

DECLARATION

Non-tuberculous mycobacteria in tuberculosis epidemic settings in South Africa

by

CLARISSA KRUGER

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**Supervisor: Dr. Tania Botha
Co-supervisor: Dr. Carmen Pheiffer**

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DECLARATION

I, Clarissa Kruger, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.



Signed

12/02/2008

Date

ABSTRACT

Non-tuberculous mycobacteria (NTM) are often isolated from Human Immunodeficiency Virus (HIV) infected individuals, but there is very little information documented about the prevalence of NTM in community settings. An increase in NTM infection is also noted in HIV-negative people. Although it is as yet unknown whether the organisms cause disease in HIV-negative individuals or whether they are merely commensal organisms, their affect on HIV-positive individuals is unquestionable. *Mycobacterium avium* is the most common cause of disease in Acquired Immunodeficiency Syndrome (AIDS) patients and account for 85% of NTM isolated from HIV-infected patients. Several studies have reported that infection with NTM (*M. xenopi*) may enhance HIV-replication. It has also been demonstrated that infection with NTM may aggravate the tuberculosis (TB) epidemic. Infection with NTM reduces the protective efficacy of the anti-tuberculosis vaccine, *M. bovis* Bacille Calmette-Guérin (BCG). Furthermore, due to microbiological similarities, individuals infected with NTM may be wrongfully diagnosed with TB. These individuals may then be inappropriately treated with anti-tuberculosis drugs, possibly exacerbating the TB drug resistance epidemic. The high HIV and TB infection rates worldwide, especially in Africa, underscore the need for NTM research.

This study was initiated due to the preliminary findings of a much larger study, the Zambia South Africa TB and AIDS Reduction trial (ZAMSTAR). ZAMSTAR is a collaborative study between South Africa and Zambia and aims to reduce the prevalence of TB and HIV in communities in South Africa and Zambia using community-based interventions. In South Africa, the first part of ZAMSTAR involved conducting TB prevalence surveys in two high incidence TB communities in the Western Cape Province. Although the prevalence surveys were focused on the identification of *M. tuberculosis* and not specifically designed to detect NTM, *M. tuberculosis* identification tests of positive cultures identified an unexpected large number of NTM, initiating further investigation. The aims of this study were therefore to determine the species distribution of NTM in these settings using DNA sequencing of the 16S ribosomal ribonucleic acid (rRNA) gene and also to determine the accuracy of a panel of laboratory tests that differentiate *M. tuberculosis* complex from NTM, using DNA sequencing as the gold standard.

For this study, 6042 spot sputum samples were collected from randomly selected participants who were 15 years or older. Samples were digested, decontaminated and then cultured in the BACTEC™ MGIT™ 960 liquid culture system and on Löwenstein-Jensen slants. In order to differentiate between *M. tuberculosis* complex and NTM, acid-fast positive cultures were subjected to the following tests: Capilia® TB/TAUNS, niacin reagent test,

p-Nitrobenzoic acid slant test and spoligotyping. The 16S rRNA gene was used to sequence 105 cultures which were most probably NTM, based on a negative spoligotype and a negative Capilia® TB/TAUNS result. 16S rRNA speciation was performed by amplification of the 16S rRNA gene and then subjecting the product obtained to DNA sequencing. The accuracy of the identification tests to identify NTM was determined using 16S rRNA as the gold standard.

Dr. Carmen Pineda

Almost one quarter of all positive cultures in this study were identified as NTM. *Arsenicococcus bolidensis* and *M. monacense* was found to be the most abundant organisms recovered in this study accounting for 11.43% and 9.52% of isolates, respectively. The accuracy of each test to correctly identify NTM was determined using 16S rRNA sequence, and was as follows: spoligotyping and the Capilia® TB/TAUNS, 96.2%; *p*-nitrobenzoic acid slants, 88.6% and the niacin reagent test, 76.2%.

Dr. Nalini Johnson

Most of the patients in this study were placed onto TB treatment, based on conventional culturing techniques. This would not only lead to the unsuccessful treatment of the patient as well as unnecessary side-effects, it could also cause drug-resistance in the future. This study concludes that the diagnostic tests used in our clinical laboratories in South Africa today are not very specific in differentiating between *M. tuberculosis* complex and other mycobacteria. Hopefully, the data generated from this project will be able to influence future decisions made within the Department of Health in order to diagnose and treat more patients successfully.

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Dr. Rabia Johnson

For my parents.

DEDICATION

For my parents.

"Far and away the best prize that life offers is the chance to work hard at work worth doing."

- Theodore Roosevelt

GLOSSARY

Abbreviations	Definition
μ	Microline
16S rRNA	16S ribosomal ribonucleic acid (S represents Svedberg units)
AFB	Acid-fast bacillus
AIDS	Acquired Immunodeficiency Syndrome
ATS	American Thoracic Society
BCG	Bacillus Calmette-Guérin
BLAST	Basic local alignment search tool
bp	Base pair
CDC	Centers for Disease Control and Prevention
CFU	Colony forming units
ddH ₂ O	Double distilled water
dNTP	Dideoxy-nucleotide triphosphate
dH ₂ O	Distilled water
	“Far and away the best prize that life has to offer is the chance to work hard at work worth doing.”
dNTP	Dideoxy-nucleotide triphosphate
DOTS	Directly observed treatment short-course
DTH	Delayed type hypersensitivity
DVR	Dried variable repeat
G	Gravitas
HIV	Human immunodeficiency virus
IFN γ	Interferon-gamma
IUATLD	International union against tuberculosis and lung diseases
LJ	Lowenstein-Jensen
MAC	Mycobacterium avium complex
MAS	Mycobacterium avium-intracellulare-Scrofulaceum complex
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacterial growth indicator tube
ml	Milliliter
mm	Millimeter
NALC	N-acetyl-L-cysteine
ng	Nanogram
NHL	National Health Laboratory Services
nm	Nanometer
NTM	Non-tuberculous mycobacteria
ODC	Oxalic acid, albumin, dextran and catalase

- Theodore Roosevelt

GLOSSARY

Abbreviations	Definition
µl	Microlitre
16S rRNA	16S ribosomal ribonucleic acid (S represents Svedberg units)
AFB	Acid-fast bacillus
AIDS	Acquired Immunodeficiency Syndrome
ATS	American Thoracic Society
BCG	Bacille Calmette-Guérin
BLAST	Basic local alignment search tool
bp	Base pair
CDC	Centres for Disease Control and Prevention
CFU	Colony forming units
ddH ₂ O	Double distilled water
ddNTP	Dideoxy-nucleotide triphosphate
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxy-nucleotide triphosphate
DOTS	Directly observed treatment short-course
DTH	Delayed type hypersensitivity
DVR	Direct variable repeat
G	Gravities
HIV	Human immunodeficiency virus
IFN γ	Interferon gamma
IUATLD	International union against tuberculosis and lung diseases
LJ	Löwenstein-Jensen
MAC	<i>Mycobacterium avium</i> complex
MAIS	<i>Mycobacterium avium-intracellulare-scrofulaceum</i> complex
MDR-TB	Multi-drug resistant tuberculosis
MGIT	Mycobacterial growth indicator tube
ml	Millilitre
mM	Millimolar
NALC	<i>N</i> -acetyl-L-cysteine
ng	Nanogram
NHLS	National Health Laboratory Services
nM	Nanomolar
NTM	Non-tuberculous mycobacteria
OADC	Oleic acid, albumin, dextrose and catalase

PANTA	Lyophilised mixture of anti-microbial agents: Polymixin B, Amphotericin B, Naladixic acid, Trimethoprim and Azlocillin	
PBMC	Peripheral blood mononuclear cell	i
PCR	Polymerase chain reaction	ii
pNB	p-Nitrobenzoic acid	iv
PPD	Purified protein derivative	v
rpm	Revolutions per minute	vii
rRNA	Ribosomal ribonucleic acid	
SB buffer	Sodium boric acid (electrophoresis) buffer	
SSPE	Saline/sodium phosphate/EDTA	
TB	Tuberculosis	1
T _m	Melting temperature	4
TNF- α	Tumour necrosis factor alpha	4
TST	Tuberculin skin test	5
TTP	Time to positivity	6
U	Unit	7
USA	United States of America	10
WHO	World Health Organisation	
XDR-TB	Extensive drug resistant tuberculosis	
ZAMSTAR	Zambia and South African Tuberculosis and AIDS Reduction Trial	
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CHAPTER ONE

LITERATURE REVIEW

1.1 The genus *Mycobacterium*

Mycobacterium is the only genus in the family *Mycobacteriaceae*. Mycobacteria are unique in the composition of their cell wall. Almost 60% of the mycobacterial cell wall is made up of mycolic acid, a waxy lipid. The cell wall also contains peptidoglycan, glycolipids and arabinogalactan (**Figure 1.1**).

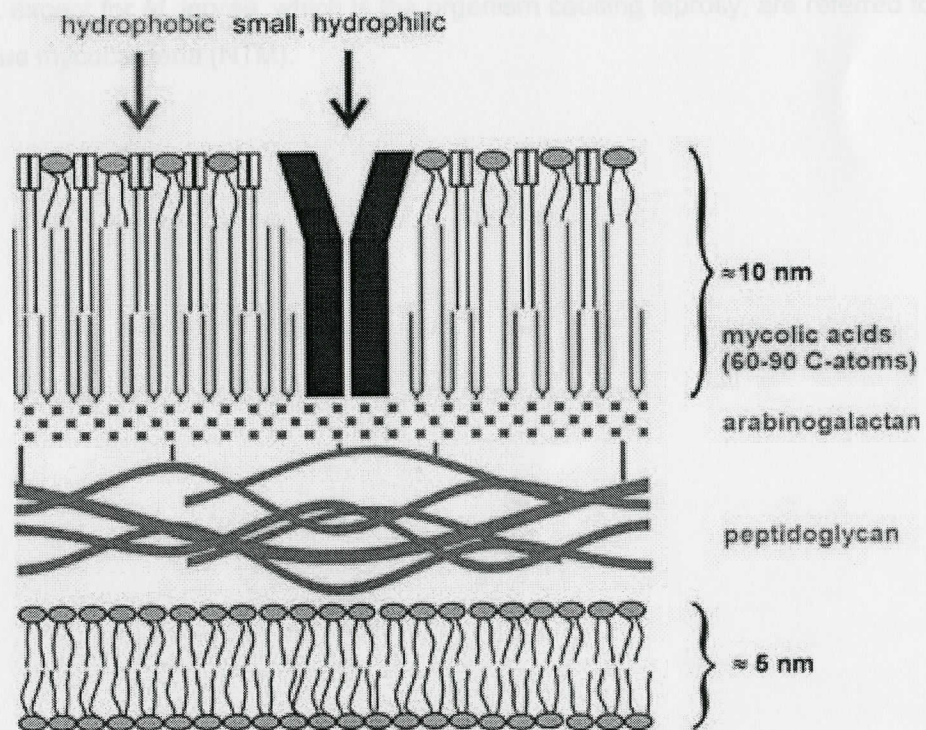


Figure 1.1 An illustration of the mycolic cell wall showing the different layers containing mycolic acids, arabinogalactan and peptidoglycan (www.biologie.uni-erlangen.de).

The high lipid content of the cell wall makes mycobacteria resistant to penetration by ordinary dyes and although considered Gram positive, these organisms are not readily stained by Gram's method (Bartholomew & Mittwer, 2003). The cell wall is also responsible for the acid-fastness of mycobacteria, the ability to retain a basic fuchsin dye when treated with an acidified alcohol solution, and has also been implicated in resistance to certain antibiotics (Falkinham, 1996). Mycobacteria can be straight or curvy bacilli, approximately 0.2-0.6µm by 1.0-10µm in size (Nolte & Metchock, 1996). They are non-spore-forming, non-motile, aerobic

bacilli and their colony morphologies range from smooth to rough and from pigmented to non-pigmented. Temperatures for optimal growth vary from 30°C to almost 45°C, depending on the species (Falkinham, 1996).

The genus *Mycobacterium* currently consists of 95 species (Katoch, 2004) that are divided into two groups according to their growth time on solid media (**Figure 1.2**). Fast growers require less than seven days to form colonies, while slow growers form visible colonies after only seven days (Shinnick & Good, 1994). Slow growers include most of the pathogenic mycobacteria, with *Mycobacterium tuberculosis* being arguably the most important. *M. tuberculosis* forms part of the *M. tuberculosis* complex that consists of *M. microti*, *M. africanum*, *M. canettii*, *M. tuberculosis* and *M. bovis* (Nolte & Metchock, 1996). All other mycobacteria, except for *M. leprae*, which is the organism causing leprosy, are referred to as non-tuberculous mycobacteria (NTM).

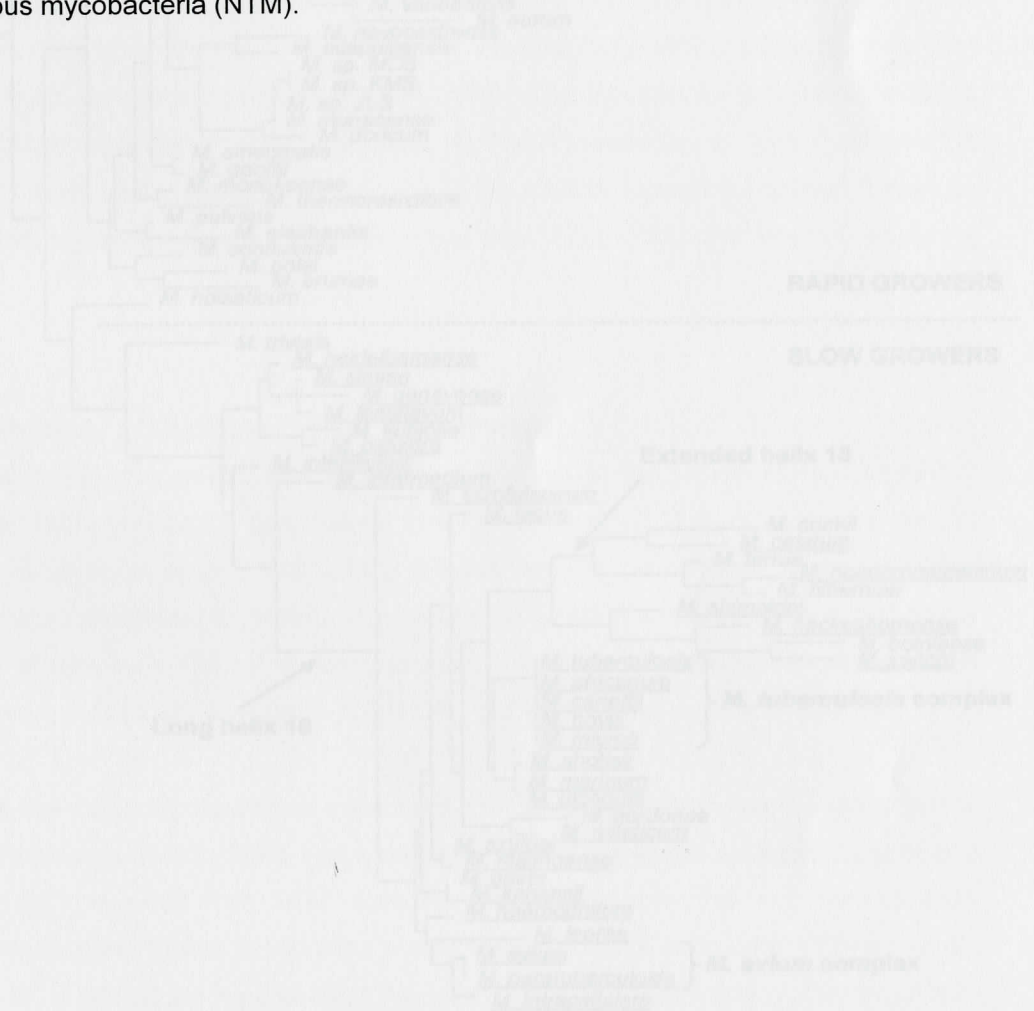


Figure 1.2 Phylogenetic tree of the genus *Mycobacterium*. The phylogenetic tree was constructed using 16S rRNA sequence information. *Mycobacterium* classified as rapid or slow growers are indicated. *M. fortuitum* is a slow-growing mycobacterium. Phylogenetic species are underlined. Species belonging to the *M. tuberculosis* or *M. avium* complex are indicated. Figure reproduced with kind permission by Dr. NC Gey van Pittius, Department of Medical Biochemistry, University of Stellenbosch, originally adapted from Shinnick and Good, 1994 and Springer et al., 1999.

