Biotyping of clinical *Mycobacterium tuberculosis* isolates using MALDI-TOF MS

by

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I, Dr Patrick Govender as supervisor of the MSc study hereby consent to the submission of this MSc Thesis.

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SUMMARY

Tuberculosis continues to be a major threat in public health; 8.8 million incidence of TB has been reported and 2 million deaths every year. Diagnosis of TB is impeded by slow growth of an organism with a generation time of 21 days. The emergence of multidrug-resistant TB isolates which are resistant to rifampicin and isoniazid worsened the treatment programme. Furthermore, surfacing of extensively drug-resistant TB isolates especially in HIV positive patients has led to a treatment failure. Currently available diagnostic methods are time consuming and laborious. Polymerase chain reaction-based assay proved to have a better resolution for TB strain discrimination, nevertheless are costly and cannot be routinely employed in resource poor settings. As a result there is an urgent need of cheap, cost effective and rapid diagnostic methods that will reduce a turnaround time. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry potentially offers an alternative rapid and cheaper method for discrimination of TB isolates.

Proper discrimination of TB isolates depends on the sample preparation method that is capable of yielding high protein content. Conventional formic/ethanol sample preparation was investigated for mycobacteria MALDI-TOF mass spectrometric analysis. Poor quality of spectra was obtained due to a complex cell wall structure of mycobacteria which released less amounts of proteins. Further attempts were made to optimize the sample preparation method by introducing glass beads for maximum cell wall disruption. Non-consistent spectra were obtained in some mycobacterial strain; therefore it was not used as a method of choice. Introduction of delipidation step using chloroform/methanol (1:1, v/v) before formic/ethanol sample preparation step, led to a generation of reproducible and consistent spectra. This newly developed method was selected to extract protein content from large number of clinical TB isolates.

With MALDI-TOF MS and chloroform/methanol-based method, all mycobacterial isolates used in the proof-of-concept were correctly identified and clustered. Fifty six of sixty clinical TB isolates were correctly identified using Biotyper software. Four were incorrectly identified; it might be possible that they carry mutations in unknown regions in their genome which led to a translation of proteins that affected the overall spectra profile. MALDI-TOF MS showed the potential to be used in the clinical laboratories for discrimination of TB isolates at lower costs

This dissertation is dedicated to my family and the Qoloqolo community

BIOGRAPHICAL SKETCH

Pride Siyanda Myende was born in Mtwalume (Qoloqolo) on the 17th January 1987. Following Matriculation from Mtwalume High School in 2006, he pursued (2007-2010) a Bachelor of Agricultural Science degree specializing in Microbiology at the Pietermaritzburg Campus of the University of KwaZulu-Natal. His fascination with Microbiology and allied disciplines motivated him to enroll for this MSc research study in 2011 at the Westville Campus of UKZN. He has undergone training under Bruker Daltonics approved instructors with respect to the use and maintenance of the MALDI-TOF MS. Furthermore he received hands-on training at Inkosi Albert Luthuli Hospital with respect to the safety procedures for the safe handling and storage of clinical specimens that presumably contain *Mycobacterium tuberculosis*. He is aiming to complete Doctors of Philosophy (PhD) in a related discipline.

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PREFACE

This dissertation is presented as a compilation of five chapters. Each chapter i s introduced separately.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**
Biotyping of *Mycobacterium tuberculosis*

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Chapter 1

INTRODUCTION AND PROJECT AIMS

1. INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Tuberculosis is an airborne disease caused by *Mycobacterium tuberculosis* (MTB). Approximately one third of the world population is latently infected with MTB (WHO, 2010). Eight million cases of tuberculosis have been recorded every year, while two million deaths per year have been reported (He & Zahrt, 2005). Human migration does have an effect on the spread of TB. However, the occurrence of TB remains high in low income economies (Hettick, *et al.*, 2006). Latently infected individuals serve as a reservoir for future spread of TB in uninfected populations (Liu, *et al.*, 1995). Moreover, the emergence of multidrug-resistant TB strains present the major challenges in the public health facilities (Sepkowitz, *et al.*, 1995). Persistence and coexistence of *M. tuberculosis* enables it to overcome the immune system and adapt inside the host environment.

Spontaneous mutations within the drug target sites of *M. tuberculosis* have been increased over the past years. The emergence of MDR-TB in patients is due to non-compliance to treatment, incompletion and the use of inappropriate treatment (Bahk, *et al.*, 2004). This has led to the increased incidence of MDR-TB as well as extensively drug resistant (XDR) TB. Furthermore, dual infections of HIV and drug resistant TB have severely threatened national TB control programmes. In 2009, South Africa was ranked fifth with the total highest estimated number of MDR-TB (WHO, 2011). MDR-TB is resistant to rifampicin and isoniazid whereas XDR-TB is an MDR that is resistant to at least one of the fluoroquinolones (ofloxacin) plus an aminoglycoside/cyclic peptide (kanamycin, capreomycin) (Salmoniere, *et al.*, 1997). In the1960's rifampicin was the last developed drug for the treatment of TB. The emergence of XDR-TB has led to the need to use second line treatment. These treatment drugs have greater side-effects, more expensive and require an extended treatment period is required (Olano, *et al.*, 2007).

Treatment and diagnosis of *M. tuberculosis* presents a major challenge globally. Current delays in the diagnosis of *M. tuberculosis* are impeded by the slow growth of the organism on agar culture plates. Rapid detection of infectious TB is essential in order to offer immediate and appropriate treatment (Brosch, *et al.*, 2002). Conventional methods are time consuming and require a four to six week turnaround time. Therefore, there is a great need for the availability of alternative methods of quick detection of *M. tuberculosis* (Gandhi, *et al.*, 2006). Various tools and techniques are available to diagnose *Mycobacterium* species not only for medically important purposes but also for strain differentiation (Abomoelak, *et al.*, 2009). This includes polymerase chain reaction-based approaches however, these methods are costly and cannot be

implemented in poor resource settings (Romanus, *et al.*, 2011). GeneXpert MTB/RIF has been applied as a rapid method for diagnosis of tuberculosis; however it can diagnose strains that are only resistance to rifampicin and does not provide any information about XDR *M. tuberculosis* strains and isozianid. This becomes a limiting factor for decision making on treatment selection for XDR infected patients. Recently, matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been shown to be a useful alternative approach for bacterial identification and classification (Gandhi, *et al.*, 2006).

MALDI-TOF MS is an analytical method that is capable of vaporizing and ionizing biological samples embedded on a UV-absorbing matrix through a single non-destructive step. As a result, it became the preferred choice for analysis of sensitive biological samples (Hettick, *et al.*, 2006). The MALDI-TOF MS is designed to distinguish between microorganisms based on their unique protein profiles. This instrument has been used for analysis, identification and characterization of biological samples and cell components (Romanus, *et al.*, 2011). MALDI-TOF MS has been used for the rapid detection of *Mycobacterium* species in an attempt to compensate the ineffectiveness of conventional diagnostic methods (Hettick, *et al.*, 2006). Although this tool has not been extensively used for differentiation of *M. tuberculosis* to a strain level, it might be useful for the diagnosis of TB which may result in more successful treatment and management programme. During the application process of MALDI-TOF MS, the UV-laser is irradiated to the matrix embedded sample. Thereafter the matrix transfers the energy to the sample analyte to be ionized and desorbed from the target plate. As a result, the ion sources accelerate desorbed ions into a flight tube (time of flight) where they are analysed and separated according to their mass to charge ratio. Smaller molecules travel faster and reach the detector earlier than larger molecules. These ions are captured and counted by a detector and represented as signals for each mass-to-charge value that is proportional to the number of ions captured (Romanus, *et al.*, 2011). In comparison to other diagnostic methods, MALDI-TOF MS is rapid, requires minimal sample preparation and fewer reagent (Hettick, *et al.*, 2006).

1.2 AIMS OF THIS STUDY

I. To optimize a MALDI-TOF MS sample preparation protocol that will enable the identification of *Mycobacterium* species.

II. To create a mass spectral database that is representative of typed and locally-based clinical mycobacterial strains.

III. To determine the potential of MALDI-TOF MS analysis to discriminate between fully susceptible *Mycobacterium tuberculosis,* multidrug-resistant and extensively drug-resistant tuberculosis strains.

IV. To identify unique mass spectral signal of clinically isolated TB isolates

1.3 REFERENCES

Abomoelak B, Hoye EA, Chi J, *et al.* (2009) mosR, a novel transcriptional regulator of hypoxia and virulence in *Mycobacterium tuberculosis*. *Journal of Bacteriology* **191**: 5941–5952.

Bahk YY, Kim SA, Kim J, Euh H, Bai G, Cho S & Kim YS (2004) Antigens secreted from *Mycobacterium tuberculosis*: Identification by proteomics approach and test for diagnostic marker. *Proteomics* **4**: 3299–3307.

Brosch R, Gordon SV, Marmiesse M, *et al.* (2002) A new evolutionary scenario for the Mycobacterium tuberculosis complex. *Proceedings of the National Academy of Science.* **99**: 3684-3689.

Gandhi NR, Moll A, Sturm AW, *et al.* (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet Infectious Diseases* **368**: 1575–1580.

He H & Zahrt TC (2005) Identification and characterization of a regulatory sequence recognized by *Mycobacterium tuberculosis* persistence regulator MprA. *Journal of Bacteriology* **187**: 202–212.

Hettick JM, Kashon ML, Slaven JE, *et al.* (2006) Discrimination of intact mycobacteria at the strain level: A combined MALDI-TOF MS and biostatistical analysis. *Proteomics* **6**: 6416-6425.

Liu J, Rosenberg EY & Nikaido H (1995) Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proceedings of the National Academy of Science.* **29**: 11254-11258.

Olano J, Lo´pez B, Reyes A, *et al.* (2007) Mutations in DNA repair genes are associated with the Haarlem lineage of *Mycobacterium tuberculosis* independently of their antibiotic resistance. *Tuberculosis* **87**: 502–508.

Romanus II, Eze AE, Egwu OA, Ngozi AF & Chidiebube NA (2011) Comparison of matrix-assisted laser desorption ionization-time of flight mass spectrometry with conventional culture and biochemical method of bacteria identification to species level. *Journal of Medical Laboratory and Diagnosis* **2**: 1-4.

Salmoniere YOG, Torrea H, Bunschoten A, Embden JDA & Goquel B, . (1997) Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. *Journal of Clinical Microbiology* **35**: 2210-2214.

Sepkowitz KA, Raffalli J, Riley L & Kiehn TE (1995) Tuberculosis in the AIDS era. *Clin.Microbiol.Rev* **8**: 180-199.

WHO (2010). *Global Tuberculosis Control*. www.who.int

WHO (2011). *World Health Statistics 2011*. www.who.int

Chapter 2

LITERATURE REVIEW

Identification of *Mycobacterium tuberculosis*

2 IDENTIFICATION OF *MYCOBACTERIUM TUBERCULOSIS*

2.1 INTRODUCTION

2.1.1 *Mycobacterium Tuberculosis:* The Pathogen

Mycobacteria are obligate aerobes, non-motile, non-spore formers, rod shape acid fast bacilli with a cell size ranging from 0.2 to 0.4 x 2 to 10 µm. *Mycobacterium tuberculosis* belongs to the *Mycobacterium tuberculosis* complex (MTBC) which comprises of pathogenic members such as *M. microti*, *M. africanum*, and *M. bovis*. These members are genetically related and are of the same medical importance (Nelson and Williams, 2007). The members of MTBC are found in well aerated body parts such as the lungs (Mahon & Manuselis, 2002). These organisms are facultative intracellular parasites of macrophages that exhibit a slow generation time of approximately 15 to 20 hours. Slow growth of MTBC members contributes to their virulence (Macia, *et al.*, 2007). The MTBC continues to be a major problem in health care facilities and globally is the major cause of death. Mycobacterial species are currently classified based on their phenotypic characteristics, nutritional requirements, growth temperature, growth rate and pigmentation. Furthermore, biochemical tests, cellular free fatty acids, and the range of pathogenicity in animal experiments are used to classify *Mycobacteria* (Nelson and Williams, 2007). Molecular methods such as polymerase chain reaction-based approaches and species specific RNA and DNA sequences have been used to expand the classification process (Tiwaria, *et al.*, 2007).

2.1.2 Tuberculosis: The disease

Tuberculosis (TB) is an airborne disease caused by *M. tuberculosis;* however, mycobacterial species have wide range of host selection from animals to humans. *M. tuberculosis* persists inside the host under natural physiological conditions. Disease development has been suggested to be the result of two types of host reactions against tubercle bacilli. The first reaction is termed delayed-type hypersensitivity (DTH) which is due to mycobacterial proteins that cause the destruction of non-activated macrophages. The second reaction is called cell mediated immunity (CMI) which activates the macrophages and results in the destruction of mycobacterial cells encased within the macrophage cytoplasm (Jacobs, *et al.*, 1987).

In spite of the availability of anti-TB treatment therapy, TB remains global threat amongst other infectious diseases. Annually eight million TB cases are reported globally (Dasgupta & Menzies, 2005). Latently infected populations act as the reservoir for the future spread of TB. Active infection results from reactivation of tubercle bacilli from latently infected individuals due to a reduced immune system (He & Zahrt, 2005); however, replication of tubercle bacilli into large numbers inside the macrophages results in an active infection which

induces the inflammatory host response (Macia, *et al*., 2007). As a result, the symptoms due to active tuberculosis are not obvious in that they might overlap with a number of pulmonary and systematic diseases (Nelson & Williams, 2007). TB can be diagnosed with symptoms such as a mild sputum, fatigue, anorexia, weight loss, sweating, chills, fever, and chest pains (Oduwole, 2008).

The mechanism for virulence, pathogenesis and persistence of *M. tuberculosis* remain major challenge that needs detailedscientific investigation(Målen, *et al*., 2011). Extensive understanding of the *Mycobacterium* biology can bring the spread of disease to a halt and enable effective control of the disease transmission. TB is normally contracted by inhalation of aerosol droplets that carry the tubercle bacilli. Although a single cough from an infected individual can generate as many as 3 000 infected droplet nuclei, fewer than 10 bacilli are sufficient to initiate pulmonary infection in a susceptible individual (Nelson & Williams, 2007). Currently, the major challenges faced by clinical laboratories are based on the characterization and identification of virulent determinants in *M. tuberculosis* which are believed to be related to the disease (Todar, 2008). An attenuated TB strain such as H37Rv has been used in an attempt to highlight the pathogenicity of *M. tuberculosis;* however, it has been observed that during the infection process of mice, the attenuated strain exhibited a dormant state in the macrophages (Målen, *et al.*, 2011).

2.2 TRANSMISSION AND EPIDEMIOLOGY OF TUBERCULOSIS

TB is widely spread by social gatherings, poverty, poor hygiene and economic disruption (Oduwole, 2008).TB attacks the lungs, respiratory tract, as well as other organs of the body. Most patients are infected with pulmonary tuberculosis and few immune-compromised patients are found to be infected with extra-pulmonary TB (Nelson & Williams, 2007). High morbidity and mortality rates are largely observed in human immunodeficiency virus (HIV) co-infected patients located in sub-Saharan Africa (Saleeb, *et al*., 2011). Research studies and control strategies have been conducted globally; however, the TB pandemic remains high and continues to cause the premature death of young adults (He & Zahrt, 2005). In 1993, the World Health Organization (WHO) considered TB a global threat. In the 1980's and 1990's the incidence of TB was observed to increase with the HIV incidence (Nelson & Williams, 2007). Furthermore, the threat has been worsened by the emergence of multidrug-resistant-TB strains.

In 2010 MDR-TB accounted for 650 000 cases worldwide of which 150 000 cases were fatal. Recently there has been an increase in the number of patients with extensively drug resistant (XDR) TB, which is defined as MDR-TB together with resistance to one or more quinolones and an injectable aminoglycoside. Currently, South Africa has been ranked the second among the highest TB burdened countries in the world, with the second largest number of MDR-TB cases (WHO, 2010).

2.3 MYCOBACTERIUM CELL WALL STRUCTURE

Mycobacterial species are intrinsically resistant to commonly used treatment therapy. The resistance is related to a unique cell wall structure that acts as an impermeable barrier to chemicals and antibiotics (Figure 2.1). Mycolic acids account for approximately 40-60% total dry weight of the cell wall structure and are long, branched chains of lipids that contain 70 to 90 carbon atoms. In addition the cell wall of mycobacterial species have a unique peptidoglycan that contains N-glycolymuramic acid instead of the normal N-acetylmuramic acid, linked to arabinogalactan (Laval, *et al.*, 2001).

Mycobacterium species are resistant to acids, alkalis, oxidative lysis, detergents and lysis by antibiotics (Oduwole, 2008). Weakly attached lipids on the cell wall are extractable with organic solvents (Liu, *et al.*, 1995). Research has been focussed on the exploration of the mycobacterial cell wall for the development of new drug therapies that will bypass the cell wall barriers and attack the important target sites within the cell (Charles, *et al.*, 1998). The lipid fraction of the *M. tuberculosis* cell wall structure consists of three major components that are covalently linked to each other namely; the mycolic acid, cord factor and wax D fractions.

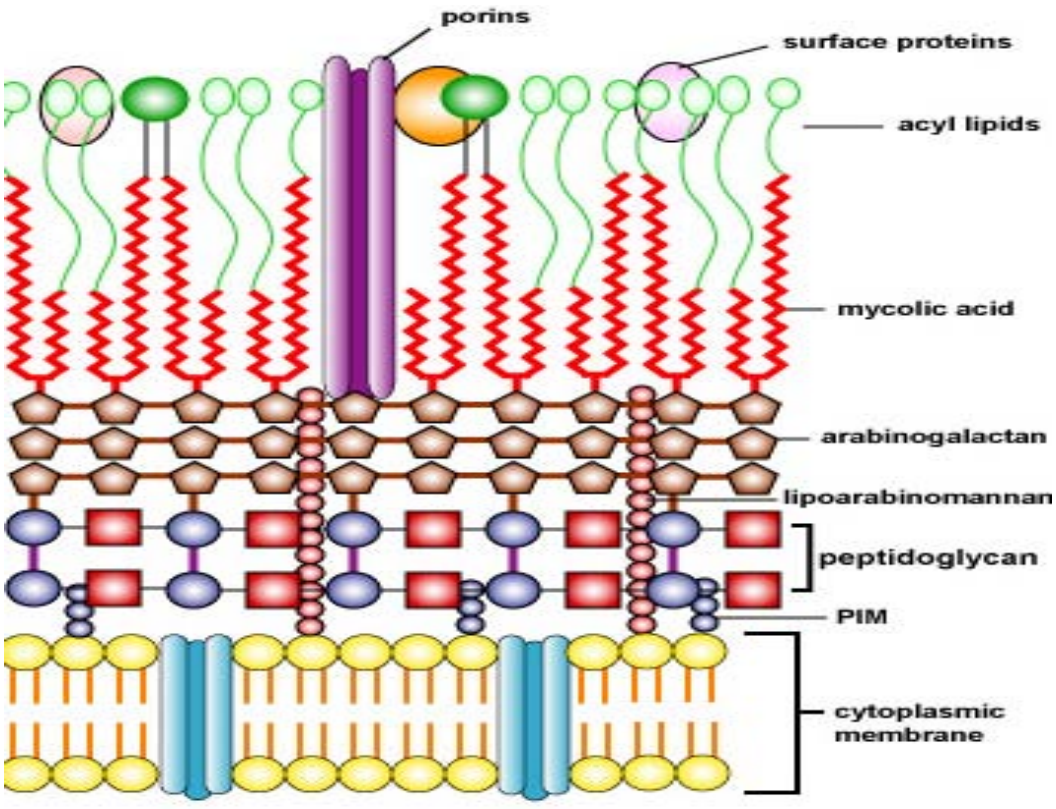


Figure 2.1 General cross sectional area of mycobacterial cell wall structure (Kaiser, 2011).

2.3.1 Mycolic acids

Mycolic acids are distinctive alpha-branched hydrophobic lipids. These form a strong waxy layer around the cells which results in low cell wall permeability in *Mycobacterium* and C*orynebacterium* species. It has been suggested that mycolic acid contributes to virulence in TB strains. This presents difficulties for the treatment of TB and other related diseases as cells are protected from antibiotic attack (Butler & Guthertz, 2001).

