	1934	1	Absent
Frappier	Montreal	1937	1	Absent
Connaught	Toronto	1948 (from Frappier)	1	Absent
Phipps		1938	1	Absent
Pasteur	Paris	Lyophilised 1961	1	Absent

(Ritz et al. 2009, Behr 2002)
*Currently used in South Africa

Other uses for the BCG vaccine include its effectiveness as an adjunctive therapy for some forms of bladder cancer. While in most cases the therapy is well tolerated it can however result in local and or systematic BCG complications, most notably BCG infection, since the vaccine is a live attenuated

M. bovis strain. It should be noted that the incidence of BCG-related complications is lower than 5%. In 2014, Pérez-Jacoiste Asín and colleagues, reviewed 282 patients who developed BCG infection while being treated for bladder cancer using the BCG vaccine as an adjunctive therapy. Their analysis found that 34.4% of the cases involved disseminated BCG infection and 23.4% of the cases involved localised (genitourinary) infection (Pérez-Jacoiste Asín et al. 2014).

In 1996 the CDC recommended that the BCG vaccine should not be administered to children and adults that are HIV infected in the United States of America (USA) (Centers for Disease Control and Prevention 1996). In 2007 Anneke Hesseling and colleagues at the Desmond Tutu TB Centre based at Stellenbosch University found that children with HIV who are vaccinated with BCG may be at risk of developing disseminated BCG disease (Hesseling et al. 2007). WHO in 2007, based on a request from the Global Advisory Committee on Vaccine Safety (GACVS), had recommended that in high TB burden areas the BCG vaccine should be given to all healthy infants as soon as possible after birth but that it should not be given to children presenting with HIV infection. This is due to the fact the children with HIV that are given the BCG vaccine are at a greater risk of developing disseminated BCG disease (World Health Organization 2007).

According to the South African National Department of Health, the BCG vaccine should be given at birth and is given intradermally preferably on the right arm. It is strongly recommended that the vaccine should not be given to children older than 12 months. Furthermore the vaccine may not be given to children whose mothers are currently on a course of anti-TB drugs, rather it is suggested that these children should be given TB prophylaxis and that later they may be given the vaccine (National Department of Health 2010).

1.2.4 Other Mycobacteria

A group of mycobacteria that is rarely discussed are the environmental mycobacteria, also known as nontuberculous mycobacteria (NTM) or mycobacteria other than tuberculosis (MOTT). These names arise due to the fact that this group of mycobacteria can be found in the environment and were thought not to cause tuberculosis, although there have been some MOTT's that can cause disease in humans and animals. Most MOTTs are found in waterlogged environments including rivers, lakes, marshes and in some cases MOTTs have been found in municipal water sources (Adjemian et al. 2012, Schaaf et al. 2009). MOTTs that have been isolated from water sources (fresh water, swimming pools and fish tanks) include *M. kansasii*, *M. xenopi*, *M. simiae* and *M. marinum* (also in salt water). Interestingly *M. kansasii* has not yet been recovered from natural water supplies or soil but it has however been recovered from tap water and can survive in that environment for up to 12 months (American Thoracic Society 1997). Since DNA sequencing has become available, this has resulted in over 125 different species of MOTTs being discovered. Unlike members of the MTBC which can spread from human to human, the pathogenicity of MOTTs is not yet fully understood

resulting in most MOTT infections being difficult to diagnose and treat. Some of the features that differentiate MOTTs from their MTBC counterparts are the increased range of pathogenicity and relative drug resistance to some antimicrobial drugs. This has resulted in the need for increased species-specific MOTT identification due to differences in antimicrobial susceptibility and treatment options. Currently MOTTs can be separated into two groups: the slow growing MOTTs (isolates that form colonies after seven days with some that may require up to eight – 12 weeks) and the fast growing MOTTs (isolates that form colonies within seven days) (Schaaf et al. 2009, Daley 2009). Table 1.3 contains the slow growing MOTTs as well as their sites of infection.

Table 1.3 Slow growing MOTT and sites of infection

Species	**Site of infection**
M. avium	Pulmonary, Lymph nodes, Bacteraemia
M. doricum	CSF
M. kansasii	Skin, Pulmonary, Bacteraemia
M. intracellulare	Pulmonary, Bacteraemia

(Schaaf et al. 2009)

Table 1.4 contains the fast growing MOTTs as well as their site of infection.

Table 1.4 Fast growing MOTT and sites of infection

Species	**Site of infection**
M. abscessus	Pulmonary, Soft tissue
M. alvei	Pulmonary
M. boenickei	Wounds, Soft tissue, Pulmonary
M. smegmatis	Lymph nodes

(Schaaf et al. 2009)

Before the acquired immune deficiency syndrome (AIDS) epidemic, most MOTTs caused mostly pulmonary and skin disease. Most patients that were affected by MOTTs were sexagenarians or individuals with predisposing lung conditions, chronic lung conditions and individuals working in dusty environments (e.g. farming and mining) (Falkinham 1996).

Since the beginning of the AIDS epidemic, there has been a rise of MOTT infections especially in individuals diagnosed with HIV. Previous estimates indicate that, in the United States and Europe, approximately 25 to 50% of patients diagnosed with HIV are infected with MOTT (Falkinham 1996). This picture holds true with MOTT infected miners in the Free State province. A MOTT infection that is a co-infection with HIV usually presents with atypical clinical symptoms which further increases difficulties in reaching a definitive clinical diagnosis. In the Corbett study, which was conducted in 1999, it was found that most miners with MOTT infection were identified and treated for *M. kansasii*. Currently it is difficult to determine incidence, since tests required for MOTTs are too expensive to be implemented in resource limited laboratories and developing countries (Corbett et al. 1999a, Corbett et al. 1999b).

As can be seen in both *Table 1.3* and *Table 1.4*, while MOTTs can affect lymph nodes, soft tissue and skin, the majority of the diseases caused by MOTTs are pulmonary (Adjemian et al. 2012).

Ultimately, once an individual becomes infected with MOTT, there are three possible outcomes: the body's immune system will clear the infection, the bacteria will proliferate in the airways or the bacterial infection will cause disease (Schaaf et al. 2009).

1.3 MTB genome

In 1998 the full genome sequence of the H37Rv of MTB, which is the reference laboratory strain, was published. The genome of MTB consists of 4,411,529 base pairs with a 65.6% guanine-cytosine (G+C) content. The genome of MTB H37Rv has abundant repetitive DNA sequences such as the IS. While the G+C content of the genome is relatively constant there are regions that display higher than normal G+C content and these sequences correspond to a large gene family which includes the polymorphic G+C-rich sequences. The genome contains 50 genes that code for functional RNA molecules. There are 16 copies of the IS*6110* as well as six copies of the relatively stable IS*1081*. Further analysis of the genome revealed an additional 32 different IS elements, which had not been previously described. It is apparent that most insertion sequences found in MTB H37Rv are inserted in non-coding regions and in some cases near transfer ribonucleic acid (tRNA) genes. The genome further reveals that there are at least two prophages present which could be the reason why MTB has shown persistent low-level lysis in culture. The MTB H37RV contains 3,924 open reading frames which account for approximately 91% of the potential coding capacity (Cole et al. 1998).

The MTBC genome contains numerous genetic loci which are polymorphic and this property allows for molecular typing and evolutionary studies. With molecular techniques becoming routine in the standard microbiology laboratory, this has allowed for numerous techniques that can assist in the molecular typing of MTBC. These techniques include: IS*6110* restriction fragment length polymorphism (RFLP), spacer oligonucleotide typing (spoligotyping) and the mycobacterial interspersed repetitive unit-variable numbers of tandem repeats (MIRU-VNTR) (Brudey et al. 2006, Otlu et al. 2009). Figure 1.1 depicts the MTB genome including the approximate distribution of the IS*6110*, direct repeat (DR) locus/region and MIRU. RFLP is described as the gold standard for genotyping MTB isolates. The technique is based on the presence and distribution of the IS*6110*. Most patients that are infected with TB should have different RFLP patterns unless there was an outbreak at the time of investigation. Strains that contain fewer than six IS*6110* copies are said to be difficult to type and for these strains, it is best to use other typing methods. Unlike the IS*6110* RFLP method which is labour intensive, MIRU-VNTR can be automated and allow a greater number of strains to be investigated. Spoligotyping, which is based on the absence or presence of spacers in the DR region, has advantages over RFLP such as: only small amounts of DNA are required allowing for this technique to be used directly on clinical samples and spoligotyping results can be

expressed in a digital format for further studies. The only disadvantage of spoligotyping is that it has a low discriminating power compared to RFLP (Barnes et al. 2003).

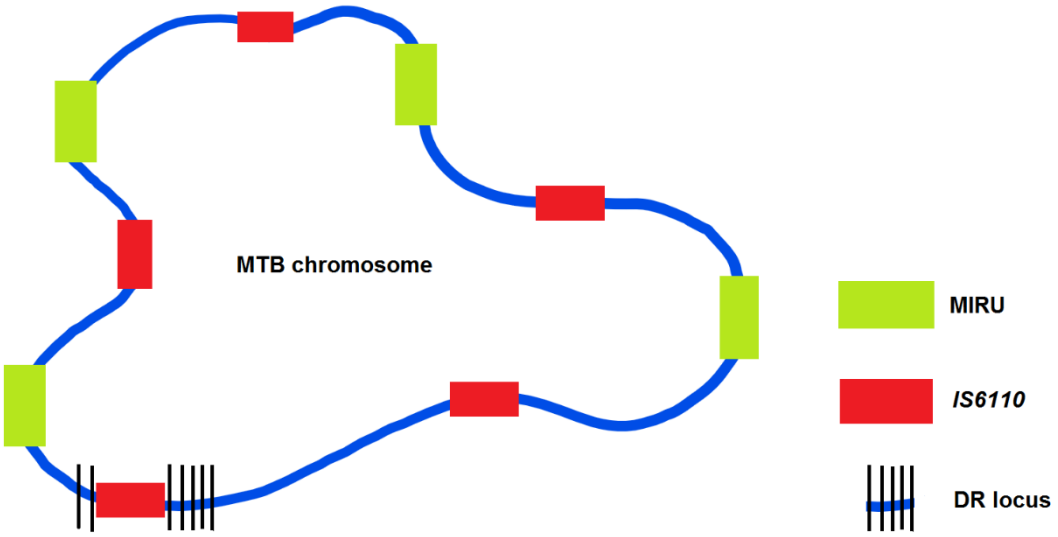

Figure 1.1 Schematic of the MTB genome

The MTB Genome contains *IS6110* which are found throughout the genome. The restriction fragment length polymorphism (RFLP) which is used to type MTB targets the *IS6110*. MIRU which is the mycobacterial interspersed repetitive unit consists of variable numbers of tandem repeats (VNTR), and like the *IS6110* are also found throughout the MTB genome. MIRU-VNTR is used to type MTB by targeting the MIRU. DR locus consists of the direct repeats which are the target for spoligotyping.

Spoligotyping has allowed for the identification of 36 potential subfamilies or subclades of MTBC, which are sometimes referred to as spoligotypes in part due to the technique used to make the identification. The spoligotype families include: Beijing, T (T1 to T4), Haarlem (Haarlem 1 to Haarlem 3), X (X1 to X3), East Africa-India/EAI (EAI 1 to EAI 5), Africa/AFRI (AFRI 1 to AFRI 3), Central Asian/CAS (CAS 1 and CAS 2), Latin America and Mediterranean/LAM (LAM 1 to LAM 10), S, as well as members of the MTBC such as *M. bovis*-BCG, *M. microti*, *M. canetti*, and the reference strain H37Rv (Otlu et al. 2009, Filliol et al. 2002, Brudey et al. 2006, Orduz et al. 2015). A common terminology used to differentiate different MTBC strains is the MTBC lineages. There are seven lineages consisting of different spoligotypes. Lineage 1 consists of EAI with the strains being mostly isolated from East Africa, Southeast Asia and Southern India. Lineage 2 consists of Beijing with the strains being isolated from East Asia, Russia and South Africa. Lineage 3 consists of CAS which is mostly isolated from East Africa and Northern India. Lineage 4 consists of Haarlem, LAM, T and X and these are mostly isolated from the Americas, Europe, North Africa and the Middle East. Lineage 5 and 6 consist of the AFRI strains and these are mostly isolated from Central Africa. Lineage 7, which was recently discovered, consists of strains mostly isolated from Ethiopia with the name

Aethiops vetus being proposed for the lineage (Firdessa et al. 2013, Yimer et al. 2016, Gagneux et al. 2007).

1.3.1 IS*6110* gene

The IS*6110* is an insertion sequence like element originally found in MTB and *M. bovis* (see Appendix B)(Thierry et al. 1990). It was later discovered that the IS*6110* is only present in the TB disease causing MTBC. It is believed that the exclusivity of the IS*6110* within the MTBC could be a result of the lack of genetic exchange with other mycobacteria however this exclusivity has allowed the development of important diagnostic tools that have assisted in differentiation of MTBC species and other mycobacteria. Furthermore, the copy number as well as the different locations in the genome of the IS*6110* has assisted in determining different strain types allowing its use in epidemiology studies (Coros et al. 2008). Numerous molecular assays have been developed targeting the IS*6110*. Sun *et al*. developed an IS*6110* based PCR (polymerase chain reaction) with a sensitivity of 0.1pg (approximately 5 MTB bacilli) and recommended that IS*6110* based PCR can be implemented for routine diagnostics (Sun et al. 2009).

While most strains harbour multiple copies of IS*6110* it should be noted that there have been some strains that lack this element. Howard *et al.* reported the first case of a MTB isolate lacking the IS*6110* in Canada although the patient's records indicated that the patient emigrated from Laos to Canada. Other cases have been identified in San Francisco, Vietnam and Chennai. The evidence seems to suggest that these isolates are mostly found in the South East Asia region. The implications that isolates that do not contain the IS*6110* have on TB diagnosis is that techniques that are based on the IS*6110* will have no further use. While there is not enough information regarding South African MTB isolates, the last study that investigated the IS*6110* in South Africa using RFLP found that South African isolates do contain the IS*6110* (Radhakrishnan et al. 2001, Warren et al. 2002, Mathuria et al. 2008, Huyen et al. 2013, Howard et al. 1998).

1.4 Clinical manifestations

The clinical features of TB will, in most cases, be related to the pathology of the disease. The site of infection can either be pulmonary or extra-pulmonary. The most common symptoms for TB include but are not limited to: fever, night sweats, tiredness, lack of appetite and weight loss (wasting). Patients presenting with EPTB should also undergo chest radiograph and sputum microbiology since it is possible for a patient to have PTB and EPTB concurrently (Schaaf et al. 2009).

1.4.1 Pulmonary tuberculosis

Most cases of TB involve the lungs with the most common symptom of PTB being coughing. Cough remains a highly sensitive method for detecting TB especially when it lasts longer than 2-3 weeks, but the same cannot be said about the specificity since a persistent cough may be caused by various

conditions including repeated acute respiratory tract infections, asthma, chronic obstructive pulmonary disease, bronchiectasis and lung cancer. Other symptoms that may accompany PTB include: productive cough with yellow or green sputum (sometimes accompanied with streaks of blood but rarely with large amounts of blood), night sweats, fever, feeling unwell (can be accompanied with loss of appetite), weight loss and shortness of breath with chest pain (Schaaf et al. 2009, Nardell 2003).

1.4.2 Extra-pulmonary tuberculosis

Apart from systemic features, patients with EPTB will, in most cases, present with symptoms related to the pathology of the site of infection (Schaaf et al. 2009). Table 1.5 is a summary of the EPTB sites and their respective clinical features.

Table 1.5 Extra-pulmonary tuberculosis sites and clinical features

Site of infection	Clinical features
Abdominal cavity	Fatigue, appendicitis like pain, swelling, slight tenderness
Bladder	Painful urination, blood in urine
Bones	Swelling, minimal pain
Brain	Fever, headache, nausea, drowsiness, coma and brain damage if untreated
Joints	Arthritis-like symptoms
Lymph nodes	Painless, red swelling, may drain pus
Pericardium	Fever, enlarged neck veins, shortness of breath
Female reproductive organ	Infertility, pelvic inflammatory disease
Male reproductive organ	Epididymitis (lump in scrotum)
Spine	Pain, leading to collapsed vertebrae and leg paralysis

(Schaaf et al. 2009, Nardell 2003)

EPTB accounts for approximately 20% of patients with TB, with 70% of these patients being co-infected with HIV. A study conducted by Karstaedt, involving EPTB patients at Chris Hani Baragwanath Hospital in Soweto, Johannesburg showed the different incidence rates of EPTB in Soweto. Briefly, of all the individuals above the age of 18 with EPTB the study found the following distribution: 39.1% pleural, 31.0% lymph node, 21.8% bacteraemia, 7.3% meningitis, 3.0% pus (site unspecified), 2.9% peritonitis and 1.6% other (Karstaedt 2013).

1.5 Female genital TB

The first case of genital TB (GTB) was described by Morgagni in 1744 after performing an autopsy on a 20-year-old woman. She died of TB and her post-mortem examination found that her fallopian tubes and uterus were filled with caseous material (cheese-like). Currently in developed countries, the incidence of TB and GTB have been steadily declining while the same may not be said for developing countries such as India and South Africa (Anderson 1988). Based on a 10 year study (1993-2003) conducted by Hassoun, it was reported that 1.8% of all TB cases could have a genitourinary site (Hassoun et al. 2005). A study that was conducted in South Africa, by Margolis et

al, found that South Africa has an incidence of 6.15% culture positive TB cases in the infertile population (Margolis et al. 1992). The global prevalence of GTB differs between developed and developing countries. This can be seen in Table 1.6 where the data was compared from several countries. The USA has a prevalence amongst infertile patients at 1% while Nigeria and India have a prevalence higher than 15% (Singh et al. 2008). When reporting the incidence of GTB it should be noted that the exact incidence of the disease remains unknown, this due simply to the fact that the majority of GTB cases remain undiagnosed since the presentation of GTB may be asymptomatic (Bajpai et al. 2014).

Table 1.6 Global prevalence of genital TB in infertile patients

Country	Prevalence amongst infertile patients
USA	1%
Pakistan*	23.08%
Saudi Arabia	4.2%
South Africa*	6.15%
Italy	0.8%
Nigeria	16.7%
India*	26%

(Singh et al. 2008)
*Countries that have multiple prevalence reports

GTB is usually the result of MTB infection but in some cases, may result from *M. bovis* infection. This is especially true in countries which lack milk pasteurization facilities and effective TB control programmes for cattle. GTB is in most cases a secondary infection, usually a result of a TB infection elsewhere in the body such as PTB. The spread of the bacteria from other sites to the genital tract can be lymphatic or haematogenous and occasionally through direct contiguity with a peritoneal focus. In extremely rare cases there can be a primary GTB infection (Anderson 1988, Schaaf et al. 2009, Bajpai et al. 2014).

GTB presents a dilemma for diagnosis due to the varied clinical presentation, while another dilemma is that tests used for GTB diagnosis could produce different or conflicting results. The paucibacillary (low bacterial load) nature of GTB results in the laboratory having difficulties to isolate and diagnose the infection (Singh et al. 2008).

Furthermore, GTB poses a diagnostic dilemma because there is no consensus on whether to use tissue samples or endometrial fluid samples for laboratory testing. In addition, there is debate as to which methods (i.e. culture, PCR or the skin test) are best suited for the quick and accurate diagnosis of GTB (Neonakis et al. 2011, Kulshrestha et al. 2011). A compelling argument for the use of menstrual fluid for GTB diagnosis is that the procedure is non-invasive and causes minimal discomfort for the patient. Essentially approximately 10 to 20ml of saline is instilled into the vagina; this will be mixed with the menstrual blood and finally it will be collected and sent to the laboratory

for testing (Botha et al. 2008). Culture is the gold standard for TB diagnosis and has also been shown to accurately diagnose GTB. However, the drawback for culture is that it has a long turnaround time of 2-4 weeks which makes molecular based techniques desirable because of their high sensitivity and specificity as well as a short turnaround time of 1-2 days (Botha et al. 2008, Bajpai et al. 2014, Neonakis et al. 2011).

It is important to have methods that can assist in the early diagnosis of GTB since this can prevent the patient from undergoing invasive diagnostic or therapeutic procedures. Early diagnosis can assist in timely therapy and avoid fibrosis since restoration of fertility becomes difficult once fibrosis is established (Neonakis et al. 2011).

1.5.1 Pathology

In nearly all cases of GTB it was found that the fallopian tubes were affected. The endometrium was found to be involved in more than 50% of cases while the cervix, vulva and vagina were rarely affected. Table 1.7 indicates the frequency of the genital organs affected by TB. Epithelioid granulomas were found in the endometrium especially in the superficial layers (Schaaf et al. 2009).

Table 1.7 Frequency of genital organs affected by TB

Organ	%
Fallopian tubes	90-100
Uterus	50-60
Ovaries	20-30
Cervix	5-15
Vagina	1

(Anderson 1988)

1.5.1.1 Fallopian tube tuberculosis and infertility

One of the leading symptoms of GTB is infertility. This is due to the involvement of the fallopian tubes and combined with endometrial involvement, this results in patients becoming infertile. Studies have indicated that 40-80% of women with GTB will become infertile. This is why GTB should be considered as a possible cause when diagnosing infertility. The prevalence of GTB is higher in countries (most notably developing countries) with a higher incidence of TB. While there is a good cure rate with GTB as it responds well to treatment, the rate of conception following treatment is low at roughly 10-38%, with the live birth rate at 7-17%. This holds true even if patients undergo in vitro fertilisation (Anderson 1988, Kulshrestha et al. 2011, Schaaf et al. 2009).

1.5.1.2 Endometrial tuberculosis

Endometrial TB can often go undiagnosed since it may be asymptomatic. For women in their reproductive cycle, the most common symptoms may include oligo-amenorrhoea, menstrual disturbances and pelvic pain. Post-menopausal women can present with leucorrhoea, pyometra or postmenopausal bleeding (Schaaf et al. 2009). The endometrium has an unremarkable appearance

but this could be due to the cyclic menstrual shedding. It should be noted that occasionally there could be fungating, ulcerative or granular lesions present. The standard histological lesion of endometrial tuberculosis is the appearance of non-caseating granulomas that are composed of epithelial cells. The location of these granulomas are usually throughout the endometrium but their density seems to be greater in the superficial layers. In some cases, total destruction of the endometrium has been found (Anderson 1988, Nogales-Ortiz et al. 1979).