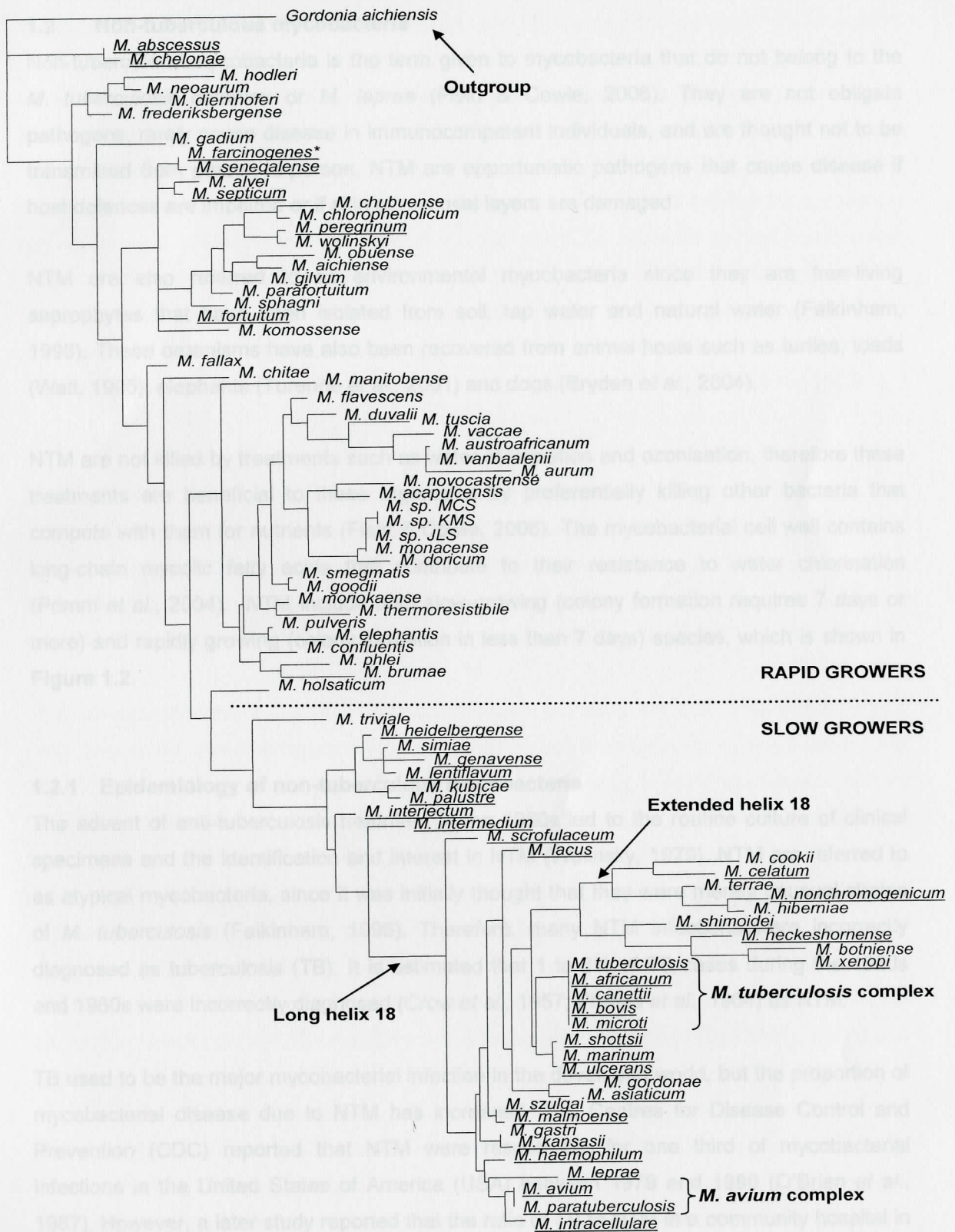


Figure 1.2 Phylogenetic tree of the genus *Mycobacterium*. The phylogenetic tree was constructed using 16S rRNA sequence information. Mycobacteria classified as rapid or slow growers are indicated. **M. farcinogenes* is a slow-growing mycobacterium. Pathogenic species are underlined. Species belonging to the *M. tuberculosis* or *M. avium* complex are indicated. Figure reproduced with kind permission by Dr. NC Gey van Pittius, Department of Medical Biochemistry, University of Stellenbosch, originally adapted from Shinnick and Good, 1994 and Springer *et al.*, 1996.

1.2 Non-tuberculous mycobacteria

Non-tuberculous mycobacteria is the term given to mycobacteria that do not belong to the *M. tuberculosis* complex or *M. leprae* (Field & Cowie, 2006). They are not obligate pathogens, rarely cause disease in immunocompetent individuals, and are thought not to be transmitted from person-to-person. NTM are opportunistic pathogens that cause disease if host defences are impaired or if skin or mucosal layers are damaged.

NTM are also referred to as environmental mycobacteria since they are free-living saprophytes that have been isolated from soil, tap water and natural water (Falkinham, 1996). These organisms have also been recovered from animal hosts such as turtles, toads (Watt, 1995), elephants (Turenne *et al.*, 2001) and dogs (Bryden *et al.*, 2004).

NTM are not killed by treatments such as water chlorination and ozonisation, therefore these treatments are beneficial to these organisms by preferentially killing other bacteria that compete with them for nutrients (Field & Cowie, 2006). The mycobacterial cell wall contains long-chain mycolic fatty acids that contribute to their resistance to water chlorination (Primm *et al.*, 2004). NTM include both slow-growing (colony formation requires 7 days or more) and rapidly growing (colony formation in less than 7 days) species, which is shown in **Figure 1.2**.

1.2.1 Epidemiology of non-tuberculous mycobacteria

The advent of anti-tuberculosis treatment in the 1950s led to the routine culture of clinical specimens and the identification and interest in NTM (Wolinsky, 1979). NTM are referred to as atypical mycobacteria, since it was initially thought that they were merely unusual strains of *M. tuberculosis* (Falkinham, 1996). Therefore, many NTM infections were incorrectly diagnosed as tuberculosis (TB). It is estimated that 1 to 2% of TB cases during the 1950s and 1960s were incorrectly diagnosed (Crow *et al.*, 1957; Phillips *et al.*, 1964) as NTM.

TB used to be the major mycobacterial infection in the developed world, but the proportion of mycobacterial disease due to NTM has increased. The Centres for Disease Control and Prevention (CDC) reported that NTM were responsible for one third of mycobacterial infections in the United States of America (USA) between 1979 and 1980 (O'Brien *et al.*, 1987). However, a later study reported that the ratio of TB to NTM in a community hospital in South Carolina, USA, decreased from 3.2:1 between 1976 and 1981 to 1:1.6 between 1986 and 1991 (Cox *et al.*, 1999). The prevalence of NTM has also increased in other countries (Marras & Daley, 2002). The increase of NTM could be due to 1) increased prevalence of immunocompromised individuals, 2) increased clinical awareness and investigation,

3) improved culture methods and identification tests, 4) reduced immunity to mycobacteria in the population due to decreased prevalence of TB and Bacille Calmette-Guérin (BCG) vaccination, 5) increased NTM virulence, and 6) greater exposure to NTM due to changes in personal hygiene habits from bathing to showering (Marras & Daley, 2002). NTM often occur in tap water and are easily aerosolised by shower heads, possibly resulting in infection. The *M. avium* complex (MAC) consisting of *M. avium* and *M. intracellulare* is the most common cause of lung disease. It is thought that MAC is more resistant to chlorination and ozonisation than other mycobacterial species (Primm *et al.*, 2004). Other NTM that are often isolated from patients with NTM lung disease include *M. kansasii*, *M. malmoense*, *M. xenopi*, *M. abscessus* and *M. fortuitum*. *M. gordonae* is the second most common NTM isolated from respiratory specimens, but rarely causes disease (Butler & Crawford, 1999).

1.2.2 Disease due to non-tuberculous mycobacteria

NTM causes lung disease similar to pulmonary TB (Crow *et al.*, 1957; Phillips & Larkin, 1964; Christensen *et al.*, 1981). Patients who present with lung disease due to NTM are often older than TB patients since older patients tend to have more pre-existing lung disease, an important risk factor for NTM colonisation and infection (Matos *et al.*, 2004).

NTM disease is more prevalent in males who smoke and drink and who have an underlying lung disease. *M. avium-intracellulare* has been recovered from tobacco, cigarette paper, and cigarette filters from several cigarette brands, thus providing a likely explanation for the association between smoking and pulmonary disease (Miguez-Burbano *et al.*, 2006). The symptoms of NTM disease is cough and sputum production, but shortness of breath, haemoptysis, fatigue, weight loss, fever and night sweats may also occur. If untreated, NTM disease may lead to respiratory failure and death (Matos *et al.*, 2004).

NTM are often overlooked as potential infecting organisms, but it has been reported that they are responsible for 20 to 50% of pulmonary mycobacterial infections (Ellis, 2004). Matos and colleagues reported that NTM infection was present in 8.2% of patients attending a multi-resistant TB clinic in Bahia, Brazil. Only 5% of these patients were Human Immunodeficiency Virus (HIV)-positive, all of them had prior TB treatment, while 95% had pre-existing lung disease (Matos *et al.*, 2004). Seventy four percent of the NTM infections were clinically significant using American Thoracic Society (ATS) guidelines (American Thoracic Society, 2007). Although the percentage of clinically significant isolates in this study may have been an over-estimation due to the study design, other studies have also reported high frequencies of clinically significant NTM isolates (Matos *et al.*, 2004), thus highlighting the increasing incidence of NTM disease worldwide.

NTM are associated with many disease states, of which bronchiectasis and cystic fibrosis predominate. Bronchiectasis is both a consequence of NTM infection as well as a risk factor (Barker, 1997). MAC and *M. abscessus* infections are common causes of bronchiectasis (Wickremasinghe *et al.*, 2005). NTM infection was first identified in a patient with cystic fibrosis (Boxerbaum, 1980). Studies done in Europe and the USA have reported that NTM occur in 4% to 20% of cystic fibrosis patients (Esther *et al.*, 2005; Pierre-Audigier *et al.*, 2005; Olivier *et al.*, 2003). However, the prevalence of NTM in these studies may be underestimated since NTM are often masked and overgrown by other organisms in cultures of cystic fibrosis patients.

Other diseases associated with NTM include the Buruli ulcer, an ulcerative skin disease caused by *M. ulcerans*, and the third most prevalent mycobacterial disease. *M. avium* subspecies *paratuberculosis* has also been implicated in human inflammatory bowel disease (Zumla & Grange, 2002). Crohn's disease is an example of such an idiopathic chronic inflammatory bowel disease and was recently reported to be caused by *M. paratuberculosis* (Katoch, 2004). Shafran *et al.*, (2002) showed that 37 of 60 patients (61.7%) with Crohn's disease produced antibodies to the p35 and p36 protein of *M. paratuberculosis*.

M. scrofulaceum has been associated with cervical lymphadenitis for many years (Falkinham, 1996). Different species of NTM may cause immunocompetent persons to present with localised pulmonary disease, adenitis, soft tissue infections, and infections of joints or bones, bursae, skin ulcers and generalised disease in individuals like leukaemia and transplant patients (Katoch, 2004).

1.2.3 Immunodeficiency and non-tuberculous mycobacteria

Although global increases in NTM disease have been observed in both immunocompetent and immunocompromised individuals (Miguez-Burbano *et al.*, 2006), it is believed that the increase in NTM infections is largely due to the increased number of patients with immunosuppression. Most notably, NTM have emerged as a major cause of opportunistic infections in Acquired Immunodeficiency Syndrome (AIDS) patients. Infection with HIV, the causative agent of AIDS, decreases CD4⁺ lymphocytes, and predisposes patients to disseminated NTM disease when their CD4⁺ lymphocyte count is severely depleted (May *et al.*, 1995). *M. avium* is the most common cause of disease in AIDS patients and account for 85% of NTM isolated from HIV-infected patients. However, other NTM may also cause disease (Jones & Havlir, 2002).

A study of South African gold miners suggested that *M. kansasii* is more pathogenic than *M. avium*, and causes disease in AIDS patients with less immunosuppression, i.e. patients that have relatively high CD4⁺ lymphocyte counts (Corbett *et al.*, 2003). Disease due to *M. avium* is the second leading cause of death in AIDS patients, with AIDS wasting syndrome being the most common cause of death (Covert *et al.*, 1999). Miguez-Burbano *et al.*, (2006) studied *M. tuberculosis* and NTM infections in 521 HIV-positive patients hospitalised in a large metropolitan hospital in Miami, USA and reported that *M. tuberculosis* and NTM accounted for 9% and 11% of respiratory disease, respectively. Ten percent of patients with disseminated NTM disease died, whereas none of the TB patients died during the course of the study. These findings highlight the significance of NTM infections in HIV-infected individuals.

Reports have shown that *M. avium* (Dezzutti *et al.*, 1999; Birkness *et al.*, 1999) and *M. xenopi* (Swords *et al.*, 2006), an NTM initially isolated from the clawed frog *Xenopus laevis* in 1957, may enhance HIV-1 replication. *M. avium* and *M. xenopi* are frequently isolated from water. Using a peripheral blood mononuclear cell (PBMC) model the authors showed that these organisms replicate in human macrophages and induce HIV-1 replication. Moreover, they demonstrated that *M. xenopi* enhances HIV-1 replication by increased production of the inflammatory cytokine, tumour necrosis factor alpha (TNF- α) (Swords *et al.*, 2006), while *M. avium* induces HIV-1 replication through a cytokine independent pathway (Dezzutti *et al.*, 1999).

It is estimated that without antiretroviral treatment up to 43% of AIDS patients will acquire NTM infection during their lifetime (Miguez-Burbano *et al.*, 2006). The AIDS pandemic is rampant in the African continent, and in 2004 it was estimated that the adult HIV infection rate in South Africa was 21.5% (WHO, 2004). NTM infections are common in Africa (Buijtels *et al.*, 2005), therefore these high HIV infection rates, and the possible interaction between NTM and HIV, are a great source for concern for health care programmes.

Opportunistic infections with NTM also occur in other forms of immunodeficiency, such as transplant patients, patients who are treated with TNF- α , and some AIDS patients receiving antiretroviral therapy who have a restored capacity to mount an inflammatory response (Shelburne *et al.*, 2002; Lawn *et al.*, 2005; Hirsch *et al.*, 1994).

1.2.4 Tuberculosis and non-tuberculous mycobacteria

In 1993 the World Health Organisation (WHO) declared TB a global health emergency (WHO, 1993). Despite continuing efforts to control the epidemic, TB is still a major cause of

mortality and morbidity worldwide, resulting in approximately 2 million deaths and 8 million new cases per year (Corbett *et al.*, 2003).

After clinical suspicion, NTM disease is diagnosed using microscopy and culture techniques. It is often difficult to distinguish between disease due to TB and disease due to NTM, especially in HIV-infected patients and in HIV-negative patients with advanced NTM disease. NTM also cause upper-lobe disease and cavitation, and often the symptoms are similar to TB (Bock *et al.*, 1996). Careful analysis of patient history and laboratory methods are required to distinguish TB from NTM.