2.3.2 Cord factor

The cord factor (trehalose-6,69-dimycolate) is the surface glycolipid or toxic trehalose-mycolate present in virulent TB strains (Laval, *et al.*, 2001). This glycolipid induces DTH. Furthermore, it is responsible for serpentine cord formations which are only observed in virulent mycobacterial strains (Saito, *et al.*, 1975).

2.3.3 Wax D fraction

The wax D fractions are active glycolipids that are principally responsible for adjuvant activity which induces DTH and the production of humoral antibodies (Saito, *et al.*, 1975).These fractions are present in all mycobacterial species and are soluble in ether, insoluble in acetone and extractable with chloroform (White, *et al.*, 1963). These fractions induce the adjuvant effect in a similar manner as whole killed cells of *M. tuberculosis* in Guinea pigs. Wax D fractions added to water-in-oil emulsion of oval albumin antigen in mineral oil and injected in humans, increases serum antibody production (Saito, *et al.*, 1975). The wax D extracts from bovine, *M. avium* and saprophytic *Mycobacteria* were found to be inactive from previously conducted experiments (White, *et al.*, 1963). It was observed that the difference in the specificity between the clinical and saprophytic strains was the presence or absence of the peptide moiety between these strains. This peptide moiety contains the *meso*-α, α'-diaminopimelic acid, D-glutamic acid, and D and L-alanine (White, *et al.*, 1963). It was concluded that the presence of the peptide moiety was essential for the adjuvant effect in the clinical *Mycobacterum* strains (Saito, *et al.*, 1975).

2.4 VIRULENT PROTEINS OF *MYCOBACTERIUM TUBERCULOSIS*

Exploitation of mycobacterium genetics is essential to explain the virulent mechanism and pathogenicity. TB strains have a wide range of structural and physiological properties that has been suggested to contribute to virulence and pathogenesis (Ioerger, *et al.*, 2010). Below are suggested virulent determinants which are believed to contribute to pathogenicity in virulent *M. tuberculosis*.

2.4.1 The 19 kDa protein

A 19 kDa antigenic protein is secreted and recognized by serum-based T cells. It has been indicated, with no valid evidence, that TB mutants are unable to produce 19 kDa proteins. Previously research has shown that a similar gene of a wild-type strain inserted in mutants allows growth in the lungs. Therefore it was assumed that the 19 kDa protein might contributed to the virulence in mycobacteria (Todar, 2008).

2.4.2 Glutamine synthase

The glutamine synthase enzyme is involved in nitrogen metabolism and the synthesis of poly-L-glutamate-glutamine cell wall constituents that are present in virulent mycobacterial species. It has been targeted as an important determinant of pathogenesis in *Mycobacterium tuberculosis* (Harth*, et al.*, 1994)**.**This enzyme is not secreted in the culture media during the growth phase; however, it can escape during leakage and lysis of the cell. Previous research studies, have indicated that this enzyme is a potential target for new drug development (Todar, 2008).

2.4.3 Fibronectin-binding proteins (fbp's)

The fbp'sare essential for cell wall biosynthesis through esterification of mycolic acid within the cell wall (Todar, 2008). Activities of these enzymes are partially overlapping and these proteins are considered as major antigens. Interestingly, 85 complex antigens which were discovered to be fbp's were previously assumed to be involved in the phagocytosis of macrophages (Garbe*, et al.*, 1996). The analysis of *M.tuberculosis* mutants containing fbp C showed that the coding gene of this protein is essential for growth and the formation of the cell wall structure in mutants. The cell wall structure of mutants contains the measurable amount of mycolic acid methyl esters (MAME's). Previously these enzymes became a target for the development of new vaccines that were formulated through the introduction of the fbp B gene in *Mycobacterium bovis* BCG (Garbe*, et al.*, 1996).

2.4.4 Two-component signal transduction system

This signal transduction system induces the persistence of TB strains during fluctuating conditions within the host. This subunit consists of the coupling of a histidine kinase sensor and a cytoplasmic cognate response regulator protein. Changes of the conditions within the host trigger the adaptive transcriptional programs in the mycobacterial cells. The signalling process is accomplished at through phosphotransferase reaction (He & Zahrt, 2005). As a result, mycobacteria invade different harsh habitats through inducing the coordinated stress response. Furthermore, heat shock proteins have been considered to be responsible for stress survival of pathogenic mycobacteria (Pang & Howard, 2007).

2.5 DRUG RESISTANCE

The emergence of MDR-TB in industrialized countries caused major problems in public health facilities (Jun Liu, *et al.*, 1995). *M. tuberculosis* persists and coexists within the host environment by overcoming the host immune system and attack by antibiotics. Therefore resistant TB strains present a challenge for treatment. The selection of drug resistant strains is due to chromosomal mutations which result in single nucleotide polymorphisms (SNP's), deletions and insertions. Mutations have great impact on the drug target sites and the encoding genes for drug activation enzymes (Olano, *et al.*, 2007). Inappropriate applications of TB regimens result in the emergence of drug resistance strains. Thus adherence to treatment must be emphasized to patients in order to suppress the emergence of strain resistance and promote an effective TB control programme. Non-compliance to treatment, wrong prescription, incorrect dosage and poor quality of drugs has led to a propagation of naturally occurring drug resistant strains (Oduwole, 2008). Slow progress in terms of drug development allows resistant strains to emerge while existing drugs become ineffective.

Approximately 12 genes have been identified to be associated with antibiotic resistance in *M. tuberculosis*. Mutations in the *kat G*gene promote strain resistance to isoniazid. The *katG* gene codes for catalase and peroxidase enzymes that catalyses the isoniazid activation (Richardson, *et al.*, 2002). Resistance to rifampicin is due to mutations in the *rpoB* gene that codes for the B -subunit of ribonucleic acid polymerase (Streicher, 2007). In the 1960's, rifampicin was the last drug developed for the treatment of TB. MDR-TB is considered to be resistant to first line drugs (isoniazid and rifampicin). Therefore second line drugs are emphasized, although these have side effects, are expensive and requires a longer treatment duration (Giffin & Robinson, 2009).

M. tuberculosis strains that are resistant to rifampicin are likely to be resistant to other anti-TB drug therapies therefore they are a good indicator of the incidence of MDR-TB. Although the emergence of mono-resistant TB strains is uncommon 90% of such resistant strains have been found to be resistant to isoniazid. Based on that observation, different molecular techniques have been developed to detect mutations in rifampicin and isoniazid coding regions (Telenti, *et al.*, 1993).

2.5.1 Multidrug-resistant tuberculosis (MDR-TB)

MDR-TB is considered to be resistant to rifampicin and isoniazid. Most patients who are co-infected with HIV are at high risk of developing active TB due to a compromised immune system (Daley, *et al.*, 1998). The number of TB cases indicate that the fatality rate is high in African countries compared to the rest of the world (Dasgupta & Menzies, 2005). Mycobacterial

species are spontaneously mutating to develop drug resistance. However, mutation rates differ between drug treatment regimens (Gillespie, 2002). Streptomycin mono-therapy results in an increased incident of MDR-TB strains. Detection of resistant TB strains at an early stage of infection will result in a more positive impact in control programmes. Resistance ratio, minimum inhibitory concentrations (MIC) or proportion method in liquid media are traditionally used to screen the MDR-TB strains. Molecular methods have been extensively employed to identify TB strains based on the genes encoding the drug resistance determinants (Gillespie, 2002).

2.5.2 Extensively Drug-Resistant tuberculosis (XDR-TB)

The first outbreak of XDR-TB was announced in South Africa in the Western Cape, where patients were suffering from a severe infection. *M. tuberculosis* was found to be resistant to known effective treatment regimens (isoniazid and rifampicin). During 1993 to 2006, the spread of MDR-TB was evaluated globally and 49 cases were found to correlate with the global definition of XDR-TB (Magnus, 2009). XDR-TB is considered as an MDR-TB that is resistant to at least one of the fluoroquinolones (ofloxacin) plus an aminoglycoside/cyclic peptide (kanamycin, capreomycin) (Salmoniere, *et al.*, 1997). Resistance to fluoroquinolones is due to substitution of amino acids in the putative fluoroquinolone binding sites of *gyr*A and *gyr*B. Whilst resistance to kanamycin and amikacin is associated with mutation in A1401G in the *rrs* gene coding for 16S rRNA (Silva & Palomino, 2011). MDR-TB can mutate into XDR-TB under the selection pressure of the treatment regimens. Isoniazid and rifampicin are potentially administered TB treatment regimens, particularly in developing countries. Isoniazid is an effective bactericidal that acts against actively dividing tubercle bacilli, while rifampicin is active against slowly dividing tubercle bacilli which result in the sterilization of infected sites (Gillespie, 2002). Kanamycin, amikacin and capreomycin are injectable second line drugs that are administered when the first line drugs fail to cure MDR-TB (Oduwole, 2008). Currently, the outbreak of MDR and XDR-TB were observed to increase linearly with HIV in public health facilities (Kaufmann & Walker, 2009).

2.6 TB PANDEMIC CONTROL STRATEGIES

As previously mentioned, infected individuals serve as the reservoir for the future spread of TB. During the reactivation state of tubercle bacilli, latently infected individuals are at higher risk of developing the active TB which is transmitted before sterilization with recommended drugs. Rapid diagnosis and treatment is urgently needed to reduce the rate of infection and transmission of tuberculosis. Combined application of drugs reduces the risk of emerging resistant TB strains (Nelson and Williams, 2007).

Direct observed therapy (DOT) is extensively used by health care workers in developed countries where they directly monitor the patients and stress the treatment adherence. DOT has been shown to be a promising strategy in reducing the incidence of tuberculosis and the emergence of drug resistance strains in communities. Sixty seven percent disease reduction has been achieved through the DOT strategy. It has been highlighted as one of the effective control strategies, although it success is below the expected WHO standard of 85% (Gandhi, *et al.*, 2006). The application of DOT, however, requires an established registration system, stable drug supply, microscopy unit, and adequate staff to effectively supervise the patients for at least three months (Murray & Salomon, 1998).

TB is currently managed by embracing the vaccination strategy in young children and treatment regimens. BCG vaccination is emphasized in developing countries although it does not provide protection in latently infected individuals (Nelson and Williams, 2007). Despite the availability of live attenuated BCG vaccines and chemotherapeutic regimens, TB infection has remained a global threat (He & Zahrt, 2005). Rifampicin and isoniazid are collectively used for duration of six to nine months in patients who are infected with pulmonary TB. Streptomycin or ethambutol is used within the initial course of two to eight weeks. Emphasis on second line drugs is stressed when the first line drugs are not effective against TB strains (Gandhi, *et al.*, 2006). Although TB presents challenges, it is treatable and curable with appropriate treatment regimens as listed in Table 2.1 (Corbett, *et al.*, 2003).

Table 2.1 Currently administered first line drugs with their mode of action against virulent mycobacterial species (Nelson and Williams, 2007).

Drug	Mode of Action	Effect
Isoniazid (H)	Bactericidal	Kills metabolically active *mycobacteria*
Rifampicin (R)	Bactericidal	Kills metabolically active and inactive *mycobacteria*
Pyrazinamide (Z)	Bactericidal	Kills *mycobacteria* in acidic pH (within cells)
Streptomycin (S)	Bacteriostatic	Inactivates cell growth and multiplication; does not kill
Ethambutol (E)	Bacteriostatic	Inactivates growth and multiplication; does not kill
Thiacetazone (T)	Bacteriostatic	Inactivates growth and multiplication; does not kill

2.7 CURRENTLY USED DIAGNOSTIC TECHNIQUES

Latently infected patients display no symptoms until the tubercle bacilli are activated. This presents a challenge for early diagnosis and treatment of TB. Point of care testing is used for the presumptive diagnosis of TB by tracing the symptoms of infection or serological reactivity against TB antigens (Shinnick & Good, 2011). Initially, radiography was predominantly used for diagnosis of tuberculosis infection. The calcified lesion was used to detect calcified lesions for diagnosis of TB. However, this diagnostic technique showed low sensitivity and specificity, therefore it was not considered as an effective diagnostic tool. Serological identification of tubercle bacilli has also been used although the sensitivity of serological reactivity is limited by the low level of tubercle bacilli in the sample or in latently infected individuals (Drobniewski, *et al.*, 2003). Furthermore, the immune response has been used to monitor TB infection through the induction of DTH with a tuberculin skin test (TST) (Nelson and Williams, 2007). Ineffective diagnosis and treatment of TB pose a challenges, as a result the extended treatment time is emphasized particularly for MDR-TB and XDR-TB infection (Ferreira, *et al.*, 2010).

Delayed diagnosis is current impeded by slow growth of tubercle bacilli using the culturing methods. Various tools and techniques have been used to diagnose mycobacterium species not only for medical purposes, but also for strain differentiation (Oduwole, 2008). PCR based techniques are extensively used for mycobacterial identification. However, these techniques are costly and cannot be implemented in resource poor settings (Romanus, *et al.*, 2011). High performance liquid chromatography (HPLC), enzyme linked immunosorbent assay (ELISA), TST, microscopy, chest X-rays, culture method, gamma interferon (IFN-γ) release and histopathology of diseased tissue have been used in an attempt to diagnose mycobacterial species they are subject to limitations, particularly for rapidity, sensitivity and cost implications (Oduwole, 2008).

2.7.1 Culturing methods

Culturing methods are the first steps taken prior to further diagnosis of TB with other methods. This method is based on the maintenance of viable cells for physical detection. Further diagnostic approaches such as genotyping, screening for drug resistant strains and biochemical testing can be done directly on viable cells (Huggett, *et al.*, 2003). Cultivation on solid media is time consuming and can extend up to three to six weeks to obtain results. Middlebrook is the well-known agar based media that contains antibiotics to inhibit Gram negative and positive bacteria as well as fungi. Middlebrook media is available as Middlebrook 7H10, 7H11 and 7H9, and differ in their chemical composition and formulation. Lowenstein-Jensen (egg based media) is also used as liquid media for the cultivation of mycobacteria. Formulated liquid media are largely used to measure the secreted secondary metabolites by viable cells in the media. BACTEC® 460 and BACTEC® 960 system MGIT™systems are mostly used as the culturing systems for liquid media to shorten the time of incubation and cell recovery to 1-15 days (Drobniewski, *et al.*, 2003).

2.7.1.1 BACTEC™ MGIT™ 960 Mycobacterial Detection Systems

The incidence of TB has led to an emphasis on quick detection of the TB pathogen in order to control the infection. The BACTEC® 960 MGIT™ systems have been developed for rapid culturing and recovery of mycobacteria. This culturing technique is the leading automated system for detection of mycobacterium species in most TB diagnostic laboratories (Drobniewski, *et al*., 2003). Oxygen quenching fluorescence technology and Middlebrook media supplemented with growth enhancers are used in *Mycobacterium* Growth Indicator Tubes (MGIT). The microbial growth is monitored through the change in oxygen concentration which results in fluorescence(Drobniewski, *et al.*, 2003). This system is mainly used for the cultivation of repository mycobacterium ATCC® strains and clinical as well as laboratory adapted mycobacterial strains. It combines the quality and reliability of the MGIT for optimum mycobacterial detection (Oduwole, 2008).

2.7.1.2 BACTEC™ 460TB System Mycobacterial Culture Media

The BACTEC™460 detection system is currently employed for isolation, susceptibility testing and discrimination of TB from non-TB mycobacteria (Nelson and Williams, 2007). This tool is a semi-automated radiometric system that uses liquid based media supplemented with growth inhibitors. BACTEC 12 B media is usually used for quantitative measurement of $^{14}CO_2$ released from carbon labelled substrates such as palmitic acid, during cellular metabolism (Tiwaria, *et al.*, 2007). The recovery rate of the organism is shortened to a minimum of five to 10 days before samples are discarded however, the incubation time can be extended to six weeks for confirmation purposes BACTEC™460 systems are also used in combination with p-nitro-alpha-acetylamino-beta-hydroxypropiophenone (NAP) to identify *M.tuberculosis* from other mycobacterial species through growth inhibition properties (Oduwole, 2008).

2.7.2 Molecular Methods

In the 1990's molecular methods were extensively used by researchers to explore the genetics of TB strains (Olano, *et al.*, 2007). Genomic manipulations such as deletion, insertion, and SNPs have been explored to study the pathogenesis and virulence of TB strains. Gene expression has been used to elucidate the phenotypic characteristics of mycobacterial species (Målen, *et al*., 2011). A restricted number of species specific nucleic acid probes have been designed and used to identify some mycobacterial isolates. These probes are used in combination with PCR to increase sensitivity, however, the effectiveness of these methods are limited by the number of species which can be identified (Russo, *et al.*, 2006). Molecular methods are dependent on PCR

to amplify specific sequences of nucleic acid (Roth, *et al.*, 1997). Nucleic acid tests (NATs) are used for various purposes with different extent of success. NAT's have been successfully used for the detection of smaller amount of DNA targets with low level of contamination (Oduwole, 2008). Although NATs are used regularly in clinical laboratories, it has been noted that one challenge of controlled optimum hybridization is due to high density arrangements of nucleic acids which results in mismatched sequence binding thereby generating incorrect data (Ohrmalm, *et al.*, 2010).

Different approaches such as comparative genomics and saturation mutagenesis have been implemented to identify essential genes in the genome of *M. tuberculosis.* Furthermore, laboratory conducted research studies have revealed less than 1 000 essential genes and more than 3 000 non-essential genes encoded in the genome (Yesilkaya, *et al.*, 2005). Virulent genes responsible for mycobacterium pathogenicity and host invasion have been identified by comparing the effect of induced mutations to wild-type strains. Reviewing molecular methods, it has been observed that mycobacterial strains containing the specific modifications in mycolic acid are virulent due to disruption of *hma* and *pcaA* genes. The *pcaA* genecodes for mycolic acid cyclopropane synthase (Dubnau, *et al.*, 2002).

Currently, molecular methods are considered to be rapid and sensitive. However, these methods are costly and cannot be implemented in resource poor laboratory settings often encountered in South Africa. Consequently, there is an urgent need for the development of cheaper, reliable and rapid TB diagnostic methods.

2.7.2.1 Spoligotyping

Molecular typing is a well-adapted method to predict the distribution of TB both nationally and regionally. Genomic sequencing provides information for the genetic capability of *M. tuberculosis* to adapt in a particular geographic region (Yesilkaya, *et al.*, 2005). Mycobacterial interspersed repetitive unit-variable number tandem-repeat (MIRU-VNTR) typing and spoligotyping have shown to have the same specificity and sensitivity for detection of transmission chains in mycobacterial species. These methods are PCR-dependant and are used for detecting a small amount of samples, non-culturable microbes and cell extracts from clinical samples. Spoligotyping has been used to predict the distribution of MTBC through amplification of direct repeats (DRs) on the bacterial chromosome. The DRs are composed of 36 bp repetitive sequences separated by non-repetitive interspersed spacers, as indicated in Figure 2.2.

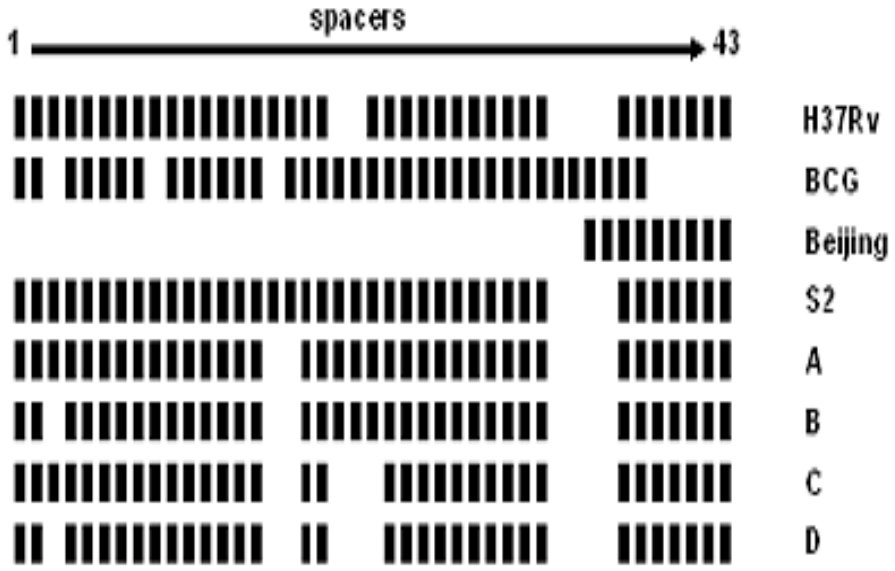


Figure 2.2 Schematic representation of spoligotyping pattern for each organism according to their geographic distribution (Ruelle, *et al.*, 2004).