1.5.1.3 Cervical tuberculosis

TB of the cervix is rare and has most likely gone underdiagnosed. Just like with other parts of the genital tract, there seem to be little to no macroscopic changes in the cervix that will be specific for TB. Cervical inspection of necrosis and ulceration are easily confused with cervical carcinoma. Caseation may sometimes be seen. Growths on the cervix are presumed to be cervical cancer but a histological examination is highly recommended (Schaaf et al. 2009, Anderson 1988).

1.5.1.4 Vulval and vaginal tuberculosis

Comprising less than 2% of cases involving genital tract disease, tuberculosis of the vulva and vagina are uncommon. In most cases it is a result of a secondary infection which originated higher up the genital tract. There is however a possibility that the disease could have originated from a male sexual partner who might have had an infected epididymis or seminal vesicles. Vulval TB in most cases begins as a nodule on the labia or sometimes in the vestibular region. At this point an irregular, ragged ulcer forms sometimes with sinuses discharging caseous material and pus. In rare cases, TB involving the vulva presents with a hypertrophic, irregular wart like growth baring resemblance to elephantiasis. Vaginal TB may, in its gross appearance, simulate carcinoma (Anderson 1988).

1.5.2 Treatment and prognosis

Treatment for GTB is with the same standard TB treatment utilized for PTB. Most authors recommended the four drug treatment therapy consisting of H, E, R and Z for the first two months. Total treatment duration should range from six months to 12 months (Schaaf et al. 2009). While the prognosis of patients that have undergone treatment is good in terms of treating GTB, early diagnosis is of paramount importance as it has to be noted that once fibrosis has been established, restoring fertility will become difficult (Neonakis et al. 2011).

1.5.3 The psychology of infertility

Couples and individuals who are incapable of conceiving children usually experience stress and heartbreak. Infertility can have a negative social and psychological effect on the individual and in some situations the individual may feel ostracised/isolated which can lead to mental distress. In numerous cultures motherhood is seen as the only way in which a woman can elevate her social standing within familial and community structures. Looking at infertility as a medical issue, it can be

seen that approximately 80 million people globally are affected by infertility (Greil 1997, Cousineau et al. 2007).

Currently infertility is affecting people in developing countries more than people in developed countries. This can be attributed to factors such as infectious disease that damage the reproductive tract as well as the difficulty or lack of access to fertility services. Reproduction is seen as an important part in adulthood and identity and when conception does not occur this often leads couples to become confused and angry. Studies have shown that infertile women display higher levels of depressive symptoms when compared to fertile women. One study showed that depression, hostility and anxiety where noticeably higher in infertile women. Infertile couples have often felt isolated from the fertile world, this can be due to infertility being seen as socially unacceptable and the lack of empathy from their friends and family which brings along with it the feeling of despair. Some cultures see infertility as defectiveness (Greil 1997, Cousineau et al. 2007).

For women, there is increased pressure to bear children and when conception fails to occur they become distressed. The core issue with infertility is that it presents a challenge to the core identity of what it means to be a woman and because of this diminished identity (that is experienced by infertile women) a women may feel a low self-esteem. Some infertile women start to have beliefs that their infertility is a form of punishment for their sexual indiscretions and use of contraceptives (Greil 1997, Cousineau et al. 2007).

Men do experience psychological effects of infertility however due to norms regarding masculinity they are expected to supress their emotions, furthermore their experiences are not well represented in literature. Some women have chosen to suppress their feelings of distress from their healthcare provider. Reports indicate that approximately 13% of women experience suicidal tendencies after a failed In vitro fertilization (IVF) attempt. WHO reported that, in developing countries, women that are childless will choose suicide over being ridiculed for their lack of ability to bear children and a growing number of authors have raised the issue of the relative neglect of the social psychology of infertility in literature (Greil 1997, Cousineau et al. 2007).

1.6 Laboratory diagnosis

For TB to be treated and eliminated there is a requirement for early diagnosis and faster testing. In 2011 it was determined that the case detection rate for TB worldwide was at 66% which shows that TB management is progressing but at a slow rate. One of the main reasons for the inadequate diagnosis is that, not only are the facilities lacking in equipment and personnel but also the reliance on classical techniques such as chest radiography and smear microscopy. Currently, 90% of all TB cases affect people living in low to middle income countries (China, India, Russia and South Africa) and where diagnosis relies on classical techniques such as smear microscopy and chest radiography

(Weyer et al. 2013, Lawn et al. 2011). In 2014, the South African Department of Health released the National TB Management Guidelines in which a strong emphasis was placed on diagnosis with the following points: a functioning health care system, TB tests with excellent sensitivity and specificity, screening of people suspected of TB and ensuring that individuals with TB are treated immediately (TB DOTS Strategy Coordination 2014).

One of the barriers affecting effective TB diagnosis is the quality of the specimen submitted to the diagnostic laboratory. It is recommended that the specimens should preferably be collected from the site of infection, collected aseptically, stored appropriately and sent to the laboratory. This is done to minimize the growth of contaminating organisms. Specimens can be grouped into 3 groups. Group 1 will include specimens that are obtained aseptically such as biopsy, surgical excision and also fluids such as cerebrospinal fluid and aspirates. These specimens do not require decontamination. Group 2 will include specimens that are secretions from areas in which there is minimal to no chance of contamination. These include respiratory tract, gastric aspirates, urine, menstrual fluid and uterine specimens. These specimens can be decontaminated before performing culture. Group 3 will include specimens that are from areas of the body that are colonised by other organisms, these include skin, oropharyngeal cavity, colon, vagina and other specimens such as stool, scrapings of ulcers and draining of abscesses. These specimens are normally contaminated. Most pathogenic organisms fail to survive during prolonged storage and transportation of specimens due to low temperatures, elevated oxygen concentration and decreased pH. Mycobacteria are not killed during these circumstances since the organism is hardy (Schaaf et al. 2009).

1.6.1 Microscopy

The detection of microorganisms through microscopy is the oldest technique for laboratory diagnosis. While the cellular structure of MTB indicates that it is a Gram-positive bacteria, it requires an experienced microbiologist to observe this phenomena. The organism is however able to be stained via an acid-fast stain since the mycobacterial cell wall has a high lipid content in the form of mycolic acid. There are two common staining methods for MTB (Schaaf et al. 2009).

The Ziehl-Neelsen (ZN) stain, shown in Figure 1.2, relies on carbol fuchin entering the bacteria when it is heated and is also retained when the cells are exposed to an alcohol/hydrochloric acid mixture. This will result in the mycobacteria being stained red (red stained rods) while the background is stained with methylene blue. The ZN has been modified and an example of a modified ZN is the Kinyoun or cold stain, where phenol (at higher concentrations) is used with carbol fuchin, with no heat required (Schaaf et al. 2009).

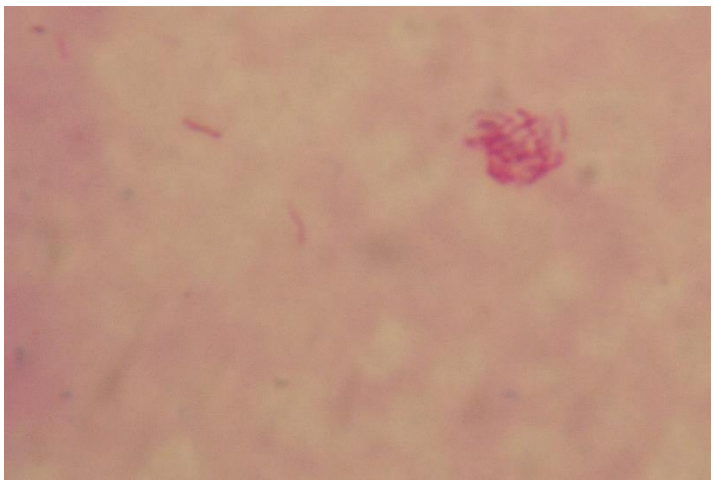

Figure 1.2 ZN stained MTB

ZN staining is used to identify acid fast bacilli. Acid fast bacilli will stain pink while non-acid fast bacilli will stain purple.

The auramine-O stain, shown in Figure 1.3, is a stain in which auramine replaces carbol fuchin. When bound to DNA the auramine fluoresces which can be observed under a fluorescence microscope. After decolourization in which non-acid-fast bacteria and other material fail to retain the auramine, only the acid fast bacteria will fluoresce (Schaaf et al. 2009).

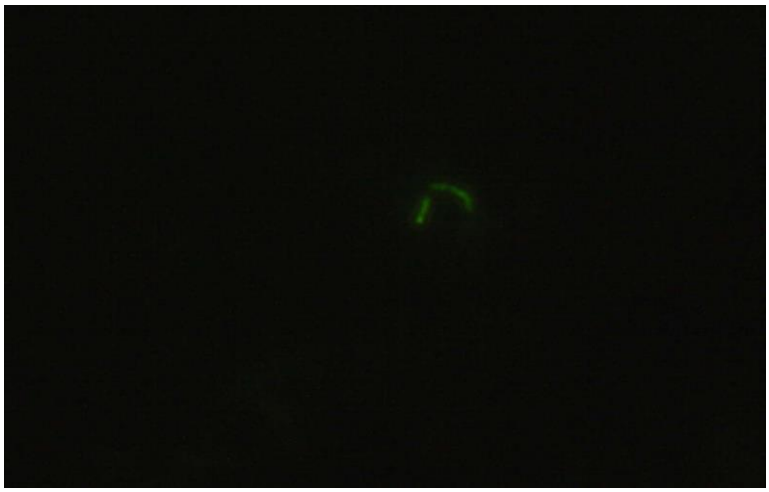

Figure 1.3 Auramine stained MTB

Auramine-O stain is used to stain acid fast bacilli. Acid fast bacilli will retain the auramine and will fluoresce. This is visualised through a fluorescent microscope.

While ZN-stained smears require magnification ranging from ×800 to ×1000, the auramine–stained smears only require magnification ranging from ×450 to ×500 since the fluorescence allows for easier detection. Advantages of the auramine stain include higher sensitivity and a lower turn-around time since the smears can be observed at lower magnifications (Schaaf et al. 2009).

1.6.2 Culture

Culture of MTB is widely accepted as the gold standard of TB diagnosis. In the International Standards of Tuberculosis Care compiled by the WHO, TB culture is recommended where possible. Culture media can be separated into two groups, namely solid media and liquid media (Tuberculosis Coalition for Technical Assistance 2006).

Culture remains important since: culture is more sensitive than microscopy, growth is required for species identification and drug susceptibility testing, and genotyping of cultured organisms may be useful for epidemiological studies. Culture has a sensitivity of 85% and a specificity of 98%. While solid culture is inexpensive, growth tends to be slower when compared to liquid culture. A disadvantage for liquid culture is that contamination is more likely (Bass et al. 2000, TB DOTS Strategy Coordination 2014, Tuberculosis Coalition for Technical Assistance 2006).

1.6.2.1 Solid media

The LJ is an egg-based medium and one of the most well-known. The LJ medium has a long shelf life but its quality may vary depending on the quality of eggs used and by the batch. Mycobacteria tend to grow better on egg based media (Bass et al. 2000, Schaaf et al. 2009).

The agar based media include the Middlebrook 7H10 and 7H11 and they tend to be more reliable for drug susceptibility testing however they have a shorter shelf life and are more expensive (Schaaf et al. 2009).

1.6.2.2 Liquid media

The most commonly used liquid media include the Middlebrook 7H9, 7H12 and the Dubos Tween albumin broths (Schaaf et al. 2009). There has been the development of automated liquid culture systems such as the BACTEC 460TB System and the BACTEC Mycobacterial Growth Indicator Tube (MGIT) 960 System developed by Becton Dickinson. The system currently in use at Universitas Academic Laboratory in Bloemfontein is the BACTEC MGIT 960 System. The system uses a modified Middlebrook 7H9 broth. At the bottom of each MGIT tube is a fluorescent compound that is embedded in silicone, which is sensitive to the presence of oxygen dissolved in the media. Oxygen quenches the fluorescence but when the oxygen is digested by actively growing mycobacteria, fluorescence will then be detected by the instrument (Goloubeva et al. 2001, Frieden et al. 2003, Schaaf et al. 2009).

1.6.3 TB antigen test

The BD MGIT™ TBc Identification Test (Becton Dickinson, Sparks, MD) is a lateral-flow immunochromatographic assay. The test is based on the detection of the 28kDa MPT64 protein secreted only by members of the MTBC (excluding the BCG strain) during culture. The assay is performed on positive MGIT tubes to differentiate MTBC from MOTTs. The assay is simple to use

with a short turnaround time of approximately 15min (Yu et al. 2011, Said et al. 2011, Kumar et al. 2011).

1.6.4 GeneXpert® MTB/RIF

Figure 1.4 GeneXpert® MTB/RIF instrument

The GeneXpert is a self-contained hemi-nested PCR unit in which both extraction and amplification/detection takes place. The GeneXpert can simultaneously detect the presence of MTB as well as its susceptibility to rifampicin.

The GeneXpert® MTB/RIF test (see Figure 1.4) is a closed-cartridge, easy to use molecular system developed by Cepheid Inc, with funding from the US National Institutes of Health (Bethesda, MD, USA) and the Bill and Melinda Gates Foundation (Seattle, WA, USA) (Weyer et al. 2013). The test follows the principle of a real-time hemi-nested PCR in which it simultaneously detects the presence of MTB and whether it is susceptible or resistant to R. The GeneXpert works by having 5 molecular beacons which cover the 81bp region of the *rpoB* gene also known as the Rifampicin Resistant Determining Region (RRDR). An added benefit to this technique is that *rpoB* core region is circumscribed by MTB specific DNA sequences (Lawn et al. 2011, Nhu et al. 2014). Of the mutations that result in rifampicin resistance, 95% occur in the RRDR. The end product is a fully automated diagnostic system that can easily be deployed in resource limited areas (Weyer et al. 2013). The sensitivity for culture positive TB is 90% with a high specificity for sputum samples. The sensitivity for HIV positive patients is 80%. Other studies have also shown high sensitivity and specificity for extra-pulmonary samples (Nhu et al. 2014).

According to the WHO and the South African National Department of Health, South Africa has lead the world in GeneXpert tests by conducting approximately 50% of the tests globally (National Department of Health 2016).

1.6.5 Line probe assays

The GenoType MTBDR*plus* is a line probe assay developed by Hain Lifescience in Germany. This assay can simultaneously detect the presence of MTB and whether there is resistance towards R and I. The assay is carried out in 3 steps, firstly extraction of DNA from the MTB sample (preferably from culture), secondly a multiplex PCR and thirdly the hybridization step. The assay determines R resistance by detecting mutations in the *rpoB* gene, while I resistance is determined by the detection of mutations in the *inhA* or *katG* genes. While it does have a short turnaround time compared to culture and can differentiate MTB from other mycobacteria it does have its drawbacks. These include: failure to distinguish live or dead bacilli (since it is molecular based), it can only be performed on either smear positive or culture positive specimens, the test is labour intensive and it is prone to contamination (TB DOTS Strategy Coordination 2014, Yadav et al. 2013).

1.6.6 Other tests

Other tests approved by the South African Department of Health include:

- Interferon gamma Release Assays (IGRA)
- TB LAM (lateral flow version)
- Histological examination
- Tuberculin skin test

WHO has reviewed the data and performances of serological tests for TB and has now recommended that these tests should not be conducted for TB diagnosis (TB DOTS Strategy Coordination 2014).

1.6.7 Characterizing *Mycobacterium tuberculosis* strains

Before the wide adoption of molecular techniques, it was believed that TB transmission and infectivity was dependant exclusively on host factors. Recently it has become understood that certain MTB strains/spoligotypes may play a role in infectivity and transmission but unfortunately this concept has received little attention for extra-pulmonary infections (Garedew et al. 2013, Garcia de Viedma et al. 2005). Previously identification of different mycobacteria was performed using phenotypic characteristics and biochemical reactions which have long turnaround times. With the adoption of molecular techniques, rapid and accurate results for mycobacterial identification are possible (Neonakis et al. 2008). These methods include IS*6110*-RFLP, spoligotyping and MIRU-VNTR.

1.6.7.1 IS6110-RFLP

This technique makes use of the insertion sequence IS*6110* which is found in the MTB chromosome. It works by restricting IS*6110* with the restriction enzyme *PvuIII* followed by gel electrophoresis and the pattern generated is more commonly known as RFLP. The pattern/size of the bands are used to determine the lineage. In most cases MTB strains contain between 8 to 18 copies of the IS*6110* but there have been reports of strains that lack the *IS6110* and these strains pose a problem for techniques that rely on IS*6110* as these could be reported as false negatives (Huyen et al. 2013, Howard et al. 1998).

1.6.7.2 Spoligotyping

Spoligotyping (spacer oligonucleotide typing) is a molecular based method that can both detect and type MTB. The genome of all strains that comprise the MTBC contain DR regions. This is comprised of conserved 36bp DRs that are separated by unique spacer regions which range from 35bp to 41bp in length, as is visually represented in Figure 1.5. Strains of MTB will differ in the number of DRs and also the presence or absence of spacer regions. Polymorphism is usually a result of phenomena such as homologous recombination DRs, single nucleotide polymorphisms (SNPs) and *IS6110* insertions (which are common in the DR region). All the above mutations, apart from SNPs, result in possible deletion of DRs from the region. Spoligotyping is a technique that is used to determine the presence/absence of the 43 spacer regions in MTB isolates. Drawbacks of spoligotyping is that it doesn't have the same discriminating power as MIRU-VNTR and *IS6110* RFLP when certain strains are tested but at the same time, strains with low *IS6110* numbers can be easily detected by spoligotyping when the *IS6110*-RFLP may fail to type them (Kamerbeek et al. 1997, Schaaf et al. 2009).

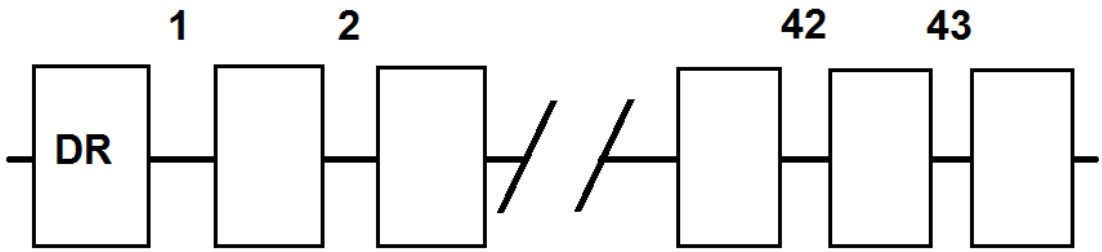

Figure 1.5 Direct repeat (DR) region

DR (direct repeat) in the genome of the *Mycobacterium tuberculosis* complex (MTBC). In between the DR are the unique spacer regions. The absence or presence of certain spacers is used to type the MTBC strain.

1.6.7.3 MIRU-VNTR

After the complete MTB genome was sequenced and analysed, it was revealed that there were 41 VNTRs present and these were called MIRUs. The DNA sequence of these repeats, ranging from 40-100bp and are repeated in a similar manner to mini-satellites found in eukaryotes. Of the 41 MIRUs, 12 display polymorphism in copy number between isolates (Supply et al. 2000). To genotype MTB isolates, a PCR is developed with primers flanking the 12 MIRU and the PCR product is used to determine the copy number present in each MIRU. This can be done with gel electrophoresis, capillary and non-denaturing high-performance liquid chromatography. MIRU-VNTR has several advantages over IS*6110* RFLP typing including: having a shorter turnaround time compared to IS*6110* RFLP typing, its simplicity and results can easily be digitized allowing for inter-laboratory collaborations. The main disadvantage of this technique is that its discrimination power is still lower when compared to IS*6110* RFLP typing, unless 24 loci are used (Supply et al. 2000, Schaaf et al. 2009, Supply et al. 2006, Mazars et al. 2001).

More recent literature has shown that the 24 loci MIRU-VNTR, especially when combined with spoligotyping, may have the same discriminating power as that of IS*6110* RFLP. Furthermore it has been suggested that the 24 loci MIRU-VNTR could be the new gold standard for molecular typing of MTBC bacteria (Narayanan et al. 2015, Jagielski et al. 2014, Jonsson et al. 2014).

1.6.8 Diagnosis of female genital TB

Diagnosing female GTB can be elusive; this can be a consequence of the bacteria being able to remain dormant thus requiring a high level of suspicion to reach a definitive diagnosis (Botha et al. 2008). Proving the existence of MTB in culture or in the biological specimen remains the gold standard. Ideally when tissue specimens are collected they should be sent for histology and culture. For histology the specimen can undergo haematoxylin and eosin (HE) staining or ZN staining. These techniques require MTB to be seen before a definitive diagnosis can be made although histological features including caseous necrosis, giant cells, epithelial cell clusters and lymphocyte infiltration are highly suggestive of MTB (Jassawalla 2006). Culture can be used to diagnose GTB such as the solid media LJ. While TB may grow better on solid media, the long turnaround time (approximately 8 weeks) has resulted in an increase in the use of liquid media which has improved sensitivity and can be automated (Botha et al. 2008, Jassawalla 2006). Some studies have made attempts at detecting GTB using molecular techniques such as the PCR. These studies have shown PCR to have comparable sensitivity while reducing the turnaround time for results to 1-2 days. It has been suggested that while PCR may not replace culture and microscopy, PCR can be implemented alongside the classical techniques. With PCR, various genes have been targeted with varying degrees of success but the IS*6110* gene remains the gene of choice for clinical samples GTB (Thangappah et al. 2011, Botha et al. 2008, Goel et al. 2013). On 12 February 2014 the National

Department of Health in collaboration with the National Health Laboratory Service (NHLS) had introduced and expanded the policy concerning which samples may be used in the GeneXpert (molecular based technique) in diagnosing TB in children and adults. The revised policy now includes: gastric washings, cerebrospinal spinal fluids, pleural biopsy and lymph node aspirates (TB DOTS Strategy Coordination 2014). Currently the GeneXpert has yet to be validated for GTB diagnosis.