NTM disease is difficult to diagnose due to the inability to distinguish between NTM disease and environmental colonisation, since not all latently infected individuals progress to active disease (Hatherill *et al.*, 2006). Strict diagnostic criteria for the diagnosis of NTM disease have therefore been recommended (American Thoracic Society, 1997).

A high degree of sequence homology has been demonstrated between the genomes of *M. tuberculosis*, the causative agent of TB, and NTM (Tobler *et al.*, 2006). Moreover, it is difficult to distinguish TB from NTM lung disease on radiological and clinical features alone (Hatherill *et al.*, 2006). In the following section some of the complications of TB associated with NTM infections are discussed.

The tuberculin skin test (TST), or Mantoux test, is widely used as an epidemiological tool to identify latent TB. The test involves intradermal injection of purified protein derivative (PPD), a crude mixture of *M. tuberculosis* extracellular proteins (Seibert & Glenn, 1941), and measurement of delayed type hypersensitivity (DTH) responses (local skin induration and erythma) after 48 to 72 hours (Huebner *et al.*, 1993). However, the TST is prone to false-positivity. It is believed that one of the factors contributing to false-positivity is exposure to NTM. Cross species reactivity between proteins from NTM and those of *M. tuberculosis* may result in false-positive TST results due to reactions to antigens derived from NTM (Brock *et al.*, 2001).

A study done in New York City, USA showed that 23.1% of navy recruits had a skin test reaction to PPD from *M. intracellulare* (Edwards *et al.*, 1969), thus highlighting the potential for false-positive TST results. A recent study reported that an immunological test measuring interferon gamma (IFN- γ) responses to the ESAT-6 and CFP-10 proteins, that are specific to the *M. tuberculosis* complex, and not secreted by most species of NTM, was more accurate in identifying subjects with latent TB than the TST (Codecasa *et al.*, 2006). In developed countries, subjects with false-positive reactions do not have TB and will thus be given

unnecessary prophylactic treatment, which may cause toxicity, particularly in the liver. Also, false-positive reactions result in inaccurate epidemiological survey data. A recent review of 24 studies published since 1966 reported that false-positive TST results from NTM cross-reactivity ranged from 0.1 to 2.3% in different regions of the world (Farhat *et al.*, 2006). The review showed that false-positive TST results are only clinically significant in populations with a high prevalence of NTM and a low prevalence of TB.

In developing countries sputum smear microscopy is the primary method to diagnose TB. The Ziehl-Neelsen (ZN) method is the most commonly used method and exploits the acid-fastness of mycobacteria. *M. tuberculosis* appears as long, slender, slightly curved rods, whereas, NTM appear as short, thick, smooth walled acid-fast bacilli (Weyer, 1998). The sputum of TB patients and those of patients with NTM disease may be smear-positive for acid-fast bacilli (Chung *et al.*, 2006), making it difficult to distinguish between *M. tuberculosis* and NTM. Recently, a study reported that ZN slides may be contaminated by dust, preparation of staining solutions and washing of slides with tap water that may contain NTM, or by using contaminated immersion oil (Selvakumar *et al.*, 2006). The above situations may lead to false-positive ZN results, incorrect diagnosis of TB, inaccurate TB statistics, but more importantly may result in unnecessary treatment. Incorrect diagnosis of NTM infection as TB results in anti-TB treatment (Corbett *et al.*, 2003) that may select for drug resistant *M. tuberculosis* strains, a major problem facing the world today.

It has been suggested that disease due to NTM may incorrectly be diagnosed as drug resistant TB due to the fact that NTM are generally resistant to conventional anti-TB treatment (Matos *et al.*, 2004). Moreover, the prevalence of drug resistant strains is increasing due to inadequate treatment regimens and patient non-compliance. Multidrug-resistant TB (MDR-TB) is a form of drug-resistant TB that occurs when *M. tuberculosis* is resistant to isoniazid and rifampicin, the two most powerful anti-TB drugs available. Extensive drug resistant TB (XDR-TB) is MDR-TB that is resistant to any fluoroquinolone, and at least one of three injectable second-line drugs (capreomycin, kanamycin, and amikacin) (WHO, 2006). In September 2006, the WHO announced the discovery of a deadly strain of XDR-TB in Tugela Ferry, KwaZulu-Natal, South Africa. Two-hundred and twenty one patients were diagnosed with MDR-TB, of whom 53 were diagnosed with XDR-TB. Forty-four of the XDR-TB patients were tested for HIV infection. All of these patients were found to be HIV-infected. It is estimated that at least 30 new cases of XDR-TB are detected in Tugela Ferry every month (Singh *et al.*, 2007).

Despite the widespread use of the anti-TB vaccine, *M. bovis* BCG, the protective efficacy of BCG ranges from 0 to 80 % (Fine, 1994). It is thought that this variability is due to exposure to NTM. Studies have shown that the protective efficacy of BCG is lowest in countries with

high levels of NTM infection (Fine, 1994). Animal models, where animals were infected with NTM prior to BCG vaccination and subsequently challenged with *M. tuberculosis*, have suggested that exposure to NTM masks the protective effect of BCG by inducing a prior level of protection, and therefore making the level of BCG vaccination indistinguishable from that measured in the non-vaccinated population (Howard *et al.*, 2002). Alternatively, these animal models have suggested that immunity induced against NTM can result in early clearance of BCG from the host (Brandt *et al.*, 2002). Brandt *et al.* showed that *M. avium*, *M. fortuitum* and *M. vaccae* interfere with BCG efficacy if they were administered prior to vaccination. They suggested that infection with NTM may inhibit BCG multiplication and thus prevent the induction of an efficient BCG-mediated immune response and protection against TB infection (Brandt *et al.*, 2002).

Recently, Flaherty *et al.* (2006) were the first to show that infection with NTM after BCG vaccination also negatively impacted the protective efficacy of BCG. These authors vaccinated mice with BCG, thereafter infected them with repeated oral doses of *M. avium*, and subsequently challenged them with *M. tuberculosis*. They showed that BCG vaccinated mice that were infected with *M. avium* had a reduced capacity to reduce the bacterial load following *M. tuberculosis* infection compared to BCG vaccinated mice that were not infected with *M. avium*. These findings have implications for TB control programmes since current WHO guidelines recommend that BCG be given at birth (WHO, 2004). Exposure to NTM after BCG vaccination may lead to waning of the protective efficacy of BCG against *M. tuberculosis* infection in the adult population.

A study measuring IFN- γ responses to a variety of mycobacterial antigens in individuals living in rural areas of northern Malawi, with no scar evidence or history of BCG vaccination, showed considerable exposure to NTM (Black *et al.*, 2001). Moreover, these authors showed that BCG vaccination did not protect against the development of TB in this population, and attributed the non-protectiveness of BCG vaccination to exposure to NTM.

1.3 Aim of this study

Research was done to investigate and characterise NTM recovered during prevalence surveys of the Zambia and South African Tuberculosis and AIDS Reduction trial (ZAMSTAR) study. ZAMSTAR, a study funded by the Bill and Melinda Gates Foundation, aims to reduce the prevalence of TB and HIV in communities in South Africa and Zambia using community-based interventions. Initial prevalence surveys conducted in two communities in the Western Cape Province of South Africa revealed that a large number of participants were infected with NTM, initiating further investigation. The aims of this study were to 1) determine the

species distribution of NTM in these settings using DNA sequencing of the 16S ribosomal ribonucleic acid (16S rRNA) gene, and 2) determine the accuracy of a panel of laboratory tests to differentiate *M. tuberculosis* complex from NTM, using DNA sequencing as the gold standard.

2.1 Sample collection

A total of 8042 spot sputum samples were collected over a period of one and a half years from participants in the ZAMSTAR prevalence survey. These samples were collected from patients older than 15 years of age in two communities, Scottsville and Site B, Khayelitsha, in the Western Cape Province of South Africa. Sputum samples were collected in the two local communities and transported to the sample manager at the Desmond Tutu TB Center, Faculty of Health Sciences, University of Stellenbosch on the day of collection. In order to retain the anonymity of patients, sputum samples were collected in barcoded containers. Samples were stored in a refrigerator before being sent to a biosafety level 3 laboratory (Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, University of Stellenbosch) for processing. Samples were usually processed within a day of sample collection or within three days if sputum samples were collected on a Friday.

2.2 Preparation and staining of direct sputum smears

Ziehl-Neelsen (ZN) staining is the most common method of diagnosing TB in developing countries (Enarson et al., 2000). The unique cell wall of mycobacteria is responsible for their acid-fastness, the ability to retain a basic fuchsin dye when treated with an acidified alcohol solution. Therefore, mycobacteria will appear as pink, rod-shaped bacteria after ZN staining, while the background, other host cells, debris and fungal or bacterial contaminants will appear blue (Figure 2.1). *M. tuberculosis* appears as long, slender, slightly curved rods, and often forms distinct cord-like structures. NTM appear as short, thick bacilli (Weyer, 1998). Due to their similar morphologies, much experience is required to distinguish between *M. tuberculosis* and NTM.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Sample collection

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2.2 Preparation and scoring of direct sputum smears

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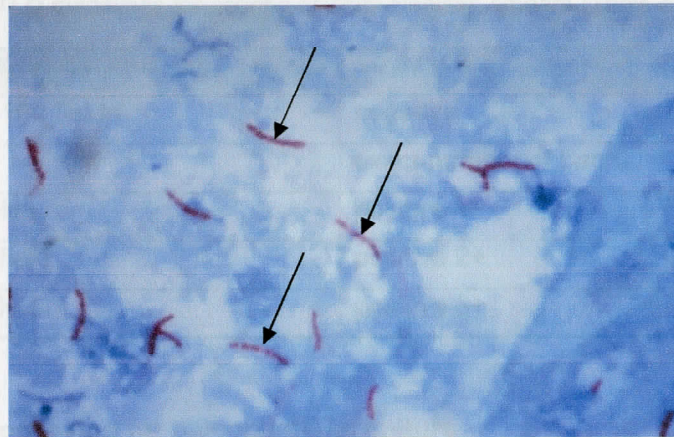


Figure 2.1 A Ziehl-Neelsen stain illustrating *Mycobacterium tuberculosis*. A smear was made from sputum of a TB patient, stained with the ZN method, and viewed under 100x magnification using a light microscope. *M. tuberculosis* appears as pink, rod-shaped bacilli, as indicated by the arrows (www.biotopics.co.uk).

Direct sputum smears were done on all sputum samples that arrived at the bio-safety level 3 laboratory. Preparation of sputum smears and staining was done according to the standard World Health Organisation/International Union Against Tuberculosis and Lung Diseases (WHO/IUATLD) method (Enarson *et al.*, 2000). Smears were made in a class 2 biological safety cabinet. Microscope slides were clearly labelled with a barcode and laboratory number and care was taken to ensure that these numbers would not be rinsed off during the staining process. Smears were made by taking a loopful of sputum and making a smear on a glass slide, ideally 1 - 2cm by 2 - 3cm in size. Thereafter, slides were placed on a hotplate at 100°C to heat fix the smears and kill mycobacteria. After approximately two hours, the hotplate was switched off and the slides were left to cool down on the hotplate. When the slides were cool enough to be handled easily, they were transported to the general laboratory for ZN staining. ZN staining was done as follows (Enarson *et al.*, 2000): Slides were covered with carbol fuchsin (1% basic fuchsin, 5% phenol in ethanol) for 5 minutes and flamed intermittently until steam came off. Care was taken to ensure that the solution did not boil during heating which could produce artefacts. Thereafter, slides were rinsed with tap water and decolourised by adding 3% acid alcohol (3% HCl in ethanol) for two minutes. Slides were again rinsed with tap water and counterstained by flooding with 0.1% methylene blue (C₁₆H₁₈ClN₃S) (NHLS, Rietfontein, South Africa) for one minute. Slides were again rinsed with tap water and placed in an upright position in a rack to air dry. Thereafter, slides were scored by reading under a 100x magnification oil-immersion lens of an OLYMPUS CX31 microscope (Olympus Corporation, Tokyo, Japan). Slides were scored according to the WHO/IUATLD guidelines (Enarson *et al.*, 2000) (Table 2.1).

Table 2.1 WHO/IUATLD guidelines for scoring of Ziehl-Neelsen smears (Table adapted from Enarson *et al*, 2000).

Number of acid-fast bacilli (AFB)	Score
No AFB in at least 100 fields	negative
1-9 AFB/100 fields	exact number of bacilli
10-99 AFB/ 100 fields	positive 1+
1-10 AFB in every field	positive 2+
>10 AFB in every field	positive 3+

2.3 Sputum processing for mycobacterial culture

Sputum processing for mycobacterial culture was performed in a class 2 biological safety cabinet. After preparation of direct sputum smears, the remainder of the sample was carefully transferred to a 50ml tube labelled with the barcode and laboratory number. In order to minimise the risk of cross-contamination, only one tube was opened at a time. An equal volume of MycoPrep™ (Becton, Dickinson and Company, Sparks, USA) (1.5%NaOH and NALC) solution was added to the sputum in the 50ml tube. Tubes were securely tightened, inverted to ensure that the entire tube was coated with the sputum-MycoPrep™ solution and then mixed by vortexing for at least 20 seconds to ensure complete homogenisation. Samples were left to stand in the biological safety cabinet to allow aerosols to settle. Exactly 15 minutes after the addition of the MycoPrep™ solution, digestion was stopped by adding sterile phosphate buffer (pH 6.8) to the 30ml mark and inverting tubes three times. Tubes were placed into capped centrifuge buckets in a class 2 biological safety cabinet. Thereafter, centrifuge buckets were transferred to a centrifuge and bacteria pelleted by centrifuging tubes at 3000rpm (1800xG) for 15 minutes at 21°C in an Eppendorf 5810 R centrifuge (Eppendorf, Hamburg, Germany). To avoid contamination in the event of possible tube leakage during centrifugation, tubes were removed from the centrifuge buckets within the class 2 biological safety cabinet. The supernatant was removed from the pellet of digested sputum and discarded into a bottle containing Hycolin disinfectant (Coventry chemicals, Coventry, England). The cell pellet was resuspended in 2 ml of sterile phosphate buffer.

2.4 *In vitro* culturing methods

2.4.1 The BBL™ MGIT™ 960 mycobacterial culture system

The BBL™ MGIT™ 960 system is a fully automated, non-radiometric system for the rapid detection of slow-growing bacilli from clinical specimens (**Figure 2.2**).



Figure 2.2 The automated BBL™ MGIT™ 960 system (Becton, Dickinson and Company, Sparks, USA) (www.bd.com).

The principle of the BBL™ MGIT™ 960 procedure lies in the fluorescent compound that is embedded in silicone on the bottom of the mycobacterial growth indicator tube (MGIT) tube. This fluorescent compound is sensitive to dissolved oxygen in the growth medium. Initially, very little fluorescence can be detected because oxygen quenches emissions from the compound. Later, fluorescence is detected because actively respiring mycobacteria consume the available oxygen. The amount of fluorescence detected is inversely proportional to the oxygen level in the culture medium, and is therefore an indicator of the mycobacterial growth (BACTEC® MGIT™ 960 System User's Manual, revision: E, 2002/08).