Spoligotyping is performed by using either one of two currently available methods. The first method is considered to be the "gold standard" which detects the 43 amplified and highly conserved spacer sequences of DRs. The obtained PCR products are subsequently hybridized to artificial oligonucleotide probes that are specific for each spacer region on a nylon membrane. The presences of spacer's are located with a selective chemiluminescent membrane stain. Therefore, the presence of a particular spacer and the number of DRs result in a strain specific pattern due to hybridization on the membrane (Das, *et al.*, 1995). A second method was established to replace the time consuming membrane steps of the first method. Beijing/W TB resistant strains are of medical importance in research studies and susceptible strains have been found to be globally distributed (Streicher, 2007). Beijing strains are identified by a particular spoligotype pattern based on their spoligotype spacers 34-35 (Oduwole, 2008).

2.7.2.2 IS6110 restriction fragment length polymorphism (RFLP) typing

This method offers the quality control in bacteriological laboratories as it is used to describe the rate of infection and trace the original source of cross contamination that would result in false positive results (Streicher, 2007). The IS6110 sequence is present in all members of the MTBC even though they are not epidemiological related due to DNA variability (Haas, *et al.*, 1997). The RFLP approach is largely used in combination with PCR where PCR products are digested followed by RFLP analysis to specify the grouping of each member in the MTBC (Asiimwe,

2008). RFLP typing is used to predict the distribution pattern of tuberculosis, and allows the description of how the infection has been spread throughout the population. The RFLP typing is effective for biotyping of multidrug-resistant strains. The effectiveness and success of this method depends on the extent of infection in a population, stability and wide variety of RFLP type. The IS6110 probes have been successfully used to biotype *M. tuberculosis* in European population, North American and Hong Kong populations. Nevertheless, a lower extent of discrimination and heterogeneity has been reported among isolated strains in Africa and Vietnam (Das, *et al.*, 1995).

2.7.3 Immunological Techniques

The TST method is the oldest immunological method that was developed by Robert Koch using boiled extract of tubercle bacilli (Frieden, *et al.*, 2003). The test results are obtained within 48 to 72 hours and they are considered positive if the diameter of the lesion is approximately 10 mm or greater (Haas, 2000). The tuberculin protein precipitates were then developed into a purified protein derivative (PPD). The PPD is a simple solution prepared from cultures of tubercle bacilli which are used to suppress the *M. tuberculosis* (Nelson and Williams, 2007). However, a regular form was developed into a standardized purified protein derivative (PPD-S) (Frieden, *et al.*, 2003). The BCG vaccine is another immunological-based method which is used as a TB control strategy that can last 15 years in a vaccinated population. This method interferes with the result interpretation of TST through the induction of the immune response against PPD (Saltin, 2006). TST can produce false negative results when the cellular immune system is impaired. Variation in the mode of action between the multiple puncture technique and the intracutanous injection are the most limiting factor for TST. Low cellular response in immune-compromised patients, inter operator variability, cross contamination and false negative tests decreases the sensitivity of TST (Nelson and Williams, 2007).

Patients who are co-infected with HIV or cancer mount less of the response to any skin test therefore results must be interpreted with caution. Malnutrition and deficiency of micronutrients in patients interferes with immunity and can also impede the cellular response to the TST (Frieden, *et al.*, 2003). The application of the TST in latently infected patients is challenging due to a lack of effective gold standard method for diagnosis. This test is usually conducted to the volar surface of the forearm and interpreted by trained workers (Nelson and Williams, 2007). Most immunological methods are sensitive and specific to their reactions. These methods measure the gamma interferon released (IFN-Y) by memory cells and effector T-cells in the blood of the infected patients. Unfortunately immunological methods are expensive, requires extensive training and proper handling of samples to avoid cross infections (Saltin, 2006).

2.7.4 Microscopic Detection Methods

In 1882 Robert Koch demonstrated the microscopic staining of *M.tuberculosis*. Thereafter, microscopy became a "gold standard" method for phenotypic identification of biological samples. Currently, it is used by many mycobacterial laboratories to confirm the presence of acid fast bacilli (AFB) in sputum samples. This method is considered cheap and rapid although it has low sensitivity (Drobniewski, *et al.*, 2003). The standard Gram stain procedure is ineffective in staining Mycobacterium species due to the high lipid content of their cell walls. The lipid content acts as an impermeable barrier for commonly used basic aniline dyes at ambient temperature during staining reaction (Oduwole, 2008). As a result, the waxy layer of the (Jun Liu, *et al.*, 1995). Mycobacterium cell wall presents a challenge for identification using Gram stain procedure. Poor retention and adsorption of staining dye on the cells are major obstacles during the staining process. Alternatively, acid fast staining procedures such as Ziehl-Neelsen (ZN) is recommended for microscopic detection of mycobacteria (Oduwole, 2008). The sample smear is fixed on the slide, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. The smear is then counterstained with methylene-blue or other recommended dyes. Stained acid-fast bacilli appear pink against the blue background when observed under the light microscope, as shown in Figure 2.3.

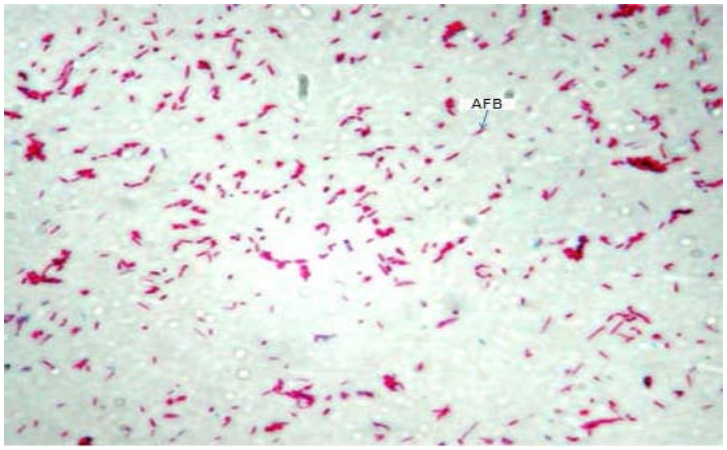


Figure 2.3 Microscopic visualization of stained *Mycobacterium tuberculosis* with Ziehl-Neelson staining procedure (Wiley, 2012).

Concentrated samples are recommended provided they increase the sensitivity of the method. Respiratory secretions such as sputum and bronchial aspirates are the predominantly used specimens. Tubercle bacilli have a low specific gravity ranging from 1.07 to 0.7 and therefore the bacilli float instead of settling. The sputum samples compensate for the lack of a sedimentation effect at a specific gravity greater than 3 000 x g (Connie & George, 2002).

Sputum is normally pre-treated with a solution containing mucolytic agents such as N-acetyl-L-cysteine (NALC) and sodium hydroxide (NaOH).

The NALC and NaOH solution acts as the liquefying agents with a non-inhibitory effect on the mycobacterial cells. NALC digests the mucoproteins to release the bacilli and so enables the sedimentation of cells (Connie & George, 2002). The NaOH component decontaminates the sputum through the elimination of unwanted organisms other than the mycobacteria. Timing of the treatment process is essential to avoid destruction of the tubercle bacilli, particularly when the process is extended for more than 20 minutes. Phosphate buffer is usually added to the reaction mixture to terminate a decontamination process after addition of equal volumes of sputum and NALC (Katoch, 2004). A minimum of 10 000 cells/ml of the sputum is required for successful visual detection with 100X microscope objective (Todar, 2008).

Ziehl-Neelsen and Kinyoun staining procedures use the carborfuschin solution as a primary staining reagent and acid alcohol for decolourization as well as a methylene blue counter-staining reaction. During Ziehl-Neelsen straining, heat is applied whereas the Kinyoun acid fast stain is a cold-based stain (Connie & George, 2002). Alternatively, fluorescence auramine-O microscopy uses the specialized fluorescence microscope with 40 X lower magnification. In this staining method the stained bacilli appear yellow or orange-red in contrast to the dark background. This staining method is more sensitive than the Ziehl-Neelsen method as it scans a large surface area of the slide per unit time (Koneman, *et al.*, 1997). Microscopy is labour intensive and samples are not considered to be negative until 300 high power fields are examined. As a result highly trained staffs are required for result interpretation. The detection limit of microscopy is approximately $5x10^3$ to 10^4 bacilli/ml of sample and the sensitivity ranges from 22 to 78% compared to known culture methods (Cooksey, 2003).

Successful detection of *M. tuberculosis* depends on several parameters of various tedious processes if are not properly monitored. These parameters include the analysis, type of specimen, liquefaction and quantity of the sample required. Sample preparation method as well as the experience of the staff preparing the slides plays an important role in the successful detection of an organism. Dead cells can also fluorescence which poses a challenge in the microscopic differentiation between live and dead cells (Koneman, *et al.*, 1997).

2.8 GENERAL OVERVIEW OF MASS SPECTROMETRY

Mass spectrometry is an analytical technique employed to measure the mass to charge ratio of electrically charged ions. Some mass spectrometric tools have been employed as an alternative to conventional methods for analysis of mycobacterial lipid components and molecules are measured in Daltons (Da) due to their minute size (Oduwole, 2008). A mass spectrometer consists of three component parts:

i) An ion source to convert molecules from the gas phase into ions.

ii) A mass spectral analyser that arranges ions according to their mass to charge ratio through an electromagnetic field. Analysers differ according to the type of mass spectrometry. In the mass analyser, ions are separated according to their mass to charge ratio through filters or dispersion. Ions are moving through a vacuum of 10^{-4} torr or less so as to prevent collision of ions with air particles (Van-Baar, 2000). Some commonly used mass spectral analysers include:

- Sector instruments which employs magnetic or an electric field to accelerate the charged ions. Bundles of ions separate according to their mass to charge ratio as they bend through the mass analyser therefore, the fast-moving, lighter and charged ions are turned aside (Heubner, *et al.*, 2006).

- A time-of-flight (TOF) analyser uses the electric field to accelerate the ions through the vacuum with a constant potential difference. Similarly charged ions travel with the same kinetic energy but separate according to their different molecular weight sizes (Frieden, *et al.*, 2003).

- A quadrupole mass filter ensures that the path of oscillating ions is either destabilized or stabilized by a vibrating electric field. A particular range of mass to charge ions pass through the system at any time, therefore it acts as a selective mass filter. Altering the potential difference however, changes the mass range of ions that pass through the system (Willoughby, *et al.*, 1998).

- A Fourier mass analyser transforms ion cyclotron resonance: The mass analyser measures the molecular weight through detection of the current image caused by accelerating ions in the presence of the magnetic field. Ions form part of the circuit through injection into a static electric or magnetic ion trap (Willoughby, *et al.*, 1998).

- A reflectron mass spectrometry analyser is a time of flight mass spectrometry (TOF-MS) that uses the static electric field to reverse the ions in the opposite direction (Willoughby, *et al.*, 1998).

iii) **Detector:** The ions are induced by ultraviolet light on the samples and the electric current released from the ions that pass or hit the surface, are recorded by the detector. Mass spectra are generated in the form of signals due to the mass-to-charge ratio during the course of the instrumental operation. Detectors differ according to the type of mass spectrometry. Mass spectrometry that uses detectors as one of their component parts is also coupled with electron multipliers, Faraday cups, ions-to-photon detectors and micro channel plate detector (MCP) (Oduwole, 2008).

Mass spectrometers are generally connected to data handling units, particularly computer systems that control, store, acquire and present data. This computer-based system is responsible for quantitative and data analysis, as indicated in Figure 2.4.

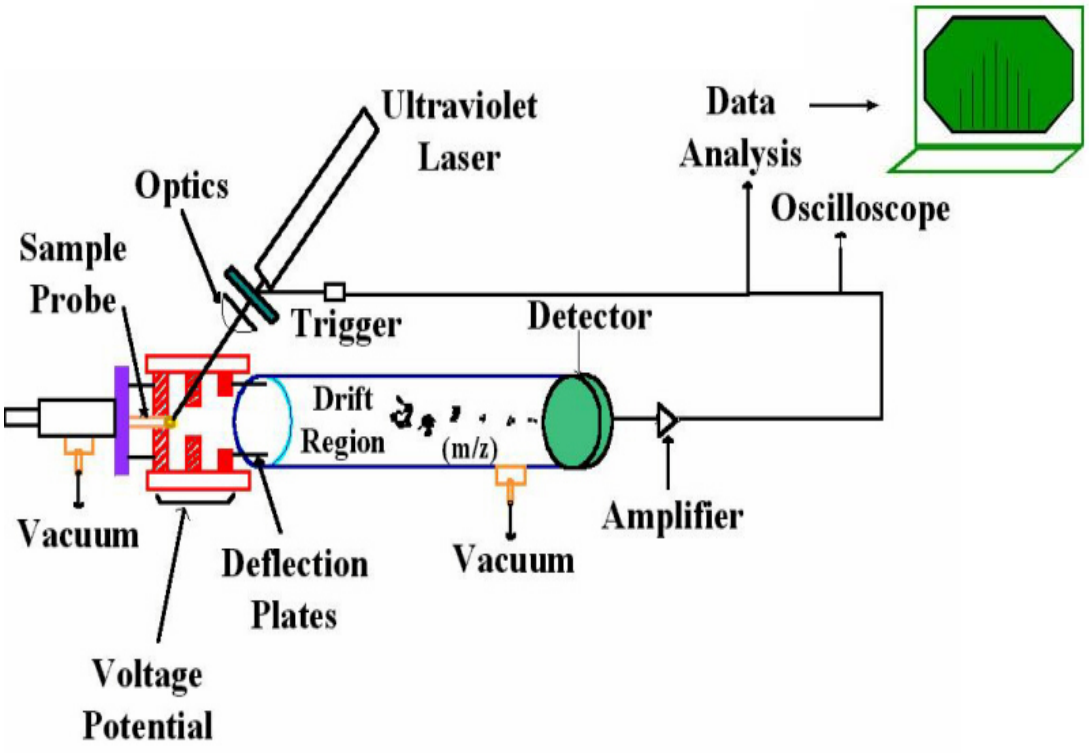


Figure 2.4. Schematic representation of the components of mass spectrometry (USM, 2012).

The computerized system enables the identification of an unknown organism both on and off spectra comparison to a spectral database (Maier & Kostrzewa, 2007). There are different kinds of "soft" ionizing instruments which include the chemical ionization (CI), which is mainly used to detect smaller molecules less than 1kDa, electrospray ionization (ESI) which is used to detect peptides and proteins with a molecular weight less than 200 kDa, and as atomic bombardment (FAB) which is used to detect the carbohydrates, organometallics and peptides with molecular weight less than 6 kDa (Oduwole, 2008). Several mass spectrometric applications have been established and MALDI TOF-MS and ESI have been suggested for the analysis of thermo-labile biological samples(Gustafsson, *et al.*, 2011).

2.8.1 Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI –TOF MS)

"Soft laser desorption" (SLD) was first invented by Tanaka and Fenn in 1987(Romanus, *et al.*, 2011). The SLD technology was subsequently improved through the introduction of Matrix-Assisted Laser Desorption/Ionization (MALDI) (Hillekamp, *et al.*, 1986). In 1988, the first spectrum of a higher molecular weight compound was obtained using MALDI (Hillenkamp & Karas, 2000). Hillenkamp and Karas (2000) showed that a mixture of alanine and tryptophan could easily ionize when irradiated with a 266 nm laser. It was observed that tryptophan absorbed the laser energy and transferred it to non-energy absorbing alanine molecules (Karas, *et al.*, 1987). Thereafter, SLD became an important tool that was exploited for analysis of sensitive biomolecules.

Molecular methods such as Mycobacterium species-specific gene sequences are used to discriminate between Mycobacterium species; however, there is a need for rapid diagnostic tools in the mycobacteriology laboratories. Challenges by conventional methods are due to genetic invariance of Mycobacterium species in their target loci and as a result they are hardly discriminated at the sub-species level (Huard, *et al.*, 2003). MALDI-TOF MS has been recommended as a rapid, sensitive and cheaper diagnostic tool in bacteriology laboratories. During the MALDI-TOF MASS analytic process, the ions are emitted at the ion source. Subsequently, these are absorbed by a UV-absorbing matrix which energizes the biomolecules. These biomolecules are then desorbed from the solid plate to a vacuum "field free" tube towards the detector. These biomolecules separate according to their mass to charge ratio as they move toward the detector (Lay, 2001). Separated ions are captured and presented as the spectral image representing the amount of ions captured by a detector, as indicated in Figure 2.5.

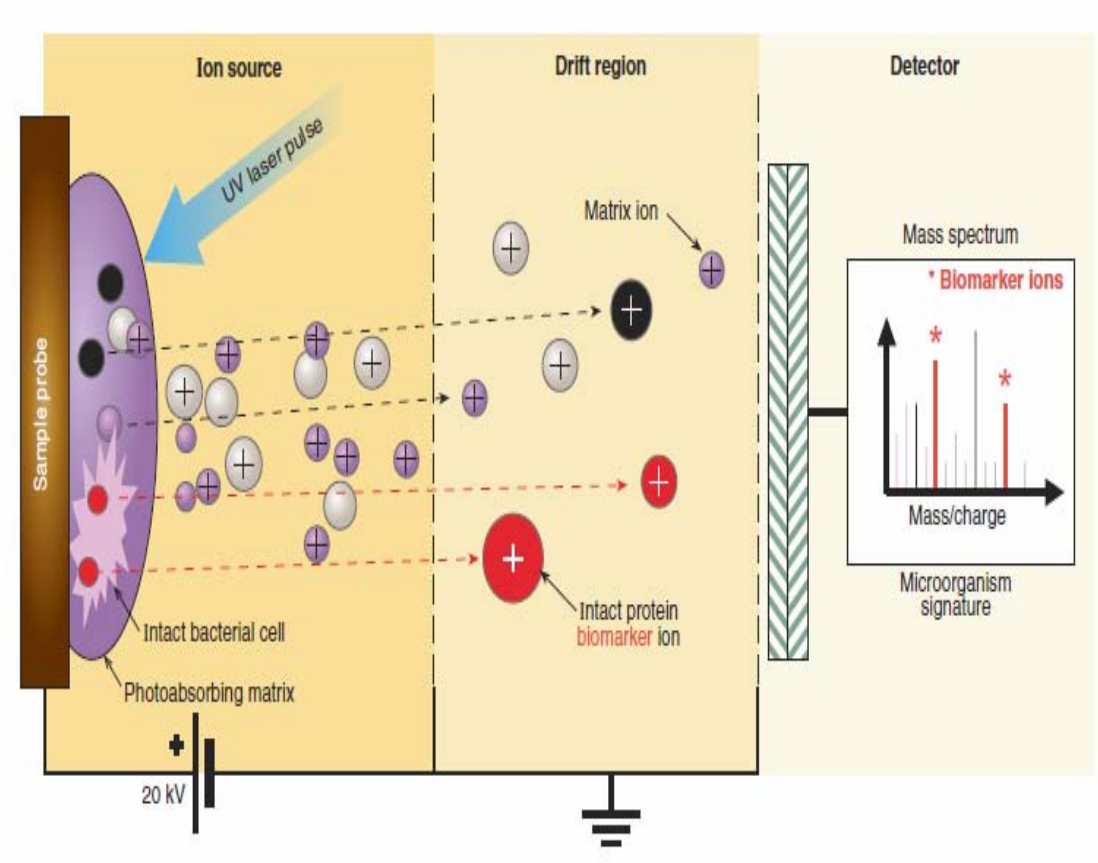
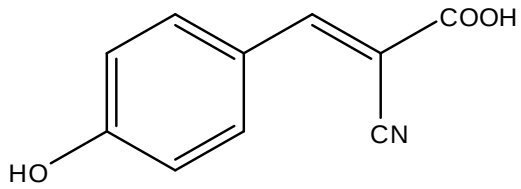
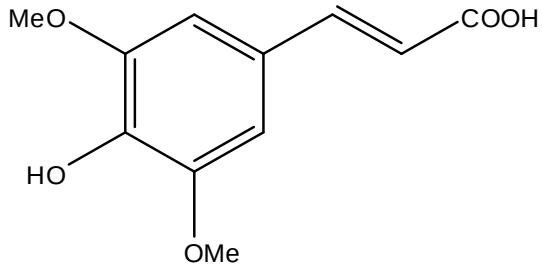
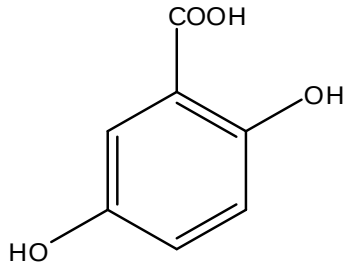


Figure 2.5 Schematic representation of MALDI-TOF MS for the conversion of ions (m/z) into spectral signals (USM, 2012).