1.7 Treatment

The National Department of Health of South Africa has set up a standard TB treatment protocol with the aim of curing TB, decreasing transmission, preventing drug resistance, preventing relapse and preventing deaths resulting from TB (TB DOTS Strategy Coordination 2014) Table 1.8 contains the antibiotics that are recommended for uncomplicated TB treatment.

Table 1.8 Antibiotics recommended for uncomplicated TB treatment

Drug	Target
Isoniazid (H)	Rapid and intermediate growing bacilli
Rifampicin (R)	All populations including dormant bacilli
Pyrazinamide (Z)	Slow growing bacilli
Ethambutol (E)	All bacterial populations

(TB DOTS Strategy Coordination 2014)

Table 1.9 lists the standard treatment regimen which is based on WHO recommendations.

Table 1.9 Standard treatment regimen

TB patient	Intensive phase	Continuous phase
New TB patient	HRZE for 2 months	HR for 4 months
New TB patient in high TB burden areas or with high H resistance	HRZE for 2 months	HRE for 4 months
Patients requiring re-treatment	Drug-susceptibility testing should be conducted	
Patients failing treatment	Should be started on an empirical MDR regimen	

(World Health Organization 2017, World Health Organization 2010)

1.8 Problem identification

In contrast to active PTB where infected individuals would produce sputa containing detectable levels of MTB bacilli, diagnosis of EPTB is particularly difficult due to the low levels of acid fast bacilli present at affected sites and the frequent atypical clinical presentation. This is a drawback for early diagnosis of EPTB by conventional techniques such as staining and culture. Furthermore, lower detectable levels of MTB bacilli by conventional techniques are also observed in patients infected with HIV. Molecular techniques however, hold promise of more rapid and accurate diagnosis of EPTB in the early stages of infection. In addition to high specificity, sensitivity and the theoretical potential to amplify minute amounts of nucleic acid, nucleic acid amplification assays (NAATs) may

be beneficial for the diagnosis of infection in samples with non-viable infectious agents. This project focused on the laboratory diagnosis of female genital TB, which results in infertility especially when left untreated. Currently there is a lack of information on the different diagnostic tests for detecting genital TB, especially South Africa.

1.9 Aim and objectives

1.9.1 Aim

The aim of this project was the development and validation of an in-house nested PCR assay and validation of the GeneXpert® MTB/RIF for the laboratory diagnosis of GTB.

1.9.2 Objectives

1. Development of a nested PCR technique targeting the IS*6110* insertion sequence of *Mycobacterium tuberculosis* for the diagnosis of genital TB.

2. Assessing the efficacy of the in-house PCR and a commercial PCR (GeneXpert® MTB/RIF) assay for the detection of genital TB with comparison to the gold-standard culture method.

3. Characterisation of TB strains responsible for genital TB using spoligotyping and mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) typing.

Chapter 2 Development and validation of a nested PCR and validation of the GeneXpert® MTB/RIF assay for the diagnosis of genital tuberculosis

2.1 Introduction

One of the problems encountered with TB diagnosis is that while microscopy is rapid and inexpensive it requires a high bacterial load which is not present in EPTB samples. These samples tend to have a low bacterial load which further impacts on the low sensitivity of microscopy. Culture, which is the gold standard for TB diagnosis, shows a good sensitivity but its long turnaround time should be taken into account. The turnaround time for MGIT culture to produce a positive result ranges from 2 to 27 days with no growth detected after 42 days being considered as a negative result (Sun et al. 2009, Liu et al. 2007). Nucleic acid amplification tests have been gaining momentum in terms of pathogen detection. This holds true especially for the PCR which has been utilised for TB diagnosis in part due to its short turnaround time and improved specificity. The sensitivity of nucleic acid amplification tests for paucibacillary samples ranges from 53% to 84% (Choi et al. 2014). Several researchers have implemented the nested PCR technique for the rapid diagnosis of TB and while each researcher has had varying degree of success, they all concluded that the nested PCR can be implemented for the rapid detection of TB (Torrea 2005, Miyazaki et al. 1993, Liu et al. 2007, Choi et al. 2014).

The nested PCR is, as Legrand and colleagues describe it, slightly different from the standard PCR in that it is a successive double PCR technique that has resulted in its deployment in fields relating to biomedicine and parasite detection (Legrand et al. 2016). Unlike a standard PCR which only has one pair of primers, the nested PCR has two pairs of primers. These are known as the “outer primers” and the “inner primers/nested primers”. The outer primers will amplify a product which will become the target for the inner primers, to produce a final product that can be visualised through gel electrophoresis (Wei et al. 2016). This process can be seen in a simplified version in Figure 2.1. The nested PCR shows improved sensitivity because of the two successive PCR rounds and shows improved specificity because of its two primer pairs making non-specific amplification less likely. These properties have allowed the nested PCR to become widely used since the early 1990s (Mijatovic-Rustempasic et al. 2016).

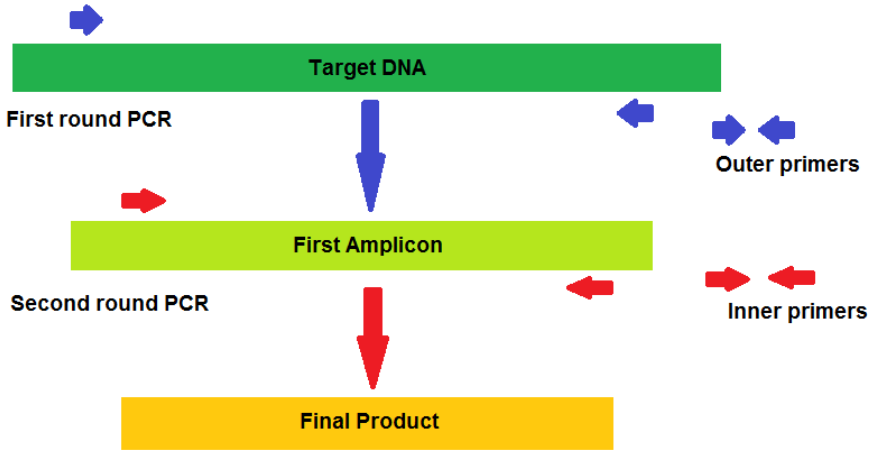

Figure 2.1 The nested PCR

Essentially the nested PCR is a 2 step standard PCR in which the first round products form the starting material for the second round. The outer primers will amplify the Target DNA which will result in the First Amplicon. The inner primers will bind to the First Amplicon and will result in the Final Product. This results in increased sensitivity and specificity.

While the nested PCR assay has been shown to provide higher sensitivity and specificity when compared to conventional PCR, it should be noted that the nested PCR does come with potential risks such as cross contamination. This is because the principal of the nested PCR in which products of the first round are starting material for the second round means that the first round amplification product from one sample could be carried over to another sample if good laboratory practice is not followed. While it seems that real-time PCR is making a push to replace the nested PCR, it should be noted that the nested PCR has been shown to have good limits of detection (usually detecting 10 genomic copies of DNA) (World Organisation for Animal Health 2008).

The IS*6110* insertion sequence was chosen as the target for the nested PCR in this study as it is only present in the genome of TB disease causing mycobacteria. This exclusivity has allowed for techniques developed at differentiating MTBC from MOTTs and other bacteria (Coros et al. 2008). This property has allowed researchers to develop molecular assays targeting the IS*6110,* such as Sun et al recommending that IS*6110* based PCR can be implemented for routine diagnostics (Sun et al. 2009).

There has been rapid development in techniques designed to detect MTB and determine its antibiotic susceptibility however these techniques have limitations such as the requirement of specialised laboratories and the high cost which may put these techniques out of reach for developing countries such as India and South Africa. The GeneXpert® MTB/RIF (Cepheid, Sunnyvale, CA, USA) is an assay that can simultaneously detect MTB in clinical samples as well as its susceptibility to rifampicin. Essentially the GeneXpert system is a sample processing, automated heminested real-time PCR and drug susceptibility testing unit where testing is performed using one simple to use

disposable cartridge. The GeneXpert can detect MTB and its susceptibility to R by amplifying the RRDR of the MTB *rpoB* gene. Approximately 95% of rifampicin resistance is a result of mutations found in the RRDR (Blakemore et al. 2010). One of the driving forces that resulted in the development of the GeneXpert was the alarming increase in the rate of the global incidence of drug-resistant MTB and the need to detect these resistant strains rapidly (Helb et al. 2010).

Since the GeneXpert is a simple to use cartridge based assay, its ease of use has seen the system being deployed outside of centralized laboratories. The GeneXpert utilises 5 different nucleic acid probes (see Figure 2.2) which overlap each other when hybridized to the RRDR of the MTB *rpoB*. The probes are designed to bind the wild type sequences which means that a mutation in the RRDR sequences will prevent the probes from binding accurately and this will be detected by the instrument as rifampicin resistance. The GeneXpert cartridge contains multiple channels that are preloaded with liquid buffers and lyophilized beads that are required for specimen processing, extraction of DNA and the hemi-nested PCR. Specimens are first treated with the sample reagent (SR), that contains sodium hydroxide and isopropanol, in a 2:1 ratio (SR:specimen) and this is incubated at ambient temperature for approximately 15 minutes. This is done to reduce the viability of MTB thereby reducing the biohazard risk and to liquefy viscous samples such as sputum. The treated specimen is transferred into the cartridge which in turn is loaded into the GeneXpert instrument (Lawn et al. 2011).

Figure 2.2 GeneXpert rpoB gene probe binding sites

The GeneXpert makes use of 5 nucleic acid probes which overlap each other covering the rifampicin resistance determining region (RRDR) region of the *Mycobacterium tuberculosis rpoB* gene. The probes are designed to bind to the wild type sequence which means that a mutation in the RRDR will result in a probe failing to bind which will be interpreted by the GeneXpert system as rifampicin resistance.

According to the manufacturer, the GeneXpert displays a sensitivity of 97.8% and a specificity of 99% with a LOD of approximately 131 CFU/ml. The analytical specificity of the assay is excellent as the assay reported all the nontuberculous mycobacteria tested as negative (Cepheid 2012). Helb and colleagues in 2010 determined the LOD by using genomic DNA added directly to the cartridge. Their results showed that the GeneXpert has a LOD of 4.5 genomic copies of MTB DNA using the H37Rv strain, where the LOD was defined as the number of genomic copies per PCR at which there

is a 95% probability of a positive assay (Helb et al. 2010). While the GeneXpert has not yet been fully validated for EPTB diagnosis, several researchers have made attempts at determining the sensitivity and specificity for EPTB specimens. In 2011 Vadwai and colleagues found that the sensitivity of the GeneXpert is 86%-100% for synovial and pericardial specimens, and 63%-73% for tissues, lymph nodes and pleural fluids. The sensitivity and specificity of the GeneXpert for genital samples have yet to be determined (Vadwai et al. 2011). In 2013 WHO, based on numerous studies conducted on EPTB samples for the GeneXpert, updated their policy concerning the use of the GeneXpert for the diagnosis of EPTB. In the updated policy, WHO recommends that the GeneXpert shall receive preference for cerebrospinal fluid (CSF) specimens in patients with suspected TB meningitis. Furthermore, the GeneXpert may be used for other non-respiratory specimens such as lymph nodes and other tissues from patients with suspected EPTB. However, these recommendations do not apply to stool, urine and blood, due to the lack of data concerning these specimens. There is still no recommendation on the use of the GeneXpert for genital samples (World Health Organization 2013).

The demonstration of acid fast bacilli (AFB) in smears is used for the preliminary diagnosis of disease associated with mycobacteria, however, the growth of mycobacteria in culture is described as the definitive diagnosis of disease associated with mycobacteria. Approximately 50%-60% of culture positive MTB samples are smear positive which is why culture still plays a key role in the diagnosis of MTB. While egg-based media such as LJ media and the agar based Middlebrook media have been used for cultivation of MTB, solid media culture is notorious for its long turn-around time with an average of 3 to 4 weeks required to recover mycobacteria from clinical specimens (Siddiqi et al. 2006). The use of liquid based media has improved the sensitivity for the detection of mycobacteria. The BACTEC™ MGIT™ 960 System (Becton Dickinson, Heidelberg, Germany) is a fully automated, liquid based culture system for the detection of mycobacteria. Previous studies have demonstrated that the BACTEC MGIT 960 System is sensitive and has a turnaround time of approximately 2 to 27 days for a positive result to be detected by the instrument (Sun et al. 2009). The MGIT tubes contain 7ml of a modified Middlebrook 7H9 broth base. Enrichment of the Middlebrook broth is required to make the medium complete, such as the MGIT 960 Growth Supplement used in this study. The MGIT Growth Supplement is essential for the growth of mycobacteria. The MGIT PANTA which contains Polymyxin B, Amphotericin B, Nalidixic acid, Trimethoprim, and Azlocillin, is added to suppress the growth of contaminants. The MGIT tube contains an oxygen-quenched fluorochrome that is embedded in silicone at the bottom of the tube. During incubation the mycobacteria will grow and in the process digest the oxygen present in the media. When the oxygen starts to become depleted, the fluorochrome is no longer inhibited and this results in a fluorescent signal being detected by the instrument or under ultraviolet (UV) light. The intensity of fluorescence detected is directly proportional to the depletion of oxygen or growth of mycobacteria. The MGIT tubes can be

incubated manually at 37°C and read with a UV light however it is recommended that the MGIT tubes be incubated in the automated MGIT 960 instrument where the instrument will check for a fluorescent signal every 60min. A sample is considered negative if there is no signal detected after 42 days (Siddiqi et al. 2006).

The South African National Accreditation System (SANAS) defines validation of an assay as the process followed to demonstrate that a specific method is suitable for the intended purpose (South African National Accreditation System 2008). For qualitative assays, validation includes the determination of the sensitivity and specificity of the assay. Analytical sensitivity, sometimes described as LOD, is the measure of the lowest concentration of a substance a particular test can detect while diagnostic sensitivity is the percentage of individuals diagnosed with a disease that the assay is able to diagnose as having that disease. Analytical specificity is the ability of a test to only detect the intended target while discriminating it from interfering substances, while the diagnostic specificity is the percentage of individuals tested negative that are truly negative for a particular disease (Burd 2010, Parikh et al. 2008).

Cohen's kappa, symbolised as κ, is a robust statistical tool used to determine how reliable results are between two raters. It can be described as the likelihood of two raters independently of each other having results that agree with each other but most importantly with the agreement by chance being factored out. This method was developed and published by Jacob Cohen in 1960 in which he described κ as "the proportion of agreement *after* chance agreement is removed from consideration" in the journal Educational and Psychological Measurement (Cohen 1960). Cohen developed the following formula $\kappa = \frac{\rho_o - \rho_e}{1 - \rho_e}$ as the coefficient of agreement.

In short ρ_o is the proportion of units in which both raters agree while ρ_e is the proportion of units that agree as expected by chance. κ follows simple rules such as:

- κ = 0 is when agreement equals chance agreement
- κ = 1 is when there is perfect agreement between the raters
- κ = -1 (below 0) while this is purely of academic interest and is unlikely to occur in practice, this result means that agreement is less than expected by chance and that it is of no further practical interest

κ upper limit is at +1 while its lower limit is at -1. Cohen has suggested that κ be interpreted as follows: values ≤ 0 there is no agreement, 0.01 to 0.20 as none to slight agreement, 0.21 to 0.40 as fair agreement, 0.41 to 0.60 as moderate agreement, 0.61 to 0.80 as substantial agreement and 0.81 to 1.00 as near perfect agreement. Marry McHugh in 2012 suggested that this is not robust enough especially for healthcare research and would result in serious quality problems. McHugh's

suggestion (see Table 2.1) would be better suited for healthcare research (McHugh 2012, Cohen 1960).

Table 2.1 McHugh's interpretation of Cohen's kappa

Value of Kappa	Level of agreement
0 - 0.20	None
0.21 - 0.39	Minimal
0.40 - 0.59	Weak
0.60 - 0.79	Moderate
0.80 - 0.90	Strong
Above 0.90	Near perfect

(McHugh 2012)

A final topic that will be explored in this chapter that can assist in determining which method is best for use as a diagnostic assay for detecting GTB from clinical samples are the predictive values. As the purpose of a diagnostic test is for the test to make a diagnosis, it can therefore be deduced that knowing the probability of the test producing the correct diagnosis will be considered helpful. Unfortunately, sensitivity and specificity do not provide this valuable information. By definition the PPV is the proportion of patients who are tested positive and are correctly diagnosed. The NPV is the proportion of patients who are tested negative and are correctly diagnosed. Unlike sensitivity and specificity, the predictive values are dependent on the prevalence of the condition being investigated. This can mean that, hypothetically if all other factors remain constant, an increasing prevalence will result in an increasing PPV and a decreasing NPV (Parikh et al. 2008, Altman et al. 1994).

In short this chapter describes the development of the nested PCR targeting the IS*6110* and validation of the GeneXpert assay. This was done to determine if the nested PCR and GeneXpert are able to detect GTB from clinical samples as compared to the gold standard, MGIT culture. Furthermore, this chapter explores which method is optimum for the detection of GTB from clinical samples by determining the sensitivity and specificity of both the GeneXpert and the nested PCR.

2.2 Materials and Methods

2.2.1 Specimen collection

Samples were collected by clinical staff at the Unit for Human Reproduction (Obstetrics and Gynaecology), Universitas Academic Hospital, Bloemfontein from women being investigated for infertility. Two types of specimens were collected. The first type of specimen was menstrual fluid/vaginal wash which was collected during the heaviest menstrual bleed. Menstrual fluid samples could only be collected if the patient visited the clinic at the correct time of her menstrual cycle. The collection of menstrual fluid is a relatively non-invasive technique which requires no special equipment, is inexpensive and has minimal risk. Approximately 20 ml of standard saline is instilled

into the vagina with a sterile syringe. The saline is then mixed with the menstrual blood, which is then aspirated and sent for testing (Botha et al. 2008). The second specimen, endometrium tissue/biopsy, was collected as part of the routine diagnostic work-up or when menstrual fluid samples could not be collected. The tissue specimens were stored in a saline solution for further processing. The women which were being investigated for infertility had to only provide endometrial tissue specimen or menstrual fluid sample. Both samples (menstrual fluid and endometrial tissue) underwent testing as laid out in Figure 2.3.

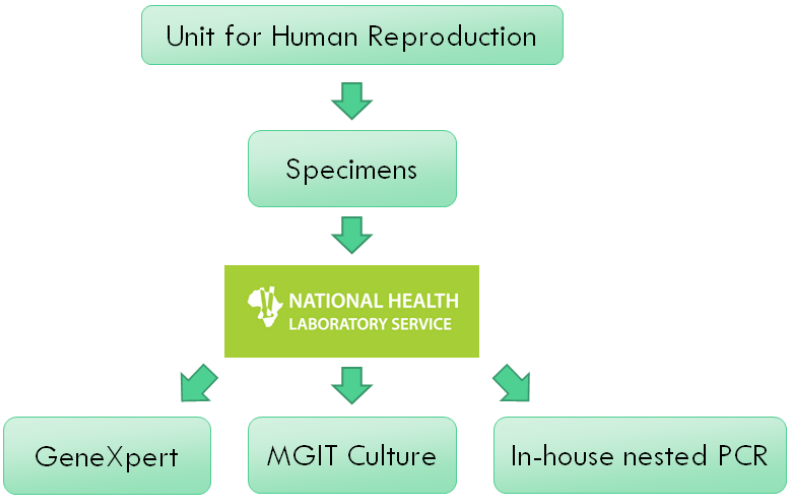

Figure 2.3 Flow diagram of the procedures followed for specimen processing

Samples were collected from the Unit for Human Reproduction. Samples included menstrual fluid specimens and endometrial tissue specimens. Each was processed for the GeneXpert, MGIT culture and the in-house nested PCR.

2.2.2 Specimen processing

The specimens were processed with the intention of optimising the yield of mycobacteria or DNA.

Menstrual fluid

Menstrual fluid specimens were first transferred to a 50ml centrifuge tube to be spun down in a centrifuge at 3000×g for 15min. The supernatant was discarded and the remaining pellet (approximately 5ml) was vortexed. The pellet was split for the 3 tests, mentioned in Figure 2.3, which are the GeneXpert, MGIT culture and the nested PCR.

Endometrial tissue

Endometrial tissue specimens were first processed with a tissue grinder. This step was done to release the mycobacteria trapped in the tissue specimen and the resulting homogenised mixture was split for the 3 tests as mentioned above.

2.2.3 Culture

Before samples could be cultured for MGIT they had to undergo decontamination, digestion and concentration inside a biosafety class 2 cabinet. Using the sodium hydroxide-N-acetyl-L-cysteine (NaOH-NALC) method for decontamination the specimens were transferred to a 50ml centrifuge tube. The NaOH-NALC-sodium citrate solution (4% concentration of NaOH) was added in a volume equal to the sample and this was vortexed lightly for 15-30s. After vortexing, the tubes were placed on a shaker and shaken for approximately 20min. Phosphate buffer (pH 6.8) was added up to the 50ml mark and lightly vortexed. This mixture was centrifuged at 3000×g for 20min with the tubes being allowed to settle for 5min. This was to allow aerosols to settle. The supernatant was carefully decanted into a waste container containing a mycobacterial disinfectant. Approximately 5ml of the pellet was left behind to be used for MGIT culture. Before the specimen could be inoculated for MGIT culture, the MGIT tubes had to first be prepared. The BBL MGIT PANTA antibiotic mixture was mixed with the 15ml BACTEC MGIT Growth Supplement and 0.8ml of the MGIT Growth Supplement/MGIT PANTA antibiotic mixture was aseptically added to the MGIT tubes. It was at this point that 0.5ml of the specimen was added to the MGIT tubes and mixed well. The inoculated MGIT tubes were then incubated in the BACTEC MGIT 960 System for 42 days or until a positive result was detected.