Sputum samples were cultured in the MGIT 960 system according to the manufacturer's recommendations (Becton, Dickinson and Company, Sparks, USA). Five hundred microlitres of the decontaminated, concentration sputum sample (section 2.3) was inoculated into a MGIT tube containing 7 ml of Middlebrook 7H9 media, supplemented with PANTA (Polymixin B, Amphotericin B, Naladixic acid, Trimethoprim and Azlocillin) (Becton, Dickinson and Company, Sparks, USA) and BBL™ MGIT™ Growth Supplement (Oleic acid, Albumin, Dextrose, Catalase (OADC) (Becton, Dickinson and Company, Sparks, USA). Oleic acid is utilised by the tubercle bacilli during metabolism, while albumin binds free fatty acids that may be toxic to mycobacteria. Dextrose is an energy source, while catalase destroys the toxic peroxides that may be produced during mycobacterial growth (Becton-Dickinson, BBL™ MGIT™ 960 package insert). After inoculation, MGIT tubes were transferred to the BBL™ MGIT™ 960 machine, scanned and incubated at 37°C. Samples were automatically monitored every hour to detect increasing levels of fluorescence. If the fluorescence rose

above the threshold value (approximately 10^5 to 10^6 colony forming units per millilitre (CFU/ml), (BACTEC® MGIT™ 960 System User's Manual, revision: E, 2002/08) the samples were flagged as positive. The growth units and the length of incubation time, also known as the time to positivity (TTP) were reported. A sample was recorded as negative if the fluorescence did not exceed the threshold value or if no fluorescence was detected within 42 days.

2.4.2 Löwenstein-Jensen slants

Löwenstein-Jensen (LJ) slants contain an egg-based culture media for the isolation and cultivation of mycobacteria (Figure 2.3). Due to the simplicity and cost-effectiveness of LJ slants it is the most widely used mycobacterial culture method (Tortoli *et al.*, 1998). However, the long time required for mycobacterial colony formation on LJ slants has led to the development of a number of improved mycobacterial culture systems, most recently, the BBL™ MGIT™ 960 system described above.



Figure 2.3 Two Löwenstein-Jensen slants after eight weeks of growth. The slant on the left shows typical buff-coloured growth of *M. bovis* ATCC strain 19210, while the slant on the right shows the photochromogenic growth of ATCC strain 14470.

LJ slants (Becton, Dickinson and Company, Sparks, USA) were inoculated with 0.25ml of the decontaminated, concentrated pellet (section 2.3). Thereafter, slants were placed with the agar face up for 30 minutes to allow bacteria to adhere to the surface of the agar. LJ slants were then transferred to a 37°C incubator and incubated for 8 weeks with weekly aeration by opening and closing the cap of each tube in a safety cabinet.

2.4.3 Confirmation of mycobacterial growth

Sputum samples were decontaminated (as described in section 2.3) before mycobacterial culture for selective elimination of bacteria other than mycobacteria. However, non-mycobacterial bacteria have been recovered from positive cultures of decontaminated sputum samples. Therefore, it is recommended that mycobacterial growth be confirmed by sub-culturing positive cultures onto blood agar plates as well as staining with the ZN method.

Growth on blood agar plates indicated that the positive culture is due to organisms other than mycobacteria, whereas, no growth within 2 days indicated a fungus or mycobacteria. The ZN stain confirmed that positive cultures are acid-fast, and depending on the experience of the reader, it may sometimes have been possible to distinguish between *M. tuberculosis* and NTM.

To confirm that positive MGIT and LJ cultures were due to mycobacterial growth and not due to contamination, all positive cultures were cultured on 2% blood agar plates (NHLS, Greenpoint Media Laboratory, Cape Town, South Africa) and stained with the ZN method (as described in section 2.2). Positive cultures were sub-cultured onto blood agar plates by inoculating with one drop of MGIT culture or one drop of a liquid suspension of the LJ slant with a disposable Pasteur pipette. A liquid suspension of the LJ slant was made by resuspending a loopful of colonies into 1ml of Tween 80 saline (refer to appendix). Blood agar plates were air dried in a safety cabinet for at least 20 minutes, and then incubated at 37°C for 2 days. They were inspected for growth after 2 days. Positive cultures were ZN stained by inoculating one drop of culture onto a glass slide and stained as described in section 2.2. Pink, rod-shaped bacteria suggested that the positive culture was due to mycobacterial growth.

2.5 Differentiation between *M. tuberculosis* complex and NTM

2.5.1 Capilia® TB/TAUNS test

The Capilia® TB/TAUNS test is a lateral flow immunochromatographic test that detects MPB64, a mycobacterial protein fraction secreted by *M.bovis* BCG (Li *et al.*, 1993). MPB64 is similar to MPT64, a protein secreted by the *M. tuberculosis* complex, but not by NTM (Oettinger & Anderson, 1994).

The Capilia® TB/TAUNS test was recently developed (Becton, Dickinson and Company/TAUNS, Tokyo, Japan) and will therefore be described in detail. One hundred microlitres of acid-fast positive MGIT cultures or a suspension of a loopful acid-fast positive LJ cultures in extraction buffer (Becton, Dickinson and Company, Sparks, USA) was added

to the specimen placing area of a Capilia® TB/TAUNS test strip (Figure 2.5). The test sample forms an immune complex with colloidal gold labelled MPB64 antibodies in the specimen placing area. The immune complex then migrates to the developing area by capillary action and is captured by the anti-MPB64 antibodies that are fixed in the reading area. Results were read within 15 minutes and care was taken not to read the results after one hour (Capilia® TB/TAUNS package insert, version 1.3). Should MPB64 antigens be present in the test sample, a purple-red line of colloidal gold will appear in the reading area T. The absence of a purple-red line indicates that the test sample does not belong to the *M. tuberculosis* complex. Excess colloidal gold-labelled MPB64 antibodies migrate through the developing area and are captured by anti-mouse immunoglobulin antibodies in reading area C. The resultant complex forms a purple-red line of colloidal gold in reading area C, and indicates that the assay was successful.

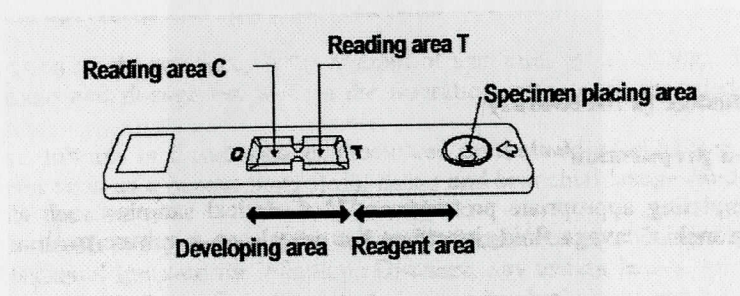


Figure 2.4 Schematic representation of Capilia® TB/TAUNS test plate.

2.5.2 Niacin test

Mycobacteria produce niacin or nicotinic acid which plays a vital role in the oxidation-reduction reactions that occur during metabolic processes. Due to a blocked pathway, *M. tuberculosis* accumulates niacin in the culture medium and can thus be differentiated from NTM that produce very little niacin.

Positive MGIT cultures were sub-cultured onto LJ slants, and incubated at 37°C for six to eight weeks until a thick, confluent growth was observed. One millilitre of sterile, distilled water was added to slants (subcultures of positive MGIT cultures or original LJ cultures), thereafter the surface of the LJ slants were punctured with a sterile glass Pasteur pipette several times. The slants were then placed in a slightly tilted horizontal position so that the water covered the entire surface of the slant, thus allowing the niacin in the LJ media to dissolve in the water. Each slant was incubated in this position at room temperature for 30 minutes, after which 0.6ml of the cloudy water was transferred to a sterile screw cap test tube. A niacin strip (Remel, Lenexa, USA) was inserted into each tube. The tube was capped

tightly and incubated at room temperature for 15 minutes with occasional side-to-side shaking to mix the fluid with the reagent at the bottom of the strip. Care was taken not to invert the tube. Colour development of the fluid was monitored by holding tubes against a white background. Any shade of yellow indicated *M. tuberculosis* complex while clear, colourless fluid was indicative of a negative result (Figure 2.5). Before being discarded, the contents of tubes were neutralised with 10% sodium hydroxide to neutralise the hazardous cyanogen chloride which accumulated in the fluid.

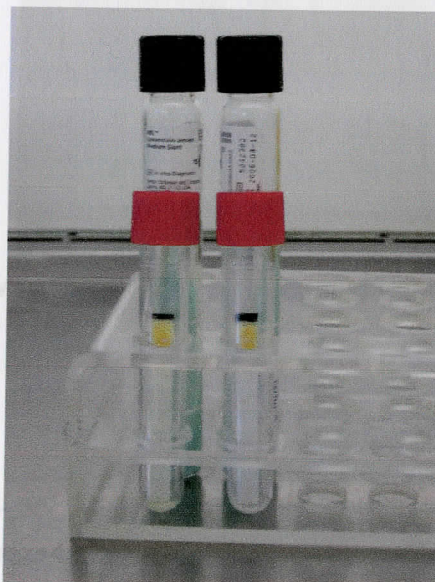


Figure 2.5 A photograph of two completed niacin reagent tests. The sample on the left, *M. tuberculosis* H37Rv, is positive and the sample on the right, *M. fortuitum*, is negative. Positivity is indicated by the yellow-coloured fluid in the left tube and negativity by the clear fluid in the right tube.

2.5.3 *p*-Nitrobenzoic acid Löwenstein-Jensen slants

p-Nitrobenzoic acid (*p*NB) slants are LJ slants that have been impregnated with *p*-nitrobenzoic acid to enable the differentiation between *M. tuberculosis* complex and NTM. *p*-Nitrobenzoic acid inhibits the growth of *M. tuberculosis* complex, while NTM growth is not affected by the acid.

Five hundred microlitres of positive MGIT culture, or alternatively positive LJ cultures suspended in 0.5ml Tween 80 saline (0.5 McFarland), were inoculated onto both a *p*NB slant and a LJ slant (NHLS, Greenpoint, South Africa). The *p*NB and LJ slants were incubated at 37°C for eight weeks and then inspected for growth. Growth on the LJ slant, but no growth on the *p*NB slant indicated *M. tuberculosis*, while growth on both the *p*NB and LJ slants indicated NTM (Figure 2.6). Growth on *p*NB slants were visible as beige, yellow or orange colonies.

Table 2.2 PCR mixture for spoligotyping

1x Reaction buffer	50 µl
MgCl ₂	0.4 mM
dNTPs	0.4 mM
Forward primer	5 pmol
Reverse primer	5 pmol
Taq	1.25 units
Boiled water	2.5 µl
Boiled DNA template	2.5 µl
Final reaction volume	50 µl

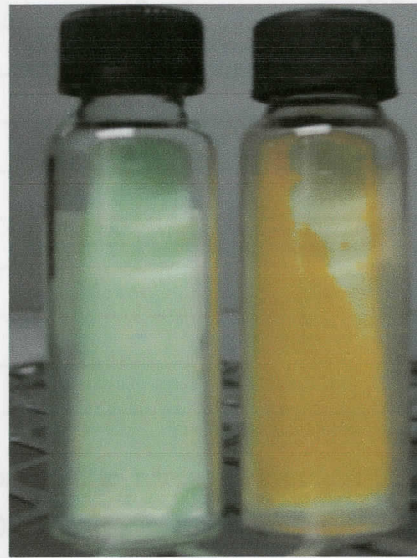


Figure 2.6 A photograph showing two pNB slants inoculated with *M. tuberculosis* H37Rv (left) and *M. fortuitum* (right), respectively.

2.5.4 Spoligotyping

Spoligotyping is a method used to simultaneously detect and genotype *M. tuberculosis* complex bacteria. This method is based on polymerase chain reaction (PCR) amplification of a highly polymorphic direct repeat locus specific to the *M. tuberculosis* complex (Gori *et al.*, 2005). Thus, spoligotyping can be used to differentiate between *M. tuberculosis* complex and NTM.

All positive MGIT and LJ cultures were spoligotyped. One millilitre of positive MGIT cultures or LJ colonies resuspended in 1 ml of 0.1% Tween 80 saline (refer to appendix) were boiled for 20 min at 100°C to kill mycobacteria. Thereafter, samples were transported from the biosafety level 3 laboratory and given to Dr. Elizabeth Streicher (DST/NRF Centre of Excellence in Biomedical TB Research, Division of Molecular Biology and Human Genetics, Faculty of Health Sciences, University of Stellenbosch) for spoligotyping. Spoligotyping was done as previously described by Kamerbeek *et al.* (1997). PCR was done in a reaction volume of 50 µl as follows; 1x reaction buffer, 0.4mM dNTPs, 5pmol forward primer [DRa (direct repeat a)] and 5pmol reverse primer [DRb (direct repeat b)] , 1.25 units enzyme (Qiagen, Hilden, Germany), 2.5µl boiled DNA template (**Table 2**). Cycling conditions were as follows; 95°C for 3 minutes, followed by 28 to 35 cycles at 94°C for 1 minute, 55°C for 1 minute, 72°C for 30 seconds, and a final extension step of 72°C for 10 minutes.

Table 2.2 PCR mixture for spoligotyping

Reagent	Final Concentration	Volume of Stock (µl)
10x Buffer	1x	5.0
MgCl ₂	2.5mM	4.0
dNTP Mix	0.4mM	8.0
Forward primer	5pmol	1.0
Reverse primer	5pmol	1.0
Taq	1.25 units	0.25
Distilled water		28.25
Boiled DNA template		2.5
Final reaction volume		50

Amplified products were hybridised to nitrocellulose membranes as follows: 20 µl of PCR products were added to 150 µl 2xSSPE/0.1%SDS buffer heat-denatured by incubating at 99°C for 10 minutes and then immediately placed on ice. Nitrocellulose membranes were washed in 2xSSPE/0.1%SDS buffer for 5 minutes at 57°C, after which membranes were placed on a support cushion in a mini blotter (Immunogenics Mini blotter 45, Isogen Bioscience) with the slots placed perpendicular to the line pattern. The residual fluid was removed from the slots by aspiration, where after denatured PCR products were added to the respective slots, taking care to avoid introducing air bubbles. It was then incubated for one hour at 57°C after which it was washed twice in 2xSSPE/0.5% SDS at 57°C. Ten microlitres of Streptavidine-peroxidase conjugate (5 units, Roche) was added to the membrane and incubated at 42°C for 45-60 minutes. Membranes were washed twice with 2xSSPE/0.5%SDS buffer at 42°C for 5-10 minutes, and rinsed with 2xSSPE buffer for 5 minutes at room temperature. The membrane was developed by adding 20ml of ECL detection reagents (Isogen Life Science, Maarsse, Netherlands) mixed in a 1:1 ratio (10ml solution 1 + 10ml solution 2) for one and a half minutes and exposed to film (Hyper Processor AM4, Amersham Pharmacia Biotech, Uppsala, Sweden) for 5-20 minutes.

2.6 PCR and DNA sequencing of the 16S rRNA gene

2.6.1 PCR

The 16S rRNA gene of acid fast positive MGIT cultures were sequenced as previously described by Turenne *et al.* (2001). A crude DNA template was prepared by boiling 500µl of cultures at 100°C for 20 minutes. Thereafter, samples were transferred to the PCR laboratory and amplified using the HotStarTaq® DNA polymerase (Qiagen, Hilden, Germany). A 577 bp fragment of the 16S rRNA gene was amplified using primers (**Table 2.3**).

Table 2.3 Primers used for PCR of 577bp product prior to 16S rRNA sequencing. (Primers were ordered from Integrated DNA Technologies, Inc., Coralville, USA; distributed by Whitehead Scientific, Cape Town, South Africa).

Primer name and orientation	Primer sequence	T _m
16S rRNA F (forward primer)	5' - AGA GTT TGA TCC TGG CTC AG - 3'	54.3 °C
16S rRNA R (reverse primer)	5' - GCG ACA AAC CAC CTA CGA G - 3'	55.9 °C

The reaction was prepared by adding 2.5 µl 10x reaction Buffer (Containing 15mM MgCl₂), 1.5µl dNTP (2.5mM), 1.5µl MgCl₂ (25mM), 1µl of each primer (forward 81.5nmol and reverse 75.2nmol), 0.1µl Taq polymerase (5 units/µl) and ddH₂O to a final volume of 20µl (**Table 2.4**). Tubes were mixed, where after 2.5µl of the crude DNA template was added to give a final reaction volume of 22.5µl. Thereafter, tubes were vortexed, briefly centrifuged and placed in the PCR machine (Eppendorf® Mastercycler, Eppendorf, Hamburg, Germany). PCR cycling conditions were as follows: 95°C for 2 minutes to activate the HotStarTaq DNA polymerase, 35 cycles of 95°C for 30 seconds to denature the DNA, 60°C for 30 seconds for primer annealing, and 72°C for 30 seconds for extension, and a final extension at 72°C for 10 minutes.