The matrix is a crystalline-like structure of weak organic acids containing an aromatic ring and carboxylic acid residue that absorbs the energy at a given wavelength. The matrix transfers and prevents decomposition of sensitive biomolecules from excessive energy (Gustafsson, *et al*., 2011). Many matrix compounds have been tested and three have been recommended for MALDI analysis, as indicated in Table 2.2.

Table 2.2 Matrix selection for MALDI-TOF MS analysis of biomolecules (Wiley).

Compound name and structure	Solvent	Mass range (Da)
α-cyano-4-hydroxy-*trans*-cinnamic acid (αCN, HCCA) 	50% acetonitrile	400-100 000
3,5-dimethoxy-4-hydroxy-trans-cinnamic-acid (sinapinic acid, SA) 	50% acetonitrile	1000-100 000
2,5-dihydroxybenzoic acid (DHB, gentisic acid) 	50% ethanol	200-5 000

Sinapinic acid (SA) ionizes larger molecules and produces fewer peaks at the lower mass range detection of 2 to 6 kDa. The matrices, 2,5-dihydroxy benzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (HCCA) produce more mass spectral signals at lower mass range detection, however, HCCA is recommended for MALDI biotyping analysis as it forms homogenous crystals (Maier & Kostrzewa, 2007).

Reproducible and reliable mass spectral fingerprints are dependent on the proper matrix selection and sample preparation method. Matrix selection is based on the crystal quality and sensitivity. Proteins and peptides are detected as positive ions from an acidified matrix (Lay, 2001). Cell extracts, taxonomic and ribosomal proteins can be analysed with MALDI-TOF MS from microorganisms (Liyanage & Lay, 2006). Spectral fingerprints are generated during the analytical process based on the composition of a particular biological sample. Different sample preparation methods yield different mass spectral fingerprints. Culture conditions such as temperature or media do not have a great effect on the conserved mass signals that define an organism. Additional peaks may be observed depending on the growth phase of an organism. Nevertheless, additional mass signals do not prevent correct identification of an organism provided the typical and stable peaks are not suppressed (Maier & Kostrzewa, 2007). The MALDI-TOF MS has three main advantages that make it a method of choice for diagnosis and identification of biological sample origins. The advantages are as follows:

i) **Detection of high mass range:** Over 300 kDa mass signals can be detected using atime of flight mass analyser (Lay, 2001).

ii) **Speed:** More spectra can be generated within a short period of time (Liyanage & Lay, 2006).

iii) **Sensitivity**: Charged ions are separated according to their mass to charge ratio which is directly proportional to time of ion transition. Therefore smaller molecules reach the detector at a faster rate than larger molecules (Liyanage & Lay, 2006). The biomolecules are converted from a solid or liquid phase into a gas phase in the flight tube. Vaporization and ionization of biomolecules are achieved through a single non-destructive step (Lay, 2001).

2.8.2 Application of MALDI-TOF MS in bacteriology

MALDI-TOF MS was invented for taxonomic identification of bacteria based on their unique mass spectral fingerprints (Lay, 2001). MALDI- TOF MS proved to have good resolution in spite of small amounts of salts and other agents that can interfere with the mass spectrometric analysis (Ruelle, *et al.*, 2004). It has been extensively used for identification and characterization of bacteria and fungi through cell extract preparations (Sherburn & Jenkins, 2003). These cell extracts might be peptides, proteins, carbohydrates, nucleic acids or synthetic polymers (Marvin, *et al.*, 2003). Preliminary isolation, culturing and protein extraction steps are conducted prior to MALDI biotyping analysis. Extraction of cellular proteins from pathogenic organisms is recommended so as to prevent disease transmission to the operator. This strategy is supported in that MALDI-TOF MS can be used to identify proteins which are poorly explained at the genomic level (Lay, 2001).

2.10 CONCLUSION

MALDI-TOF MS is able to discriminate mycobacterial species to a genus and species level, however, some mycobacterial species are closely related in such a way that MALDI can unambiguously identify them. Appropriate software needs to be integrated into MALDI-TOF MS for better resolution and discrimination of closely related organisms to a strain level. To the best of our knowledge thus far no study has been conducted to discriminate *M. tuberculosis* to a strain level using MALDI-TOF MS. The lack of a mycobacterial database has been a limiting factor for mycobacterial identification, however; it can be remediated by increasing the entries of mass spectra in the database.

2.11 REFERENCES

Asiimwe J (2008) molecular characterization of *M. bovis* isolates from selected slaughter houses in Kampala. Thesis, Makerere University.

Butler WR & Guthertz LS (2001) Mycolic acid analysis by high-performance liquid chromatography for identification of Mycobacterium Species. *American Society for Microbiology* **14**: 704–726.

Charles LD, Judith AH, Andrew RM, Philip CH & Gisela FS (1998) Incidence of Tuberculosis in Injection Drug Users in San Francisco. *American journal of respiratory and critical care medicine* **157**: 19-22.

Connie RM & George M (2002) *Diagnostic microbiology*. Saunders.

Cooksey RC (2003) Recent advances in laboratory precedures for pathogenic Mycobacteria. *Clinical Laboratory Medicine* **23**: 801-821.

Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC & Dye C (2003) The growing burden of tuberculosis. *Archives of Internal Medicine* **163**: 1009-1021.

Daley CL, Hahn JA, Moss AR, Hopewell PC & Schecter GF (1998) Incidence of tuberculosis in injection drug users in san francisco. *American Journal of Respiratory and Critical Care Medicine* **157**: 19–22.

Das S, Paramasivan CN, Lowrie DB, Prabhakar R & Narayanan PR (1995) IS6110 restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. *Tubercle and Lung Disease* **76**: 550-554.

Dasgupta K & Menzies D (2005) Cost-effectiveness of tuberculosis control strategies among immigrants and refugees. *European Respiratory Journal* **25**: 1107–1116.

Drobniewski FA, Caws M, Gibson A & Young D (2003) Modern laboratory diagnosis of tuberculosis. *Lancet Infectious Diseases* **3**: 141–147.

Dubnau E, Fontán P, Manganelli R, Appel S & Smith I (2002) *Mycobacterium tuberculosis* genes induced during Infection of human macrophages. *Infection and Immunity* **70**: 2787–2795.

Ferreira L, Castan SV, Juanes FS*, et al.* (2010) Identification of Brucella by MALDI-TOF mass spectrometry. Fast and reliable identification from agar plates and blood cultures. *PLoS ONE* **5**: 1-8.

Frieden TR, Sterling TR, Munsiff SS, Watt CJ & Dye C (2003) Tuberculosis. *Lancet* **362**: 887-889.

Gandhi NR, Moll A, Sturm AW*, et al.* (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet Infectious Diseases* **368**: 1575–1580.

Garbe TR, Hibler NS & Deretic V (1996) Isoniazid Induces Expression of the Antigen 85 Complex in Mycobacterium tuberculosis. *Antimicrobial Agents and Chemotherapy* **40**: 1754–1756.

Giffin RB & Robinson S (2009) *Addressing the Threat of Drug Resistance Tuberculosis; A realistic Assessment of the Challenge*. The National Academic Press.

Gillespie SH (2002) Evolution of drug resistance in *Mycobacterium tuberculosis*:Clinical and molecular perspective. *American Society for Microbiology* **46**: 267–274.

Gustafsson JOR, Oehler MK, Ruszkiewicz A, McColl SR & Hoffmann P (2011) MALDI imaging mass spectrometry (MALDI-IMS)-Application of spatial proteomics for ovarian cancer classification and diagnosis. *International Journal of Molecular Sciences* **12**: 773-794.

Haas DW (2000) *Principle and practice of infectious diseases*. Churchill Livingstone.

Haas WH, Bretzel G, Amthor B*, et al.* (1997) Comparison of DNA fingerprint patterns of isolates of *Mycobacterium africanum* from East and West Africa. *Journal Of Clinical Microbiology* **35**: 663–666.

Harth G, Clemens DL & Horwitz MA (1994) Glutamine synthetase of *Mycobacterium tuberculosis:* Extracellular release and characterization of its enzymatic activity. *Proceedings of the National Academy of Science* **91**: 9342-9346.

He H & Zahrt TC (2005) Identification and characterization of a regulatory sequence recognized by *Mycobacterium tuberculosis* persistence regulator MprA. *Journal of Bacteriology* **187**: 202–212.

Heubner RE, Good RC & Tokars JI (2006) Current Practice in Microbiology; Results of a Survey of State Public Health Laboratory. *Clinical Microbiology Review* **8**: 180-199.

Hillekamp F, Kara M, holtkamp D & Klusener P (1986) Energy Deposition in Ultraviolet Lasre Desorption Mass Spectrometry of Biomolecules. *International Journal of Mass Spectrometry and Ion Processes* **69**: 265-276.

Hillenkamp F & Karas M (2000) Matrix-assisted laser desorption/ionisation, an experience. *International Journal of Mass Spectrometry* **200**: 71-77.

Huard RC, Lazzarini LCO, Butler WR, Soolingen D & Ho JL (2003) PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. *Journal of Clinical Microbiology* **41**: 1637–1650.

Huggett JF, McHugh TD & Zumla A (2003) Tuberculosis; amplification based clinical diagnostic techniques. *The International Journal of Biochemistry and Cell Biology* **35**: 1407-1412.

Ioerger TR, Feng y, Ganesula k, *et al*. (2010) Variation among Genome Sequences of H37Rv Strains of Mycobacterium tuberculosis from Multiple Laboratories. *Journal of Bacteriology* **192**: 3645–3653.

Jacobs WR, Tuckman M & Bloom BR (1987) Introduction of foreign DNA into mycobacterium using a shuttle plasmid. *Nature* **327**: 532-535.

Jun Liu J, Rosenberg EY & Nikaido H (1995) Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proceedings of National Academy of Science.* **29**: 11254-11258.

Kaiser GE (2011). *Structure of an Acid-Fast Cell Wall*. www.student.ccbcmd.edu

Karas M, Bachmann D & Hillekamp F (1987) Matrix Assisted-Laser Desoption of non-volatile Compounds. *International Journal of Mass Spectrometry and Ion Processes* **74**: 63-68.

Katoch VM (2004) Infectious due to non-tuberculous mycobacteria *Indian Journal of Medical Research* **120**: 290-304.

Kaufmann S HE & Walker B D (2009) *AIDS and tuberculosis; infection biology handbook series*. Willey-Blackwell.

Koneman EW, Allen SD, Janda WM & Schreckenberger PC (1997) *Color Atlas and Textbook of Diagnostic Microbiology*. Lippincott Williams and Wilkins.

Laval F, Laneelle MA, Deon C, Monsarrat B & Daffe M (2001) Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. *Analitical Chemistry* **73**: 4537-4544.

Lay JO (2001) MALDI-TOF mass spectrometry of bacteria. *Mass Spectrometry Reviews* **20**: 172-194.

Liu J, Rosenberg E Y & Nikaido H (1995) Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proceedings of the National Academy of Science.* **29**: 11254-11258.

Liyanage R & Lay J O (2006) *An introduction to MALDI-TOF MS. Identification of Microorganisms by Mass Spectrometry*. John Wiley and Sons.

Macia A, Dainese E, Rodriguez G M, *et al.* (2007) Global Analysis of the Mycobacterium tuberculosis Zur (FurB) Regulon. *Journal of bacteriology* **189**: 730–740.

Magnus M (2009) *Essential reading in infectious disease epidemiology*. Jones and Bartlett Publisher.

Mahon CR & Manuselis G (2002) *Text Book of Diagnostic Microbiology*. SAUNDERS.

Maier T & Kostrzewa M (2007) Fast and reliable MALDI-TOF MS-based microorganism identification. chimiccs oggi. *Chemistry Today* **25**: 68-71.

Målen H, Souza GAD, Pathak S, Søfteland T & Wiker HG (2011) Comparison of membrane proteins of *Mycobacterium tuberculosis* H37Rv and H37Ra strains. *BMC Microbiology* **11**: 1-10.

Marvin LF, Roberts MA & Fay LB (2003) Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry in Clinical Chemistry. *Clinica Chimica Acta* **337**: 11-21.

Murray C JL & Salomon J A (1998) Modeling t he i mpact o f g lobal t uberculosis control strategies. *Proceedings of National Academy of Science.* **95**: 13881–13886.

Nelson KE & Wi lliams C M (2007) *Infectious D isease E pidemiology: T heory and pr actice*. Jones and Barlett publishers.

Oduwole E O (2008) G eneration o f a D atabase of M ass S pectra P atterns o f S elected Mycobacterium sp ecies Using MA LDI-TOF mass spectrometry. T hesis, U niversity of Stellenbosch.

Ohrmalm C , J obs M , E riksson R*, e t al*. (2010) H ybridization pr operties of long nu cleic aci d probes for detection of variable target sequences, and development of a hybridization prediction algorithm. *Nucleic Acids Research* **10**: 1-3.

Olano J, Lo´pez B, Reyes A*, et al*. (2007) Mutations in DNA repair genes are associated with the Haarlem lineage of *Mycobacterium tuberculosis* independently of their antibiotic resistance. *Tuberculosis* **87**: 502–508.

Pang X & Howard S T (2007) R egulation o f the -Crystallin Gene a cr2 by the MprAB T wo-Component System of Mycobacterium tuberculosis. *Journal of Bacteriology* **189**: 6213–6221.

Richardson M, Lill S & Spuy GD (2002) Historic and Recent Events Contribute to the Disease Dynamics of B eijing-like My cobacterium t uberculosis I solates in a H igh I ncidence R egion *International of Tuberculosis and Lung Disease* **6**: 1001-1011.

Romanus I I, E ze A E, E gwu O A, N gozi A F & C hidiebube N A (2011) C omparison of m atrix-assisted l aser d esorption i onization-time o f flight mass s pectrometry w ith c onventional culture and b iochemical m ethod o f b acteria identification t o sp ecies l evel. *Journal of M edical Laboratory and Diagnosis* **2**: 1-4.

Roth A, S chaberg T & M auch H (1997) M olecular di agnosis of t uberculosis; current c linical validity and future perspectives. *European Respiratory Journal* **10**: 1877-1891.

Ruelle V , M oalij B , Z orzi W , L edent P & D e-Pauw E (2004) R apid identification of enveronmental b acterial strains b y m atrix-assisted l aser de sorption/Ionization time of flight mass spectrometry. *Rapid Communication in Mass Spectrometry* **18**: 2013-2019.

Russo C, Tortoli E & M enichella D (2006) E valuation of t he new g enoType M ycobacterium assay for identification of mycobacterial species. *Journal of Clinical Microbiology* **44**: 334–339.

Saito R , T anaka A , S ugiyama K , A zuma I, Y amamura Y , K ato M & G oren M B (1975) Adjuvant effect of cord factor of a mycobacterial lipid. *American Society for Microbiology* **13**: 776-781.

Saleeb P G, D rake S K, Murray R P & Z elazyn A M (2011) I dentification of m ycobacteria in solid-culture m edia b y matrix-mssisted laser de sorption i onization-time o f tl ight m ass spectrometry. *Journal of Clinical Microbiology* **49**: 1790-1794.

Salmoniere YOG, Torrea H, Bunschoten A, Embden JDA & Goquel B, . (1997) Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. *Journal of Clinical Microbiology* **35**: 2210-2214.

Saltin C (2006) Chemotherapy and diagnosis of tuberculosis. *Respiratory Medicine* **100**: 2085-2097.

Sherburn RE & Jenkins RO (2003) A novel and rapid approach to yeast differentiation using matrix-assisted laser desorption/ionisation-time of flight mass spectrometry. *Spectroscopy* **17**: 31-38.

Shinnick TM & Good RC (2011) Diagnostic mycobacteriology laboratory practices. *Clinical Infectious Diseases* **21**: 291-299.

Silva PEAD & Palomino JC (2011) Molecular basis and mechanisms of drug resistance in M. tuberculosis: Class and new drugs. *Journal of Antimicrobial Chemotherapy* **66**: 1417-1430.

Streicher E M (2007) Application of spoligotyping in the understanding of the dynamics of *Mycobacterium tuberculosis* strains in high incident communities. Thesis, Stellenbosch University.

Telenti A, P. Imboden P, Marchesi F, T.,, Schidheimi T & Bodmer T (1993) Direct, automated detection of rifampicin-resistant *Mycobacterium tuberculosis* by polymerase chain reaction and single strand conformation polymorphism analysis. *Antimicrob Agents Chemother* **37**: 2054 - 2058.

Tiwaria EP, Hattikudura NS, Bharmalb R N, Kartikeyanc S, Deshmukhd NM & Bisene PS (2007) Modern approaches to a rapid diagnosis of tuberculosis: Promises and challenges ahead. *Tuberculosis* **87**: 193–201.

Todar K (2008). *Toda's online text book of bacteriology*. Textbook of Bacteriology.net U SM (2012). 28 March 2012. www.psrc.usm.edu

Van-Baar B LM (2000) Characterization of Bacteria by Matrix-Assisted Laser Desorption/Ionization and Electrospray Mass Spectrometry. *FEMS Microbiology Review* **24**: 193-219.

White RG, Jolles P, Samour D & Ledere E (1963) Correlation of adjuvant activity and chemical structure of wax D fractions of mycobacteria *Immunology* **7**: 158-171.

Wiley *Current Protocols*. Accessed 15 November 2012. www.currentprotocols.com

Willoughby R, Sheehan E & Mitrovich SA (1998) Global View Publishing.

Yesilkaya H, Dale JW, Strachan NJC & Forbes KJ (2005) Natural transposon mutagenesis of clinical isolates of *Mycobacterium tuberculosis*: How many genes does a pathogen need? *Journal of Bacteriology* **187**: 6726–6732.

Chapter 3

RESEARCH RESULTS I

Optimization of the sample preparation protocol for matrix assisted laser desorption ionization-time of flight mass spectrometry discrimination of *Mycobacterium tuberculosis* complex

Optimization of the sample preparation protocol for matrix assisted laser desorption ionization-time of flight mass spectrometry discrimination of *Mycobacterium tuberculosis* complex

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3.1 ABSTRACT

Rapid and accurate discrimination of most bacteria and yeast using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is possible using a standardized ethanol/formic acid sample preparation protocol. However, due to the complex cell wall structure and composition of mycobacteria, this sample preparation protocol is suboptimal for accurate strain identification purposes. KwaZulu-Natal is a hotspot region associated with the recent emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) *M. tuberculosis* strains. In this study as a proof-of-concept, typed and clinical strains of mycobacteria were initially used to evaluate variations of the ethanol/formic acid sample preparation protocol. A modified protocol that involved an initial delipidation of mycobacteria with chloroform/methanol (1:1, v/v) extraction to facilitate removal of cell wall-attached mycobacterial lipids was conceptualized. This sample preparation strategy was evaluated against the routinely employed ethanol/formic acid and glass bead-modified ethanol/formic acid sample preparation methods. Reproducible and unique mass spectra were consistently and exclusively generated using the delipidation modified sample preparation protocol. A database of American Type Culture and clinical mycobacterial strains of *M. tuberculosis* strains was established.