A purity check was performed on all positive MGIT tubes using the modified Kinyoun's staining procedure to ensure the growth of AFB as well as to determine the presence of contaminating bacteria. Approximately 1 to 2 drops of the MGIT culture was placed on glass slides and this was spread evenly with a sterile plastic loop. The slides were placed on a heated surface, in a metal container, for drying at approximately 80°C for 15 to 30min and the slide was then smeared with Kinyoun carbol fuchsin. The slides were then left to stand for 5min. The stain was washed off with clean water and excess water was removed by tilting the staining rack. The slides were then decolourised with a 3% v/v acid-alcohol for 2min with the slides being washed again with clean water and the excess was drained. The slides were then counterstained with methylene blue for 2min with the slides being rinsed with water for the final time and placed on paper towel which was then left to dry on a heating block. Finally, the dried slides were examined microscopically by making use of the 100× oil immersion lens. AFB should stain pink/red while non AFB should stain blue/purple. All positive GTB MGIT tubes had AFB that stained pink/red with no appearance of contaminating bacteria.

A TB antigen test was performed on MGIT tubes detected positive by the BACTEC MGIT 960 System using the BD MGIT™ TBc Identification Test (Becton Dickinson, Sparks, MD). Approximately 100µl of the MGIT culture was loaded in the cartridge inside a biosafety class 2 cabinet. The assay was left to incubate for 15min after which the results were ready to be interpreted. A pink/red band in the control area meant that the test was valid and a pink/red band by the test area meant that the MPT64

protein was detected (i.e. the result was positive for MTBC). The absence of a band in the test area meant that the isolate was likely a MOTT.

2.2.4 DNA extraction

For the nested PCR, 1ml of the menstrual fluid pellet or 1ml of the homogenised tissue mix was transferred into 2ml of NucliSENS® Lysis Buffer solution (bioMérieux, Marcy l'Etoile, France). This off-board lysis step was performed to lyse the bacteria and assist in releasing DNA for the NucliSENS® easyMAG® instrument (bioMérieux, Marcy l'Etoile, France). The NucliSENS® easyMAG® is an automated system that can extract nucleic acid using magnetic beads. The lysis step can either be performed before the specimen is loaded onto the instrument, which is termed off-board lysis, or on the instrument as part of the extraction protocol, which is termed on-board lysis. Both methods result in the bacteria being lysed before undergoing DNA extraction in the automated system.

The magnetic bead DNA extraction was then performed. Briefly, following off-board lysis the specimen was loaded into the sample vessel inside a biosafety class 2 cabinet. The sample vessel was loaded onto the instrument and the magnetic silica was added. The nucleic acid in the specimen was bound to the magnetic silica and was purified through a number of wash steps. The next step involved elution. To achieve efficient elution, the magnetic silica was mixed with final buffer. The final step involved purification, in which the magnetic beads were removed and the pure concentrated nucleic acid remained. The DNA was eluted in 25µl of elution buffer and this was carefully transferred into a 1.5ml microcentrifuge tube. The eluted DNA was stored at -20°C until the nested PCR was ready to be performed.

2.2.5 Preparation of positive control

A TB positive control needed to be prepared to ensure that the primers were in fact amplifying the *IS6110* from the MTB genome, as well as to assist in validating both the nested PCR and GeneXpert assays. The MTB H37Rv strain was chosen as it is the most commonly used strain for laboratory research (Bifani et al. 2000). The strain was obtained from the National Health Laboratory Service Universitas Academic Laboratories in Bloemfontein. The H37Rv strain is maintained and is available through the Trudeau Mycobacterial Collection (TMC) and the American Type Culture Collection (ATCC). The strain was sub-cultured in a MGIT tube using the following procedure. The BBL MGIT PANTA antibiotic mixture was mixed with the 15ml BACTEC MGIT Growth Supplement with 0.8ml of the MGIT Growth Supplement/MGIT PANTA antibiotic mixture being aseptically added to the MGIT tubes. Approximately 0.5ml of the H37Rv was added to the MGIT tube and was mixed well.

The MGIT tube containing the H37RV strain was incubated in the BACTEC MGIT instrument until a positive result was detected. A ZN stain was performed as a purity check to ensure that there was

TB growth and to ensure that there was no contaminating bacteria present before the positive MGIT tube underwent nucleic acid extraction. The ZN stain was performed using the modified Kinyoun's staining, following the same procedure used for the MGIT culture.

The positive MGIT tube underwent nucleic acid extraction using the NucliSENS easyMAG automated system as described above. The off-board lysis protocol was followed ensuring that the bacteria had already been lysed before entering the instrument. The extracted DNA concentration and purity was measured with the Thermo Scientific NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The NanoDrop can measure samples ranging from 0.5µl to 2µl in volume with high accuracy and reproducibility. For this project, a 2µl volume was used for all NanoDrop measurements.

The DNA of the TB positive control was stored at -20°C until required.

2.2.6 Nested PCR

2.2.6.1 DNA amplification of the human β-globin gene

To ensure that the DNA extraction was successful and to exclude PCR inhibition, the human β-globin gene was amplified as an internal control using a hemi-nested PCR with published primers (Table 2.2). The human β-globin gene forms part of the larger human β-globin gene locus which is found on chromosome 11. This locus consists of: ε-globin gene, γ-globin genes, δ-globin gene and the β-globin gene. Previous studies have used the β-globin gene as an internal control for human clinical samples. Successful amplification of the β-globin gene indicates DNA integrity as well as successful DNA extraction (Levings et al. 2002, Ritari et al. 2012, Coutlée et al. 2000).

Table 2.2 Human β-globin gene primers

Primers	Sequences	Direction	Tm (°C)	GC content (%)
PCO4	5'-CAACTTCATCCACGTTCACC-3'	Reverse	57	50
PCO3	5'-ACACAACTGTGTTCACTAGC-3'	Forward	56	45
KM29	5'-GGTTGGCCAATCTACTCCCAGG-3'	Forward	63	59

Tm and GC content were calculated using open source cloud computing from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

Table 2.3 shows the different primer combinations used to amplify the human β-globin gene and the expected amplicon size for each. For the hemi-nested PCR, the first round PCR used primers KM29/PC04 while the second round PCR used primers PC03/PC04 (see Appendix A). A hemi-nested PCR is a slight variation from the nested PCR in which 3 primers are used instead of 4, however the same principle of two rounds of amplification still applies.

Table 2.3 Primer combinations for human β-globin gene hemi-nested PCR

Primer combination	Amplicon size (bp)
KM29/PC04	205
PC03/PC04	110

The PCR reaction was performed using KOD Hot Start DNA Polymerase (Merck Millipore, Billerica, MA) according to the manufacturer's instructions. The components of the master mix for both first and second rounds of PCR are shown in Table 2.4.

Table 2.4 First and second round PCR master mix for human β-globin gene

Master Mix	Per 50µl reaction (µl)
Nuclease free H_2O	30
10x Buffer	5
25 mM $MgSO_4$	2
2 mM dNTP's	5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
KOD polymerase (1 U/µl)	1
Total	**45**

A volume of 5µl of template DNA was added to the master mix before undergoing PCR. A negative control was included with each PCR run. For the negative control, 5µl of nuclease free water was added to the reaction instead of template DNA.

Both first round and second round PCR followed the same thermal-cycling conditions as shown in Table 2.5.

Table 2.5 Thermal cycling conditions for the human β-globin gene hemi-nested PCR

Temperature (°C)	95	95	55*/60**	70	70	4
Time (min:sec)	2:00	0:20	0:10	0:15	5:00	∞
		30 cycles				

* indicates 55°C for the first round
** indicates 60°C for the second round

Both rounds of the hemi-nested PCR underwent agarose gel electrophoresis for visualisation of results as described in section 2.2.6.3.

2.2.6.2 Amplification of the IS6110 insertion sequence

To amplify the IS*6110* insertion sequence, two sets of primers were used following a nested PCR protocol. The outer primers, TBF1 (forward primer) and TBR1 (reverse primer), were designed using open source cloud computing from the National Center for Biotechnology Information (NCBI)

(https://www.ncbi.nlm.nih.gov/tools/primer-blast/) while the inner primers, TBF2 (forward) and TBR2 (reverse), were based on primers published by Choi *et al* (Choi et al. 2014). Both sets of primers were aligned with the IS*6110* (see Appendix B) using Clustal Omega multiple sequence alignment tool from European Molecular Biology Laboratory and European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/Tools/msa/clustalo/). Table 2.6 contains the primers for the IS*6110* insertion sequence and the anticipated amplicon size.

Table 2.6 IS*6110* insertion sequence primers

Primers	Sequence	Direction	Amplicon size	Tm(°C)	GC (%)
TBF1	5'-CGATGGCGAACTCAAGGA-3'	Forward	330bp	57	56
TBR1	5'-TAGGCGTCGGTGACAAAGG-3'	Reverse		60	58
TBF2	5'-GTCGAACGGCTGATGACCAAACT-3'	Forward	120bp	63	52
TBR2	5'-TCCGAAGCGGCGCTGGACGA-3'	Reverse		69	70

Tm and GC content were calculated using open source cloud computing from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

The PCR for amplifying the IS*6110* insertion sequence was performed following the KOD Hot start protocol and enzymes according to the manufacturer's instructions, the master mix of which is shown in Table 2.7.

Table 2.7 IS*6110* gene PCR master mix

Master Mix	Per 50µl reaction(µl)
Nuclease free water	30
10x Buffer	5
25 mM $MgSO_4$	2
2 mM dNTP's	5
Forward Primer (10 µM)	1
Reverse Primer (10 µM)	1
KOD polymerase (1 U/µl)	1
Total	**45**

A volume of 5µl of template DNA was added to the master mix before undergoing PCR. Positive and negative controls were included with each PCR run. For the positive control, 5µl of the TB positive control DNA described in section 2.2.5 was added as template. For the negative control, 5µl of nuclease free water was added to the reaction instead of template DNA.

Prior to the validation, the nested PCR was optimised by using the positive TB control to test the annealing temperature at various intervals. It was determined that to achieve optimal results, a first round annealing temperature of 55°C and a second round annealing temperature of 60°C would be optimum for the detection of the IS*6110*. Both first round and second round PCR followed the thermal-cycling conditions as indicated in Table 2.8.

Table 2.8 IS*6610* gene PCR thermal cycling conditions

Temperature (°C)	95	95	55*/60**	70	70	4
Time (min:sec)	2:00	0:20	0:10	0:15	5:00	∞
		30 cycles				

* indicates 55°C for the first round
** indicates 60°C for the second round

2.2.6.3 Agarose gel-electrophoresis

The TB nested PCR as well as the β-globulin human gene hemi-nested PCR amplicons underwent agarose gel-electrophoresis. A 1% Seakem® LE agarose gel (Lonza, Basel, Switzerland) was prepared in a 1× tris-acetate-disodiumethylenediaminetetraacetate (TAE) buffer at pH 8.0 (see Appendix D).

After the PCR was performed, 5µl of the PCR product was mixed with 1µl of a custom made loading dye containing GelRed™ (Biotium Inc, Fremont, CA) (see Appendix E).This mixture was loaded into the gel well. The ladder mix consisting of GeneRuler™ DNA Ladder Mix SM0331 (Merck Millipore, Billerica, MA) was mixed with the GelRed loading dye and loaded into the gel. The GeneRuler™ DNA Ladder Mix contains DNA fragments ranging from 100bp to 10000bp and was used to assist in determining the PCR amplicon sizes.

The process of gel electrophoresis was performed using BioRad PowerPac Basic Systems (BioRad, Hercules, CA) at constant 100V and approximately 400A for 35 minutes, followed by visualization with the Gel Doc XR+ System (BioRad, Hercules, CA).

2.2.7 GeneXpert® MTB/RIF

For both specimen types, 2ml of the GeneXpert Sample Reagent buffer was mixed with either 1ml of the concentrated menstrual fluid sample or 1ml of the homogenized tissue mix in a 50ml tube to give a 2:1 ratio inside a biosafety class 2 cabinet. The mixture was shaken vigorously and left to incubate for 10min at ambient temperature. After 10min, the tubes were shaken vigorously again and left to incubate for an additional 5min. Approximately 2ml of the processed sample was transferred into the GeneXpert cartridge which was then be loaded into the instrument.

2.2.8 Analytical Sensitivity (Limit of Detection)

The analytical sensitivity or LOD is defined as the lowest concentration at which the assay is able to detect a positive result for all replicates tested (Sales et al. 2014). With regards to the GeneXpert, while the instrument was designed extract DNA from the organism within the cartridge, for this project DNA was loaded into the instrument instead since this would allow for a more fair comparison of the LOD for both the nested PCR and the GeneXpert. It is noted that this could affect the LOD of the

GeneXpert however a similar procedure was performed Helb *et al* and their study found that the LOD for the GeneXpert (using genome DNA) was 4.5 genomes per reaction (Helb et al. 2010).

For both the nested PCR and the GeneXpert, the LOD was determined as follows. The TB positive control DNA was measured with the NanoDrop to determine the concentration which was given in µg/µl. The TB positive control was first diluted down to 1 ng/µl, from that point 10-fold serial dilutions were performed ranging from 1 ng/µl to 1fg/µl. The nested PCR and the GeneXpert were performed at these concentrations to determine the preliminary LOD. This was repeated 4 times and the preliminary LOD was the lowest concentration in which the TB positive control was detected 4 out of 4 times. Once the preliminary LOD was established, a 2 fold dilution was performed ranging down from the preliminary LOD. The final LOD was determined to be the lowest concentration of the 2-fold dilutions in which the TB positive control was detected 4 out of 4 times. While both rounds of the nested PCR were performed and visualised, the LOD was determined with the results obtained from the second round nested PCR only.

Because the nested PCR made use of 5µl of template, the same volume was added for the GeneXpert to ensure a similar comparison. A 5µl aliquot of TB positive control DNA was added to 2ml of the GeneXpert Sample Reagent buffer in a 10ml tube. The tube was shaken vigorously and were left to incubate for 10min at ambient temperature. After 10min, the tube were again shaken vigorously and incubated at ambient temperature for a further 5 min. The 2ml sample was then transferred to the GeneXpert cartridge and loaded into the instrument. This was performed for all dilutions and repeated 4 times.

Since 5µl of template was used per reaction, the following formula was used to calculate the weight of DNA that was loaded in the nested PCR and the GeneXpert:

$$DNA\ weight = Concentration\ of\ DNA \times 5\mu l$$

For both the nested PCR and the GeneXpert, the concentration of DNA was converted to the weight of DNA to assist in determining the LOD. While the DNA concentration ranged from 1ng/µl to 1fg/µl, the actual total TB DNA added to the reactions for the 10 fold dilutions therefore ranged from 5ng to 5fg using the above mentioned formula.

It has been determined that approximately 5fg of DNA is equivalent to 1 genome of MTB (Bhanu 2005, Boyle et al. 2014, Mehta et al. 2012). To calculate the molecular weight, a few basics need to be established. The average weight of a DNA bp = 650 daltons (Da) with 1 Da = 1.67 x 10^{-24}g. The molecular weight of double stranded DNA can be calculated with the following formulae obtained from Integrated DNA Technologies (Integrated DNA Technologies 2011):

$$Molecular\ weight(Da) = xbp \times 650Da$$

$$Molecular\ weight(g) = molecular\ weight(Da) \times (1.67 \times 10^{-24})$$

The MTB H37Rv genome contains 4,411,529bp, therefore using the above formulae it can be calculated that the molecular weight = 4,411,529bp × 650Da = 2,867,493,850Da. Since 1Da is equivalent to 1.67×10^{-24}g then the molecular weight can be determined as follows 2,867,493,850Da × 1.67×10^{-24}g = 4.788×10^{-15}g = 4.788fg (Cole et al. 1998, Burd 2010, Boyle et al. 2014).

2.2.9 Analytical specificity

The analytical specificity was defined as the ability of the assays to detect only the intended target (MTB) in the presence of other bacteria as well as closely related mycobacteria such as MOTTS. For this project, the analytical specificity was determined by performing the nested PCR and GeneXpert® MTB/RIF on the following bacteria which may be part of the vaginal biota: *Proteus mirabilis, Klebsiella oxytoca* and *Escherichia coli*. Furthermore the following MOTTs underwent testing to ensure that the assays did not detect closely related mycobacteria: *M. avium, M. intracellulare, M. gordonae* and *M. scrofulaceum* (Burd 2010, Boyle et al. 2014).

The MOTTs and other bacteria were obtained from the National Health Laboratory Service Universitas Academic Laboratories in Bloemfontein. The MOTTs were identified using the GenoType Mycobacterium CM (Hain Lifescience GmbH, Nehren, Germany). Similar to the GenoType MTBDRplus which is used to identify MTBC and its resistance to R and I, the GenoType Mycobacterium CM is used to identify MTBC as well as 27 other clinically relevant MOTTs. The other bacteria were isolates that have been stored by the laboratory for academic purposes. These bacteria were streaked onto MacConkey agar plates (to prevent the growth of Gram positive bacteria) and were incubated overnight at 37°C. Following incubation the colonies were transferred into 2ml NucliSENS Lysis Buffer solution following the off-board lysis protocol. The bacterial nucleic acid was extracted using the NucliSENS easyMAG automated system. The extracted DNA concentration and purity was measured with the NanoDrop and the DNA was stored at -20°C until it was needed for the GeneXpert and nested PCR validation. Similar to the TB positive control preparation (described in section 2.2.5), the MOTTs were first sub cultured in MGIT tubes and were incubated in the BACTEC MGIT 960 instrument until a positive result was detected. The positive MGIT tubes containing the MOTTs underwent nucleic acid extraction using the NucliSENS easyMAG automated system. The offboard lysis protocol was followed ensuring that the MOTTs had already been lysed before entering the instrument. The MOTT DNA concentration was measured with the NanoDrop to determine the DNA concentration and purity. The MOTT DNA was stored at -20°C until it was needed for the validation of both the nested PCR and the GeneXpert. For the nested PCR, 5µl of DNA was added per PCR reaction for each of the organisms. For the GeneXpert, 5µl of DNA was added to 2ml of the GeneXpert Sample Reagent buffer and processed as for the TB control DNA.

2.2.10 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 project, using MGIT culture as the reference method, diagnostic sensitivity was measured by determining if a test (GeneXpert or nested PCR) could detect GTB in clinical samples that MGIT culture was able to detect. The following formula was used:

$$Sensitivity = \frac{true\ positive}{true\ positive + false\ negative}$$

Diagnostic specificity is defined as the ability of a test to accurately identify people who do not have the disease as being disease free. For this project, similar to diagnostic sensitivity, MGIT culture was used as the reference method. Diagnostic specificity was measured by determining if a test (GeneXpert or nested PCR) could successfully not detect GTB in clinical samples that MGIT culture determined as negative for GTB. The following formula was used:

$$Specificity = \frac{true\ negative}{true\ negative + false\ positive}$$

The prevalence of GTB was determined as the percentage of GTB positive samples from the samples submitted for GTB screening.

2.2.11 Predictive Values

The PPV is defined as the percentage of patients who tested positive that actually have the disease. The closer the PPV is to 100% the closer it is to the gold standard. The PPV for this project was measured for both the GeneXpert and the nested PCR using the following formula:

$$PPV = \frac{true\ positive}{true\ positive + false\ positive}$$

Similar to the PPV the NPV is defined as the percentage of patients who tested negative that actually don't have the disease. The closer the NPV is to 100% the closer it is to the gold standard. The NPV for this test, similar to the PPV, was measured for both the GeneXpert and the nested PCR using the following formula:

$$NPV = \frac{true\ negative}{true\ negative + false\ negative}$$

2.2.12 Cohen's Kappa

As explained in section 2.1, κ is the measurement of agreement between two raters with agreement by chance being factored out. Cohen's formula is as follows:

$$\kappa = \frac{\rho_o - \rho_e}{1 - \rho_e}$$

In this project κ was calculated using the Microsoft Excel (Microsoft Corporation, Redmond, WA) spreadsheet program. The spreadsheet calculation formula was self-designed using Cohen's formula and using Charles Zaiontz idea of performing statistics calculations on Microsoft Excel (Zaiontz 2015).

For this project κ was used to determine if the GeneXpert and the nested PCR results agree with MGIT culture as the reference method. Table 2.1 was used to interpret the result obtained from determining κ.

2.3 Results

2.3.1 Specimen collection

In total, during the study period of July 2015 to August 2016, 54 samples were received from the Unit for Human Reproduction. Of the 54 samples that were tested, 10 samples were menstrual fluid specimens while 44 samples were endometrial tissue specimens. The median age of the women being investigated was 32 years (ranging from 23 to 42 years).

2.3.2 Culture

MGIT culture detected 2 positive samples from the 54 that were screened. Table 2.9 contains the results of the types of specimens that were received and screened for GTB. A total of 10 menstrual fluid specimens were received and screened with only one specimen being culture positive. A total of 44 endometrial tissue specimens were received and screened with only one specimen being detected as GTB positive with MGIT culture.

Table 2.9 Types of specimen screened for genital tuberculosis

Specimen screened	Total specimen screened	GTB Positive
Menstrual fluid	10	1
Endometrial tissue	44	1

The TB antigen test was performed on MGIT cultures that were positive by the BACTEC MGIT 960 System using the BD MGIT™ TBc Identification Test. Both MGIT positive samples tested were identified as MTBC with the antigen test.

2.3.3 Nested PCR

A positive result on the nested PCR can be seen with a band of expected size appearing on the agarose gel after electrophoresis. All the samples had the human β-globin gene amplified to determine if the DNA extraction was successful. A positive result was indicated by a band appearing

at approximately the 205bp region for the first round hemi-nested PCR, seen in Figure 2.4, and at approximately the 110bp region for the second round of the hemi-nested PCR which is seen in Figure 2.5. The human β-globin gene was successfully amplified for all 54 samples, which indicates that the DNA extraction was a success and that there were no inhibitors present.