Table 2.4 PCR mixture for amplification of the 16S rRNA gene

Reagent	Final Concentration	Volume of Stock (µl)
10x Buffer	1x	2.5
dNTP Mix	0.75µM	1.5
MgCl ₂	1.5mM	1.5
Forward primer	785ng	1
Reverse primer	705ng	1
Taq	0.5U	0.1
Distilled water		12.4
Boiled DNA template		2.5
Final reaction volume		22.5

Amplified products were visualised by agarose gel electrophoresis and staining. When an electric field is applied across an agarose gel, DNA, which is negatively charged, migrates toward the anode at a rate proportional to its size (Sambrook *et al.*, 1989). Agarose gel electrophoresis was performed using 1.5% agarose gels. Agarose (Whitehead Scientific, Johannesburg, South Africa) was dissolved in 1x sodium boric (SB) acid buffer by heating in a microwave oven until the solution became clear. It was cooled to approximately 50°C before adding 2µl of GelStar® nucleic acid stain (Lonza, Basel, Switzerland) and being

poured into a horizontal mould and allowed to solidify for 30-60 min. The loading dye used was 5µl bromophenol blue (refer to appendix) (Merck, Darmstadt, Germany) and 5µl of sample was added to the loading dye before 5µl of this mixture was loaded onto the gel submerged in a buffer tank containing 1x SB buffer. A voltage of approximately 250V was applied to the gel, and the gel was allowed to run until the dye lines were clearly visible and had migrated approximately two-thirds of the distance of the gel. DNA was visualised by transillumination on a SynGene G:BOX (Syngene, Cambridge, United Kingdom). The GeneRuler™ 100 bp DNA ladder (Fermentas International Inc., Ontario, Canada) was used as a molecular weight marker.

2.6.2 Purification of PCR products for DNA sequencing

It is important that PCR products be purified to remove unbound primers, nucleotides and other debris in the sample, which could inhibit DNA sequencing. PCR products were purified by treating with ExoSAP-IT® (USB Corporation, Ohio, USA). Five microlitres of PCR product was mixed with 2µl of ExoSAP-IT® to yield a final reaction volume of 7µl. Reaction mixtures were incubated at 37°C for 15 minutes to degrade the remaining primers and nucleotides. The reaction was terminated by incubating at 80°C for 15 minutes to inactivate the ExoSAP-IT®.

2.6.3 DNA sequencing

Purified PCR products were sequenced with the 16S rRNA forward primer. PCR products were diluted to 7ng/µl as required for DNA sequencing and sent to the Central Analytical Facility of the University of Stellenbosch for sequencing.

The DNA sequencing reaction mixture consisted of 3µl purified PCR product, 3µl primer (81.5nmol), and 4µl BigDye™ (BigDye™ Terminator v3.1 Cycle Sequencing Kit, supplier). The cycling conditions were as follows: 94°C for 5 min, and 25 cycles of 94°C for 10seconds, 55°C for 10 seconds and 60°C for 4 min. Thereafter, the machine was cooled to 4°C until the completed sequencing reactions were removed. Purification of DNA sequencing reactions was done according to the Princeton Centri Sep 96 protocol (Princeton Separations, Incorporated, New Jersey, USA). Thereafter, samples were dried, 10µl of HiDi formamide (Applied Biosystems, Foster City, USA) added and the samples denatured at 95°C for 3 minutes. Samples were run on the Load ABI 3130xl Genetic Analyser (Applied Biosystems, Foster City, USA) and then analysed using the Sequencing Analysis v5.2 software (Applied Biosystems, Foster City, USA).

CHAPTER THREE

RESULTS

3.1 Identification of *M. tuberculosis* complex bacteria by spoligotyping and Capilia® TB/TAUNS

A total of 6042 sputum samples were collected during the ZAMSTAR prevalence surveys. Of these, 388 (6.42%) were MGIT culture positive. Spoligotyping (Figure 3.1) and Capilia® TB/TAUNS (Figure 3.2) identified 283 (72.94%) of these as belonging to the *M. tuberculosis* complex, while 105 (27.06%) were identified as possible NTM.

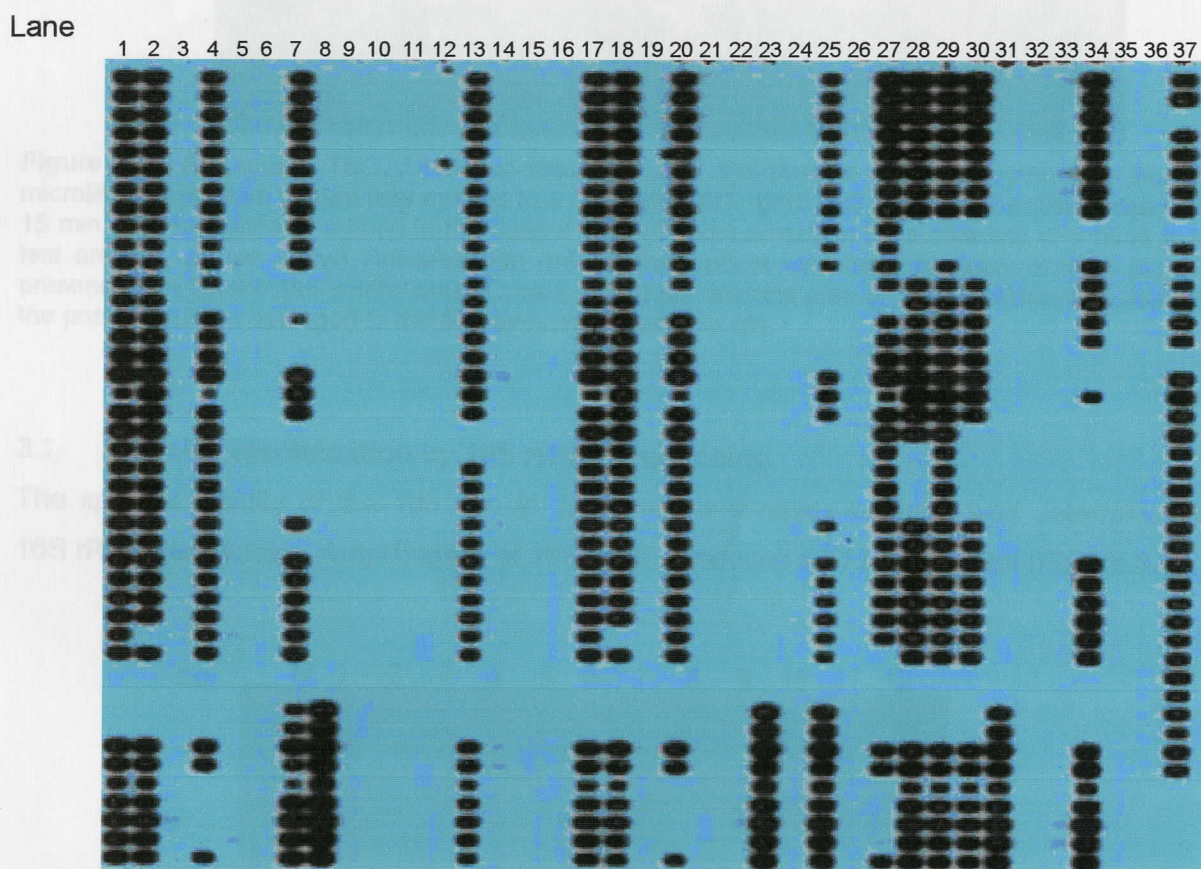


Figure 3.1 A spoligotype blot of 37 of the MGIT positive cultures analysed. MGIT positive cultures were amplified using primers specific to the *M. tuberculosis* complex, and then hybridised to a nitrocellulose membrane containing 43 unique direct variable region (DVR) repeat sequences. Hybridisation between the PCR product and the DVR region on the nitrocellulose membrane indicates *M. tuberculosis* complex (lanes 1, 2, 4, 7, 8, 13, 17, 18, 20, 23, 25, 27-31, 34, 37), while the absence of hybridisation indicated NTM (lanes 3, 5, 6, 9-12, 14-16, 21, 22, 24, 26, 32, 33, 35, 36). Positive control *M. tuberculosis* H37Rv can be seen in lane 13 and BCG is shown in lane 37.

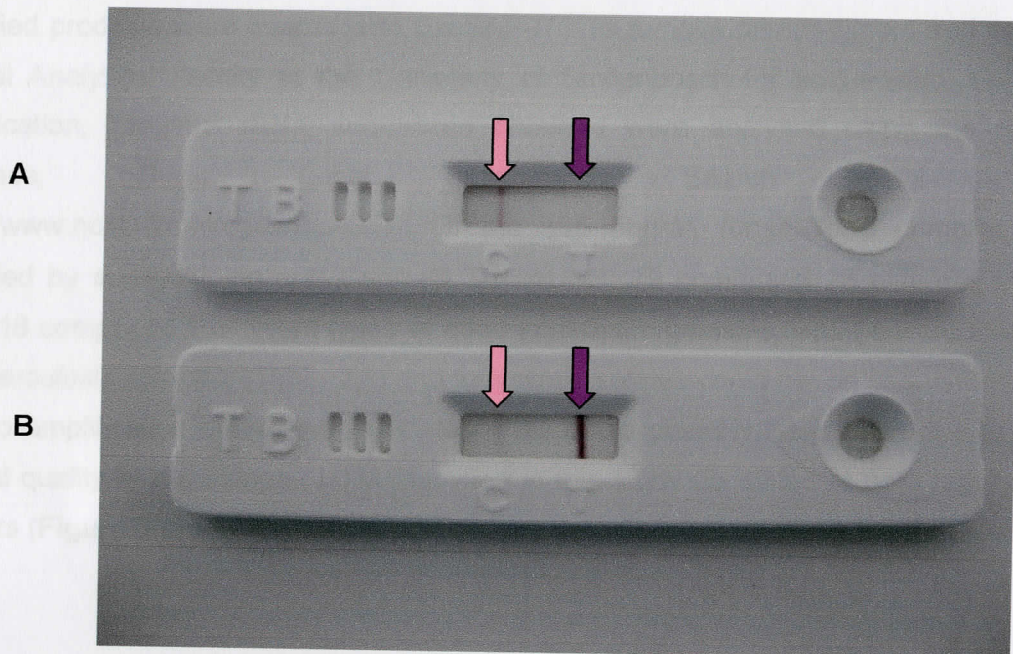


Figure 3.2 A Capilia® TB/TAUNS test result for 2 of the positive MGIT cultures. One hundred microlitres of positive culture was applied to a Capilia® TB/TAUNS test strip and the results read after 15 min. The presence of a band in the control area (C, pink arrow) and the absence of a band in the test area (T, purple arrow) indicated that the positive culture was most probably a NTM (A). The presence of a band in the control area (C, pink arrow) and the test area (T, purple arrow) indicated that the positive culture belonged to the *M. tuberculosis* complex (B).

3.2 Species identification by 16S rRNA sequencing

The species identity of the 105 non-*M. tuberculosis* complex bacteria was determined by 16S rRNA sequencing. Amplification of 16S rRNA produced a 577 bp product (Figure 3.3).

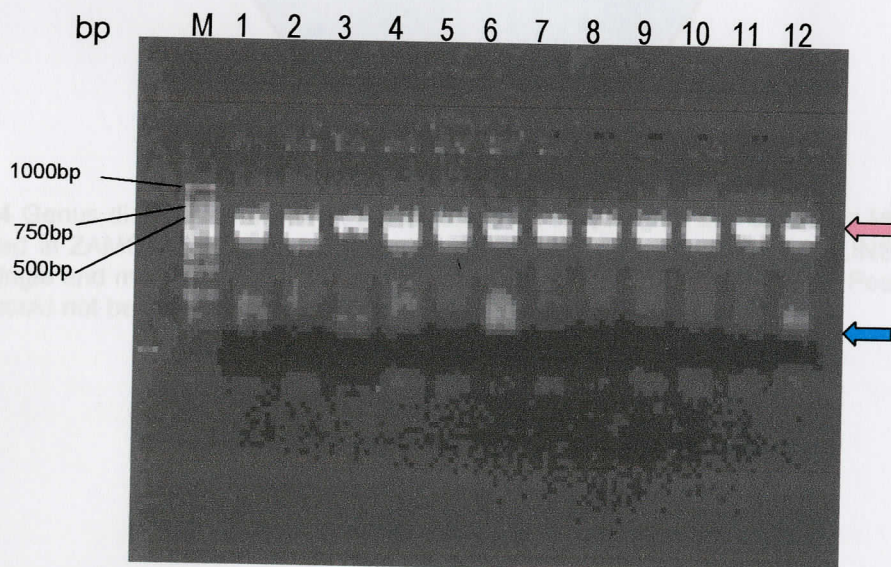


Figure 3.3 16S rRNA amplification products for 12 of the 105 possible NTM. PCR was done using crude DNA templates and primers listed in Table 2.3. Amplified products were separated with 2% agarose gel electrophoresis. Lanes 1-12 represent the amplified products of 577 bp (pink arrow). The 100 bp molecular weight marker was used as a DNA size marker (M). The blue arrow indicates primer dimers.

Amplified products were treated with ExoSAP-IT® to remove primer-dimers and sent to the Central Analytical Facility at the University of Stellenbosch for sequencing. For species identification, the 16S rRNA sequences obtained were analysed using the nucleotide database, Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov/BLAST/>). Of the 105 non-*M. tuberculosis* complex bacteria identified by spoligotyping and Capilia® TB/TAUNS, 79 comprised 28 different species of NTM, 18 comprised 7 different types of other acid-fast organisms, while 4 were identified as *M. tuberculosis* complex (Table 3.1) and four other isolates could not be sequenced as there was no amplification during the PCR step. This could possibly have been due to the sub-optimal quality of the sample. NTM identified in this study consisted of both rapid and slow growers (Figure 3.4).

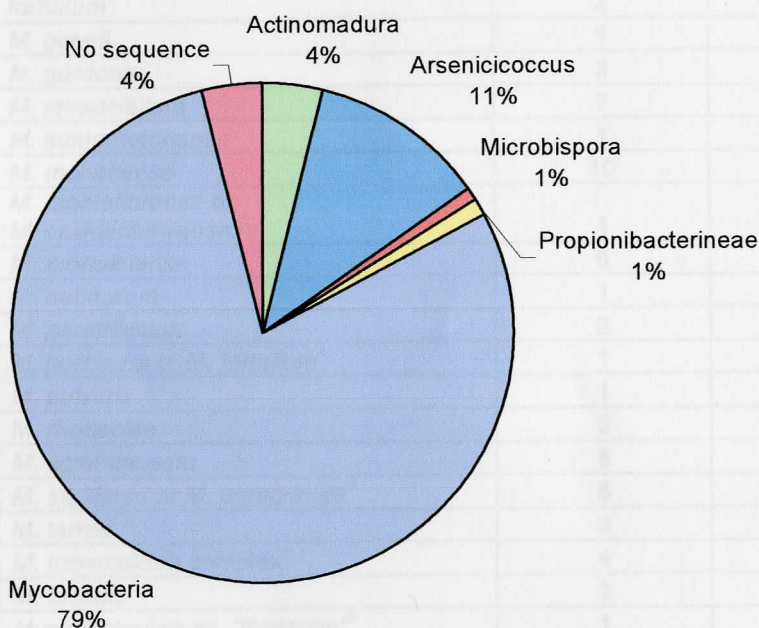


Fig 3.4 Genus distribution of the 105 possible NTM identified in this study. All MGIT positive cultures obtained in ZAMSTAR that had a negative spoligotype and Capilia® TB/TAUNS result, representing both single and multiple samples from patients were included in this analysis. Four of the 105 possible NTM could not be sequenced.