3.2 INTRODUCTION

The genus of mycobacteria comprises approximately 100 heterogeneous species, many of which are medical important. These organisms have wide range of host selection ranging from animals to humans. Human infections caused by pathogenic mycobacteria have been increased over the past years (Pignone, *et al.*, 2006). Members of *Mycobacterium tuberculosis* complex (MTBC) are the leading causative agents of tuberculosis as intracellular parasites of macrophages (Homolka, *et al.*, 2009).

Mycobacteria are uniquely characterized by the presence of long chain fatty acids on their cell wall called mycolic acids. Mycolic acids are covalently attached to the cell wall arabinogalactan or esterified to trehalose and glycerol. These fatty acids contribute approximately 40-60% cellular dry weight of the cell. It has been suggested that mycolic acids plays a significant role in the cell wall structure of mycobacteria; such as low cell permeability and highly rigid cell wall structure. As a result, mycobacteria are resistant to some chemicals and antibiotics (Laval, *et al.*, 2001).

Tuberculosis is the long existing infectious disease; however, it remain the major global threat in the public health (Ikryannikova, *et al.*, 2007). Approximately, one-third of the world population is latently infected and 1.8 million people die annually. eight million cases of active infection been reported every year (Bahk, *et al*., 2004). Furthermore, the condition has been worsened by the emergence of multidrug-resistant tuberculosisisolates (MDRTB and XDR TB). Multidrug-resistant tuberculosis (MDR TB) is considered to be resistance to isoniazid and rifampicin drug therapy. According to data obtained by world health organization (WHO), it is estimated that 490 000 multidrug-resistant TB isolatesemerge every year with more than 110 000 death rate (Homolka *et al.*, 2009). Emergence of drug resistant isolates is due to patient non-compliance, non-adherence and incorrect treatment implementation. Consequently, tubercle bacilli grow until it reaches the acute phase of infection (Bahk, *et al*., 2004). Furthermore, the diagnosis of MDR and XDR *M. tuberculosis* is obtained by performing direct susceptibility testing on culture positive specimens, with a turnaround time of approximately 6-8 weeks (Hillemann, *et al.*, 2007).

Currently, there is no rapid, reliable and cheap method for detection of *Mycobacterium tuberculosis* at an early stage of infection (Bahk, *et al.*, 2004). Conventional diagnostic methods are dependent on biochemical tests, growth pattern and morphological characteristic of an organism. These methods proved to be laborious, error prone and time consuming; therefore, cannot be implemented for urgent medical situations. PCR- based approaches have higher resolution power; however are costly for routine diagnosis, as a result cannot be implemented in

poor resourced settings (Maier & Markus, 2007). Furthermore, these approaches have limited number specific probes for known organisms; therefore cannot be used for classification of unknown bacterial sample origin (Sauer, *et al.*, 2008).

However, MALDI-TOF MS offers a potentially promising approach for rapid, reliable and cheap diagnosis of tuberculosis. MALDI-TOF MS has been extensively used for research purposes. Recently, it has been used for diagnostic purposes. MALDI mass spectrometry analysis has been successfully employed to discriminate yeast and bacteria in the clinical laboratories. Nevertheless, there has been limited studies reported the use of MALDI-TOF MS for *Mycobacterium* discrimination based on their unique protein fingerprints. Conventional ethanol/formic acid method does not yield reproducible results for mycobacterial analysis. It has been suggested that some mycobacterial species have glycopeptidolipids (GPLs) on their cell wall. As a result, the GPLs prevent the penetration of chemicals during protein extraction process; therefore, poor quality of spectra are generated (Saleeb, *et al.*, 2011).

During MALDI-TOF MS analysis, the thermolabile biomolecules or whole cells are embedded on the UV-absorbing crystal-like structure of weak organic acid called matrix. This weak organic acid prevent decomposition of biomolecules through absorbing excess energy, and enables desorption and ionization of these biomolecules through energy transfer and protonation (Bonk & Humeny, 2001). Subsequently, the analytes are desorbed and accelerated into a "field-free" vacuum region. Whilst the ions are travelling through the "field free" region they separate according to their mass to charge ratio. smaller molecules travel faster and reach the detector earlier than larger molecules, these ions are then captured by a detector and represented in the form of spectral fingerprints (Schmidt & Kallow, 2005).

Multiple software programmes have been developed to enable further identification through comparison of generated spectral fingerprints with reference spectra in the established databases (Bienvenut, *et al.*, 1999). Unsupervised approach such as principal component analysis (PCA) can be incorporated in the Biotyper software to reduce the multi-dimensionality of data generated with MALDI-TOF mass spectrometry. Furthermore, hierarchical clustering approaches such as dendrograms and correlation matrix are also integrated in the system to enable identification (Sauer, *et al.*, 2008).

This study was used to evaluate the potential of MALDI-TOF MS and the optimized sample preparation protocol to differentiate closely related members of *Mycobacterium tuberculosis* complex. Further discrimination of *Mycobacterium tuberculosis* at the strain level was also evaluated.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains

American Type Culture Collection (ATCC) and two clinical isolates of mycobacterial cultures were used in this study (Table 3.1). These clinical TB isolates were rejuvenated from the previously identified and stored TB cultures by routine conventional methods.

Table 3.1 Mycobacterial strains used for optimization and comparison of protein extraction protocols.

Strain	Source
M. *tuberculosis* H37Rv	American Type Culture Collection
M. *tuberculosis* ATCC 25177	American Type Culture Collection
M. *bovis* ATCC 19210	American Type Culture Collection
M. *gordonae* ATCC 23409	American Type Culture Collection
M. *smegmatis* ATCC 21293	American Type Culture Collection
Fully susceptible MTB strain	Clinical specimen
MDR-TB strain	Clinical specimen
XDR-TB strain	Clinical specimen

3.3.2 Media and cultivation conditions

The American Type Culture mycobacteria and clinically-isolated TB isolates were rejuvenated at 37 ^{o}C for seven to fourteen days in Middlebrook 7H9 broth. Thereafter isolateswere transferred onto drug free Middlebrook 7H11 agar plates in triplicates and incubated at 37°C for four and twenty one days to cultivate fast (*M. smegmatis)* and slow (*M. bovis, M. gordonae* and *M. tuberculosis*) growing isolates, respectively. A single loop-full of the culture grown on solid medium was transferred into a MicroBank (Pro-Lab Diagnostics, Canada) for long term storage at -75°C.

3.3.3 Standard ethanol-formic acid (EFA) sample preparation protocol

A single colony of culture isolate was harvested using disposable 10 µl inoculating loop on drug free Middlebrook 7H11 agar plate and suspended in a 1.5 ml screw cap Eppendorf tube containing 600µl of high pressure liquid chromatography (HPLC) grade distilled water. The cell suspension was vortexed for 1 minute and heat inactivated at 103°C for 30 minutes, whilst maintaining an internal tube temperature of 98°C. The sample was centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 300 µl of HPLC grade distilled water and 900 µl of absolute ethanol (HPLC grade). The suspension was vigorously vortexed at 13,000 rpm for 2 minutes and the supernatant was discarded. Centrifugation was repeated to completely remove ethanol residues. Thereafter, the ethanol exposed pellet was air dried at room temperature for 5 minutes. Five to eighty µl of formic acid was added and thoroughly vortexed for 30 seconds. Five to eighty µl of absolute acetonitrile (HPLC grade) was added to a suspension and homogenized. The suspension was centrifuged for 2 minutes and the supernatant was transferred into a screw cap Eppendorftube. One µl of supernatant was spotted onto MTP 384 ground steel target plate (Bruker Daltonics, Germany) and allowed to air-dry. Subsequently, dried sample was covered with a 1 µl aliquot of daily prepared portioned matrix solution (10 mg of α-cyano-4-hydroxycinnamic acid dissolved in 1 ml of a solvent mixture containing 50% acetonitrile, 47.5% water and 2.5 % trifluoroacetic acid).

3.3.4 Ethanol-formic acid-glassbead (EFAGB) sample preparation protocol

The EFAGB method that facilitates mycobacterial cellular disruption by micro-glass beads was also used for MALDI-TOF MS sample preparation. Cell biomass from each mycobacterium strain was harvested from Middlebrook 7H11 agar plates with a disposable inoculating loop (10 µl) and transferred into 600 µl of high pressure liquid chromatography (HPLC) grade distilled water contained in a 1.5 ml screw-cap microcentrifuge tube. The cell suspensions were vortexed for one minute and heat inactivated at 103°C for 30 minutes, whilst maintaining an internal tube temperature of 98°C. A micropestle was used to disperse mycobacterial cellular aggregates and the suspension was washed twice with HPLC grade distilled water (300 µl) and centrifuged at 13,000 rpm for 2 minutes. The pellet was re-suspended in 300 µl HPLC grade distilled water and 900 µl of absolute ethanol. Thereafter, the suspension was centrifuged at 13,000 rpm for two minutes and the supernatant was discarded. The pellet was briefly centrifuged and residual ethanol was completely removed with a pipette. Thereafter, the pellet was air-dried. Depending on the volume of the pellet, 10-50 µl of HPLC grade acetonitrile was added. The pellet was re-suspended by vigorous vortexing and an equal volume of silica beads (0.1 mm) was added and microcentrifuge tubes were vortexed for 5 minutes. Based on the volume of acetonitrile, an equal volume of 10-50 µl of a 70% formic acid was added into the mixture and vortexed for 5

minutes. The suspension was centrifuged at 13,000 rpm for one minute and 1 µl of the supernatant was spotted on a MTP 384 ground steel target plate (Bruker Daltonics, Germany). The sample was allowed to air-dry and was subsequently coated with a 1 µl aliquot of daily prepared portioned matrix solution (see above).

3.3.5 Chloroform-methanol ethanol-formic acid (CMEFA) sample preparation protocol

A loopful (10 µl) of mycobacterium culture from a Middlebrook 7H 11 agar plate was suspended in 600 µl of distilled water contained in a 1.5 mls crew-cap Eppendorf tube. *Mycobacterium* suspensions were heat inactivated as described above. The microcentrifuge tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatants were discarded. The delipidation of cells was effected by addition of 600 µl of a chloroform/methanol (1/1, v/v) solvent mixture. The cell suspensions were vigorously vortexed for 60 seconds and then centrifuged at 13,000 rpm for 5 minutes. The delipidation treatment was repeated twice to efficiently remove the lipids; the pellet was then re-suspended in 300 µl of HPLC grade distilled water followed by addition of 900 µl of HPLC grade ethanol. The mixture was vigorously vortexed for 30 seconds and the tubes were then centrifuged at 13,000 rpm for 5 minutes. The supernatant was discarded and the pellet was air dried at room temperature for 20 minutes to remove residual ethanol. Depending on the volume of the pellet, 5-80 µl of 70% formic acid was added and the suspension was rapidly vortexed for 30 seconds. This was followed by the immediate addition of an equal volume (5-80 µl) of HPLC grade acetonitrile and the contents of the tubes were rapidly vortexed for 30 seconds. Samples were then centrifuged at 13,000 rpm for 5 minutes. An aliquot (1 µl) of the supernatant was spotted onto a MTP 384 ground steel target plate. The sample was allowed to air-dry and covered with matrix solution as described in the previous method.

3.3.6 MALDI-TOF MS settings

MALDI target plates were processed in an Autoflex III smartbeam MALDI-TOF MS (Bruker Daltonics, Germany) using smart beam laser frequency at a maximum of 200 Hz. Spectra were acquired in a linear positive mode. Mass signals were detected in the mass-to-charge (m/z) range of 2,000 to 20,000. Ion source one (IS1), two (IS2) and the lens were adjusted to 20.08 kV, 18.57 kV, and 6.02 kV, respectively.

3.3.7 Mass spectral data analysis

Twenty four spectra were accumulated per mycobacterium sample from 40 laser shots summed to 240 in different regions per sample spot. The instrument was externally calibrated with the Bruker bacterial test standard (BTS) (Bruker Daltonics, Germany) with a mass range of 3.6 to

17 kDa prior to sample analysis. Mass spectra were analyzed with the flexAnalysis 3.3 software program (Bruker Daltonics, Germany). The "MBT FC.par" standard flexControl method was selected for both analysis and internal calibration of BTS. The maximum deviation was always observed to be below ± 300 ppm as recommended by the manufacturer. FlexAnalysis was employed to evaluate the quality of spectra through elimination of the mass-to-charge signals with a mass deviation above 3 Da, particularly for peaks that occurred at mass range between 6-7 kDa. Mass spectra projections (MSP's) or libraries were created using Biotyper 3.0 software (Bruker Daltonics, Germany). Mass spectrometry-based dendrograms were also created with Biotyper 3.0 software. Principal component analysis (PCA) was generated using ClinProTools 2.2 software (Bruker Daltonics, Germany) that is externally integrated to MATLAB software.

3.4 RESULTS

In a proof-of-concept study the routinely employed EFA MALDI-TOF MS sample preparation method was evaluated against modified sample preparation methods that included the newly developed EFAGB and CMEFA methods for their potential to yield mass signals that can be used to generate reliable and consistent mass spectral profiles from ATCC-typed mycobacterial strains.

The employment of the approved and routinely used EFA extraction procedure to isolate proteins from ATCC-typed mycobacterial strains was deemed inefficient as the generated mass spectral profiles were very weak and inconsistent (results not shown). These mass spectral fingerprints were unreliable and as such this method was eliminated as a potential candidate for precise discrimination of ATCC-typed and clinically isolated mycobacteria.

We also assessed a modified version of the EFA-based extraction method that incorporated micro-glass bead disruption step (EFAGB method). The EFAGB method was devised to effect mechanical disruption of mycobacterial cell wall that would promote release of mycobacterial-related cellular proteins. MALDI-TOF MS analysis of EFAGB prepared samples yielded protein mass spectral profiles that were characterized by low intensities and noise peaks for some ATCC mycobacterial organisms (Figure 3.1). Therefore, this method was not used for MALDI-TOF MS analysis of communicable clinical *M. tuberculosis* isolates.

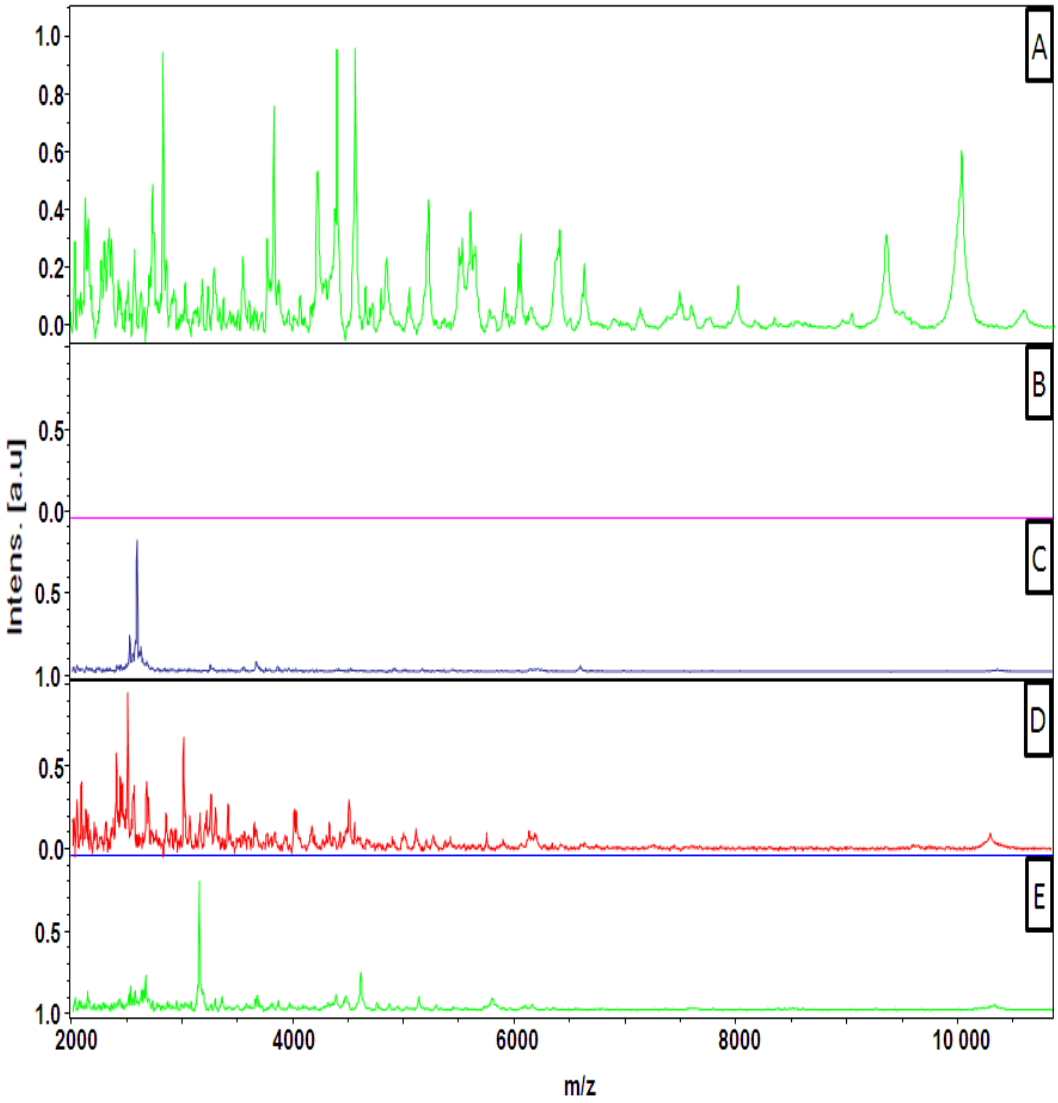

Figure 3.1 Mass spectra of *M. tuberculosis* H37Rv (A), *M. gordonae* ATCC 23409 (B), *M. bovis* ATCC 19210 (C), *M. tuberculosis* ATCC 25177 (D), *M. smegmatis* ATCC 21293 (E) generated using EFGB protein extraction method. Although mass spectra were generated using this method, they were not consistent and more laser shots were required for generation of spectra. When using this method, it was observed that *M. gordonae* species yielded poor quality spectra.

However, the newly devised sample preparation method (CMEFA) seems to have yielded high protein content for MALDI-TOF MS analysis that consistently produced reproducible and strong mass spectral signals. This protocol yielded spectra with enhanced spectral qualities signified by high peak intensities and an extremely low signal-to-noise ratio as illustrated of some of the strains used in this study (Figure 3. 1). Culture-free chloroform/methanol (1:1, v/v) and distilled water were conducted in parallel as negative controls during the sample preparation process to monitor the effect of chloroform/methanol for noise peak generation. The MALDI-TOF MS analyses of chloroform/methanol and distilled

water revealed no mass signals or contaminants (results not shown). This enabled reliable strain differentiation of closely related members of *M. tuberculosis* complex (MTBC) and *M. tuberculosis* isolates (Figure 3.2). Given the superiority of the CMEFA method to yield the strongest and most consistent mass spectral profiles in comparison to the EFA and EFAGB extraction protocols it was implemented as the extraction method of choice.