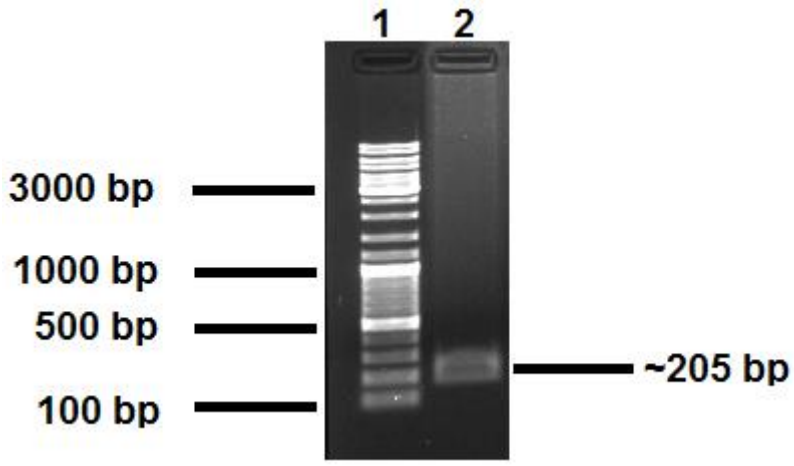

Figure 2.4 Human β-globin gene first round amplification

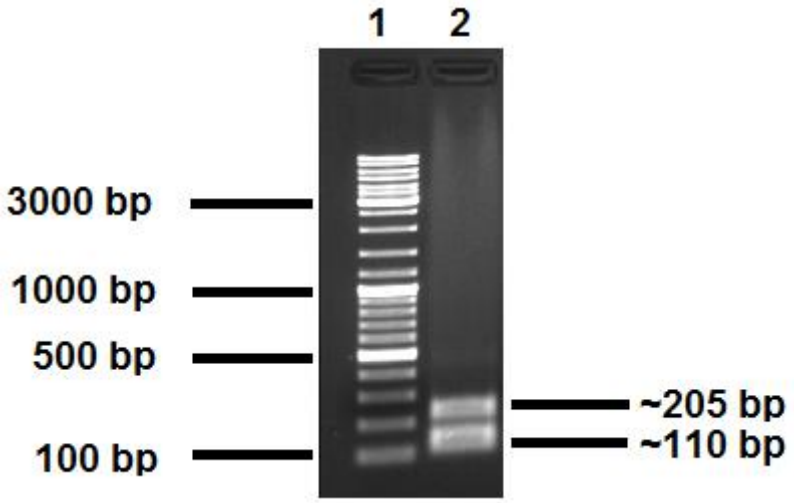

Figure 2.5 Human β-globin gene second round

Gel images of the heminested PCR conducted to detect the human β-globin gene. Figure 2.4 is a gel image of the first round heminested PCR. A successful amplification of the human β-globin in the first round will result in a product size of approximately 205bp. Figure 2.5 is a gel image of the second round heminested PCR. A successful amplification of the human β-globin in the second round will result in a product size of approximately 110bp. For both the first round and second round heminested PCR gels for the human β-globin gene, the GeneRuler™ DNA Ladder was chosen to assist in estimating the PCR product sizes. For both Figure 2.4 and Figure 2.5 the lanes contained the following: 1) GeneRuler™ DNA Ladder. 2) Human β-globin gene.

For the nested PCR targeting the IS*6110* insertion sequence, a first round positive result will have a band appearing at approximately 320bp while a second round positive result will have a band appearing at approximately 120bp. Figure 2.6 is an example of how a positive first round result would appear on an agarose gel. Figure 2.7 shows a positive second round result.

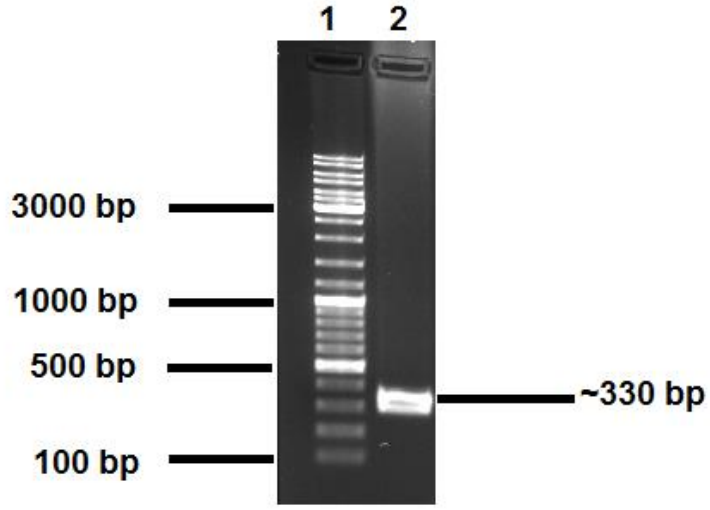

Figure 2.6 Agarose gel electrophoresis of 1st round nested PCR

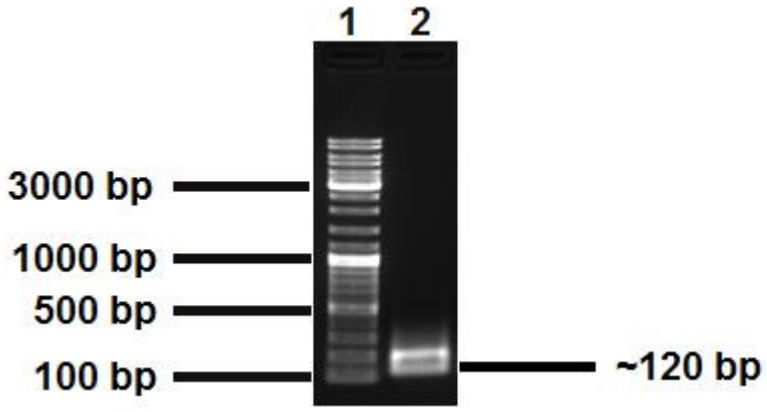

Figure 2.7 Agarose gel electrophoresis of 2nd round nested PCR

The gel images are of the IS*6110* nested PCR. Figure 2.6 is an image of the first round nested PCR gel. Successful amplification of the IS*6110* will result in a first round product size approximately 330bp. Figure 2.7 is an image of the second round nested PCR gel with the second round product size approximately 120bp. *The* GeneRuler™ DNA Ladder was chosen to assist in estimating the PCR product sizes. For both Figure 2.6 and Figure 2.7 the lanes contained the following: 1) GeneRuler™ DNA Ladder. 2) TB IS*6110* positive control.

Of the 44 tissue specimens, only 1 tested positive with the IS*6110* nested PCR, while only 1 of the 10 vaginal wash samples tested positive.

2.3.4 Analytical Sensitivity (Limit of Detection) and Specificity for the nested PCR

The TB positive control was used to determine the LOD of the nested PCR. Figure 2.8 is a gel image of the 10-fold serial dilution with the DNA template ranging from 500pg to 5fg. The preliminary LOD was determined as the lowest concentration in which the nested PCR was able to detect the TB

positive control. The preliminary LOD was determined to be 500fg, as was confirmed with the assay being repeated 4 times and achieving the same result.

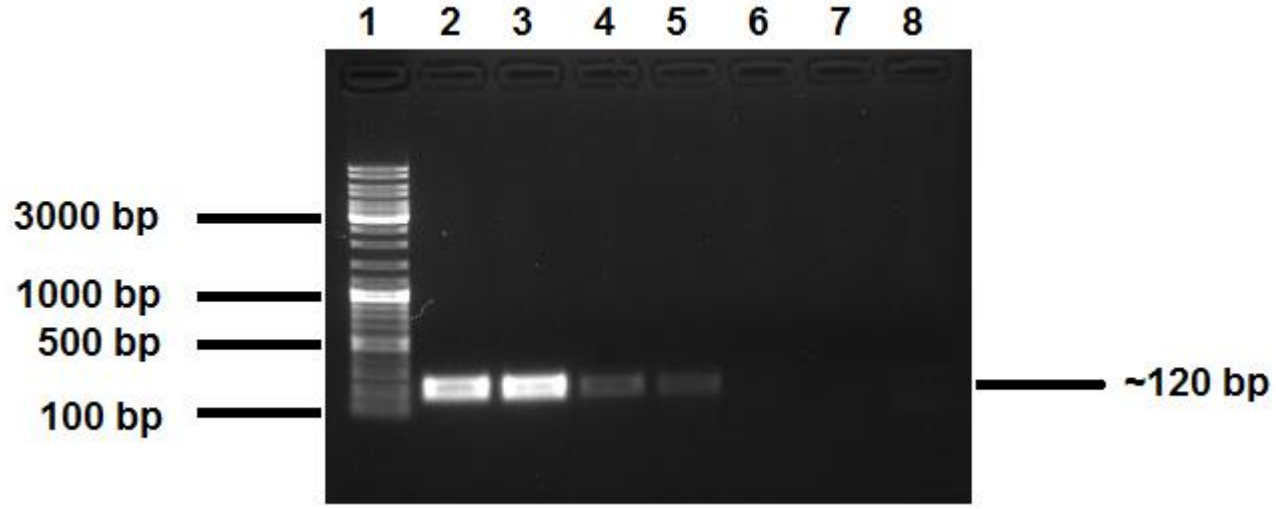

Figure 2.8: Agarose Gel Electrophoresis of the 10-fold dilution validation

The TB positive control DNA underwent a 10-fold serial dilution from 5ng to 5fg to determine the preliminary limit of detection for the nested PCR. The lanes marked were: 1) GeneRuler™ DNA Ladder. 2) 500pg. 3) 50pg. 4) 5pg. 5) 500fg. 6) 50fg. 7) 5fg. 8) Negative control.

Table 2.10 contains the results of the 10 fold serial dilution of the nested PCR as well as the preliminary LOD.

Table 2.10 Limit of detection for IS*6110* nested PCR 10 fold serial dilution

DNA amount	**500pg**	**50pg**	**5pg**	**500fg**	**50fg**	**5fg**
IS*6110* positive/tested	4/4	4/4	4/4	4/4 Preliminary LOD	3/4	1/4

To determine the final LOD, a 2-fold serial dilution was performed starting from the preliminary LOD. Based on the results obtained from the 10 fold dilution, the final LOD would be between 500fg and 50fg per reaction. Figure 2.9 is a gel image of the 2-fold serial dilution ranging from 500fg to 15.6fg of DNA.

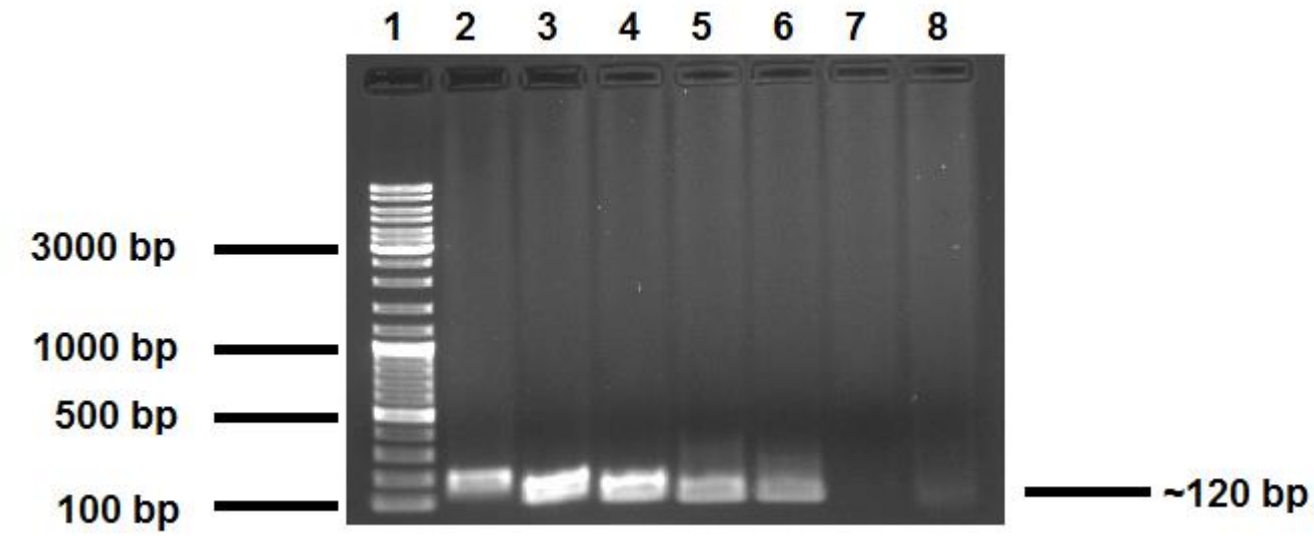

Figure 2.9 Agarose Gel Electophoresis 2-fold dilution validation

The TB positive control DNA underwent a 2-fold serial dilution from 500fg to 5fg to determine the final LOD for the nested PCR. The lanes marked were: 1) GeneRuler™ DNA Ladder. 2) 500fg. 3) 250fg. 4) 125fg. 5) 62.5fg. 6) 31.3fg. 7) 15.6fg. 8) Negative control.

The final LOD was determined as the lowest DNA dilution at which the nested PCR targeting the IS*6110* insertion sequence was positive in all 4 reactions. It was found by performing the 2 fold dilution that the final LOD was 62.5fg which is equivalent to approximately 12 MTB genomic equivalents as shown in Table 2.11.

Table 2.11 Limit of detection for IS*6110* nested PCR two fold serial dilution

DNA amount	**500fg**	**250fg**	**125fg**	**62.5fg**	**31.3fg**	**15.6fg**	**5fg**
IS*6110* positive/tested	4/4	4/4	4/4	4/4 LOD	2/4	0/4	1/4

The analytical specificity was determined by ensuring that the TB positive control was the only sample that was detected by the nested PCR. The first part of the specificity validation was the MOTTs (Figure 2.10). The nested PCR included the TB positive control and was tested against: *M. avium, M. intracellulare, M. gordonae* and *M. scrofulaceum*. The nested PCR was successful in detecting only the TB positive control and not the closely related MOTTs.

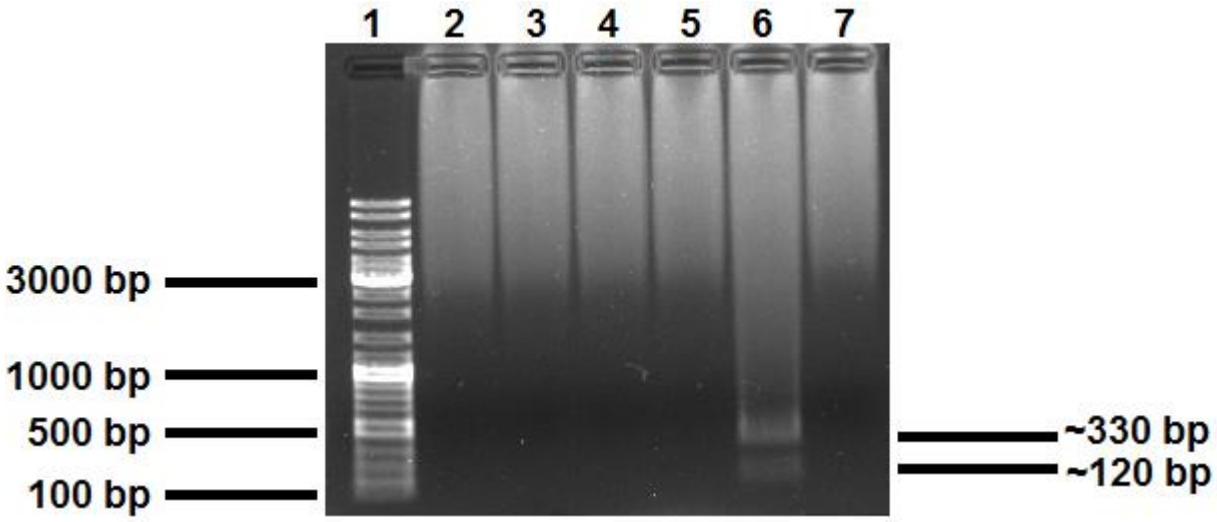

Figure 2.10 Second round nested MOTT validation

The nested PCR underwent a MOTT validation to ensure that the assay was able to discriminate TB positive control DNA from the closely related MOTTs. The lanes: 1) GeneRuler™ DNA Ladder. 2) *M. avium*. 3) *M. intracellulare*. 4) *M. gordonae*. 5) *M. scrofulaceum*. 6) MTB control. 7) Negative control.

The second part of the specificity validation was the other bacteria that form part of the vaginal biota (Figure 2.11). The nested PCR included the TB positive control and was tested against: *Proteus mirablis, Klebsiella oxytoca* and *Escherichia coli*. The nested PCR was successful in detecting only the TB positive control and not the bacteria that formed part of the vaginal biota.

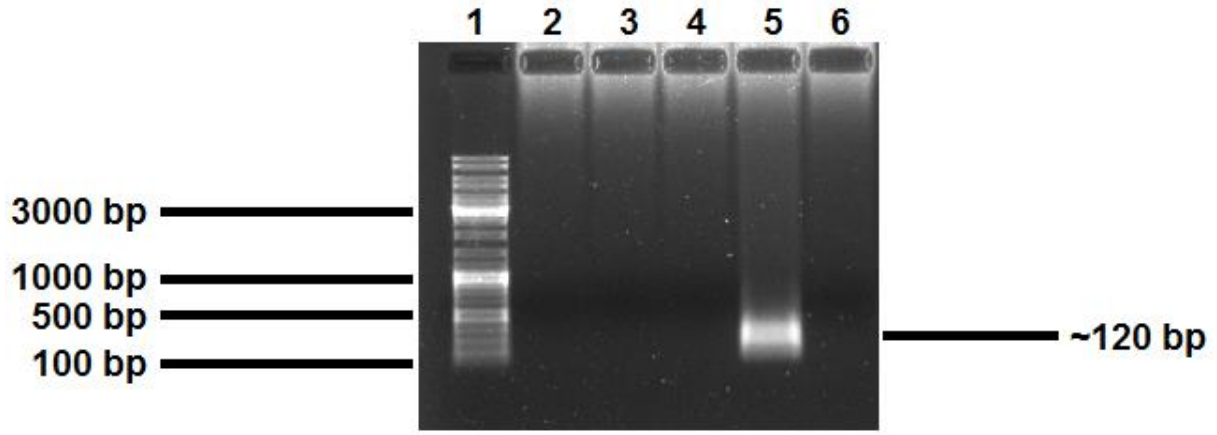

Figure 2.11 Second round nested bacterial validation

The nested PCR underwent other bacteria validation to ensure that the assay was able to discriminate TB positive control DNA from the other bacteria that form part of the vaginal biota. The lanes: 1) GeneRuler™ DNA Ladder. 2) *Proteus mirabilis*. 3) *Klebsiella oxytoca*. 4) *Escherichia coli*. 5) MTB. 6) Negative control.

The nested PCR was successful in discriminating TB DNA from all the MOTTs and bacteria tested. The results from both Figure 2.10 and Figure 2.11 indicate that the nested PCR displayed an excellent analytical specificity.

2.3.5 GeneXpert® MTB/RIF

The GeneXpert instrument releases the following possible results: MTB Not Detected; MTB Detected: R resistance not detected; MTB Detected: R resistance detected; and MTB Detected: R resistance indeterminate. Table 2.12 describes the results generated by the instrument and the interpretation of these results. Briefly, a “MTB Not Detected” was interpreted as a negative result while “MTB Detected” (irrespective of its resistance to the R antibiotic) was interpreted as a positive result.

Table 2.12 GeneXpert result interpretation

GeneXpert Result	Interpretation
MTB Not Detected	Negative result
MTB Detected: rifampicin resistance not detected	Positive result
MTB Detected: rifampicin resistance detected	Positive result
MTB Detected: rifampicin resistance indeterminate	Positive result

Of the 54 samples that were screened for GTB with the GeneXpert, the assay only detected 1 menstrual fluid sample as positive for TB. Although R resistance was not the main focus of this study, the specimen which tested positive with the GeneXpert was not R resistant.

2.3.6 Analytical Sensitivity (Limit of Detection) and Specificity for the GeneXpert

The GeneXpert validation was performed by determining the LOD and the analytical specificity. The LOD was determined by first performing a 10-fold serial dilution of TB positive control DNA, followed by a 2-fold serial dilution to determine the final LOD.

Table 2.14 shows the results obtained from the LOD validation. The preliminary LOD for the GeneXpert was determined to be 500pg of TB positive control DNA, as this was the smallest amount of DNA in the 10-fold serial dilution in which the GeneXpert instrument was able to consistently display a positive result.

Table 2.13 GeneXpert limit of detection 10 fold serial dilution

DNA amount	5ng	500pg	50pg	5pg	500fg
IS*6110* positive/tested	4/4	4/4 Preliminary LOD	2/4	1/4	0/4

The 2-fold serial dilution was performed from 250pg of DNA to 62.5pg of DNA to determine the final LOD for the GeneXpert. The final LOD of the GeneXpert was determined to be 250pg of DNA. The LOD for the GeneXpert can be seen in Table 2.14.

Table 2.14 GeneXpert limit of detection two fold serial dilution

DNA amount	**250pg**	**125pg**	**62.5pg**
***IS6110* positive/tested**	4/4 LOD	3/4	3/4

The analytical specificity of the GeneXpert was determined as to whether the instrument can successfully discriminate TB DNA from other bacterial DNA as well as MOTT DNA. Table 2.15 describes the bacterial and MOTT DNA used to determine the analytical specificity of the GeneXpert as well as the result. The GeneXpert was successful in discriminating TB DNA from other bacterial and closely related MOTT DNA.

Table 2.15 GeneXpert analytical specificity

Test	**Result**
M. avium	MTBC Not detected
M. intracellulare	MTBC Not detected
M. gordonae	MTBC Not detected
M. scrofulaceum	MTBC Not detected
Proteus mirabilis	MTBC Not detected
Klebsiella oxytoca	MTBC Not detected
Escherichia coli	MTBC Not detected

2.3.7 Diagnostic sensitivity and specificity

The diagnostic sensitivity and specificity was determined on both the GeneXpert and the nested PCR by using MGIT culture as the reference method. This was done to determine the suitability of these methods for detecting GTB in clinical samples.

The GeneXpert detected GTB in 1 of the 54 samples that were screened, furthermore the GeneXpert determined the sample was susceptible to rifampicin. The nested PCR, similar to MGIT culture, detected GTB in 2 of the 54 samples that were screened. Table 2.16 displays the results obtained from screening GTB from clinical samples using MGIT culture, GeneXpert and the nested PCR.