Table 3.1 16S rRNA sequencing results of the 105 possible NTM recovered in this study. These isolates included all MGIT positive cultures obtained in ZAMSTAR that had a negative spoligotype and Capilia® TB/TAUNS result and represented both single and multiple samples from patients.

Genus	Species	No. of isolates (101) ¹	Percentage (%)
<i>Actinomadura</i>	<i>A. cremea</i>	1	0.95
	<i>A. formosensis</i>	1	0.95
	<i>A. meyerii</i>	1	0.95
	<i>Actinomadura sp.</i> ²	1	0.95
<i>Arsenicococcus</i>	<i>A. bolidensis</i>	12	11.4
<i>Microbispora</i>	<i>M. corallina</i>	1	0.95
<i>Propionibacterineae</i> ³		1	0.95
<i>Mycobacterium</i>	<i>M. acapulcensis</i> or <i>M. flavescens</i> ⁴	1	0.95
	<i>M. alvei</i>	1	0.95
	<i>M. arupense</i>	1	0.95
	<i>M. avium complex</i>	1	0.95
	<i>M. duvali</i>	1	0.95
	<i>M. elephantis</i>	2	1.9
	<i>M. flavescens</i>	2	1.9
	<i>M. florentinum</i>	1	1.0
	<i>M. fortuitum</i>	6	5.7
	<i>M. fortuitum acetomidolyticum</i> or <i>fortuitum</i> ⁴	2	1.9
	<i>M. goodii</i>	1	0.95
	<i>M. gordonae</i>	3	2.85
	<i>M. intracellulare</i>	7	6.7
	<i>M. kumamotoense</i>	1	0.95
	<i>M. monacense</i>	10	9.5
	<i>M. montefiorensis</i> or <i>M. saskatchewanense</i> ⁴	1	0.95
	<i>M. moriokaense</i>	6	5.7
	<i>M. neoaurum</i>	1	0.95
	<i>M. paraffinicum</i>	2	1.9
	<i>M. porcinum</i> or <i>M. fortuitum</i> ⁴	1	0.95
	<i>M. pulveris</i>	1	0.95
	<i>M. rhodesiae</i>	3	2.85
	<i>M. scrofulaceum</i>	5	4.8
	<i>M. septicum</i> or <i>M. peregrinum</i> ⁴	8	7.6
	<i>M. terrae</i>	3	2.85
	<i>M. tuberculosis complex</i>	4	3.8
	<i>M. tusciae</i>	2	1.9
	<i>Mycobacterium sp. "Bavariae"</i> ⁵	1	0.95
	Unknown mycobacterium species ⁶	5	4.8

¹Four of the 105 possible NTM could not be sequenced.

²Member of the *Actinomadura* genus. The species could not be identified.

³Member of the *Propionibacterineae* genus. The species could not be identified.

⁴Blast searches using 16S rRNA sequence data showed similarity to both organisms

⁵This is a new species of the genus *Mycobacterium* which has been given a provisional species name.

⁶This is an unknown species closely related, but not identical to *M. moriokaense*.

The NTM species recovered in this study are showed in **Figure 3.5** as they are grouped according to their growth rates.

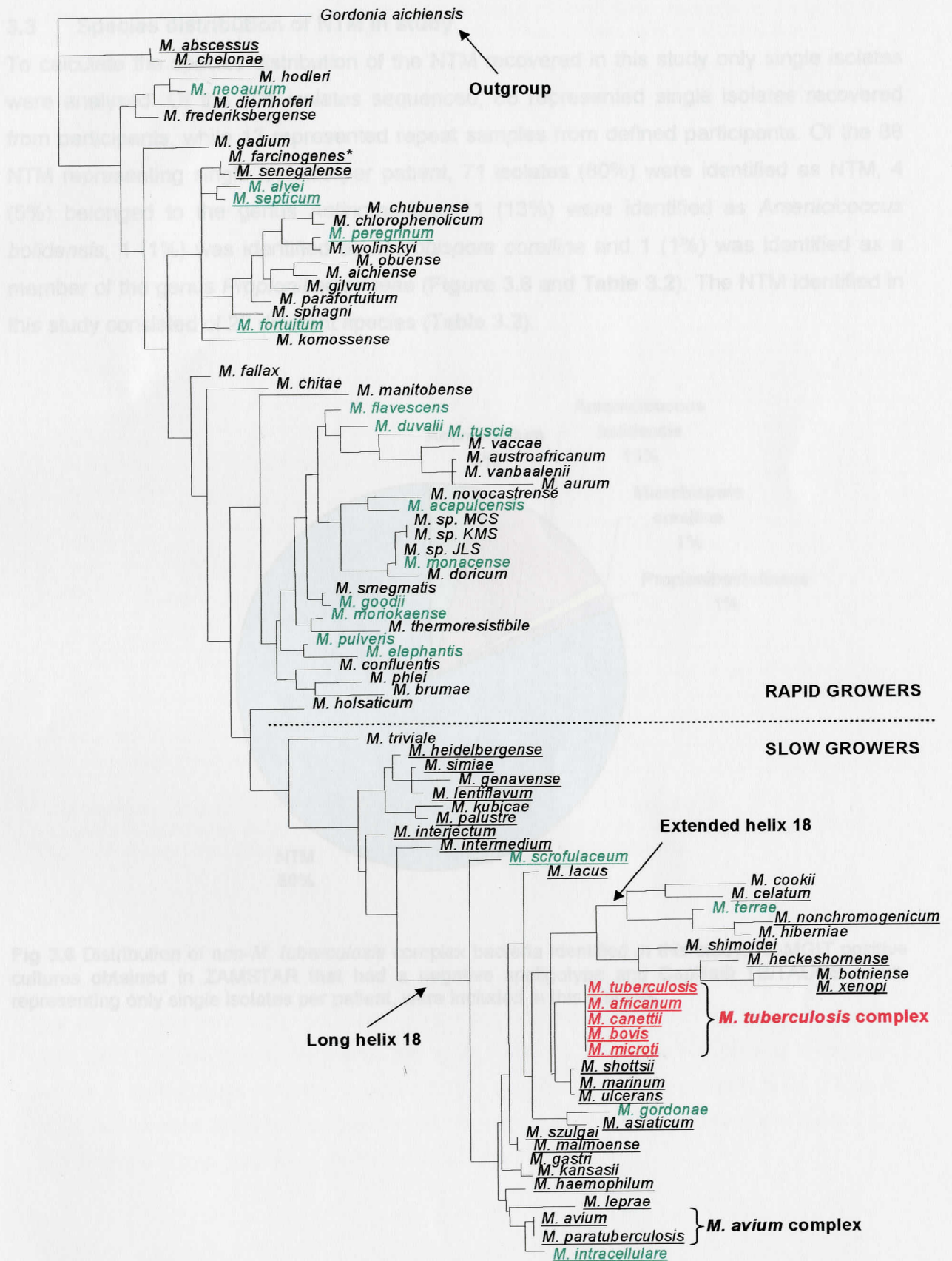


Figure 3.5 Phylogenetic tree of the genus *Mycobacterium*. The phylogenetic tree was constructed using 16S rRNA sequence information. Mycobacteria classified as rapid or slow growers are indicated. Mycobacterial species identified in this study are indicated in green (NTM) and red (*M. tuberculosis*). Figure reproduced with kind permission by Dr. NC Gey van Pittius, Department of Medical Biochemistry, University of Stellenbosch, originally adapted from Shinnick and Good, 1994 and Springer *et al.* 1996.

3.3 Species distribution of NTM in study

To calculate the species distribution of the NTM recovered in this study only single isolates were analysed. Of the 101 isolates sequenced, 88 represented single isolates recovered from participants, while 13 represented repeat samples from defined participants. Of the 88 NTM representing single isolates per patient, 71 isolates (80%) were identified as NTM, 4 (5%) belonged to the genus *Actinomadura*, 11 (13%) were identified as *Arsenicicoccus bolidensis*, 1 (1%) was identified as *Microbispora corallina* and 1 (1%) was identified as a member of the genus *Propionibacterineae* (Figure 3.6 and Table 3.2). The NTM identified in this study consisted of 27 different species (Table 3.2).

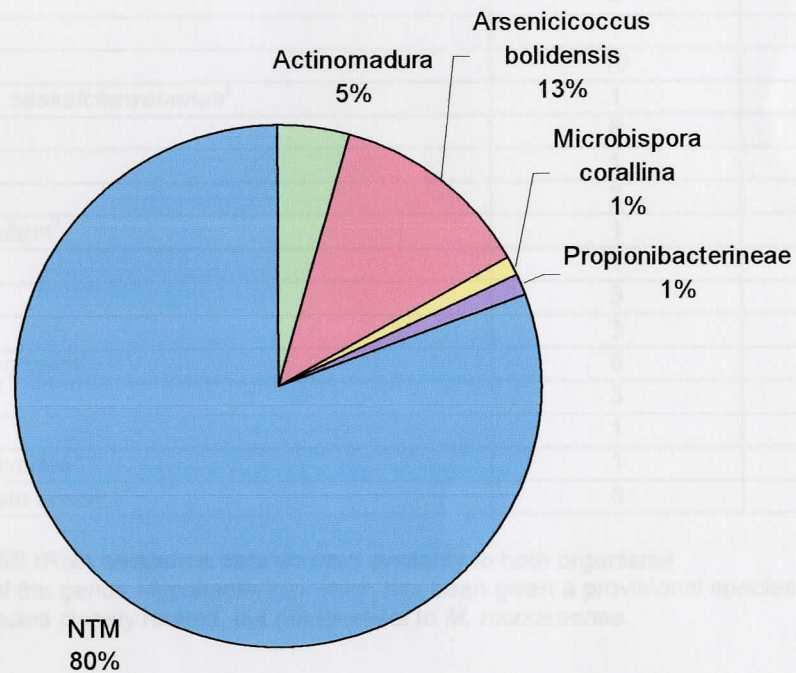


Fig 3.6 Distribution of non-*M. tuberculosis* complex bacteria identified in this study. All MGIT positive cultures obtained in ZAMSTAR that had a negative spoligotype and Capilia® TB/TAUNS result, representing only single isolates per patient, were included in this analysis.

Table 3.2 Species distribution of the 71 NTM identified by 16S rRNA sequencing. These NTM represented only single isolates recovered from patients. These isolates were all MGIT culture positive and had a negative spoligotype and Capilia® TB/TAUNS result.

Species	No. of isolates (71)	Percentage (%)
<i>M. acapulcensis</i> or <i>M. flavescens</i> ¹	1	1.4
<i>M. alvei</i>	1	1.4
<i>M. arupense</i>	1	1.4
<i>M. duvali</i>	1	1.4
<i>M. elephantis</i>	2	2.8
<i>M. flavescens</i>	1	1.4
<i>M. florentinum</i>	1	1.4
<i>M. fortuitum</i>	6	8.5
<i>M. fortuitum acetomidolyticum</i> or <i>fortuitum</i> ¹	2	2.8
<i>M. goodii</i>	1	1.4
<i>M. gordonae</i>	3	4.2
<i>M. intracellulare</i>	4	5.6
<i>M. kumamotoense</i>	1	1.4
<i>M. monacense</i>	10	14.1
<i>M. montefiorensis</i> or <i>M. saskatchewanensis</i> ¹	1	1.4
<i>M. moriokaense</i>	6	8.5
<i>M. neoaurum</i>	1	1.4
<i>M. paraffinicum</i>	2	2.8
<i>M. porcinum</i> or <i>M. fortuitum</i> ¹	1	1.4
<i>M. pulveris</i>	1	1.4
<i>M. rhodesiae</i>	3	4.2
<i>M. scrofulaceum</i>	3	4.2
<i>M. septicum</i> or <i>M. peregrinum</i> ¹	8	11.3
<i>M. terrae</i>	3	4.2
<i>M. tusciae</i>	1	1.4
<i>Mycobacterium</i> sp. "Bavariae" ²	1	1.4
Unknown mycobacterium species ³	5	7.0

¹Blast searches using 16S rRNA sequence data showed similarity to both organisms.

²This is a new species of the genus *Mycobacterium* which has been given a provisional species name.

³This is an unknown species closely related, but not identical to *M. moriokaense*.

3.4 Percentage of NTM recovered

To calculate the percentage of NTM recovered in MGIT positive cultures only single isolates per patient were considered and follow-up samples were excluded from the analysis. Of the 249 MGIT positive isolates recovered per patient, 155 (62.2%) were identified as belonging to the *M. tuberculosis* complex, while 92 (36.1%) were identified as possible NTM (Figure 3.7). Two isolates could not be classified as *M. tuberculosis* complex or possible NTM due to the absence of spoligotype results for these isolates.

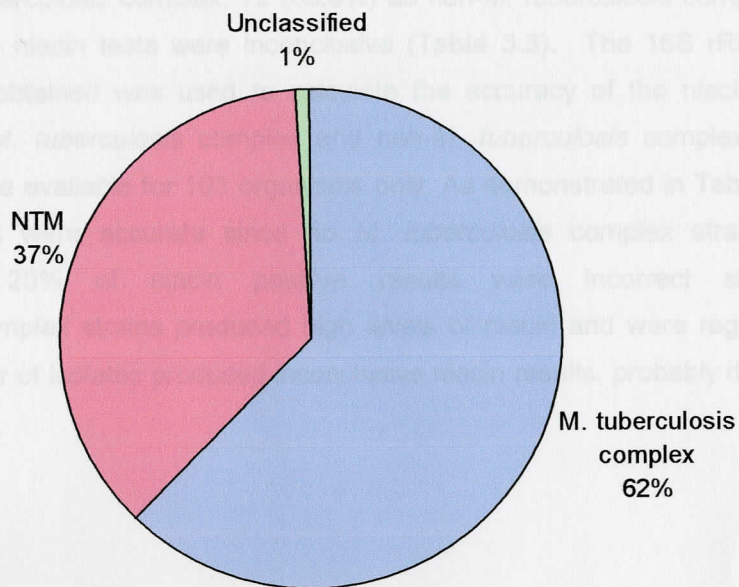


Fig 3.7 Percentage of NTM recovered in MGIT positive cultures. All MGIT positive cultures obtained in ZAMSTAR that had a negative spoligotype and Capilia® TB/TAUNS result, representing only single isolates per patients were included in this analysis.

3.5 Niacin test

The niacin test is a biochemical test that was used to determine whether MGIT positive cultures belonged to the *M. tuberculosis* complex. Due to a blocked metabolic pathway, strains belonging to the *M. tuberculosis* complex produce more niacin than NTM, and will therefore produce a yellow colour when a niacin reagent test strip is inserted in the culture medium (**Figure 3.8**).

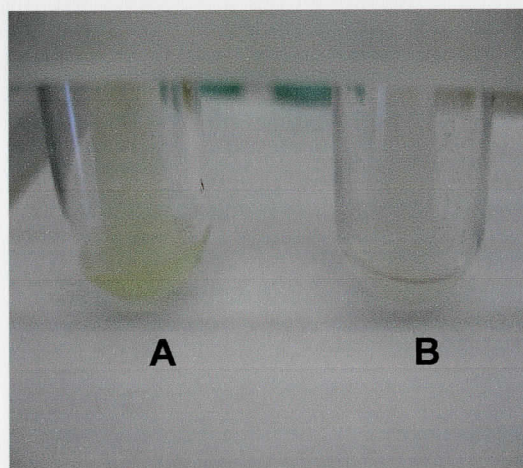


Figure 3.8 Niacin result for two of the 105 possible NTM. Positive cultures were subcultured onto LJ slants, incubated at 37°C for 6 weeks and the amount of niacin production measured with a niacin test strip. Bacteria belonging to the *M. tuberculosis* complex will produce a yellow colour (A), while non-*M. tuberculosis* complex bacteria will yield a colourless result (B).