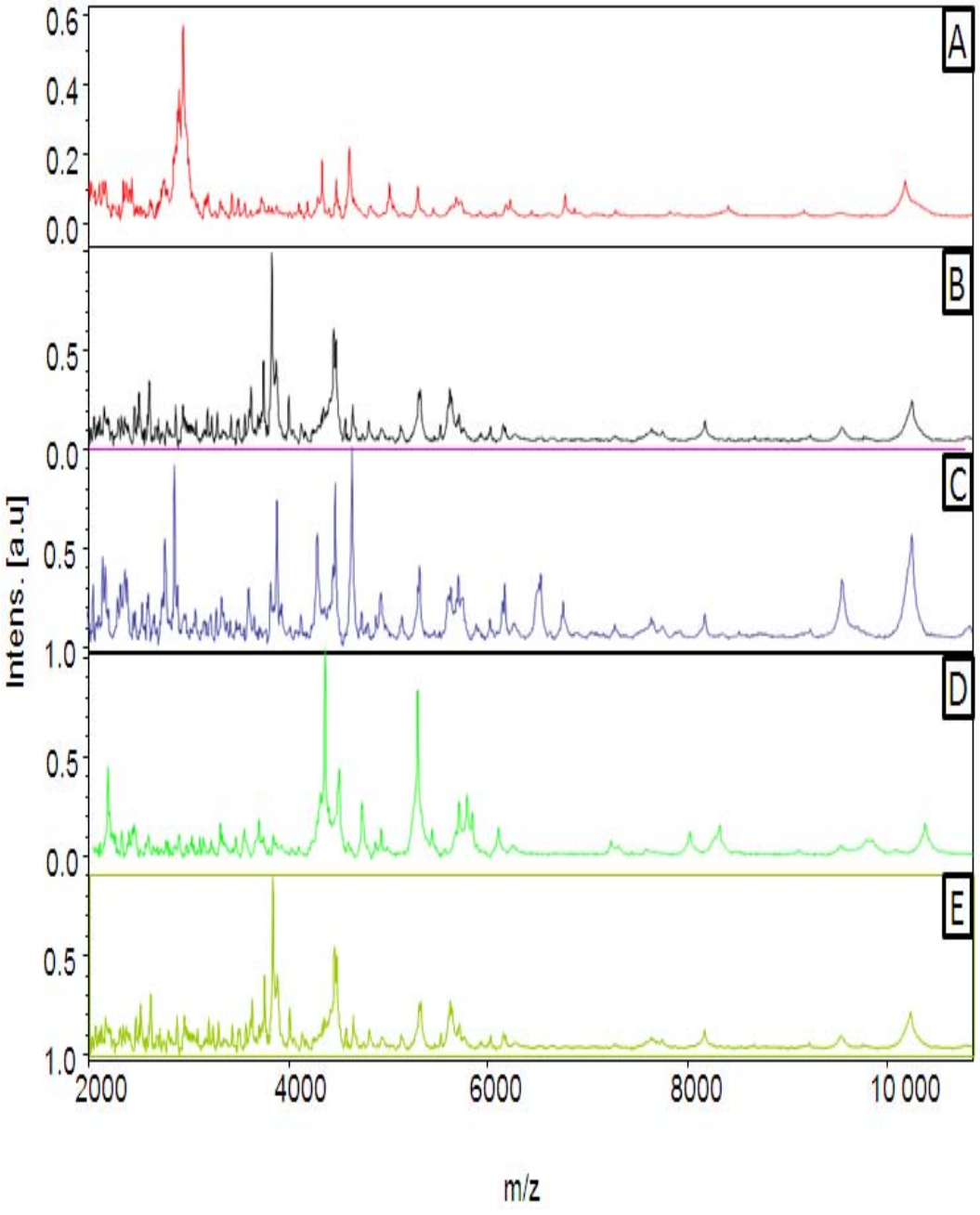

Figure 3.2 Mass spectra yielded from *M. gordonae* ATCC 23409 (A), *M. tuberculosis* ATCC 25177 (B), *M. tuberculosis* H37Rv (C), *M. bovis* ATCC 19210 (D) and *M. smegmatis* ATCC 21293 (E) under standardized conditions using the CMEFA protein extraction method. All spectral profiles have different characteristic peak patterns corresponding to each organism.

The mass spectral data yielded from CMEFA extracted samples of the ATCC typed strains *M. gordonae, M. smegmatis*, *M. tuberculosis* H37Rv, *M. tuberculosis* 25177 and clinically isolated fully susceptible (MTB), MDR and XDR-TB isolates were analysed by MALDI- Biotyper 3.0 software to create a dendrogram (Figure 3.3) using default software settings (distance measure, correlation; linkage, average) under default software as signed analysis parameters (distance measure, correlation; linkage, average). Importantly, members of MTBC (red) were differentiated and correctly clustered away from *M. gordonae* and *M. smegmatis*. Interestingly the algorithm was incapable of resolving distance level between susceptible *M. tuberculosis* isolates.

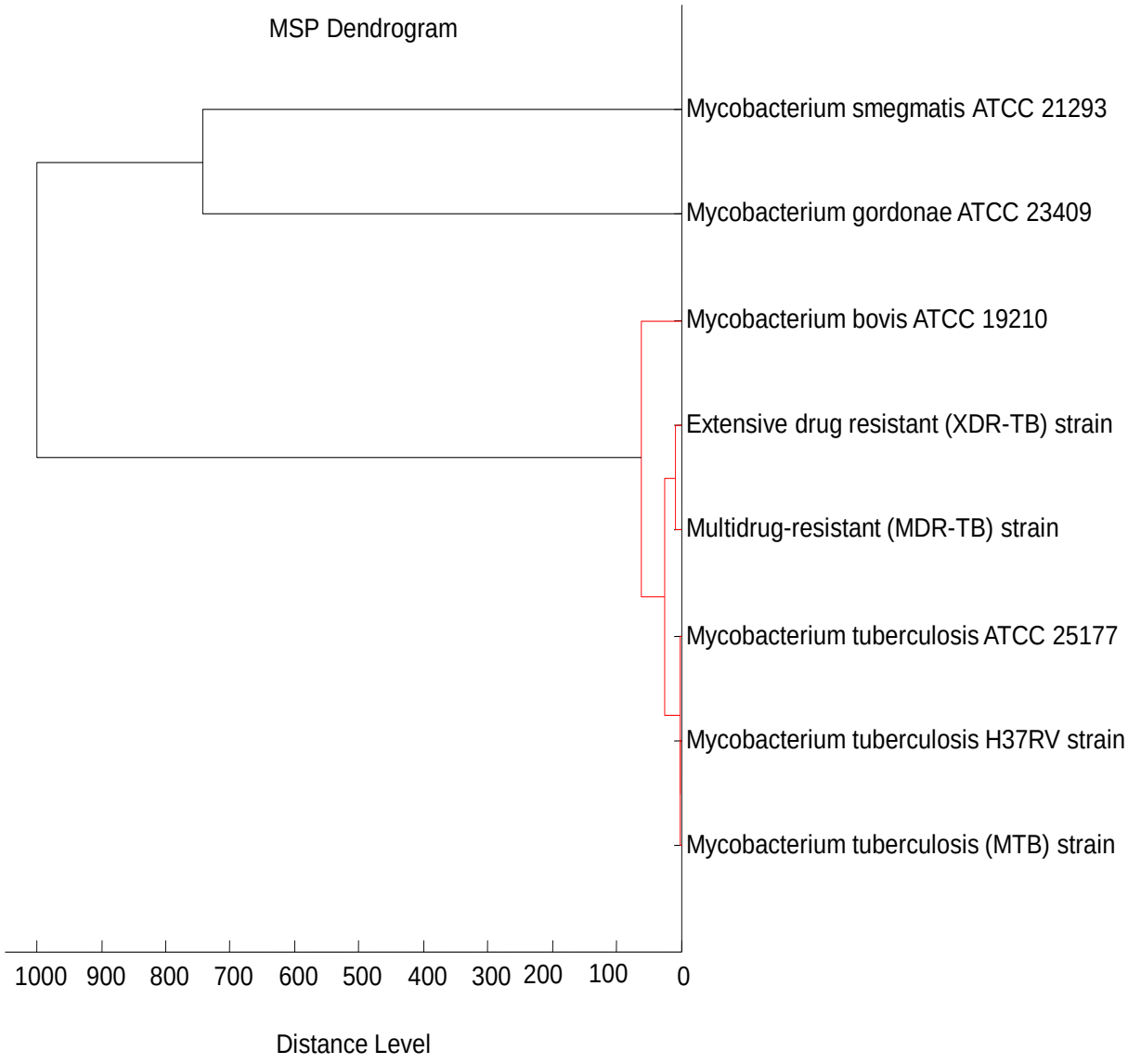

Figure 3.3 MSP dendrogram of MTBC members (red cluster) and outliers (black cluster). Employed software resolved distance levels between M. *Bovis*, MDR and XDR TB isolates and failed to resolve distance levels between fully susceptible TB isolates.

Biotyper 3.0 software (Bruker Daltonics, Germany) was further used to identify blind-coded isolates based on the MSP's match against created local reference library. Unambiguous identification of each blind-coded isolates was achieved with score values ≥ 2 as indicated in Table 3.2. Biotyping of blind-coded isolates was performed three times from different extracts of the same isolate cultured in triplicates to ascertain the reproducibility of the mass spectra.

Table 3.2 Log score values generated from blind-coded isolates through pattern matching algorithm integrated on Biotyper 3.0 software. Blind coded samples were correctly identified according to the matching organism in the database.

Bacterial species	Overall score values
M. *tuberculosis* H37Rv	≥ 2
M. *tuberculosis* ATCC 25177	≥ 2
M. *bovis* ATCC 19210	≥ 2
M. *smegmatis* ATCC 19293	≥ 2
M. *gordonae* ATCC 23409	≥ 2
Susceptible (MTB) strain	≥ 2
Multidrug-resistant (MDR-TB) strain	≥ 2
Extensively drug-resistant (XDR-TB) strain	≥ 2

Log scores ≥ 2.000 indicate correct identification, 1.700 to 1.999 indicate genus identification and <1.700 indicate no reliable identification.

3.5 DISCUSSION

The recent emergence of drug resistant *M. tuberculosis* (MDR and XDR) isolates has necessitated the development of robust *M. tuberculosis* strain identification protocols that will aid either in the initial diagnosis or verify the findings of other identification protocols so as to facilitate appropriate healthcare interventions and management practices that would contribute to the effective treatment of infected individuals and prevent the dissemination of these potentially devastating infections. *M. tuberculosis* and *M. bovis* isolates are members of the *Mycobacterium tuberculosis* complex (MTBC) that share 99 % nucleotide similarity with identical 16S rRNA (Brosch, *et al*., 2002). The MTBC members have the same host selection range. Consequently, many similar proteins may be expressed for adaptation in a well-oxygenated environment inside the host and for pathogenesis (Maeda, *et al*., 2001). The results

obtained using MALDI-TOF MS illustrated the potential of this technology to discriminate *M. tuberculosis* complex up to a species and strain level. The MSP-dendrogram did not clearly discriminate a local clinically isolated fully-susceptible MTB strain from ATCC-typed susceptible isolates at a distance level below 25. This suggests the effectiveness of MALDI-TOF MS for obtaining robust results regardless of the geographical isolation of the same *M. tuberculosis* strain type.

MALDI-TOF MS-based biotyper technology can correctly identify some bacteria to the genus, species, and subspecies levels (Welker & Moore, 2011). The mass spectral profiles that are critical to the purpose of precise bacterial taxonomic prediction are primarily representative of ribosomal proteins. These cellular proteins are synthesized in abundance under all growth conditions (Jarman, *et al.*, 2000). However, in this study it was hypothesized that the relatively high lipid component (40-60%) of mycobacterial cell walls could potentially impede the extraction of proteins for accurate MALDI-TOF MS-based differentiation of clinically isolated *M. tuberculosis* isolates. It has been previously hypothesized that chemicals do not penetrate their cell walls easily for optimum protein extraction, and as a result poor spectra are obtained when cells are not pre-treated (Saleeb, *et al.*, 2011). We evaluated three MALDI-TOF MS sample preparation methods (EFA, EFGB and CMEFA). The CMEFA method was found to be more efficient than EFGB and EFA extraction protocols. This optimized method showed the ability to produce spectra with high peak intensities. It has been reported that protein isolation, solubilisation and efficient generation of positively charged ions for mass spectrometric analysis is impeded by lipids that bind proteins (Mirza, *et al.*, 2007). Chloroform/methanol increases the quality of mass signals through lipid removal and physiological salts, both of which are likely to interfere with matrix crystallization and spectral quality (Gustafsson, *et al.*, 2011). Non-removal of these contaminants as evidenced when the EFGB and EFA extraction methods were used yielded low quality mass spectra characterized by low peak intensities and noise peak generation. The data of this study seems to indicate that initial delipidation of cells with chloroform/methanol efficiently extracts these cellular components thereby promoting optimum protein extraction and generation of consistently strong spectra. Due to the extremely infectious nature of *M. tuberculosis* isolates, whole cell MALDI-TOF MS analysis was not considered as a viable option from a personnel safety point of view. The newly developed CMEFA sample preparation protocol is cost effective and time saving when compared to other methods.

It has been reported that different extraction protocols contribute to different protein mass spectral profiles (Saleeb, *et al.*, 2011). The current available Bruker mass spectral reference library was established using the conventional ethanol/formic acid extraction method and contains 3998 reference organisms for microbial biotyping. However, it was clearly evident

in this study, that the standard EFA extraction method although suitable for other bacteria was ineffective when used for mycobacteria sample preparation. This finding is further supported in that Bruker Daltonics (Bremen, German) have initiated the generation of a new mycobacteria mass spectral database that has been acquired from samples prepared using a microbead-based method. The CMEFA generated spectra of this study were analysed by Bruker Daltonics (Bremen, German). They reported both species confirmation and log score values (≥ 2.0) for *M. tuberculosis* H37Rv, *M. tuberculosis* 25177, *M. bovis*, *M. gordonae* and *M. smegmatis* isolates. Importantly, this signifies that spectra generated with CMEFA method contained extensive coverage of the characteristic peaks of bead generated spectra obtained by Bruker Daltonics for the same organisms.

Mass spectrometry-based dendrograms have been observed to correlate with the 16S rRNA sequence-based dendograms. This has led to a presumptive conclusion that MALDI mass spectrometry analysis is based on the ribosomal proteins which are conserved, constantly and abundantly expressed within the cell (Maier & Markus, 2007). Genome sequencing of *M. tuberculosis* and *M. bovis* isolates using synonymous single nucleotide polymophisms (sSNP's), result to a genomic resolution of the members of *Mycobacterium tuberculosis* complex based on their genetic relationships. Thus *M. bovis* and *M. tuberculosis* isolates are different at their genomic level which is reflected at the protein level (Gutacker, *et al.*, 2002). In this study, it has been observed that protein differences between these isolates can be recognized by MALDI-TOF MS that leads to their unambigous identification as individual isolates as indicated. It has been reported that MALDI-TOF MS technology can able to discriminate *Mycobacterium* species to a strain level (Hettick, *et al.*, 2006).

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3.7 REFERENCES

Bahk YY, Kim SA, Kim J, Euh H, Bai G, Cho S & Kim YS (2004) Antigens secreted from *Mycobacterium tuberculosis*: Identification by proteomics approach and test for diagnostic marker. *Proteomics* **4**: 3299–3307.

Bienvenut WV, Sanchez JC, Karmime A, Rouge V, Rose K, Binz PA & Hochstrasser DF (1999) Toward a clinical molecular scanner for proteome research: Parallel protein chemical processing before and during Western blot. *Analitical Chemistry* **71**: 4800-4807.

Bonk T & Humeny A (2001) MLDI-TOF-MS Analysis of Protein and DNA. *Institute of Biochemistry* **7**: 6-12.

Brosch S , G ordon S V, Marmiesse M *, e t al.* (2002) A ne w e volutionary s cenario f or t he *Mycobacterium t uberculosis* complex. *Proceedings of t he National Academy of Science* **99**: 3684–3689.

Gustafsson JOR, O ehler MK, R uszkiewicz A , M cColl S R & H offmann P (2011) MALDI imaging mass spectrometry (MALDI-IMS)-Application of spatial proteomics for ovarian cancer classification and diagnosis. *International Journal of Molecular Sciences* **12**: 773-794.

Gutacker MM, Smoot JC, Migliaccio CAL*, et al.* (2002) Genome-wide analysis of synonymous single nucleotide polymorphisms i n *Mycobacterium tuberculosis* complex organisms: Resolution of genetic relationships among closely related microbial strains. *Genetics Society of America* **162**: 1533–1543.

Hettick J, Kashon M, Slaven J*, et al*. (2006) Discrimination of intact mycobacteria at the strain level: a combined MALDI-TOF MS and biostatistical analysis. *Proteomics* **6**: 6416–6425.

Hillemann D, Gerdes SR & Richter E (2007) Evaluation of the GenoType MTBDRplus assay for r ifampin a nd is oniazid s usceptibility te sting of *Mycobacterium t uberculosis* strains an d clinical specimens. *Journal of Clinical Microbiology* **45**: 2635-2640.

Homolka S , K o¨ser C , A rcher J, R u¨sch-Gerdes S & N iemann S (2009) S ingle-nucleotide polymorphisms i n Rv2629 a re specific f or *Mycobacterium t uberculosis* genotypes B eijing a nd Ghana but not associated with rifampin resistance. *Journal of Clinical Microbiology,* **47**: 223–226.

Ikryannikova LN, A fsanas'ev MV, A kopian TA, Il'ina EN, C hernousova LN & Govorum VM (2007) M ass-spectrometry ba sed m inisequensing method f or t he r apid detection of drug resistance in *Mycobacterium tuberculosis*. *Journal of Microbiological Methods* **70**: 395-405.

Jarman KH, Cebula ST, Saenz AJ, P etersen CE & V alentine NB (2000) An algorithm for automated b acterial id entification u sing matrix-assisted l aser d esorption/ionization m ass spectrometry. *Analitical Chemistry* **72**: 1217–1223.

Laval F , L aneelle MA, D eon C , Monsarrat B & D affe M (2001) A ccurate molecular m ass determination of m ycolic a cids by MALDI-TOF m ass spectrometry. *Analitical Chemistry* **73**: 4537-4544.

Maeda M K, R hee JT & G ingeras TR (2001) C omparing genomes w ithin t he species *Mycobacterium tuberculosis*. *Genome Research* **11**: 547-554.

Maier T & M arkus K (2007) F ast and r eliable MALDI-TOF M S-based m icroorganism identification. *Chemistry Today* **25**: 68-71.

Mirza SP, Halligan BD, Greene AS & Olivier M (2007) Improved method for the analysis of membrane proteins by mass spectrometry. *Physiol Genomics* **30**: 89-94.

Pignone M, Greth KM, Cooper J, Emerson D & Tang J (2006) Identification of mycobacteria by Matrix-assisted l aser desorption/ionization-time-of-flight mass sp ectrometry. *Journal Of Clinical Microbiology* **44**: 1963-1970.

Saleeb P G, D rake S K, Murray R P & Z elazyn A M (2011) I dentification of m ycobacteria in solid-culture m edia b y matrix-mssisted laser de sorption i onization-time o f tl ight m ass spectrometry. *Journal of Clinical Microbiology* **49**: 1790-1794.

Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R & Kostrzewa M (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS ONE* **3**: 1-10.

Schmidt O & Kallow W (2005) Differentiation of indoor wood decay fungi with MALDI-TOF mass spectrometry. *Holzforschung* **59**: 374–377.

Welker M & Moore ERB (2011) Applications of whole-cell matrix-assisted laserdesorption/ionization time-of-flight mass spectrometry in systematic microbiology. *Systematic and Applied Microbiology* **34**: 2–11.

Chapter 4

RESEARCH RESULTS II

Collection and identification of clinical *Mycobacterium tuberculosis* isolates

Collection and identification of clinical *Mycobacterium tuberculosis* isolates

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4.1 ABSTRACT

South Africa has a high incidence of tuberculosis and the emergence of multidrug-resistant TB isolates which limit the treatment success. Diagnosis of tuberculosis relies on biochemical and culture techniques. However these methods are time consuming. PCR-based and have high resolution for TB isolates discrimination. The BACTEC™ MGIT™ 960 System was used to culture acid fast bacilli and the GenoType MTBDRplus assay as well as drug susceptibility testing were used to screen multidrug-resistant TB isolates. Twenty fully susceptible isolates, twenty MDR and twenty XDR-TB isolates were isolated from the sputum samples obtained from patients suspected of tuberculosis. *M. tuberculosis* isolates that were resistant to first line drugs were considered as MDR-TB isolates, while those that were resistant to first-line and at least to one of the second-line drugs were considered XDR-TB isolates. These isolates were successfully screened and were selected to further the present study.