Table 2.16 Diagnostic screening for female genital tuberculosis

Sample name	Age	Sample type	TB antigen	β-globin	MGIT culture	GeneXpert	Nested PCR
AE1/GTB_1	23	Fluid	MTBC	Present	Positive	Positive	Positive
E1	39	Fluid	N/A	Present	Negative	Negative	Negative
E4	34	Tissue	N/A	Present	Negative	Negative	Negative
E5	31	Tissue	N/A	Present	Negative	Negative	Negative
E6	32	Tissue	N/A	Present	Negative	Negative	Negative
E7	30	Tissue	N/A	Present	Negative	Negative	Negative
E8	29	Tissue	N/A	Present	Negative	Negative	Negative
E9	42	Tissue	N/A	Present	Negative	Negative	Negative
E10	31	Tissue	N/A	Present	Negative	Negative	Negative
E11	34	Fluid	N/A	Present	Negative	Negative	Negative
E12	33	Tissue	N/A	Present	Negative	Negative	Negative
E13	35	Tissue	N/A	Present	Negative	Negative	Negative
E14	29	Fluid	N/A	Present	Negative	Negative	Negative
E15	35	Tissue	N/A	Present	Negative	Negative	Negative
E16	41	Tissue	N/A	Present	Negative	Negative	Negative
E17	26	Tissue	N/A	Present	Negative	Negative	Negative
E18	39	Fluid	N/A	Present	Negative	Negative	Negative
E19	34	Tissue	N/A	Present	Negative	Negative	Negative
E20	32	Tissue	N/A	Present	Negative	Negative	Negative
E21	28	Tissue	N/A	Present	Negative	Negative	Negative
E22	32	Tissue	N/A	Present	Negative	Negative	Negative
E23	39	Tissue	N/A	Present	Negative	Negative	Negative
E24	32	Tissue	N/A	Present	Negative	Negative	Negative
E25	32	Tissue	N/A	Present	Negative	Negative	Negative
E26	36	Tissue	N/A	Present	Negative	Negative	Negative
F1	24	Fluid	N/A	Present	Negative	Negative	Negative
F2	35	Tissue	N/A	Present	Negative	Negative	Negative
F3	34	Tissue	N/A	Present	Negative	Negative	Negative
F4	38	Tissue	N/A	Present	Negative	Negative	Negative
F5	29	Tissue	N/A	Present	Negative	Negative	Negative
F6	24	Fluid	N/A	Present	Negative	Negative	Negative
F7	24	Tissue	N/A	Present	Negative	Negative	Negative
F8	24	Tissue	N/A	Present	Negative	Negative	Negative
F9	30	Tissue	N/A	Present	Negative	Negative	Negative
F10	33	Fluid	N/A	Present	Negative	Negative	Negative
F11	35	Tissue	N/A	Present	Negative	Negative	Negative
F12	34	Tissue	N/A	Present	Negative	Negative	Negative
F13	36	Tissue	N/A	Present	Negative	Negative	Negative
F14	40	Tissue	N/A	Present	Negative	Negative	Negative
F15	37	Fluid	N/A	Present	Negative	Negative	Negative
F16	33	Tissue	N/A	Present	Negative	Negative	Negative
F17	31	Fluid	N/A	Present	Negative	Negative	Negative
F18	31	Tissue	N/A	Present	Negative	Negative	Negative
F19	23	Tissue	N/A	Present	Negative	Negative	Negative
F20	36	Tissue	N/A	Present	Negative	Negative	Negative
F21/GTB_2	24	Tissue	MTBC	Present	Positive	Negative	Positive
F22	40	Tissue	N/A	Present	Negative	Negative	Negative
F23	37	Tissue	N/A	Present	Negative	Negative	Negative
F24	32	Tissue	N/A	Present	Negative	Negative	Negative
F25	29	Tissue	N/A	Present	Negative	Negative	Negative
F26	33	Tissue	N/A	Present	Negative	Negative	Negative

G1	26	Tissue	N/A	Present	Negative	Negative	Negative
G2	31	Tissue	N/A	Present	Negative	Negative	Negative
G3	37	Tissue	N/A	Present	Negative	Negative	Negative

Table 2.17 shows the results obtained for the diagnostic sensitivity and specificity for both the GeneXpert and the nested PCR. The calculations are shown in Appendix F. In short, the results show that the diagnostic sensitivity and specificity for the GeneXpert were 50% and 100% respectively, while the diagnostic sensitivity and specificity for the nested PCR were 100%.

Table 2.17 Diagnostic sensitivity and specificity for genital tuberculosis screening

Diagnostic test	Diagnostic sensitivity (%)	Diagnostic specificity (%)
GeneXpert	50	100
Nested PCR	100	100

Using the reference method, 2 of the 54 samples tested positive for MTB indicating a prevalence of GTB in this study of approximately 3.7%.

2.3.8 Predictive values

The PPV and NPV for both the GeneXpert and the nested PCR were determined as follows: the GeneXpert achieved a PPV of 100% and a NPV of 98.1% while the nested PCR achieved a PPV of 100% and a NPV of 100%.The full calculations are shown in Appendix F.

2.3.9 Cohen's Kappa

Cohen's kappa was used to determine if the GeneXpert and the nested PCR results agree with the MGIT culture (with the agreement by chance factored out). The result, seen in Figure 2.12, indicates that the κ for the GeneXpert and MGIT culture (i.e. level of agreement) is at 0.66. The GeneXpert and MGIT culture results therefore show a "moderate" level of agreement.

	A	B	C	D	E
1	**Cohen's Kappa**				
2					
3		**MGIT culture**			
4	**GeneXpert**	Positive	Negative	Total	
5	Positive	1	0	1	
6	Negative	1	52	53	
7	Total	2	52	54	
8					
9	p_o (observed agreement)			0,981481	
10	p_e (agreement by chance)			0,945816	
11					
12					
13	κ	0,658228			
14					

Figure 2.12 Cohen's kappa calculated for the GeneXpert and MGIT culture on a Microsoft Excel spreadsheet

κ is the measurement of agreement between two raters with agreement by chance being factored out. κ upper limit is at +1 while its lower limit is at -1. κ=0 means that agreement is by chance and κ=+1 means that there is total agreement. Using Jacob Cohen's formula, κ was calculated with Microsoft Excel.

The result, seen in Figure 2.13, indicates that the κ for the nested PCR and MGIT culture (i.e. level of agreement) is at 1. The nested PCR and MGIT culture therefore have a "near perfect" level of agreement.

	A	B	C	D	E
1	**Cohen's Kappa**				
2					
3		**MGIT culture**			
4	**Nested PCR**	Positive	Negative	Total	
5	Positive	2	0	2	
6	Negative	0	52	52	
7	Total	2	52	54	
8					
9	p_o (observed agreement)			1	
10	p_e (agreement by chance)			0,928669	
11					
12					
13	κ	1			
14					

Figure 2.13 Cohen's kappa calculated for the nested PCR and MGIT culture on a Microsoft Excel spreadsheet

κ is the measurement of agreement between two raters with agreement by chance being factored out. κ upper limit is at +1 while its lower limit is at -1. κ=0 means that agreement is by chance and κ=+1 means that there is total agreement. Using Jacob Cohen's formula, κ was calculated with Microsoft Excel.

2.4 Summary

Molecular techniques have been gaining popularity in terms of rapid pathogen identification and diagnosis. This holds true for TB diagnosis and MTBC identification. While traditional techniques such as smear microscopy and TB culture will remain in the TB diagnostic laboratory in the foreseeable future, these techniques have their drawbacks that can be a hindrance to rapid and accurate EPTB diagnosis. Microscopy, while it is rapid and cost effective, fails to detect EPTB from clinical samples since it requires a high bacterial load and EPTB samples are paucibacillary. TB culture is the gold standard of TB diagnosis but the long turnaround time makes culture undesirable. Molecular techniques may therefore solve the problem that is facing EPTB diagnosis with increased accuracy and rapidity.

A total of 54 samples were received from the Unit for Human Reproduction, including 44 endometrial tissue specimens and 10 menstrual fluid specimens. All samples underwent GTB screening with MGIT culture, nested PCR and GeneXpert® MTB/RIF. The samples were processed with the goal of increasing the recovery of MTB using concentration by centrifugation for menstrual fluid samples and homogenization with a tissue grinder for the endometrial tissue samples.

The nested PCR was designed to target the *IS6110* insertion sequence. This target was chosen since it is only found in members of the MTBC which are known to be pathogenic in humans. The outer primers for this assay were designed using open source cloud based software while the inner primers were based on previously published primers (Choi et al. 2014). To ensure that the DNA extraction was successful and to ensure there were no PCR inhibitors present, the human β-globin gene was chosen as an internal control. Successful amplification of the β-globin gene for all samples confirmed DNA integrity as well as successful DNA extraction.

Both the nested PCR and the GeneXpert underwent validation to determine their analytical sensitivity (LOD) and analytical specificity. The LOD for both the techniques was performed using H37Rv DNA which underwent serial dilutions with the LOD being the lowest amount of the DNA that the assay could successfully detect in all replicates. The LOD for the nested PCR was determined to be 62.5fg. With a single MTB genome weighing approximately 5fg, the nested PCR can therefore reliably detect 12 MTB genomes per reaction. The lowest dilution of 5fg, which is one MTB genome equivalent, was successfully detected in 25% of reactions. The LOD for the GeneXpert was determined to be 250pg this means that the GeneXpert can detect approximately 50,000 MTB genomes. This LOD is significantly higher than that determined in previous studies, this is likely due to the low number of replicates run and the definition of LOD used in this study which was the DNA input at which there was 100% probability of a positive assay. It should be noted that the GeneXpert LOD was determined with genomic DNA instead of whole organism as originally designed. It is possible that this slight modification in determining the LOD could explain the obtained results. The nested PCR displayed

a better LOD when compared to the GeneXpert, indicating that the nested PCR is more likely to detect TB from paucibacillary samples than the GeneXpert.

The analytical specificity of both the GeneXpert and the nested PCR was performed to ensure that both techniques could discriminate TB DNA from other bacterial DNA and MOTTs. Both the nested PCR and the GeneXpert were successful in discriminating TB DNA from other DNA. This indicates that both techniques display excellent analytical specificity and that a false positive from any DNA other than TB DNA is unlikely.

MGIT culture detected 2 of the 54 samples as positive for GTB, indicating a prevalence of 3.7% in this study population. The nested PCR obtained similar results, achieving a diagnostic sensitivity of 100% and a diagnostic specificity of 100%. The GeneXpert only reported 1 of the 54 samples as positive for GTB, yielding a sensitivity of 50% and specificity of 100%. The better performance of the nested PCR compared to the GeneXpert in detecting GTB from clinical samples is likely due the nested PCR having a better LOD.

Predictive values were utilised to assist in determining which method was best at detecting GTB from clinical samples. In short, the closer the predictive value is to 100% the closer it is to the gold standard. The GeneXpert obtained a PPV of 100% and a NPV of 98.1%. The nested PCR achieved both a PPV and NPV of 100%. It can be seen from the predictive values that the nested PCR performed slightly better than the GeneXpert, although both molecular techniques are close to the gold standard. The high negative predictive value for both the GeneXpert and nested PCR was the result of the low GTB prevalence found in this study. A higher prevalence would result in a decreased NPV meaning that negative results would start to become less reliable.

While the nested PCR performed better than the GeneXpert, the drawback of the nested PCR is that this method requires specialised laboratory facilities and equipment while the GeneXpert was designed for the purposes of TB diagnosis in laboratories lacking such facilities. Furthermore, since the collection of menstrual fluid specimens is a relatively non-invasive procedure, this combined with the versatility of the GeneXpert means that GTB screening can be performed on a larger scale in resource limited areas.

Chapter 3 Characterisation of TB strains responsible for genital TB using spoligotyping and mycobacterial interspersed repetitive unit-variable number of tandem repeats (MIRU-VNTR) typing.

3.1 Introduction

MTB is considered to be a medically important pathogen because of the millions of deaths that have resulted from TB infection. TB has gained more attention in modern times due to the HIV co-epidemic as well as the emergence of antibiotic resistant MTB strains (Allix-Beguec et al. 2008). With molecular techniques becoming more common in the TB laboratory, there has been an emergence of molecular based epidemiological studies of MTB. The purpose of these studies is the genotyping of MTBC strains. With this information researchers can distinguish between a relapse and a reinfection, detect or exclude laboratory errors, detect unrecognized outbreaks and determine the transmission of strains in secondary infections. Furthermore genotyping has assisted in unravelling the strains that make up the MTBC by determining the differences in the phylogenetic lineages. These can be further characterised by differences in virulence, immunogenicity, drug resistance as well as their global distribution (Allix-Beguec et al. 2008, Pang et al. 2012). Commonly used MTB genotyping techniques include spoligotyping, MIRU-VNTR and IS*6110* RFLP. IS*6110* RFLP is considered the gold standard for the genotyping of MTB however the technique is labour intensive and requires the culturing of MTB which is time consuming. Furthermore, IS*6110* RFLP requires a substantial copy number of the IS*6110* and there have been reports of some MTB strains lacking the IS*6110* (Oelemann et al. 2007, Mathuria et al. 2008, Howard et al. 1998).

Both spoligotyping and MIRU-VNTR are PCR based techniques designed for the simultaneous detection of MTB as well as the typing of MTB, with both methods being considerably less labour intensive compared to IS*6110*-RFLP (Mathuria et al. 2008).

Spoligotyping is a PCR based technique that can detect and type MTBC from culture and clinical samples by making use of the DR region found exclusively in the MTBC genome. The MTBC genome contains identical conserved DRs which are 36bp in length. The number of DRs can vary between strains with MTB H37Rv containing 48 DRs while *M. bovis* BCG contain 49 DRs. In 2000, van Embden *et al* described 51 novel spacers in addition to the 43 standard spacers allowing for a total of 94 spacers that can be used to further identify strains of MTB isolates. Interspersed within the DRs are non-repetitive spacer sequences which can vary from 34 to 41bp in length. Different strains have been known to vary in the number of DRs as well as the absence or presence of certain spacers. Most MTB strains contain one or more IS*6110* within the DR region (see Figure 1.5). The process of spoligotyping detects the absence or presence of 43 spacers. This is done by detecting whether the spacers have hybridised to the set of synthetic immobilised oligonucleotides on a

membrane (van Embden et al. 2000, Kamerbeek et al. 1997, Ocimum Biosolutions 2016, Mathuria et al. 2008). Spoligotyping has identified 36 potential subfamilies of the MTBC. Some of the most common spoligotypes include: Beijing, LAM, Haarlem and X (Filliol et al. 2002, Brudey et al. 2006).

MIRU-VNTR, similar to spoligotyping, is a PCR based technique that can detect as well as type MTBC (Mathuria et al. 2008, Supply et al. 1997). MIRU was originally described by Supply and colleagues in 1997, who found that there were 40 to 100bp tandem repeats which were spread throughout the MTB genome particularly in 41 loci. Twelve loci were shown to be excellent for the genotyping of MTB (Supply et al. 1997). MIRU-VNTR works by amplifying the specific MIRU loci by PCR. The copy number of each MIRU locus is used to differentiate MTB strains. While the original MIRU-VNTR made use of twelve loci, progress has seen an increase in the loci number with recommendations for the use of 15 MIRU loci and 24 MIRU loci. The 24 MIRU loci has been recommended for phylogenetic studies and consists of the 15 MIRU loci and 9 additional loci. The original 12 MIRU loci were used for MTBC strains that had low copy numbers of IS*6110* instead of IS*6110* RFLP, however IS*6110* RFLP had better discriminating power for strains with a high IS*6110* copy number. However, as more loci were included in MIRU-VNTR, this resulted in an increase in its discriminating power. The 24 MIRU loci assay is currently recommended for Beijing strains (Kato-Maeda et al. 2011, Narayanan et al. 2015, Jagielski et al. 2014, Allix-Beguec et al. 2008).

The 24 MIRU loci, when combined with spoligotyping, has been shown to have the same discriminating power as IS*6110* RFLP which has long been considered the gold standard of MTBC genotyping. Recently there have been a growing number of researchers that have determined that the 24 MIRU-VNTR is the new gold standard for MTBC genotyping replacing IS*6110* RFLP (Jonsson et al. 2014, Jagielski et al. 2014, Narayanan et al. 2015).

Figure 3.1 contains data which was obtained from the freely accessible SITVITWEB database http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/description.jsp# and shows the distribution of spoligotypes in South Africa. The SITVITWEB database is an online database containing genotyping information from over 62,000 MTBC clinical isolates, from over 153 countries. The database is managed by the Institut Pasteur de Guadeloupe (Demay et al. 2012). The database includes the percentage of the spoligotype distribution. Using the SITVITWEB database, the most common spoligotypes found in South Africa are (in decreasing order): LAM (22.71%), Beijing (19.08%), X (15.45%), T (13.55%) and S (5.23%) with the rest of the spoligotypes making up the remaining 12.8%.

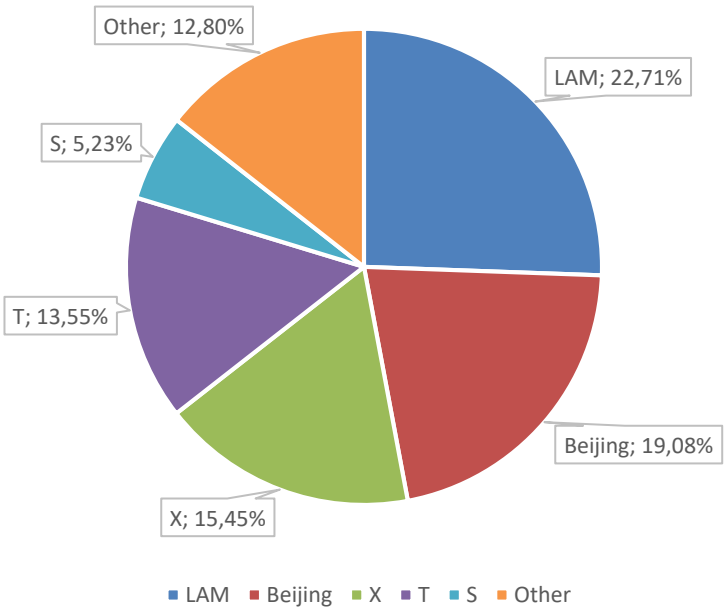

Figure 3.1 Prevalence of spoligotypes in South Africa

The spoligotypes include: AFRI, Beijing, CAS, EAI, Haarlem, LAM, S, T and X. From this figure it can be seen that the most common spoligotypes found in Southern Africa region include the LAM, Beijing, X, T and Haarlem (data obtained from SITVITWEB database http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/description.jsp#).

A study conducted by Stavrum *et al.* in 2009 determined that the T spoligotype was the most common MTB strain in South Africa, making up 25.8% of isolates. Furthermore, the same study found that the T strain was also the most prevalent strain in the Free State province making up 32% (Stavrum et al. 2009). However Van der Spoel van Dijk *et al.* in 2016 found that it is the LAM strain that is the most prevalent strain in the Free State province at approximately 33%, while the T strain was at 31% (Van der Spoel van Dijk et al. 2016). Insufficient information exists concerning the genotyping of GTB isolates. Most studies discussing the genotyping of GTB describe transmission of congenital TB, which is rare considering GTB in most cases results in infertility. Flibotte *et al.* in 2013 described 5 cases of congenital TB in which genotyping was used to determine if the mothers and babies shared the same TB strain to confirm a congenital TB diagnosis. The study isolated different strains from the 3 mothers, namely *M. africanum*, EAI and EA. These cases were identified in North America however the mothers originated from India (Flibotte et al. 2013). This lack of information regarding which strains are associated with GTB is one of the reasons why more studies involving the genotyping of GTB isolates are necessary.

3.2 Materials and Methods

MIRU-VNTR and spoligotyping were used to identify the MTBC strains collected during the project and to determine which strains of MTBC are responsible for GTB. Since spoligotyping and MIRU-

VNTR required specialised facilities and equipment, both of these techniques were performed at the Centre for Tuberculosis (CTB) at the National Institute for Communicable Diseases (NICD) in Johannesburg.

3.2.1 Sub-culturing of MTB for genotyping

For genotyping assays to be performed, both GTB isolates that were positive with MGIT culture were sub-cultured in MGIT tubes and underwent purity checks. The 2 GTB samples were sub-cultured from the positive MGIT tubes using the same method employed for culturing the TB positive control. Approximately 0.5ml of the MGIT positive GTB sample was added to the MGIT tubes and mixed well. The MGIT tubes were then incubated in the BACTEC™ MGIT™ 960 instrument until a positive result was detected (Siddiqi et al. 2006). Following the sub-culturing of MGIT tubes, the GTB isolates underwent ZN purity check to ensure that no contaminating bacteria was present in the MGIT tubes. As with previous ZN staining techniques, the modified Kinyoun's staining was performed.

DNA extraction of the GTB MGIT sub-cultured isolates was performed with the NucliSENS® easyMAG® as described previously. The off-board lysis protocol was performed to ensure that the GTB was lysed before undergoing nucleic acid extraction. The DNA elution volume used was 75µl and this was carefully transferred into a 1.5ml microcentrifuge tube. The eluted DNA had its purity and concentration checked with the NanoDrop and was stored at -20°C until the MIRU-VNTR and spoligotyping were ready to be performed. The DNA from the GTB isolates was later transferred to the CTB at the NICD where the equipment and facilities necessary for the MIRU-VNTR and spoligotyping were available.

3.2.2 Spoligotyping

The primers DRa and DRb were used to amplify the spacers found within the DR region. Since the primer DRa is biotinylated, this resulted in the PCR products being labelled with biotin. Sequences of the primers are as follows; DRa (GGTTTTGGGTCTGACGAC, 5' biotinylated) and DRb (CCGAGAGGGGACGGAAAC). Approximately 20ng/µl or less of chromosomal MTB DNA was required for the PCR. (Kamerbeek et al. 1997).

The PCR for the spacer DNA was performed using the PCR reaction master mix as described in Table 3.1. DNA from both MTB H37Rv and *M. bovis* BCG strains were included as controls. The PCR was performed in a 96 well plate.

Table 3.1 PCR master mix for spoligotyping

Master Mix	**Per 50µl reaction (µl)**
Template DNA	5
DRa primer (20 pmol)	4
DRb primer (20 pmol)	4
dNTP mixture	4
10x Super T^{th} buffer	5
Super T^{th} polymerase (5U/µl)	0.1
Nuclease free water	27.9

A drop of mineral oil was added to prevent evaporation during amplification with PCR thermal cycling conditions set as described in Table 3.2.

Table 3.2 Thermal cycling conditions for spoligotyping

Temperature (°C)	96	96	55	72	72	4
Time (min:sec)	3:00	1:00	1:00	0:30	5:00	∞
		25 cycles				

With the PCR product hybridization step, the biotin-labelled PCR products were hybridized to spacer oligonucleotides immobilized on a membrane. The membrane contains 43 spacer oligonucleotides and the absence or presence of the spacers was used to genotype the MTBC.