Table 3.3 Niacin results for the 105 possible NTM strains identified in this study. MGIT positive cultures with negative spoligotypes and negative Capilia® TB/TAUNS results were used as selection criteria.

Niacin result	No. of isolates	Species
Positive	25	<i>M. tuberculosis</i> complex n=4 <i>Arsenicococcus bolidensis</i> , n=9 <i>M. flavescens</i> , n=1 <i>M. fortuitum</i> , n=2 <i>M. fortuitum acetomidolyticum</i> or <i>fortuitum</i> , n=2 <i>M. monacense</i> , n=3 <i>M. moriokaense</i> , n=2 <i>M. septicum</i> or <i>M. peregrinum</i> , n=1 <i>M. tusciae</i> , n=1
Negative	72	<i>Actinomadura meyerii</i> , n=1 <i>Actinomadura</i> sp., n=1 <i>Arsenicococcus bolidensis</i> , n=3 <i>M. acapulcensis</i> or <i>M. flavescens</i> , n=1 <i>M. alvei</i> , n=1 <i>M. arupense</i> , n=1 <i>M. avium</i> complex, n=1 <i>M. duvali</i> , n=1 <i>M. elephantis</i> , n=1 <i>M. fortuitum</i> , n=4 <i>M. goodii</i> , n=1 <i>M. gordonae</i> , n=3 <i>M. intracellulare</i> , n=8 <i>M. kumamotoense</i> , n=1 <i>M. monacense</i> , n=7 <i>M. montefiorensis</i> or <i>M. saskatchewanese</i> , n=1 <i>M. moriokaense</i> , n=4 <i>M. neoaurum</i> , n=1 <i>M. paraffinicum</i> , n=1 <i>M. porcinum</i> or <i>M. fortuitum</i> , n=1 <i>M. pulveris</i> , n=1 <i>M. rhodesiae</i> , n=5 <i>M. scrofulaceum</i> , n=4 <i>M. septicum</i> or <i>M. peregrinum</i> , n=6 <i>M. terrae</i> , n=3 <i>M. tusciae</i> , n=1 Mixed infection, maybe <i>M. flavescens</i> , n=1 <i>Microbispora corallina</i> , n=1 <i>Mycobacterium</i> species "Bavariae", n=1 <i>Propionibacterineae</i> bacterium clone, n=1 Unknown mycobacterial sequence closely related to <i>M. moriokaense</i> , n=4 No sequence, n=2
Inconclusive	8	<i>Actinomadura cremea</i> , n=1 <i>Actinomadura formosensis</i> , n=1 <i>M. elephantis</i> , n=1 <i>M. interjectum</i> , n=1 <i>M. septicum</i> or <i>M. peregrinum</i> , n=1 Unknown mycobacterial sequence closely related to <i>M. moriokaense</i> , n=1 No sequence, n=2

3.6 *p*-Nitrobenzoic acid Löwenstein-Jensen slants

The accuracy of the *p*-nitrobenzoic acid test to differentiate between *M. tuberculosis* complex and non-*M. tuberculosis* complex strains was investigated by inoculating the 105 non-*M. tuberculosis* complex strains identified by spoligotyping and Capilia® TB/TAUNS onto *p*NB slants. The *p*NB test identified 12 of the 105 possible NTM samples as *M. tuberculosis* complex, while 93 of the possible NTM organisms were confirmed as being non-*M. tuberculosis* complex (Table 3.3).

Figure 3.9 illustrates the *p*NB test results for two of the 105 samples analysed in this study.



Figure 3.9 *p*-Nitrobenzoic acid Löwenstein-Jensen slant result for two of the 105 possible NTM. Positive cultures were sub-cultured onto *p*NB slants and incubated at 37°C for 6 weeks, or until *p*NB slants became positive. *M. tuberculosis* complex bacteria are unable to grow on *p*NB slants (A), while non-*M. tuberculosis* complex bacteria form yellow, crumb-like colonies (B).

Table 3.4 *p*-Nitrobenzoic acid Löwenstein-Jensen slants results for 105 possible NTM strains identified in this study.

pNB result	No. of isolates	Species
No growth	12	<i>M. tuberculosis</i> complex n=1 <i>Actinomadura</i> sp., n=1 <i>Arsenicococcus bolidensis</i> , n=1 <i>M. flavescens</i> , n=1 <i>M. intracellulare</i> , n=1 <i>M. paraffinicum</i> , n=1 <i>M. rhodesiae</i> , n=1 <i>M. scrofulaceum</i> , n=1 <i>M. tusciae</i> , n=1 <i>Mycobacterium</i> species "Bavariae", n=1 Not sequenceable, n=2
Growth	93	<i>M. tuberculosis</i> complex, n=3 <i>Actinomadura cremea</i> , n=1 <i>Actinomadura formosensis</i> , n=1 <i>Actinomadura meyerii</i> , n=1 <i>Arsenicococcus bolidensis</i> , n=11 <i>M. acapulcensis</i> or <i>M. flavescens</i> , n=1 <i>M. alvei</i> , n=1 <i>M. arupense</i> , n=1 <i>M. avium</i> complex, n=1 <i>M. duvali</i> , n=1 <i>M. elephantis</i> , n=2 <i>M. fortuitum</i> , n=6 <i>M. fortuitum acetomidolyticum</i> or <i>fortuitum</i> , n=2 <i>M. goodii</i> , n=1 <i>M. gordonae</i> , n=3 <i>M. interjectum</i> , n=1 <i>M. intracellulare</i> , n=6 <i>M. kumamotonense</i> , n=1 <i>M. monacense</i> , n=10 <i>M. montefiorensis</i> or <i>M. saskatchewanese</i> , n=1 <i>M. moriokaense</i> , n=6 <i>M. neoaurum</i> , n=1 <i>M. paraffinicum</i> , n=1 <i>M. porcinum</i> or <i>M. fortuitum</i> , n=1 <i>M. pulveris</i> , n=1 <i>M. rhodesiae</i> , n=4 <i>M. scrofulaceum</i> , n=3 <i>M. septicum</i> or <i>M. peregrinum</i> , n=8 <i>M. terrae</i> , n=3 <i>M. tusciae</i> , n=1 <i>Microbispora corallina</i> , n=1 <i>Propionibacterineae</i> bacterium clone, n=1 Unknown mycobacterial sequence closely related to <i>M. moriokaense</i> , n=5 Not sequenceable, n=2

3.7 Accuracy of spoligotyping, Capilia® TB/TAUNS, niacin and p-Nitrobenzoic acid Löwenstein-Jensen slant tests to identify *M. tuberculosis* complex bacteria

The accuracy of spoligotyping, Capilia® TB/TAUNS, niacin and pNB tests to identify *M. tuberculosis* complex bacteria was calculated by comparing test results to 16S rRNA sequencing data (Table 3.5).

Table 3.5 Results of comparative analysis of the spoligotyping, Capilia® TB/TAUNS, niacin and pNB tests using 16S rRNA sequence as the gold standard.

Test	Result (16S rRNA results)	Accuracy of tests to identify NTM using 16S rRNA as the gold standard (%)
Spoligotyping	<i>M. tuberculosis</i> complex, n=0 (4)	101 (96.2%)
	Non- <i>M. tuberculosis</i> complex, n=105 (101)	
Capilia® TB/TAUNS	<i>M. tuberculosis</i> complex, n=0 (4)	101 (96.2%)
	Non- <i>M. tuberculosis</i> complex, n=105 (101)	
Niacin	<i>M. tuberculosis</i> complex, n=25 (4)	80 (76.2%)
	Non- <i>M. tuberculosis</i> complex, n=80 (101)	
pNB	<i>M. tuberculosis</i> complex, n=12 (4)	93 (88.6%)
	Non- <i>M. tuberculosis</i> complex, n=93 (101)	

Spoligotyping was unable to identify 4 *M. tuberculosis* complex strains.
 Capilia® TB/TAUNS was unable to identify 4 *M. tuberculosis* complex strains.

3.8 Direct Ziehl-Neelsen and Löwenstein-Jensen culture

Direct smears were prepared for all sputum samples received in the laboratory. Thereafter, sputum was decontaminated and cultured in MGIT system and on LJ slants. The 105 isolates investigated in this study were all MGIT positive, while 20 were LJ positive and only 8 were smear positive (Table 3.6).

Table 3.6 Direct smear and LJ results of the 105 MGIT positive samples investigated in this study.

Isolate	Direct smear		LJ	
	Positive (n)	Negative (n)	Positive (n)	Negative (n)
<i>M. tuberculosis</i> complex, n=4	4	0	3	1
<i>Actinomadura cremea</i> , n=1	0	1	0	1
<i>Actinomadura formosensis</i> , n=1	0	1	0	1
<i>Actinomadura meyerii</i> , n=1	0	1	0	1
<i>Actinomadura sp.</i> , n=1	0	1	0	1
<i>Arsenicococcus bolidensis</i> , n=12	0	12	2	10
<i>M. acapulcensis</i> or <i>M. flavescens</i> , n=1	0	1	0	1
<i>M. alvei</i> , n=1	0	1	0	1
<i>M. arupense</i> , n=1	0	1	0	1
<i>M. avium</i> complex, n=1				
<i>M. duvali</i> , n=1	0	1	0	1
<i>M. elephantis</i> , n=2	0	2	1	1
<i>M. flavescens</i> , n=1	0	1	0	1
<i>M. fortuitum</i> , n=6	1 (1+)	5	2	4
<i>M. fortuitum acetomidolyticum</i> or <i>fortuitum</i> , n=2	1 (0-9 bacilli)	1	1	1
<i>M. goodii</i> , n=1	0	1	0	1
<i>M. gordonae</i> , n=3	0	3	1	2
<i>M. interjectum</i> , n=1	0	1	1	
<i>M. intracellulare</i> , n=7	2 (1+)	6	3	5
<i>M. kumamotoense</i> , n=1	0	1	0	1
<i>M. monacense</i> , n=10	0	10	1	9
<i>M. moriokaense</i> or <i>M. saskatchewanese</i> , n=1	0	1	1	1
<i>M. moriokaense</i> , n=6	0	6	0	6
<i>M. neoaurum</i> , n=1	0	1	0	1
<i>M. paraffinicum</i> , n=2	0	2	0	2
<i>M. porcinum</i> or <i>M. fortuitum</i> , n=1	0	1	1	
<i>M. pulveris</i> , n=1	0	1	0	1
<i>M. rhodesiae</i> , n=5	0	5	0	5
<i>M. scrofulaceum</i> , n=4	0	4	1	3
<i>M. septicum</i> or <i>M. peregrinum</i> , n=8	0	8	1	7
<i>M. terrae</i> , n=3	0	3	1	2
<i>M. tusciae</i> , n=2	0	2	0	2
<i>Microbispora corallina</i> , n=1	0	1	0	1
<i>Mycobacterium species "Bavariae"</i> , n=1	0	1	0	1
<i>Propionibacterineae</i> bacterium clone, n=1	0	1	0	1
Unknown mycobacterium species closely related to <i>M. moriokaense</i> , n=5	0	5	0	5
No sequence, n=4	0	4	0	4

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

NTM are ubiquitous in nature and usually do not cause disease. However, NTM infection may have consequences for immunocompromised individuals and in those infected with *M. tuberculosis*. In this study, NTM were recovered from participants in a TB surveillance study conducted in two high-incidence TB communities in the Western Cape Province of South Africa were investigated.

The ZAMSTAR trial aims to reduce the prevalence of TB and HIV by using community based intervention studies in South Africa and Zambia. Pilot studies to determine the prevalence of TB in local communities were performed in both countries. In South Africa, these pilot prevalence surveys were conducted in high-incidence TB communities, Scottsdene and Site B, Khayelitsha. Both communities have high unemployment and poverty rates. In 2004, the two most common causes of death in Khayelitsha were HIV/AIDS and pulmonary TB (Dr. Azevedo, personal communication).

During ZAMSTAR, a total of 6042 spot sputum samples were recovered from randomly selected participants. These included both single and multiple samples from the same patient, since follow up samples were requested from patients whose first sample was MGIT culture positive. Direct smear analysis was done on all sputum samples received in the laboratory, thereafter they were cultured in the MGIT system and on LJ slants. All positive cultures were subjected to a panel of different laboratory tests to differentiate between *M. tuberculosis* complex and NTM. Of the 6042 samples received in the laboratory, 388 were MGIT culture positive (confirmed by culture ZN as being acid-fast bacteria). A negative spoligotype and a negative Capilia® TB/TAUNS test result most probably indicated that an isolate does not belong to the *M. tuberculosis* complex. Of the 380 MGIT positive cultures recovered in this study, 105 were identified as not belonging to the *M. tuberculosis* complex using spoligotyping and Capilia® TB/TAUNS results as inclusion criteria. The 16S rRNA gene of these 105 isolates was sequenced and the accuracy of the laboratory tests (including pNB slants, Capilia® TB/TAUNS tests, spoligotyping and niacin tests) to correctly identify NTM was established using 16S rRNA gene sequences as the gold standard. Only single isolates per patient were included in an analysis to detect the proportion of NTM identified in positive MGIT cultures and to calculate the species distribution of NTM in the study. Furthermore, growth in the MGIT system and on LJ slants, and direct smear analysis were compared to determine the sensitivities of these methods to detect NTM.

Of the 5743 single isolates recovered from participants, 71 were identified as NTM. This is quite high and implies that at least 1.2% of individuals in these communities are infected with NTM. Importantly, the high numbers of NTM recovered in this study may be an underestimation since the sputum processing methods used here selected for the culture of *M. tuberculosis* complex strains and may have been too harsh for NTM. South Africa has one of the highest HIV infection rates in the world, therefore the high NTM infection rates reported here is a source for concern. For example, it has been reported that infection with NTM may increase the progression to AIDS, as studies by Dezzutti and Swords have shown that certain NTM may enhance HIV-1 replication (Dezzutti *et al.*, 1999; Swords *et al.*, 2006).

TB is diagnosed by smear microscopy, culture or clinical and radiological symptoms (Foulds & O'Brien, 1998). However, it is often difficult to distinguish between *M. tuberculosis* and NTM, therefore often hindering accurate diagnosis of TB. In this study, 4 of the 87 (from the total amount of 6042 samples) NTM had positive direct smears. Smear microscopy is the most widely used method to diagnose TB in developing countries (Van Deun & Portaels, 1998), therefore, these results imply that in certain cases NTM infection may incorrectly be diagnosed as TB since it is difficult to distinguish between NTM and *M. tuberculosis* by microscopy.

Culturing TB on LJ slants is the most popular *M. tuberculosis* culture method in developing countries (Foulds & O'Brien, 1998). Even though it takes much longer to culture *M. tuberculosis* than in liquid culture media, it is not technically difficult, only requires a simple incubator and is more accessible to developing countries where technology, like the BACTEC™ MGIT™, is not available. The MGIT system has replaced traditional culture systems such as LJ in developed countries. This is a liquid based culture method for mycobacteria from sputum or other clinical samples.

In this study, only eight samples had a positive LJ slant without also having a positive MGIT and of the 105 possible NTM in this study, 20 had positive LJ slants, of which 15 were NTM. In this context, LJ seems to be the better option to culture *M. tuberculosis* complex bacteria. Very few NTM were recovered from LJ culture, whereas many were isolated by MGIT culture. Some authors suggest the MGIT, and other liquid culture methods similar to it, are too sensitive, sometimes culturing slight contaminants, which would not have been recovered by less sensitive methods. LJ solid media, being a simple culturing method, is not as sensitive as liquid culture and will thus only show growth of organisms that are present in large amounts.