4.2 INTRODUCTION

The genus mycobacteria comprises of more than 100 species of which many are pathogenic to humans and animals. Mycobacteria are uniquely characterized by the presence of unusual lipids which form part of the cell wall structure, these lipids offer intrinsic resistance to antibiotics and chemicals. Mycolic acid contributes a major fraction of these lipids (Liu, *et al.*, 1995). Most pathogenic mycobacterial species, particularly the causative agents of tuberculosis (TB) disease have been observed to be members of *Mycobacterium tuberculosis* complex (MTBC).

TB is one of the most threats particularly in the third world countries such as South Africa. Eight million cases of TB infection and two million deaths have been reported every year (Bahk, *et al.*, 2004). The emergence of multidrug-resistance TB isolates pose challenges on TB control programmes. Multidrug-resistance (MDR) TB isolates are considered to be resistant to first line drugs, particularly isoniazid (INH) and rifampicin (RIF). The care rate of MDR-TB is lower and patients are subjected to treatment regimens of 18-24 months. Furthermore, expensive and toxic second line drugs are used; therefore immediate and appropriate control of MDR-TB pandemic is urgently needed (Moodley, *et al.*, 2011).

In KwaZulu Natal (South Africa), researchers announced the emergence of resistant TB isolates not only to isoniazid, and rifampicin but also to at least three classes of second line-drugs. This form of resistant TB was named as extensively drug-resistant (XDR). TB has been observed to emerge linearly with human immunodeficiency virus (HIV) (Gandhi, *et al.*, 2006). This became a major threat in developing countries. Therefore there is a need of effective treatment programmes for TB through invention of cheaper and rapid diagnostic tools.
Development of standardized method for detection of *M. tuberculosis* in clinical samples will enable scientist and researchers to manage disease epidemic throughout the world (Warren, *et al.*, 2005). Currently, detection of *M. tuberculosis* relies on culturing, biochemical and microscopy. Drug susceptibility testing (DST) is used for further identification process, however this process can extend to a maximum of two months for acquisition of results. Therefore, it does not provide urgent detection of MDR-TB in highly affected communities. BACTEC MGIT 960 system has reduced detection rate (5-10 days), this technique require costly equipment (Dixit, *et al.*, 2012).

Culture method is a "gold standard" for detection of *M. tuberculosis* in routine diagnostic laboratories in South Africa. Automated systems using liquid media are available to speed up the diagnostic process of *M. tuberculosis* (Dowdy, *et al.*, 2008). Diagnostic tests such as nitrate reductase assay (NRA), microscopy, microscopic observed drug susceptibility

(MODS) test and molecular techniques are used for TB diagnosis. Current global TB diagnosis is largely dependent on sputum smear microscopy which exhibit high sensitivity. However, sensitivity of this method is reduced in HIV infected patients about 35% and 45% in HIV negative patients (Dowdy, *et al.*, 2008).

M. tuberculosis produces the characteristic roping-like structures which can be seen under microscope; therefore this allows the initial detection of an organism for further identification process. A MODS is a liquid-based media assay conducted in OADC-supplemented 7H9 broth on tissue culture plates. A mixture of antibiotics such as polymyxin B, amphotericin B, nalidixin acid, trimethoprim and azlocillin are added in the media to eliminate contaminating microbes and fungi. Thereafter, isoniazid and rifampicin are incorporated for direct detection of MDR-TB. The growth in the presence of each drug indicates MDR-TB isolates and the absence of growth indicate the susceptible TB isolates. This assay can be conducted on the smear positive and negative sputum samples, however it is laborious and time consuming (Dixit, *et al.*, 2012).

Different genetic loci of MTBC such as 38 kDa (PhoS), 23 kDa (MPB64), 65 kDa (GroEL) and insertion sequence (IS) 611 are used to discriminate members of the complex from other mycobacteria. The IS6110 has proven to be effective diagnostic marker of *M. tuberculosis* complex compared to other loci. The repetitive abundance of these genetic markers increases the chance of identification from amplified DNA. It has been reported that these sequences are present only in the members of *M. tuberculosis* complex. Therefore they serve as potential targets for diagnosis (Khorshidi, *et al.*, 2009).

It has been suggested that polymerase chain reaction (PCR)-based assays may be limited by contamination, tedious sample preparation procedures and DNA extraction. Successful PCR reaction is greatly dependant on the potential extraction method to lyse resistant cell wall structure of mycobacteria. furthermore, real-time PCR has been developed for a rapid detection of target sequences including those that confer drug-resistance in MTBC (Causse, *et al.*, 2011). The sensitivity and reliability of PCR methods are greatly dependent on DNA extraction methods. Lysis of mycobacterial cells wall is impeded by complex impermeable cell wall structure. As a result, poor results may be obtained due to an incomplete lysis of cells and low DNA yield. However some studies have approved PCR-based methods for routine diagnosis based on their sensitivity and specificity. Detection systems used to quantify the yield of amplified DNA products, clinical samples, DNA extraction and pair primer selection differ

from one method to another (Amita, *et al*., 2002). Recovery of tubercle bacilli during DNA extraction process has been reported; this might expose a technicians and technologist at risk of infection. Therefore it is suggested that DNA extraction process should be carried out in biosafety level II or III practices (Warren, *et al.*, 2005).

Amplification nucleic acid tests (NATs) has proven to be effective for detection of mycobacteria directly from the clinical specimens (Khorshidi, *et al*., 2009). The possibilities of false-negative reactions has brought many question, therefore the use of NATs has been investigated. Furthermore, GenoType MTBDRplus is a DNA hybridization assay for detection of common mutations in the (*katG* and *rpoB*) genes which are responsible for rifampicin (RIF) and isoniazid (INH) resistance, respectively. However, this method exists in different versions for screening of drug resistance to second line drugs and detection of mycobacteria other than tuberculosis (MOTTS). This assay rely on DNA extraction, PCR, solid phase reverse hybridization and detection of mutations on the strips (Hillemann, *et al*., 2007). The aim of this study was to screen clinical isolates of *M. tuberculosis* using conventional diagnostic methods.

4.3 MATERIALS AND METHODS

4.3.1 Screening and culturing of clinical *M. tuberculosis* isolates

Sixty well characterised MDR, XDR and fully susceptible MTB isolates were made available by the NHLS tuberculosis laboratory based at Inkosi Albert Luthuli Central hospital (IALCH) for MALDI-TOF MS analysis. The NHLS tuberculosis laboratory receives and processes approximately 800 sputum samples per day. Prior to characterisation, the sputum samples were decontaminated as per the CLSI guidelines using the N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH) method. The laboratory performs this process in a biological safety cabinet class-II equipped with high efficiency particulate air filters (HEPA). Decontamination of the sputum samples (5ml) was performed by the laboratory using an equal volume of NALC-NaOH. The samples were then allowed to stand at room temperature for 15-20 minutes. Sodium phosphate buffer (pH 6.8; 40ml) was added to samples and centrifuged at 3,000 x g for 15 minutes. The sediment was retained with approximately 5 ml of supernatant. The samples were vigorously vortexed and 100 µl of the suspension was subjected to auramine-O staining to confirm the presence of acid-fast bacilli (Salfinger & Pfyffer, 1994). In addition, a 500 µl aliquot of the suspended sediment was used to inoculate mycobacteria growth indicator tubes (MGIT) supplemented with BBL™ MGIT™ OADC enrichment and BBL™ MGIT™ PANTA™ antibiotic mixture (Becton Dickinson, USA). The MGIT tubes were incubated at 37°C in a BACTEC™ MGIT™ 960 Mycobacterial

Detection System (Becton Dickinson, USA). All positive samples from the latter assay were screened to detect mutations in the rpoB, katG and INH genes that confer drug resistance to rifampicin and isoniazid using the PCR-based GenoType MTBDRplus® assay according to instructions of the manufacturer (Hain Lifescience GmbH, Germany). The PCR data was used to identify fully susceptible and MDR-TB clinical isolates. In addition, all MDR-TB clinical isolates were assessed for the presence of XDR-TB using the agar dilution drug susceptibility protocol outlined below (Abe, et al., 1999). A ten-fold dilution of Mcfarland No. 1 inoculum obtained from the MGIT-positive cultures was cultivated on Middlebrook 7H10 agar quarter plates with each quadrant containing 1µg/ml rifampicin, 0.2 µg /ml isoniazid, 7.5 µg/ml ethambutol, 2 µg/ml streptomycin, 5 µg /ml kanamycin and 2 µg/ml ofloxacin. Cultures were incubated for three weeks at 37°C and the results were interpreted using dissecting microscopy.

4.4 RESULTS

Twenty fully susceptible TB isolates that showed all wild-type bands on the membrane strips were selected for the study. Furthermore, the *M. tuberculosis* isolates that showed some missing bands/mutations were further screened using indirect drug susceptibility testing (DST) conducted on Middlebrook 7H10 agar plates incorporated with drugs. Different banding patterns on the membrane strips were observed and interpreted according to the instructions of the manufacture. From previously identified TB isolates in that manner, twenty susceptible TB isolates susceptible to these drugs were selected and twenty TB isolates that were resistant to at least isoniazid and rifampicin were considered as multidrug-resistant isolates (MDR) and were selected for the study as well as twenty multidrug-resistant isolates were resistant to isoniazid and, rifampicin, quinolones (ofloxacin) and at least to one of injectable second line drugs (kanamycin) were considered as extensively drug-resistant (XDR) *M. tuberculosis* isolates and were also selected for the study.

4.5 DISCUSSION

Different mycobacterial DNA extraction methods has been evaluated by many researchers, however boiling method incorporated with ultra-sonication proved to be efficient and cheaper in our laboratory setting for routine application. As a result, sufficient DNA molecules were released for PCR assay. The isolation of tubercle bacilli and specimen manipulation was carried out in a biological safety cabinet Class III to prevent the risk disease transmission to working personnel. GenoType MTBDRplus strips that were hard to interpret using the manufacture's interpreting template were excluded in the study. However, those that correlate with DST results were selected to further the study and stored for future use. Samples that contained mixed population of mycobacteria were also excluded during the screening process in order to prevent the ambiguity. All the instruments were calibrated according to standard operation procedures (SOPs) to ensure optimum result.

The impermeability nature of mycobacterial cell wall nature has contributed to a low DNA yield due to incomplete cell lysis (Amita, *et al.*, 2002). According to a literature, different methods has been compared, however none has been standardized for routine application in all clinical laboratories across the globe. Therefore clinical laboratories have established different sample preparation methods suitable for their laboratory settings and specimen type (Hosek, *et al.*, 2006). Boiling method accompanied with sonication has become a suitable DNA extraction method for our routine setting, based ideal comparison to other methods this method is cost effect, requires minimum reagents, yield sufficient DNA and is rapid with high through output.

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4.7 REFERENCES

Amita J, Vandana T, Guleria RS & Verma RK (2002) Qualitative Evaluation of Mycobacterial DNA Extraction Protocols for Polymerase Chain Reaction. *Molecular Biology Today* **3**: 43-50.

Bahk YY, Kim SA, Kim J, Euh H, Bai G, Cho S & Kim YS (2004) Antigens secreted from *Mycobacterium tuberculosis*: Identification by proteomics approach and test for diagnostic marker. *Proteomics* **4**: 3299–3307.

Causse M, Ruiz P, Aroca JBG & Casal M (2011) Comparison of two molecular methods for rapid diagnosis of extrapulmonary tuberculosis. *Journal of Clinical Microbiology* **49**: 3065 - 3067.

Dixit P, Singh U, Sharma P & Jain A (2012) Evaluation of nitrate reduction assay, resazurin microtiter assay and microscopic observation drug susceptibility assay for first line antitubercular drug susceptibility testing of clinical isolates of *M. tuberculosis*. *Journal of Microbiological Methods* **88**: 122-126.

Dowdy D, Lourenc MC, Cavalcante SC, *et al.* (2008) Impact and cost-effectiveness of culture for diagnosis of tuberculosis in HIV-Infected Brazilian adults. *PLoS ONE* **3**: 1-8.

Gandhi NR, Moll A, Sturm AW, *et al.* (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet Infectious Diseases* **368**: 1575–1580.

Hillemann D, Gerdes SR & Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *Journal of Clinical Microbiology* **45**: 2635-2640.

Hosek J, Svastova P, Moravkova M, Pavlik I & Bartos M (2006) Methods of mycobacterial DNA isolation from different biological material: a review. *Veterinarni Medicina* **51**: 180-192.

Khorshidi A, Rohani M, Moniri R & Torfeh M (2009) Comparison of culture and microscopic methods by PCR for detection of *Mycobacterium tuberculosis* in sputum. *Iranian Journal of Clinical Infectious Disease* **4**: 228-232.

Liu J, Rosenberg E Y & Nikaido H (1995) Fluidity of the lipid domain of cell wall from *Mycobacterium chelonae*. *Proceedings of the National Academy of Science.* **29**: 11254-11258.

Moodley P, Shah NS, Tayob N*, et al*. (2011) Spread of extensively drug-resistant tuberculosis in KwaZulu-Natal Province, South Africa. *PLoS ONE* **6**: 1-6.

Salfinger M & Pfyffer HE (1994) The new diagnostic mycobacteriology laboratory. . *European Journal of Clinical Microbiology and Infectious Diseases* **13**: 961-979.

Warren R, Kock M, Engelke E, Myburgh R, Pittius NG, Victor T & Helden P (2005) Safe *Mycobacterium tuberculosis* DNA extraction method that does not compromise integrity. *Journal of Clinical Microbiology* **44**: 254-256.

Chapter 5

RESEARCH RESULTS III

Biotyping of clinical *Mycobacterium tuberculosis* isolates using MALDI-TOF MS

Biotyping of Clinical *Mycobacterium tuberculosis* isolates using MALDI-TOF MS

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5.1 ABSTRACT

Tuberculosis is the major cause of death in developing countries. The condition has been worsened by the emergence of combination drug-resistant tuberculosis strains. Furthermore, in 2006 the emergence of extensively drug-resistant tuberculosis was first reported in South Africa (KwaZulu Natal region) and caused high mortality in HIV co-infected patients. This has created the need for effective, cheaper and rapid diagnostic tools to control the disease pandemic. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) has been reported as a reliable and cheap method for bacterial and yeast identification. Conventional methods were used to identify sixty *Mycobacterium tuberculosis* isolates from clinical specimens. Protein analytes were extracted from each tuberculosis strain using the chloroform-methanol-ethanol-formic acid (CMEFA) sample preparation protocol for MALDI-TOF MS analysis. Reproducible spectra and log score values ≥ 2 were obtained, with 93% of tuberculosis isolates being correctly identified. The CMEFA sample preparation method for MALDI-TOF MS successfully discriminated clinical *M. tuberculosis* at the strain level with the aid of Biotyper software. The results seemingly suggest that MALDI-TOF MS analysis may be a useful tool for *M. tuberculosis* strain identification purposes in routine clinical laboratories.

5.2 INTRODUCTION

The genus mycobacteria comprises approximately 100 heterogeneous species of slow and fast growing acid fast bacilli (Pignone, *et al.*, 2006). Several members of this genus are causative agents of human and animal diseases such as pulmonary tuberculosis, skin and soft tissue infections (Saleeb, *et al.*, 2011). Mycobacteria are characterized by unique cell wall lipids called mycolic acids. These lipids are long chains of hydrophobic molecules that form a waxy layer around the cell wall structure and contribute approximately 40-60% of the total dry weight of the cell (Laval, *et al.*, 2001). This waxy layer results in a low cell wall permeability which pose challenges for therapeutic agents to penetrate. Mechanical lysis of the cell wall structure has proven to be efficient for protein extraction in mycobacteria. Different lysis buffers have been used to achieve optimum protein extraction for analysis. It has been suggested that mycobacteria are not susceptible to a single protein extraction method because of variations in the cell wall composition of different species of this genus (Gumber, *et al.*, 2007).

Mycobacterium tuberculosis is the causative agent of tuberculosis (TB). This disease is transmitted by generated aerosol-containing tubercle nuclei *via* coughing, vortexing and aerosol generating procedures in the clinical laboratory. Therefore class I or II biosafety cabinets are recommended and sealed aerosol cup centrifugation instruments (Shinnick & Good, 2011). TB has become a major threat in the public health care and correctional facilities (Hettick, *et al.*, 2006). Reactivated *M. tuberculosis* from latently infected individuals serves as a pool of infection in communities (Corbett, *et al.*, 2003). Furthermore, migration has an effect on the spread of TB especially from low income economies, therefore infection has remained high in most African countries (Dasgupta & Menzies, 2005)

M. tuberculosis is a member of the *M. tuberculosis* complex (MTBC) which also consists of *M. microti*, *M. africanum*, and *M. bovis*. These members are genetically related and are of the same medical importance (Nelson & Williams, 2007). *M. tuberculosis* is an intracellular parasite of macrophages. Furthermore, it can persist in a changing environment within the endosomal system of the host (Maciag, *et al.*, 2007). During infection, the antigenic membranes and surface proteins are involved in the host-antigen interaction. These proteins are also responsible for intracellular multiplication and host microbicidal effect. In spite of research studies that have been conducted, the pathogenicity and virulence of *M. tuberculosis* is not well understood. Therefore identification of virulent gene products and persistent determinants can be a potential target sites to combat *M. tuberculosis* (Målen, *et al.*, 2011).

TB infection has been present for a long time however, it has been curable and preventable through the administration of multiple drug therapies. Isoniazid, rifampicin, pyrazinamidine and ethambutol are mostly used as the first line anti-TB treatment. Currently, many young adults die every year because of the treatment failure caused by the emergence of drug resistant-TB strains (Blower, *et al.*, 1996). These strains are increasingly developing resistance to rifampicin due to mutations in the *rpoB* genes that code for the B-subunit of ribonucleic acid polymerase (Streicher, 2007). Furthermore, mutations in the *katG* gene promote the strain resistance to isoniazid. The *katG* gene codes for the catalase and peroxidase enzymes which are responsible for isoniazid activation (Murase, *et al.*, 2010). The emergence of multidrug-resistant (MDR) TB strains are due to non-compliance of patients to a treatment and incorrect application of treatments, as a result spontaneous mutations are triggered in the suppressed *M. tuberculosis* strains. These chromosomal mutations result in single nucleotide polymorphisms (SNPs), insertions and deletions (Olano, *et al.*, 2007). Mutations in the DNA repair mechanism of *M. tuberculosis* have proven to result in drug resistance and adaptation of the bacilli inside the host environment. Furthermore, natural variations of *M. tuberculosis* isolates are the result of the mobile insertion sequence IS6110, which leads to insertion inactivation and deletion mutations (Yesilkaya, *et al.*, 2005). The diagnosis of MDR and XDR *M. tuberculosis* is obtained by performing direct susceptibility testing on culture positive specimens, with a turnaround time of approximately 6-8 weeks (Hillemann, *et al.*, 2007).

TB control programmes has been established to stress the management of disease. First line drug therapy (isoniazid and rifampicin) has been also used to target the cell wall biosynthesis in *M. tuberculosis* to reduce the effect of disease within the population (Shi, *et al.*, 2008). Bacillus Calmette-Gue'rin (BCG) vaccine has been used to combat the infection, however TB remains a major problem (Bahk, *et al.*, 2004). The emergence of drug resistant TB was firstly reported in South Africa in 2006. These TB isolates have acquired additional resistance to fluoroquinolones and at least one second line injectable drugs (Gandhi, *et al.*, 2006). The side-effects of treatment for XDR-TB are worse than that encountered with MDR-TB and the mortality rate is markedly increased in HIV-infected patients. Furthermore, additional expenditure is required to manage patients for an extended period of time which may exhaust the patient and resources of the public health programme (Murase, *et al.*, 2010).

Diagnosis of TB is challenging due to a slow growth of the organism as the doubling time of *M. tuberculosis* is 24 hours. Current detection of MDR-TB in South Africa relies on culturing methods on the solid media which extend to a maximum of four to six weeks for results to be available. However, liquid culture media has reduced the recovery time of acid fast

bacilli to seven days (Gandhi, *et al.*, 2006). There is a great need for rapid, accurate and cheap diagnostic methods to manage the TB pandemic at an early stage of infection for effective administration of appropriate treatment.