The following buffers needed to be prepared from the concentrated stock solution and diluted with de-mineralized water:

- 2×SSPE/0.1% SDS at 42°C
- 2×SSPE/0.5% SDS at 60°C
- 2×SSPE/0.5% SDS at 42°C
- 2×SSPE at room temperature

The stock solution was obtained from Ocimum Biosolutions (Ocimum Biosolutions Ltd., Hyderabad, India).

Approximately 150 µl of 2×SSPE/0.1% SDS was mixed with 20µl of the PCR product. The diluted PCR product was then heat denatured at 99°C for 10min followed by quick cooling on ice. This was done to ensure the DNA was single stranded prior to hybridization. The membrane was washed in 250 ml of 2×SSPE/0.1% SDS at 60°C for 5min. The membrane and support cushion were placed in the miniblotter in such a manner that the channels of the miniblotter were perpendicular to the rows of the immobilized oligonucleotides. Residual fluid was removed from the slots of the miniblotter by aspiration. The channels were filled with the diluted PCR products and left to hybridize for 60min at

60°C on a flat horizontal surface. After hybridization the membrane was carefully removed using forceps and washed twice in 250ml of 2×SSPE/0.5% SDS at 60°C for 10 min. The membrane was placed in a rolling bottle and left to cool before the next step which involved inactivation of the peroxidase. 2.5 µl of streptavidin-peroxidase conjugate (500 U/ml) was mixed with 10 ml of 2×SSPE/0.5% SDS, which was incubated with the membrane for 60 min at 4°C in the rolling bottle. The membrane was then washed twice in 250 ml of 2×SSPE/0.5% SDS at 42 °C for 10min. Lastly the membrane was rinsed twice in 250 ml of 2×SSPE at room temperature for 5 min.

Detection of the hybridized DNA was performed through chemiluminescence. The membrane was incubated in 20ml of ECL detection liquid for 1 min. The membrane was then exposed to an X-ray film for approximately 1min. The presence of black squares on the developed X-ray film is indicative of the presence of spacers.

Using the standardised nomenclature proposed by Dale *et al.* in 2001, the project made use of the rational systems in which the pattern of the 43 spacers are represented in a binary form with 1 as a positive spacer and 0 as a negative spacer. This binary code could then be shortened by converting the binary code into either the octal code or hexadecimal format. The octal code was used for this project (Dale et al. 2001). The binary code was reported manually while the conversion of the binary code to the octal code was performed using the SPOLDB4 Database http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/index.jsp maintained by the Institut Pasteur de Guadeloupe (Brudey et al. 2006).

3.2.3 MIRU-VNTR

24 locus MIRU-VNTR was also used for the genotyping of the MTB. The DNA was obtained from MGIT sub-cultured GTB positive isolates as described previously (GenoScreen 2011).

Results from the MIRU-VNTR can be analysed manually through agarose gel electrophoresis or through a Genetic DNA analyser by fragment analysis. For this project the MIRU-VNTR PCR analysis was conducted with the Applied Biosystems™ 3500 Series Genetic Analyzer (Applied Biosystems, Foster City, CA). This allowed the use of a multiplex PCR with each quadruplex targeting 4 different MIRU loci (GenoScreen 2011). Table 3.3 contains the 6 quadruplex panels, the different MIRU markers as well as their respective aliases.

Table 3.3 MIRU-VNTR 24 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

(GenoScreen 2011)

Plate loading

Approximately 8µl of each quadruplex mix with 2µl of template DNA (including positive controls) was loaded into the 96-well plate based on the PCR spreadsheet in Table 3.4. Two positive controls were used, namely MTB H37Rv strain and *M. bovis* BCG strain.

Table 3.4 MIRU-VNTR PCR 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 1	S-7	Ctrl 1	S-7	Ctrl 1	S-7	Ctrl 1	S-7	Ctrl 1	S-7	Ctrl 1	S-7
B	Ctrl 2	S-8	Ctrl 2	S-8	Ctrl 2	S-8	Ctrl 2	S-8	Ctrl 2	S-8	Ctrl 2	S-8
C	S-1	S-9	S-1	S-9	S-1	S-9	S-1	S-9	S-1	S-9	S-1	S-9
D	S-2	S-10	S-2	S-10	S-2	S-10	S-2	S-10	S-2	S-10	S-2	S-10
E	S-3	S-11	S-3	S-11	S-3	S-11	S-3	S-11	S-3	S-11	S-3	S-11
F	S-4	S-12	S-4	S-12	S-4	S-12	S-4	S-12	S-4	S-12	S-4	S-12
G	S-5	S-13	S-5	S-13	S-5	S-13	S-5	S-13	S-5	S-13	S-5	S-13
H	S-6	S-14	S-6	S-14	S-6	S-14	S-6	S-14	S-6	S-14	S-6	S-14

Crtl 1 – 1st control (H37Rv)
Ctrl 2 – 2nd control (BCG)
S – Samples (1 to 14)

The 96-well plate was sealed with an adhesive PCR film and was spun down before being loaded into a thermal cycler making use of the thermal cycling conditions described in Table 3.5.

Table 3.5 Thermal cycling conditions for MIRU-VNTR

Temperature (°C)	95	94	59	72	72	4
Time (min:sec)	15:00	1:00	0:30	1:30	10:00	∞
		40 cycles				

After thermal cycling, the PCR products were protected from light and stored at 4°C until capillary electrophoresis could be performed.

Fragment Analysis

Fragment analysis was performed on the 3500 Series Genetic Analyzer following the manufacturer's instructions as well as the guidelines in the GenoScreen MIRU-VNTR Typing kit. A new master mix was prepared consisting of 9.5µl of HiDi Formamide (Applied Biosystems, Foster City, CA) and 0.5µl GeneScan™ 1200 LIZ® (Applied Biosystems, Foster City, CA) per well. A 96-well plate was loaded with 10µl of the master mix per well. The 96-well plate containing the PCR products was centrifuged to prevent cross contamination when the adhesive film was removed. The PCR film was removed and 2µl of the PCR products was added to the sequencing plate containing the master mix, using the same layout as for the PCR reaction. The sequencing plate was sealed with an adhesive film and the plate was centrifuged to remove air bubbles. DNA was denatured by placing the plate on the thermal cycler set at 95°C for 5min, after which the plate was immediately placed on ice. Lastly capillary electrophoresis was performed as described in the ABI manual. The GeneMapper® software (Applied Biosystems, Foster City, CA) was used to analyse the results of the fragment analysis.

3.3 Results

For the characterisation, the samples were given the following identification; GTB_1 – menstrual fluid specimen, GTB_2 – endometrial tissue specimen, BCG – *M. bovis* BCG control and H37Rv – MTB control.

3.3.1 Spoligotyping

As previously described, the spoligotyping results were exposed on an X-ray film (see Appendix C). The spoligo pattern was first manually converted into a binary code (1 for a spacer and 0 for an absent spacer). The SPOLDB4 Database uses a different binary code, where instead of 1 and 0, n indicates a present spacer and o indicates an absent spacer. This binary code was then converted to an octal code which, when combined with the MIRU-VNTR, was used to characterise the MTB

isolate. Table 3.6 contains the spoligotyping results as well as the SPOLDB4 identification for the two GTB isolates, the BCG and H37Rv controls.

Table 3.6 Spoligo pattern and octal code

Sample	Spoligo pattern and binary code	Octal code	SPOLDB4*
GTB_1	0000000000000000000000000000000000111111111	000000000003771	Beijing (1)
GTB_2	11100000000011111011111111111111000011111111	700076777760771	X3 (92)
BCG	11011111011111101111111111111111111100000	676773777777600	BOVIS1_BCG (482)
H37Rv	11111111111111111110011111111111000011111111	777777477760771	H37Rv (451)

*SPOLDB4 contains the spoligotype (according to SPOLDB4) as well as the spoligo-international-type number (SIT)

3.3.2 MIRU-VNTR

For the MIRU-VNTR, fragment analysis was performed on the 3500 Series Genetic Analyzer and the GeneMapper® software was used to analyse the results of the fragment analysis. The results contain the 24 loci MIRU-VNTR copy number for each isolate that was genotyped. These results were exported into an excel spreadsheet (Figure 3.2).

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
1	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
2	GTB_1	2	4	4	2	3	3	3	5	2	8	4	2	4	2	5	1	7	3	3	5	3	2	2	4
3	GTB_2	2	4	3	2	4	4	3	3	2	9	3	4	4	2	5	1	5	3	3	3	4	10	3	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	2
5	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

Figure 3.2 MIRU-VNTR 24 loci copy number

The 24 loci MIRU-VNTR results for both GTB isolates as well as the controls. Briefly GTB_1 – menstrual fluid, GTB_2 – endometrial tissue, BCG – control and H37Rv – control.

The results from the MIRU-VNTR and spoligotyping were combined using the MIRU-VNTR*plus* web application http://www.miru-vntrplus.org/MIRU/index.faces (Weniger et al. 2010). The final combined result (Figure 3.3), contains the strain name, 24 MIRU loci copy number and the spoligo pattern for each sample that was screened. Furthermore other reference strains were included in the analysis for comparison. These strains were obtained from MIRU-VNTR*plus* web application database and SPOLDB4 Database which include: *M. bovis*, EAI, S, LAM, Beijing, X1, Haarlem, T, AFRI and CAS.

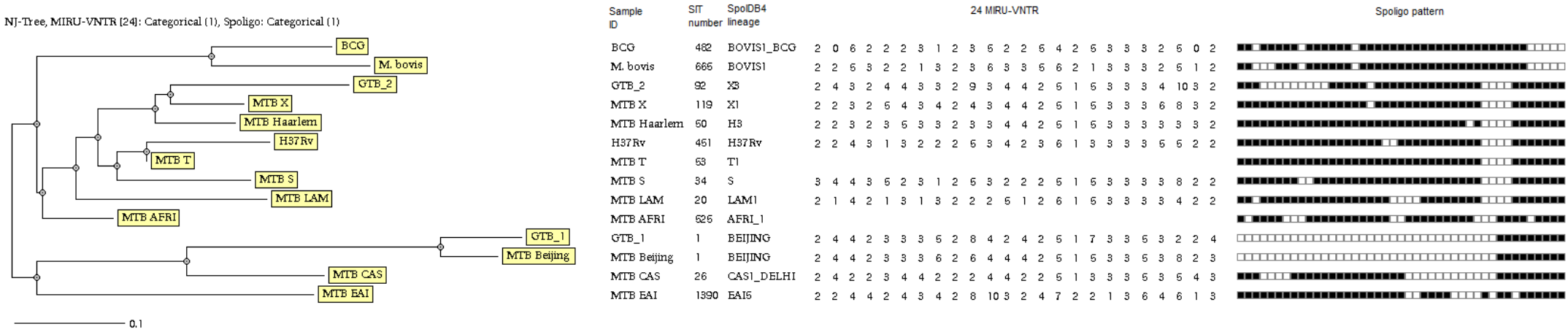

Figure 3.3 MIRU-VNTR and Spoligotyping results

The results from the MIRU-VNTR and spoligotyping were combined using the MIRU-VNTRplus web application http://www.miru-vntrplus.org/MIRU/index.faces. The genotyping involved the 2 samples from the project (GTB_1 and GTB_2) and 2 controls (H37Rv and BCG). Other MTB reference strains were included in the tree for comparison, these included *M. bovis*, EAI, S, LAM, Beijing, X1, Haarlem, T, AFRI and CAS. The figure contains (from left to right) a phylogenetic tree (constructed with the neighbour-joining clustering method/NJ), the isolate ID name, SpolDB4 type (SIT number), SpolDB4 Lineage, 24 MIRU loci copy number and the spoligo pattern.

From the combined results of the MIRU-VNTR and spoligotyping (Figure 3.3), it can be seen that both GTB_1 and GTB_2 were successfully genotyped and identified. GTB_1, which was isolated from a menstrual fluid sample, was identified as a Beijing strain. GTB_2, which was isolated from an endometrial tissue sample, was identified as an X3 strain. Based on the information obtained from the SITVIT database, the Beijing and X strains are known to be prevalent in South Africa.

3.4 Summary

As a result of the HIV epidemic as well as the emergence of drug resistant MTB strains, TB has in recent times gained more attention. This has resulted in an array of molecular techniques aimed at detecting MTB, determining the antibiotic susceptibility, and performing molecular based epidemiological studies. Genotyping of MTB has given researchers the tools necessary to identify outbreaks as well as the ability to map out strains. The most common techniques used for the genotyping of MTBC include IS*6110* RFLP, as well as the PCR based MIRU-VNTR and spoligotyping (Jonsson et al. 2014, Jagielski et al. 2014, Narayanan et al. 2015).

In this chapter, the two GTB isolates that were positive with MGIT culture underwent 24 locus MIRU-VNTR and spoligotyping. Using online databases, the spoligo pattern obtained for the GTB isolates was converted to an octal code. This code combined with the results obtained from the MIRU-VNTR was used to genotype the GTB isolates.

Both isolates were successfully genotyped, yielding a Beijing strain from the menstrual fluid isolate and an X3 strain from the endometrial tissue isolate. With only two isolates, it is not possible to conclusively determine which strains of MTB are responsible for GTB in South Africa, however the MIRU-VNTR and spoligotyping did provide valuable information by revealing that the X3 and Beijing strains are associated with GTB. A study conducted by Kong *et al.* found that the Beijing spoligotype is commonly associated with EPTB (Kong et al. 2007). Patients infected with Beijing isolates were three times more likely to have an extra-thoracic site of infection than those infected with non-Beijing isolates. The results obtained from this study therefore confirm the information found in the literature that the Beijing strain is commonly associated with EPTB.

It should be noted that GTB has not received much attention from researchers and available data regarding this condition is limited. This could be due in part to GTB being rare in countries that have the facilities necessary to carry out large scale studies and epidemiological investigations. GTB has a high prevalence in developing countries which are known to lack the facilities and funding to carry out large scale screening for GTB in infertile women.

Chapter 4 Discussion

TB is a disease that is a result of MTB infection. Clinical manifestations of TB infection include PTB and EPTB. Some commonly affected EPTB sites include the central nervous system, gastrointestinal organs and the genitalia (GTB). Culture remains the definitive method for diagnosing TB from clinical samples. EPTB samples are in most cases paucibacillary, which often presents a diagnostic challenge, hence a negative laboratory test may not be sufficient to rule out a diagnosis. A rapid technique such as microscopy may not be sensitive enough for the detection of EPTB. Molecular based techniques such as PCR offer the promise of a more rapid detection of MTB from clinical samples. Most molecular based techniques that have been used in the diagnosis of TB display high specificity but generally have a low sensitivity with EPTB samples. The high specificity indicates that molecular techniques can be used as a rapid technique to detect TB However, the low sensitivity suggests that molecular tests should be accompanied by another test to rule out TB (Lee 2015, Dinnes et al. 2007, Linasmita et al. 2012).

In this study, women being investigated for infertility were screened for GTB. A total of 54 samples were received from the Unit for Human Reproduction at Universitas Academic Hospital, including 10 menstrual fluid and 44 endometrial tissue specimens. All the samples were tested with the GeneXpert® MTB/RIF assay, an in-house nested PCR and cultured in MGIT tubes.

Culture detected 2 of the 54 samples as positive for GTB, including 1 endometrial tissue sample and 1 menstrual fluid sample. This indicates a prevalence of GTB in this study of 3.7%. Previous studies investigating TB in female infertility patients in South Africa found a prevalence ranging from 6.2% to 21.0% (Oosthuizen et al. 1990, Margolis et al. 1992). The low prevalence obtained in this study could be a result of this being a small study that was conducted within a set timeframe resulting in small sample number.

Culture remains the gold standard for TB diagnosis and this study has confirmed that culture should be used for GTB screening. However, culture has a notoriously long turnaround time (7 to 21 days). In this study the turnaround time for culture was 14 days for the menstrual fluid sample and 19 days for the endometrial tissue sample. Ideally culture should therefore be accompanied by a more rapid assay.

The GeneXpert which is a commercial molecular based assay, can detect TB as well as its susceptibility to R within 2 hours. It was not until 2013 that the WHO recommended the use of GeneXpert for EPTB samples. This recommendation was based on a meta-analysis of available data. This implementation of GeneXpert testing has assisted in the rapid detection of EPTB especially for TB meningitis which is a life threatening condition. However, this recommendation does not extend to urine, blood and stool specimens, and no guidelines have been issued regarding

the use of GeneXpert testing for the diagnosis of GTB (Denkinger et al. 2014, World Health Organization 2013, Lee 2015).

Based on the GeneXpert Report published by the National Health Laboratory Service in October 2016, the GeneXpert is currently deployed in 207 centres throughout South Africa. These centres are found in both urban and rural areas. Up until 31 October 2016, 9,695,971 specimens have been processed and screened with the GeneXpert in South Africa. Approximately 10.45% of these specimens were positive for MTBC, with only 6.9% of these being R resistant (National Health Laboratory Service 2016).

For this study, the GeneXpert was only able to detect MTB in 1 of the 54 genital samples. The positive sample was a menstrual fluid specimen. This suggests a role for the GeneXpert for diagnosis of GTB but a larger sample size is needed to confirm its routine use. Using MGIT culture as a reference method, the GeneXpert obtained a diagnostic sensitivity of 50% and a diagnostic specificity of 100%. In South Africa, a study on EPTB diagnosis with the GeneXpert conducted by Scott *et al.* in 2014, obtained an overall sensitivity of 59% and a specificity of 92%. Their work involved screening specimens such as pus, aspirate, fluids, tissue biopsies and CSF. Interestingly both this study and Scott's obtained similar sensitivities and specificities although Scott had a larger sample size of 7,916 specimens (Scott et al. 2014).

Sharma *et al.* in 2016, performed a study to determine whether the GeneXpert will be useful for diagnosing GTB in infertile women in India. The authors investigated 240 infertile women with only 7 being detected by the GeneXpert as positive for GTB. In this population, the GeneXpert obtained a sensitivity of 46.6% and a specificity of 100%. Furthermore all of the GeneXpert positive samples were rifampicin susceptible (Sharma et al. 2016). This is similar to the result obtained in the current study with none of the samples positive for GTB being rifampicin resistant by the GeneXpert. However in a separate study Sharma *et al.* in 2017 identified MDR TB in 6 women who were screened for GTB. Sharma commented that while the GeneXpert can only determine rifampicin resistance, the GenoType MTBDR*plus* can determine both rifampicin and isoniazid resistance (Sharma et al. 2017). The choice of assay will therefore depend on the particular patient population and will be influenced by factors such as the prevalence of drug resistant TB.

The nested PCR has previously been used for the diagnosis of TB from clinical samples. One of the characteristics of the nested PCR is its increased sensitivity due to additional second round amplification. Miyazaki *et al.* in 1993, achieved a 1,000 fold increase in the sensitivity of their PCR (targeting MTB from clinical samples) after performing the second round of amplification (Miyazaki et al. 1993). Liu *et al* in 2007, designed an in-house nested PCR targeting the IS*6110* for the diagnosis of TB pleurisy and obtained a sensitivity of 43.4% and a specificity of 95.5%. Furthermore

the PPV was 82% and the NPV was 77.7% (Liu et al. 2007). Similarly, in this study an in-house nested PCR was designed targeting the IS*6110*. The insertion sequence was chosen since it is restricted to members of MTBC which are pathogenic in humans and is generally highly conserved among strains. During the screening for GTB, the nested PCR detected 2 of the 54 samples as positive for GTB. These results were identical to the results obtained by MGIT culture. The nested PCR therefore displayed a diagnostic sensitivity of 100% and a diagnostic specificity of 100%. The nested PCR has a turnaround time of 24 to 48 hours and while this is longer than the GeneXpert, it is considerably shorter when compared to MGIT culture. While the high sensitivity and specificity indicate that the nested PCR demonstrated a similar performance to MGIT culture, the results obtained could be influenced by the small sample size. However, the results do indicate that the nested PCR was more sensitive than the GeneXpert.

The two molecular diagnostic techniques used in this study underwent validation to determine the LOD (analytical specificity) and analytical sensitivity. The LOD for the GeneXpert was at 250pg of DNA which is equivalent to 50,000 MTB genomes. Helb *et al.* determined that the LOD for the GeneXpert, using genomic DNA, was 4.5 MTB genomes (Helb et al. 2010). While the LOD for the GeneXpert is higher than what Helb achieved, the results do explain why the GeneXpert was only able to identify only 1 sample as positive for GTB. The false negative result obtained for the endometrial tissue sample was most likely due to the low bacterial load that was present in the sample. EPTB samples are known to be paucibacillary. Hillemann *et al.* in 2011, found that of all the EPTB samples that were screened with the GeneXpert, the tissue samples displayed a lower sensitivity compared to the other samples that were being investigated (Hillemann et al. 2011, Theron et al. 2014). Another possible cause for the false negative result obtained in this study could be that the isolate lacked target site for the GeneXpert assay within the *rpoB* gene which is the (Kim et al. 2014). To investigate this further, the isolate in question was cultured and underwent GeneXpert testing. The GeneXpert displayed a positive result with susceptibility towards rifampicin, indicating that the false negative result was not a consequence of variation in the *rpoB* target site. It can be concluded that the only limitation of the GeneXpert in this study was the LOD which resulted in a suboptimal sensitivity. It should be noted that the LOD for the GeneXpert was determined by using genomic DNA instead of whole organism. This refers to the fact that the GeneXpert was designed to extract DNA from whole organisms within the provided cartridge. This could explain why the GeneXpert has a higher LOD when compared to the nested PCR.