The inclusion criteria for this particular study was for the organisms to have both a negative spoligotype as well as a negative Capilia® TB/TAUNS test result. The niacin test, 16S rRNA sequencing and *p*-Nitrobenzoic acid Löwenstein-Jensen slants were also used to differentiate between *M. tuberculosis* complex bacteria and NTM. The accuracy of the identification tests in this study were calculated to be: spoligotyping and Capilia® TB/TAUNS, 96.2%; niacin test, 76.2% and *p*-Nitrobenzoic acid LJ slants, 88.6%. Incorrect diagnosis of NTM infection as TB may lead to incorrect treatment. Many patients may be wrongfully diagnosed as having TB, due to identification tests that are not able to discriminate between *M. tuberculosis* complex strains and NTM. These patients are then inappropriately treated, possibly exacerbating the TB drug resistance epidemic. They also have to endure the side-effects of the TB medication, which can be hepatotoxic. This highlights the importance of accurate identification tests to diagnose mycobacterial infection and disease. In this particular study, most of the participants with positive cultures were placed on TB treatment.

The test with the highest accuracy was the Capilia® TB/TAUNS which was able to correctly identify 7 NTM. However, the test failed to correctly identify 4 *M. tuberculosis* complex strains. The Capilia® TB/TAUNS test detects the MPB64 protein, a protein that is specific to the *M. tuberculosis* complex. Others have reported that mutations in the *mpb64* gene or the lack of protein expression Capilia® TB/TAUNS may also cause false-negatives. According to Hirano *et al.*, 2004, there were 12 Capilia® TB/TAUNS negative *M. tuberculosis* strains isolated between June 2001 and August 2002 in various districts in Japan. Mutations in the *mpb64* gene caused deletions of the C-terminal amino acids, thus causing the test to yield false-negative results (Hirano *et al.*, 2004). This could be a possible explanation for the false-negative results found in this study. Currently the test is only commercially available in Japan, but the ease, simplicity and accurate results of this test warrants its distribution to other parts of the world. The Capilia® TB/TAUNS test may also play a very valuable part in TB diagnosis in remote settings. Cultures from either liquid or solid media can be used and the result is available within 15 minutes. No technology is needed and minimal skill is required. This test, when commercially available, can help many clinicians to diagnose patients much sooner than previously, and the spread of TB might become less with earlier diagnosis.

The niacin test performed the weakest of all the identification tests. Although all mycobacteria produce niacin, *M. tuberculosis* produces the most niacin because of a blocked metabolic pathway (SA Health Info, 2006). Very few other species have positive niacin tests, while niacin-negative *M. tuberculosis* strains are very rare (SA Health Info, 2006). The niacin test is done by determining the amount of niacin released from bacteria cultured on LJ slants. Cultures grown on egg media have been shown to yield the most consistent results (SA

Health Info, 2006). The inaccuracy of the niacin test could possibly be due to the fact that the level of niacin secreted into the growth medium is dependent on the amount of growth on the LJ slant, which is not easily quantifiable. It has been reported that confluent growth may result in false-negative results, as the extraction fluid (in this case water) cannot come into contact with the medium (SA Health Info, 2006). However, in this study slants were punctured with a sterile glass Pasteur pipette after the water was added, and then incubated for 30 minutes, thus eliminating a major factor in causing many false-negatives. No false-negatives were observed in this study.

A further explanation for the inaccuracy of the niacin test could be its subjectivity. In some cases the colour change is very slight and different technologists may interpret the variance in colour differently. The difference in colour changes may be due to the fact that cultures may be at different stages of growth. In this study the niacin strip reagent was used. Gadre *et al.*, did a comparative test in 1995 between the older, modified Runyon method and the new paper strip method used in this study. The results showed that 90 out of 100 *M. tuberculosis* strains yielded a positive niacin result, while the other 10 strains were niacin negative with the Runyon Method (Gadre *et al.*, 1995). The strip method gave very similar results by yielding 87 positive strains and 13 negative strains, proving that the paper strip test is a good replacement of its older, more dangerous counterpart, as the Runyon method utilises cyanogen bromide for the colour reaction (Gadre *et al.*, 1995). Low accuracy is alarming since this test is the most widely used test for differential identification of mycobacteria (Gadre *et al.*, 1995).

The *p*NB test was 88.6% accurate. NTM grows well on *p*NB slants, since they are resistant to the effects of *p*-nitrobenzoic acid, while *M. tuberculosis* complex is inhibited, therefore NTM causes growth on *p*NB slants, while *M. tuberculosis* does not (SA Health info, 2006). The poor results may be due to the fact that this test cannot distinguish between mixed infection with *M. tuberculosis* complex and NTM. A slant with NTM growth could also contain a *M. tuberculosis* complex strain, but will not be seen. The test is the most cost-effective and simplest test available today. However, the inability to identify co-infection with *M. tuberculosis* complex and NTM strains is a major drawback, given the high NTM infection rates in Africa and demonstrated in this study. However, it has recently been reported that a small percentage of NTM may be susceptible to *p*-nitrobenzoic acid (Giampaglia *et al.*, 2007).

Spoligotyping is a PCR-based typing system that detects the presence or absence of 43 unique direct variable repeat (DVR) sequences in the genome of *M. tuberculosis* complex strains. DVR sequences are characterised by direct repeat sequences interspersed with

variable repeat sequences. *M. tuberculosis* complex strains are differentiated by the presence of specific variable repeat sequences (Warren *et al.*, 2002). NTM does not possess the highly polymorphic direct repeat locus that is found in the *M. tuberculosis* genome and thus there cannot be a pattern formed on the spoligotyping blot. The accuracy of spoligotyping in this study was found to be 96.2% as there were four false-negative spoligotyping patterns which belonged to the *M. tuberculosis* complex organisms. There are various reasons for a negative spoligotype, although rare. One of the reasons was described by Gori *et al.* in 2005 when they found samples to yield false-negative spoligotypes. Upon further investigation, they noted that those samples had a very high load of AFB on the smear. The bacteria were diluted 1:10 and this resulted in a positive spoligotype. Other factors that may lead to a false-negative spoligotype include inhibitors in the samples that stop the PCR or the fact that the DNA was lost or destroyed during the extraction. The strains may also have mutations which would cause them to either not contain the target or was not recognised by the primers.

To determine the species distribution of the NTM in these communities only single isolates from patients were investigated. Unfortunately sequence information was not available for 4 of the 105 isolates identified as non-*M. tuberculosis* complex strains. Thirteen of these were follow-up samples, therefore the 16S rRNA sequence of 71 NTM were used to calculate the species distribution. In total, 16S rRNA sequencing of the 105 samples identified 79 isolates as NTM strains belonging to 28 different species, 18 were identified as not belonging to the genus *Mycobacterium*, while 4 were identified as belonging to the *M. tuberculosis* complex and two samples are yet unknown, as they were not suitable for sequencing. These two samples did, however, fit the inclusion criteria.

Two case studies

Two interesting case studies were observed in this study and will be discussed. The first case is a 49-year-old HIV-negative woman who completed the prevalence questionnaire and revealed the following facts: She has lived in Scottsdale for the past ten years and was successfully treated for TB in 2004. She did not have any symptoms of pulmonary infection in November 2005 when the first questionnaire was given to her to complete. When a follow-up sample was collected from her three months later because the first specimen collected in November 2005 was positive for acid-fast bacilli, she completed another questionnaire. This time, she complained of coughing for more than three weeks with production of phlegm but no blood. She also had shortness of breath as well as fever, night sweats, chest pain and weight loss in the last month. In both cases *M. intracellulare* was recovered from her sputum sample.

The second case study is a 42-year-old woman who had also been living in Scottsdale for the past 21 years. She was successfully treated for TB in 1987 and was coughing when the first questionnaire was completed in February 2006. Two months later she completed a follow-up questionnaire and a second sample was collected. She then revealed that she was coughing for more than three weeks. The cough produced phlegm, but no blood. She consulted a private clinic and gave a sputum sample but the result was as yet unknown at that time. She complained of shortness of breath, but there were no other symptoms such as fever, night sweats, chest pain or weight loss. She did not give consent for HIV testing. The organisms recovered from her three sputum samples were *M. interjectum*, *M. scrofulaceum* and *M. avium* complex.

Chest x-rays were taken of both individuals and were examined by a clinician. It was found that the first patient had an abnormal chest x-ray, with changes due to previous TB infection and a possible reinfection of some kind. The second patient had a normal chest x-ray with no signs of pulmonary TB or current infection. Further medical investigation is needed, and all results should be taken into account before a diagnosis can be made, but it seems that patient 1 is representative of a NTM infection, while patient 2 is merely colonised by several environmental contaminants. It is increasingly difficult for clinicians to diagnose NTM disease or colonisation (Hatherill *et al.*, 2006).

All non-*M. tuberculosis* complex strains in this study were MGIT-ZN positive. 16S rRNA sequencing showed that all of the organisms isolated in this study were part of the class *Actinobacteria*. The Linnean classification of each organism found in this study is listed in **Table 4.1**. The system used today for classifying living organisms dates from 1758 when Caroli Linnaei published *System Naturae*.

Table 4.1 The Linnean classification of organisms isolated in this study (NCBI/PubMed Taxonomy Browser)

Species	Linnean classification
<i>Actinomadura</i>	cellular organisms; Bacteria; <i>Actinobacteria</i> ; <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Streptosporangineae</i> ; <i>Thermomonosporaceae</i> ; <i>Actinomadura</i>
<i>Arsenicococcus</i>	cellular organisms; Bacteria; <i>Actinobacteria</i> ; <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Micrococcineae</i> ; <i>Intrasporangiaceae</i> ; <i>Arsenicococcus</i>
<i>Microbispora</i>	cellular organisms; Bacteria; <i>Actinobacteria</i> ; <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Streptosporangineae</i> ; <i>Streptosporangiaceae</i> ; <i>Microbispora</i>
<i>Mycobacteriaceae</i>	cellular organisms; Bacteria; <i>Actinobacteria</i> ; <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Corynebacterineae</i> ; <i>Mycobacteriaceae</i>
<i>Propionibacterineae</i>	cellular organisms; Bacteria; <i>Actinobacteria</i> ; <i>Actinobacteridae</i> ; <i>Actinomycetales</i> ; <i>Propionibacterineae</i>

The new series of test kits developed by HAIN Lifescience (Hain Lifescience GmbH, Nehren) is a new molecular based diagnostic test that differentiates NTM from *M. tuberculosis* complex organisms. There are also other kits in the GenoType® range which determines drug-resistance and identifies specific NTM. Tests like this may shed light on the findings of this study. The test involves amplification of *M. tuberculosis* complex or NTM from either culture or clinical samples and thereafter the test is performed within two hours after the completed PCR reaction. The only disadvantage of this assay is the fact that the samples need to undergo PCR before it can be tested. Not many laboratories are currently able to produce these services in developing countries and PCR could be prone to false positivity when there is contamination in the laboratory, especially when amplifying the *rpob* gene of *M. tuberculosis*. In addition, the costs of most of these tests are very high and will not be feasible in developing countries where resources are limited.

Future experiments will include the investigation of the unknown mycobacteria that were recovered in this study, studying the genetics of NTM recovered in this study, and investigating why large numbers of *Arsenicicoccus bolidensis* were recovered in South Africa since these organisms have only been recovered in Sweden before. Future work will also include experiments trying to isolate NTM from various soil and water sources in the Cape Town Metropole as this may explain the high NTM infection rate in this study.

This study shows that NTM infection rates are high in HIV and TB epidemic settings in South Africa. Although it has been shown that NTM may exacerbate HIV and TB in animal models, not much is known about the impact of NTM infection on the HIV and TB epidemic in humans. We hope that this study, and planned future studies, may give insight into the role NTM infection may play in aggravating the HIV and TB epidemic in South Africa, and globally.

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APPENDIX: List of reagents and methods used

- Preparation of 0.1% methylene blue solution

Weigh 0.5 grams of methylene blue and transfer to a 1 litre glass flask.

Add 500 ml of distilled water.

Shake well to dissolve.

Store in a glass bottle with the label showing name of the reagent and date of preparation.

- Preparation of Carbol Fuchsin

Basic Fuchsin (pararosaniline chloride)	10g	25g	50g
Phenol	50g	125g	250g
Absolute Ethanol	100ml	250ml	500ml
Distilled water	1000ml	2500ml	5000ml

Melt phenol over Bunsen burner or in the microwave.

Add basic fuchsin and leave to dissolve.

Add alcohol and mix well.

Add dH₂O to make up one litre.

Filter with Whatman no. 1 filter paper.

Store in a dark glass bottle with label showing name of reagent and date of preparation.

- Phosphate buffer

BBL™ MycoPrep™ Phosphate Buffer was used.

Cut open sachet and empty contents into a clean 1000ml glass bottle.

Pour 500ml of distilled/double distilled water into glass bottle.

Screw cap on tightly, shake well and autoclave.

Unscrew cap slightly before autoclaving the bottle with buffer in it.

Store at room temperature with label stating the reagent and date of preparation.

Check bottles regularly for macroscopic contamination.

Approximate Formula per 500 ml purified water:

Disodium Phosphate (Na₂HPO₄) 2.37g

Monopotassium Phosphate (KH₂PO₄) 2.27g

Final pH 6.8

- 3% Acid-alcohol

To make 2 litre of acid alcohol:

Pour 1900ml of alcohol into a 3 litre glass beaker.

Add 100ml of distilled water.

Mix well.

Discard 60ml of the solution.

Add 60ml of HCl to make up 2000ml again.

Mix well and store in a glass bottle stating the reagent and date of preparation.

- NALC-NaOH/BD™ MycoPrep™ Reagent

Open a MycoPrep™ bottle and squeeze out all of the air.

Tighten cap and break glass vial completely.

Open lid again to let air into bottle.

Mix well.

Approximate Formula per litre purified water

NaOH 20.0g

Trisodium Citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) 14.5g

Each sealed glass ampule within the bottle contains 0.375 g NALC ($\text{C}_5\text{H}_9\text{NO}_3\text{S}$)

- Sodium boric acid (SB) electrophoresis buffer

20X SB buffer:

Weigh out 8g of NaOH and add to 45g of boric acid. Fill flask to 1ℓ with MilliQ water. Shake well to dissolve all particles and autoclave. Label bottle with contents and date of preparation.

- Tween 80 saline

NaCl 19.6g

Tween 80 0.4mℓ

Distilled water 4 ℓ

Add together, stir and autoclave. Dispense 4.5mℓ into tubes for a 1:10 dilution.

(1:10 dilution = 4.5mℓ Tween 80 saline and 0.5mℓ of the liquid culture.)

- 3% Acid-alcohol

To make 2 litre of acid alcohol:

Pour 1900ml of alcohol into a 3 litre glass beaker.

Add 100ml of distilled water.

Mix well.

Discard 60ml of the solution.

Add 60ml of HCl to make up 2000ml again.

Mix well and store in a glass bottle stating the reagent and date of preparation.

- NALC-NaOH/BD™ MycoPrep™ Reagent

Open a MycoPrep™ bottle and squeeze out all of the air.

Tighten cap and break glass vial completely.

Open lid again to let air into bottle.

Mix well.

Approximate Formula per litre purified water

NaOH 20.0g

Trisodium Citrate ($\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$) 14.5g

Each sealed glass ampule within the bottle contains 0.375 g NALC ($\text{C}_5\text{H}_9\text{NO}_3\text{S}$)

- Sodium boric acid (SB) electrophoresis buffer

20X SB buffer:

Weigh out 8g of NaOH and add to 45g of boric acid. Fill flask to 1ℓ with MilliQ water. Shake well to dissolve all particles and autoclave. Label bottle with contents and date of preparation.

- Tween 80 saline

NaCl 19.6g

Tween 80 0.4mℓ

Distilled water 4 ℓ

Add together, stir and autoclave. Dispense 4.5mℓ into tubes for a 1:10 dilution.

(1:10 dilution = 4.5mℓ Tween 80 saline and 0.5mℓ of the liquid culture.)