The nested PCR displayed a LOD of 62.5fg of DNA which is equivalent to 12 MTB genomes. There is much variation in the LODs obtained for different molecular techniques designed for the detection of TB. Kulkarni *et al.* in 2012, achieved an LOD of 1fg for their PCR targeting the *IS6110* (Kulkarni et al. 2012). In 2005, Kulkarni *et al.* designed a PCR that could detect a 38kDa protein from MTB

with a LOD of 10fg or 2 MTB genomes (Kulkarni et al. 2005). While the nested PCR had a higher LOD compared to the above mentioned studies, the nested PCR was still able to detect GTB from 1 sample more than the GeneXpert. The sensitivity of the nested PCR played a role in the better performance of the assay when compared to the GeneXpert. As EPTB samples are paucibacillary, this would lend credence to the technique with a good LOD being better suited to detecting TB from these samples. It should be noted that even though the nested PCR performed better than the GeneXpert, the results obtained indicate that both the GeneXpert and nested PCR can detect GTB from clinical samples

Statistical analysis was performed to assist in determining which method is best suited to detect GTB from clinical samples in the study population. The tools used included the PPV, NPV and Cohen's Kappa. Briefly, the predictive values for the GeneXpert were PPV of 100% and NPV of 98.1%. The nested PCR obtained a PPV of 100% and a NPV of 100%. Similar to the sensitivity, the nested PCR displayed an improved performance compared to the GeneXpert. Using Cohen's Kappa, the GeneXpert and MGIT culture obtained a 0.66 level of agreement, indicating a moderate level of agreement. The nested PCR and MGIT culture had a level of agreement of 1 indicating a near perfect agreement. Similar to previous statistical analysis, the nested PCR performed better than the GeneXpert. However both methods meet the threshold that was set by McHugh of 0.60 (McHugh 2012). This means that confidence can be placed in the results obtained by the nested PCR and GeneXpert particularly in the context of the low prevalence of GTB in the study population.

Lastly, the GTB isolates that were culture positive were sub-cultured and underwent characterisation by spoligotyping and MIRU-VNTR at the National Institute for Communicable Diseases in Johannesburg. Genotyping revealed that the 2 MTB isolates were identified as a Beijing strain for the menstrual fluid isolate and an X3 strain for the endometrial tissue isolate. The Beijing strain is the second most prevalent strain in South Africa with the X strain being the third most prevalent strain based on data obtained from the SITVITWEB database.

The Beijing spoligotype has been associated with increased morbidity and mortality around the world with Hanekom *et al.* describing Beijing as "a template for success" considering that approximately one third of TB in the world is a result of the Beijing spoligotype (Hanekom et al. 2011, Hesseling et al. 2010). Another study hypothesised that the Beijing spoligotype's ability to spread globally could suggest that the lineage is better adapted to cause infection and disease in humans. One hypothesis suggests that the Beijing spoligotype has lower susceptibility to anti-TB drugs which has resulted in the spoligotype being able to spread (Bifani et al. 2002). The Beijing spoligotype has gained popularity with regard to the amount of research done since it is a prevalent spoligotype in Asia and former Soviet Union States (including Russia, Ukraine, Armenia and Georgia).

Furthermore, vaccination with the BCG vaccine provides less protection against Beijing strains. A study conducted involving mice found that the Beijing spoligotype was more virulent compared to other spoligotypes, while another study in rabbits displayed a more severe form of TB meningitis when compared with other spoligotypes (Dormans et al. 2004, Tsenova et al. 2005). The hypervirulence of the Beijing spoligotype observed in animal studies could be as a result of the phenolic glycolipid (PGL). 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 lineages that cannot synthesise PGL (Hanekom et al. 2011, Caws et al. 2008). In South Africa, it was found that the Beijing and S were more likely to be associated with EPTB when compared to other spoligotypes such as LAM (Hesseling et al. 2010). Similarly, we identified a Beijing strain in one case of GTB despite the low overall prevalence of Beijing strains in TB cases in the Free State province (van der Spoel van Dijk et al. 2016). While little information exists concerning which strain is the main cause of GTB, this study has confirmed the association of Beijing and X strains with GTB in South Africa. To the best of our knowledge, there have been no previous reports of GTB isolates being characterised in South Africa. Therefore this is the first study to characterise GTB isolates in South Africa and it is recommended that further characterisation studies of GTB should be carried out using a larger sample size.

What does the future hold for GTB testing?

In terms of clinical detection, the future seems promising. With both the GeneXpert and nested PCR being able to detect GTB from clinical samples, future diagnosis of GTB can be more rapid. Even though the data suggest that the nested PCR performed better than the GeneXpert, the GeneXpert may still be suitable for deployment of GTB diagnosis in resource limited areas where robust, easy to perform tests are required for large scale testing. Furthermore, the nested PCR requires specialised facilities as well as technical expertise, resulting in the nested PCR being unrealistic for deployment away from centralised laboratories. The GeneXpert however, was built and designed for this very purpose with a strong emphasis on ease of use and the ability to be deployed in such areas. While the GeneXpert was not able to detect GTB from the positive endometrial tissue sample, it was able to detect GTB from the menstrual fluid sample. With the collection of menstrual fluid being a relatively non-invasive and inexpensive technique to perform, when combined with the versatility of the GeneXpert, this could result in improved availability of screening for GTB. This could mean that more infertile women, especially those from remote areas, can now be screened for GTB and receive the treatment they need. The good specificity and positive predictive value indicate that positive results obtained with this assay can reliably be used to rapidly initiate treatment. However, the poor sensitivity indicates the need to employ additional testing such as culture before the diagnosis can be excluded.

While rifampicin resistance was not detected in this study it should be noted that increasing numbers of MDR-TB cases worldwide have been documented leading to an increased risk of MDR-TB in GTB cases (Sharma et al. 2017). While rifampicin resistant GTB has not yet been reported in South Africa, the GeneXpert has the added benefit of determining rifampicin resistance directly in clinical samples. This is helpful considering that the TB drug resistance survey conducted by the NICD reported that approximately 2.8% of all TB cases detected in South Africa are MDR-TB. While the MDR-TB prevalence has remained relatively stable, rifampicin resistance has shown a steady increase. Based on the information obtained from the South African National Department of Health's Management of drug-resistant TB report, between 2004 and 2010, there were a total of 45,196 MDR-TB patients with 267 being from the Free State Province. Furthermore, during the same period there were a total of 3,128 XDR-TB patients with 7 being from the Free State. The NICD suggested that the GeneXpert will play a major role in detecting rifampicin resistant TB (Centre for Tuberculosis 2016, National Department of Health 2013).

The nested PCR would be better suited for deployment in laboratories that have the facilities necessary to carry out more elaborate PCR testing. It is strongly advised that the nested PCR be coupled with an automated extraction system such as the NucliSens EasyMAG. This is because one of the bottle necks for rapid molecular testing is the nucleic acid extraction step. This process is also one of the most labour intensive. Since the NucliSENS EasyMAG is an automated system, this has allowed a reduction of the turnaround time for the nested PCR. The system can extract 24 samples within 40 minutes meaning that large sample volumes could be processed efficiently. This is essential if the nested PCR is to be used in a centralised laboratory processing large sample volumes. In total, from sample processing to delivering a result, the turnaround time would be approximately 24 to 48 hours.

Based on a report published by Stats SA in 2015, the country is experiencing a decrease in fertility rate. This decrease is observed amongst all racial groups in the country when comparing data from 1996 to data obtained in 2011. Briefly, the national total fertility rate in 1996 was observed to be 3.23. In 2001 the national total fertility rate decreased to 2.84 and in 2011 the rate has further decreased to 2.67. Provincially in the Free State province, the total fertility rate in 1996 was 2.97 in contrast to 2011 in which the total fertility rate was 2.54. Stats SA named numerous possibilities that could cause this trend such as: changes in social norms, increased contraceptive use, higher education as well as the increased HIV prevalence during the study period. However, Stats SA failed to look at the possibility of GTB as a cause of the decrease fertility rates considering that South Africa has a high TB prevalence. Then again research into GTB is lacking and ideally a nationwide study into infertility with regards to GTB as a possible causative agent could assist in determining the cause of the observed decrease in fertility in South Africa (Statistics South Africa 2015a).

This study has certain limitations. The most notable limitation involves the small total sample number and the small number of positive samples. Based on clinic attendance preceding the study, collection of approximately 100 samples was predicted during the planned study period. The lower sample numbers obtained may have resulted from difficulty with regards to patient follow up as well as patients struggling to obtain transport to visit the unit. Another limitation is the lack of clinical information for the study cohort. Collection of this data is underway in a related study and may provide valuable information regarding clinical presentation, site of infection and response to therapy.

In conclusion, an in-house molecular assay was developed for the detection of GTB. Both the in-house assay and the GeneXpert were used for the detection of GTB from clinical samples that were obtained from infertile women. Furthermore, the two rapid molecular assays have been validated for use in screening for GTB in combination with MGIT culture. This study recommends that both molecular techniques can be implemented for the rapid detection of GTB from clinical samples.

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Appendix A Human β-globin gene

GenBank: AH001475.2

>Human β-globin gene

TCTATTTATTTAGCAATAATAGAGAAAGCATTTAAGAGAATAAAGCAATGGAAATAAGAAATTTGTAAAT
TTCCTTCTGATAACTAGAAATAGAGGATCCAGTTTCTTTTGGTTAACCTAAATTTTATTTCATTTTATTG
TTTTATTTTATTTTATTTTATTTTATTTTATTTTGTGTAATCGTAGTTTCAGAGTGTTAGAGCTGAAAGG
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TCCTCACCTGAGGAGTTAATTTAGTACAAGGGGAAAAAGTACAGGGGGATGGGAGAAAGGCGATCACGTT
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NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
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TCTGTGTGCTGGCCCATCACTTTGGCAAATAATTCACCCCACCAGTGCAGGCTGCCTATCAGAAAGTGGT
GGCTGGTGTGGCTAATGCCCTGGCCCACAAGTATCACTAAGCTCGCTTTCTTGCTGTCCAATTTCTATTA
AAGGTTCCTTTGTTCCCTAAGTCCAACTACTAAACTGGGGGATATTATGAAGGGCCTTGAGCATCTGGAT
TCTGCCTAATAAAAAACATTTATTTTCATTGCAATGATGTATTTAAATTATTTCTGAATATTTTACTAAA
AAGGGAATGTGGGAGGTCAGTGCATTTAAAACATAAAGAAATGAAGAGCTAGTTCAAACCTTGGGAAAAT
ACACTATATCTTAAA

Primers and their binding sites:

KM29 5'-GGTTGGCCAATCTACTCCCAGG-3' (outer forward primer)
PCO3 5'-ACACAACTGTGTTCACTAGC-3' (inner forward primer)
PCO4 5'-CAACTTCATCCACGTTCACC-3' (reverse primer)

Appendix B *IS6110* sequence

GenBank: Y14047.1

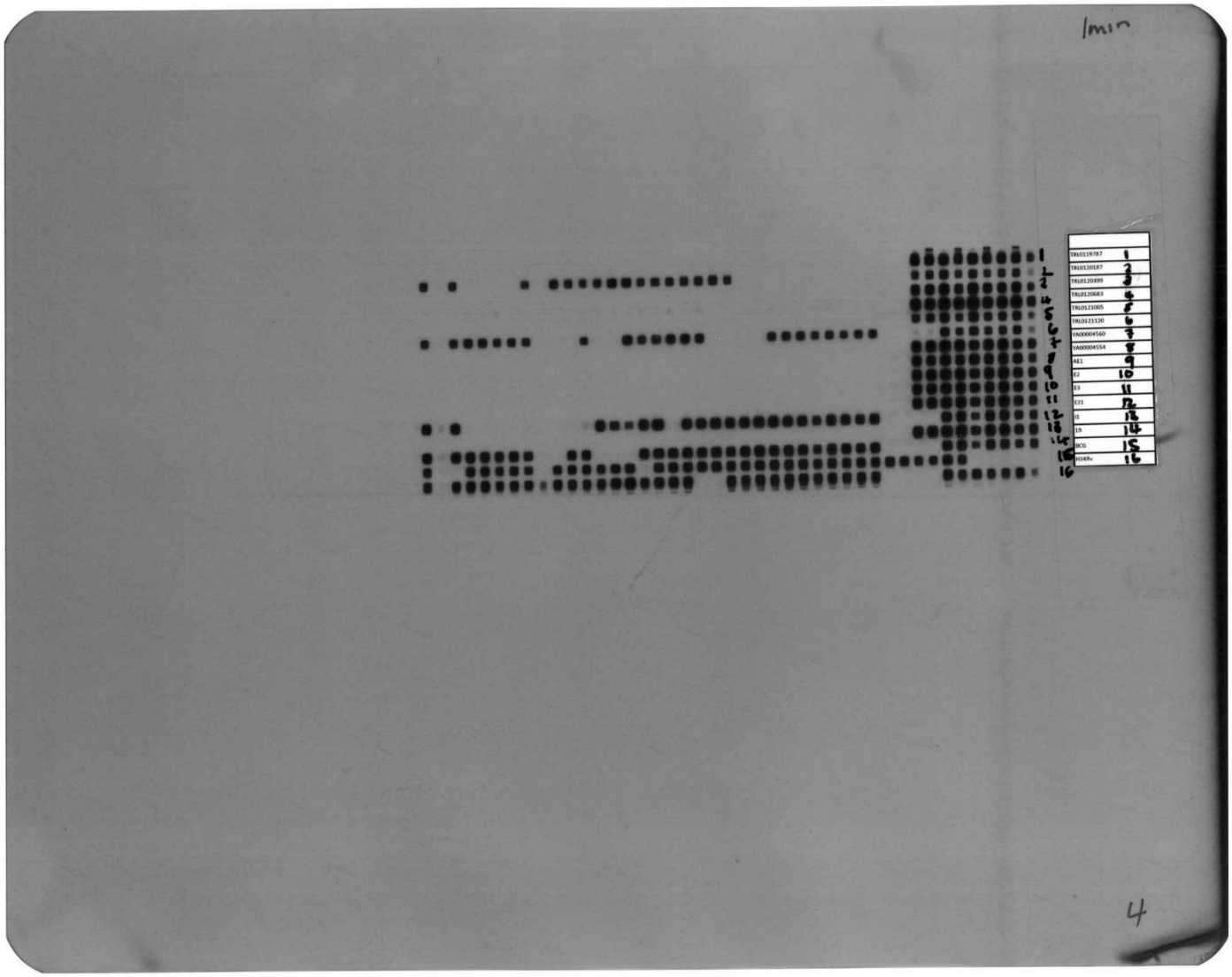
>Mycobacterium tuberculosis IS6110

TGAACCGCCCCGGCATGTCCGGAGACTCCAGTTCTTGGAAAGGATGGGGTCATGTCAGGTGGTTCATCGA
GGAGGTACCCGCCGGAGCTGCGTGAGCGGGCGGTGCGGATGGTCGCAGAGATCCGCGGTCAGCACGATTC
GGAGTGGGCAGCGATCAGTGAGGTCGCCCGTCTACTTGGTGTTGGCTGCGCGGAGACGGTGCGTAAGTGG
GTGCGCCAGGCGCAGGTCGATGCCGGCGCACGGCCCGGGACCACGACCGAAGAATCCGCTGAGCTGAAGC
GCTTGCGGCGGGACAACGCCGAATTGCGAAGGGCGAACGCGATTTTAAAGACCGCGTCGGCTTTCTTCGC
GGCCGAGCTCGACCGGCCAGCACGCTAATTACCCGGTTCATCGCCGATCATCAGGGCCACCGCGAGGGCC
CCGATGGTTTGCGGTGGGGTGTCGAGTCGATCTGCACACAGCTGACCGAGCTGGGTGTGCCGATCGCCCC
ATCGACCTACTACGACCACATCAACCGGGAGCCCAGCCGCCGCGAGCTGCGCGATGGCGAACTCAAGGAG
CACATCAGCCGCGTCCACGCCGCCAACTACGGTGTTTACGGTGCCCGCAAAGTGTGGCTAACCCTGAACC
GTGAGGGCATCGAGGTGGCCAGATGCACCGTCGAACGGCTGATGACCAAACTCGGCCTGTCCGGGACCAC
CCGCGGCAAAGCCCGCAGGACCACGATCGCTGATCCGGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGC
CGCTTCGGACCACCAGCACCTAACCGGCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGGCAGGGT
TCGCCTACGTGGCCTTTGTCACCGACGCCTACGCTCGCAGGATCCTGGGCTGGCGGGTCGCTTCCACGAT
GGCCACCTCCATGGTCCTCGACGCGATCGAGCAAGCCATCTGGACCCGCCAACAAGAAGGCGTACTCGAC
CTGAAAGACGTTATCCACCATACGGATAGGGGATCTCAGTACACATCGATCCGGTTCAGCGAGCGGCTCG
CCGAGGCAGGCATCCAACCGTCGGTCGGAGCGGTCGGAAGCTCCTATGACAATGCACTAGCCGAGACGAT
CAACGGCCTATACAAGACCGAGCTGATCAAACCCGGCAAGCCCTGGCGGTCCATCGAGGATGTCGAGTTG
GCCACCGCGCGCTGGGTCGACTGGTTCAACCATCGCCGCCTCTACCAGTACTGCGGCGACGTCCCGCCGG
TCGAACTCGAGGCTGCCTACTACGCTCAACGCCAGAGACCAGCCGCCGGCTGAGGTCTCAGATCAGAGAG
TCTCCGGACTCACCGGGGCGGTTCA

Primers and their binding sites:

TBF1 5'-CGATGGCGAACTCAAGGA-3' (outer forward primer)
TBR1 5'-TAGGCGTCGGTGACAAAGG-3' (outer reverse primer)
TBF2 5'-GTCGAACGGCTGATGACCAAACT-3' (inner forward primer)
TBR2 5'-TCCGAAGCGGCGCTGGACGA-3' (inner reverse primer)

Appendix C Spoligotyping X-ray film



Samples tested for spoligotyping:

ID	*Spol ID*	*Spol row*
GTB_1	AE1	9
GTB_2	E21	12
BCG	BCG	15
H37Rv	H37Rv	16

Appendix D TAE buffer

TAE buffer was prepared using the following steps:

- 0.5M disodium ethylenediamineteraacetate (Na_2EDTA) solution
    - Na_2EDTA 84.05g (SAARChem, Krugersdorp, South Africa)
    - Using HCl to adjust pH to 8.0 (Merck Millipore, Billerica, MA)
    - In 500ml distilled water

- 50× TAE buffer
    - 100ml aliquot of 0.5M Na_2EDTA solution
    - tris(hydroxymethyl)aminomethane 242g (Merck Millipore, Billerica, MA)
    - aliquot of glacial acetic acid 57.1ml (Merck Millipore, Billerica, MA)
    - In 1l of distilled water

- 1× TAE buffer
    - 20ml aliquot of the 50× TAE buffer in 2l distilled water

Appendix E Custom loading dye

The custom loading dye with GelRed, loading dye and ladder mix was made with the following protocol:

- Loading dye
    - Bromophenol blue (Merck Millipore, Billerica, MA) 0.012g
    - Glycerol (100%) 3.78g
    - TAE 7ml
- Loading dye containing GelRed
    - GelRed (Biotium Inc, Fremont, CA) 3.5µl
    - Loading dye 750µl
- Ladder mix
    - Loading dye containing GelRed 20µl
    - GeneRuler DNA Ladder (Merck Millipore, Billerica, MA) 20µl
    - Nuclease free water 40µl

Appendix F Calculations

Diagnostic sensitivity - GeneXpert

$$Sensitivity = \frac{true\ positive}{true\ positive + false\ negative}$$

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

$$Sensitivity = 0.5$$

Diagnostic sensitivity – nested PCR

$$Sensitivity = \frac{true\ positive}{true\ positive + false\ negative}$$

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

$$Sensitivity = 1.0$$

Diagnostic specificity - GeneXpert

$$Specificity = \frac{true\ negative}{true\ negative + false\ positive}$$

$$Specificity = \frac{52}{52+0}$$

$$Specificity = 1.0$$

Diagnostic specificity – nested PCR

$$Specificity = \frac{true\ negative}{true\ negative + false\ positive}$$

$$Specificity = \frac{52}{52+0}$$

$$Specificity = 1.0$$

Positive predictive value - GeneXpert

$$PPV = \frac{true\ positive}{true\ positive + false\ positive}$$

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

$$PPV = 1.0$$

Positive predictive value – nested PCR

$$PPV = \frac{true\ positive}{true\ positive + false\ positive}$$

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

$$PPV = 1.0$$

Negative predictive value - GeneXpert

$$NPV = \frac{true\ negative}{true\ negative + false\ negative}$$

$$NPV = \frac{52}{52 + 1}$$

$$NPV = 0.98$$

Negative predictive value – nested PCR

$$NPV = \frac{true\ negative}{true\ negative + false\ negative}$$

$$NPV = \frac{52}{52 + 0}$$

$$NPV = 1.0$$

Appendix G UFS Health Sciences Ethics Letter

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

04 March 2016

MR CM SOKHELA
DEPARTMENT OF MEDICAL MICROBIOLOGY AND VIROLOGY
UFS

Dear Mr Sokhela

ECUFS 49/2013B
PROJECT TITLE: DEVELOPMENT AND VALIDATION OF A MOLECULAR ASSAY AND EVALUATION OF THE GENEXPERT MTB/RIF ASSAY FOR THE RAPID DETECTION OF GENITAL TUBERCULOSIS

1. You are hereby kindly informed that the Health Sciences Research Ethics Committee (HSREC) approved the above project after all conditions were met when the signed permission letter from the Free State Department of Health was submitted. This decision will be ratified at the next meeting to be held on 15 March 2016.

2. The Committee must be informed of any serious adverse event and/or termination of the study.

3. Any amendment, extension or other modifications to the protocol must be submitted to the HSREC for approval.

4. A progress report should be submitted within one year of approval and annually for long term studies.

5. A final report should be submitted at the completion of the study.

6. Kindly use the ECUFS NR as reference in correspondence to the HSREC Secretariat.

7. 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 HSREC of the Faculty of Health Sciences.

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

Cc: Dr D Goedhals

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 H FS Department of Health

health
Department of Health
FREE STATE PROVINCE

26 February 2016

Mr. CM Sokhela
Dept. of Medical Microbiology and Virology
UFS

Dear Mr. CM Sokhela

Subject: Development and validation of a molecular assay and evaluation of the genexpert MTB/RIF assay for the rapid detection of genital Tuberculosis.

- Permission is hereby granted for the above – mentioned research on the following conditions:
- 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 Universitas Hospital 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: 1/3/2016

Head : Health
PO Box 227, Bloemfotein, 9300
4th Floor, Executive Suite, Bophelo House, cnr Maitland and, Harvey Road, Bloemfotein