DNA probes for identification of MTBC, *Mycobacterium avium*-complex (MAC), *M. kansasii* and *M.gordonae* are commercially available for a limited number of mycobacterial species. Biochemical tests are also used, however are time consuming, laborious, tedious and solely dependent on culturing methods (Saleeb, *et al.*, 2011). Microscopic determination of acid fast bacilli serves as an initial step of diagnosis in mycobacteriology laboratories. This method is cheap and rapid for detection of highly infected population. Basic fuschine dye or fluorochrome (auramine-rhodamine) is used to stain the acid fast bacilli. The minimum number of cells in a sample must be $5x10^3$ bacilli/ml, below this number the *M. tuberculosis* cannot be differentiated from other mycobacteria if there is no roping-like structures (Shinnick & Good, 2011).

Various molecular and biological diagnostic methods have been used to discriminate MTBC; it is challenging to differentiate members of the MTBC into a subspecies level due to genetic invariance in their target loci. Restricted SNPs in *gyrA*, *katG*, *pncA*, *oxyR*, *hsp65*, and *gyrB* gene loci have been used to define members of the MTBC and lineages. Furthermore, these loci are unable to discriminate some species of MTBC. Molecular typing methods, such as tandem repeats and mix Linker PCR, are designed to reveal inter-strain relationships, however these methods are not frequently used methods to explore differences in each member of the MTBC strain due to their inefficiency (Huard, *et al.*, 2003). The IS6110-RFLP molecular typing has been used for strain differentiation of XDR-TB from MDR-TB through genomic clustering. This method has been successfully used although it is subject to many limitations and ambiguity (Blower, *et al.*, 1996).

MALDI-TOF MS potentially offers a promising alternative for *M. tuberculosis* strain discrimination. This tool has been successfully used for identification of yeast and bacteria from protein extracts (Saleeb *et al.*, 2011). There has been limited work reported with this tool for *M. tuberculosis* strain discrimination (Seng, *et al.*, 2009). Nevertheless, there has been limited studies reported the use of MALDI-TOF MS for *Mycobacterium* discrimination based on their unique protein fingerprints. Conventional ethanol/formic acid method does not yield reproducible results for mycobacterial analysis. It has been suggested that some mycobacterial species have glycopeptidolipids (GPLs) on their cell wall. As a result, the GPLs prevent the penetration of chemicals during protein extraction process; therefore, poor quality of spectra are generated (Saleeb, *et al.*, 2011).

During MALDI-TOF MS analysis, the thermo-labile biomolecules or whole cells are embedded on the UV-absorbing crystal-like structure of weak organic acid called matrix. This weak organic acid prevent decomposition of biomolecules through absorbing excess energy, and enables desorption and ionization of these biomolecules through energy transfer and protonation (Bonk & Humeny, 2001).The analytes are then ionized and desorbed from the target plate into a flight tube (vacuum field free region) where ions separate according to mass-to-charge (m/z) ratio. Smaller molecules travel at a faster velocity and reach the detector earlier than the larger molecules. These ions are captured by a detector and represented as a spectrum (intensity against mass- to-charge ratio). The quality of the spectra depends on the sample preparation method and bacterial culture (Gustafsson, *et al.*, 2011).

Multiple software programmes have been developed to enable further identification through comparison of generated spectral fingerprints with reference spectra in the established data bases (Bienvenut, *et al.*, 1999). Un supervised approach such as principal component analysis (PCA) can be incorporated in the Biotyper software to reduce the multi-dimensionality of data generated with MALDI-TOF mass spectrometry. Furthermore, hierarchical clustering approaches such as dendrograms and correlation matrix are also integrated in the system to enable identification (Sauer, *et al.*, 2008).

The aims of this study were to evaluate the potential of the optimized sample preparation protocol for MALDI-TOF MS to discriminate closely related members of *M. tuberculosis* complex as a proof of concept, subsequently to discriminate clinically isolated *M. tuberculosis* at the strain level and to screen the unique mass signals between TB isolates.

5.3 MATERIALS AND METHODS

5.3.1 Clinical *M. tuberculosis* isolates

Sixty clinical *M. tuberculosis* isolates were used in this study. These clinical TB isolates were rejuvenated from the previously identified and stored TB cultures.

5.3.2 Media and cultivation conditions

Clinically-isolated TB isolates (section 4.4; Table 4.1) were rejuvenated at 37^{o}C for seven to fourteen days in Middlebrook 7H9 broth. Thereafter isolates were transferred onto drug free Middlebrook 7H11 agar plates in triplicate and incubated at 37°C for twenty one days. A single loop-full of the culture grown on solid medium was transferred into a MicroBank (Pro-Lab Diagnostics, Canada) for long term storage at -75°C.

5.3.3 MALDI-TOF MS analysis of clinical isolates

Samples from clinical culture isolates for MALDI-TOF MS analysis were prepared by the CMEFA extraction method as described earlier in section 3.3.5 and spectral data was captured and processed as described in sections 3.3.6, and 3.3.7 respectively.

5.4 RESULTS

The CMEFA sample preparation method generated consistent and mass reproducible spectra. Samples consisting of chloroform/methanol (1:1) or distilled water without culture was analysed in parallel as negative controls to assess the effect of chloroform/methanol for noise peak generation. Importantly, distilled water and chloroform/methanol controls showed similar results with no contaminants detected. Of significance, differences (Figure 5.1) were clearly evident between the spectra of susceptible MTB, MDR-TB and XDR-TB isolates.

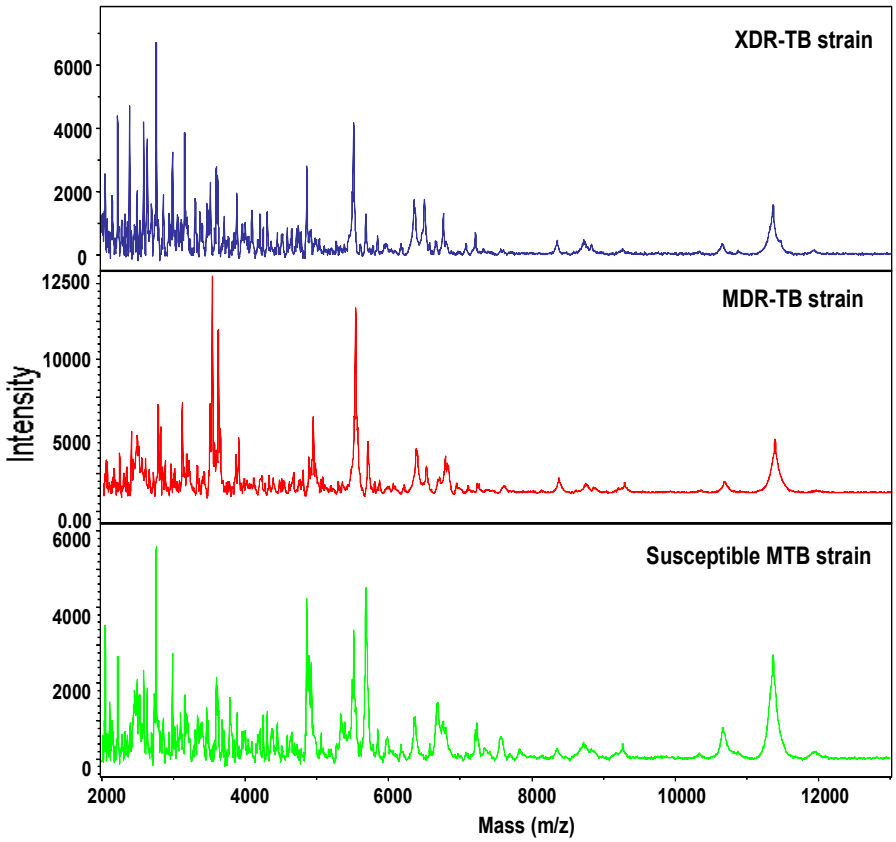

Figure 5.1 MALDI-TOF mass spectra obtained from fully susceptible (MTB), multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB isolates using CMEFA sample preparation method. Spectral pattern of fully susceptible MTB and MDR-TB isolates are likely similar at 8000-10 000 mass to charge (m/z) ratio range than that of XDR-TB isolates.

The principal component analysis (PCA) was used to reduce the multi-dimensionality of spectral data sets into most dominant components, whilst preserving the most relevant variances between the spectral data sets of TB isolates (Figure 5.2). Fully susceptible MTB and MDR-TB isolates clustered separately but on the same plane, whilst XDR-TB isolates clustered separately on the other plane of the plot.

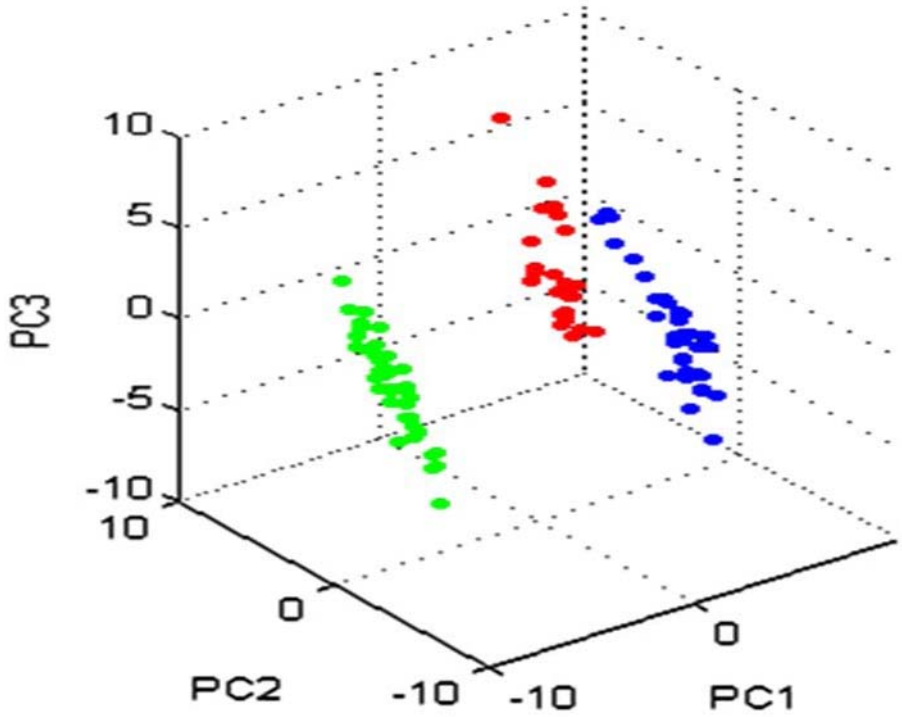

Figure 5.2 Three dimensional principal component analysis (PCA) derived from MSP's of MTB (green), MDR-TB (red) and XDR-TB (blue) isolates using Biotyper 3.0 software. The CMEFA sample preparation method was used for protein extraction from TB isolates.

In an initial hierarchical cluster analysis attempt, a dendrogram was used to illustrate the relatedness between susceptible MTB, MDR-TB and XDR-TB isolates (Figure 5.3). Interestingly, susceptible MTB and MDR-TB isolates were grouped in the same branch but in separate clusters which signifies closer relatedness when compared to XDR-TB strain. The *M. bovis* species was used as an outlier strain control and as can be observed from dendrogram, it distinctively clustered away from *M. tuberculosis* isolates employed in this study. Thereafter, an expanded cluster analysis of clinically isolated *M. tuberculosis* isolates was performed. Importantly this more extensive analysis (Figure5.3) correlated with the initial analysis (Figure 5.2). Furthermore; the Biotyper software tool was also used to generate log score values from blind-coded clinical TB isolates and matched against the newly created local database (Table 5.1).

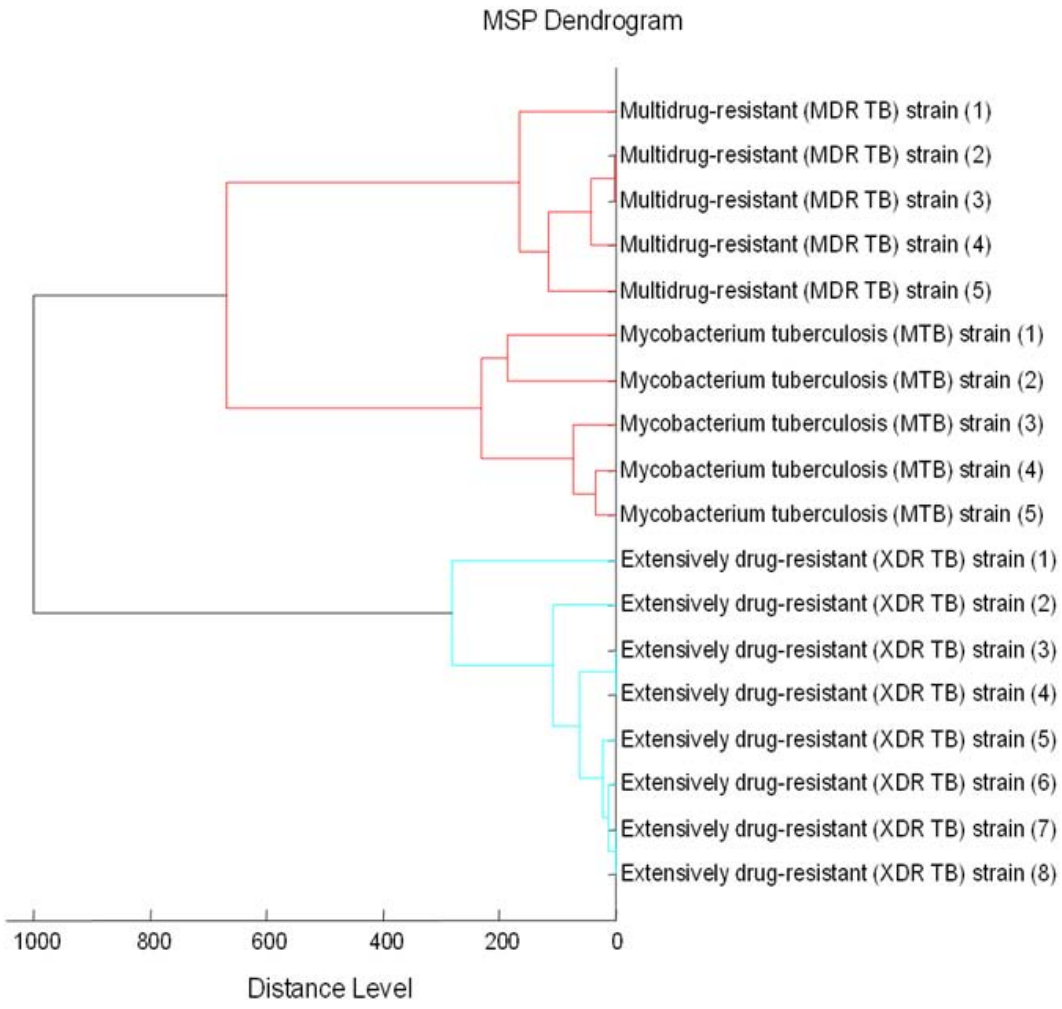

Figure 5.3 Mass spectral projections (MSP)-based dendrogramderived from fully susceptible MTB, MDR and XDR-TB isolates as well as an ATCC typed *M. bovis* straingenerated using Biotyper 3.0 software. Zero distance levels indicate that the system failed to resolve these isolates due to high level of similarity of spectra between the isolates based on the peak location and intensities. Some isolates may produce extra peaks due to varying growth phases in a culture.That do not change the clustering of the same isolates type in a dendrogram. Log score values are generated as similarity scores at the background by the system and ranked according to the similarity of the spectra generated, which results in grouping of organisms as dendrogram.

Table 5.1 Log score values generated by Biotyper 3.0 software from sixty blind-coded isolates of *M. tuberculosis*.

Identification	Susceptible-MTB	MDR-TB	XDR-TB	Log score values
Correct	16	20	20	≥ 2.0
Incorrect	4	0	0	≥ 2.0

Log scores ≥2.000 indicating correct identification, 1.700 to 1.999 indicate genus identification and <1.700 indicate no reliable identification.

An effort was made to identify the prominent biomarkers that confer unique protein profiles as was observed for the three types of *M. tuberculosis* isolates. In this regard, ClinProTool 2.2 software was used to generate pseudo-gel images for the purpose of identifying unique mass signals through visual inspection (Figure 5.4).Selection of unique mass signals based on these gel images was challenging. Most mass signals particularly in a lower mass range were shared between the three types of *M. tuberculosis* isolates. Although, some mass signals may be measured by the system but it was difficult to clearly demarcate them these pseudo-gel view images, particularly if they are present in low intensities. As such it was deemed that visual inspection of pseudo-gel view images was not a suitable tool to identify unique biomarkers of susceptible MTB, MDR-TB and XDR-TB isolates.

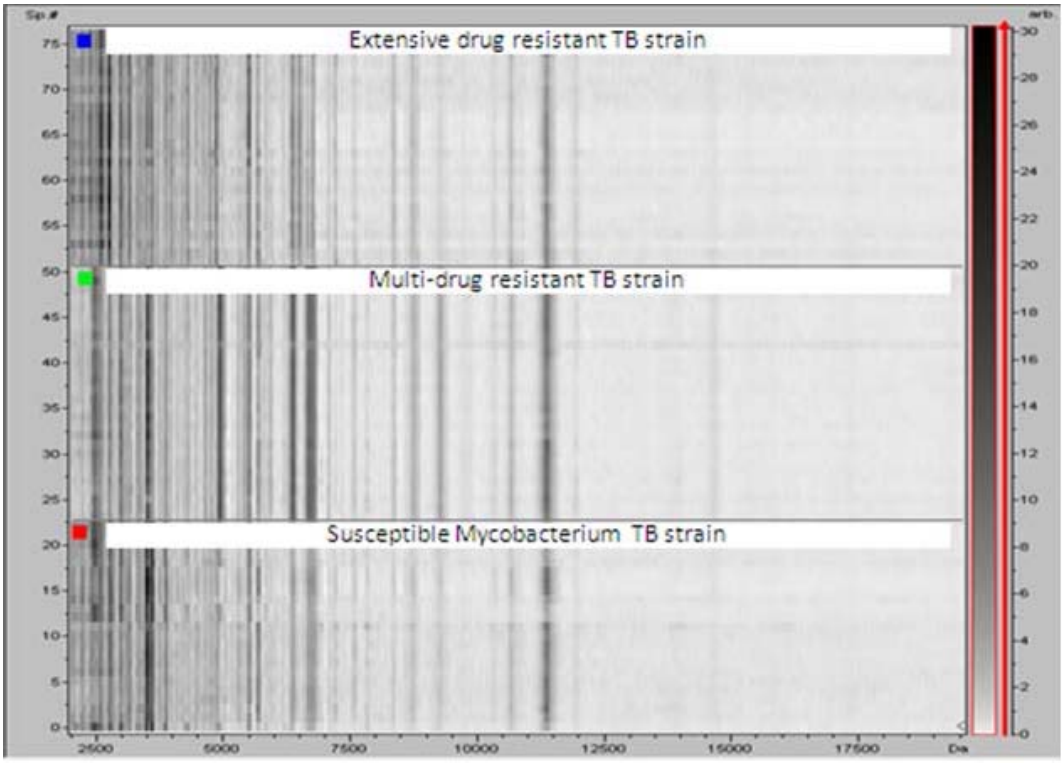

Figure 5.4 Pseudo-gel view image of clinical fully susceptible MTB (red), MDR (green) and XDR (blue) TB isolates generated with ClinProTool 2.2 software.

As a result, intra and inter-strain comparison of conserved mass signals was manually conducted using signal mass lists as generated by the Biotyper software. Thirty six mass signals were conserved in all susceptible TB isolates, 36 were also conserved in all MDR-TB isolates as well as 34 signals in all XDR-TB isolates. Variant mass signals were not employed for intra-strain comparison.

Table 5.2 Unique mass to charge signals (m/z) of proteins/peptides that occur between conserved mass signals of clinically susceptible MTB, MDR and XDR-TB isolates captured by time of flight analyser.

Susceptible-MTB isolates **(m/z)**	**MDR-TB** isolates **(m/z)**	**XDR-TB** isolates **(m/z)**
375 ± 7	375(±7)	-
554 ± 1	554(±1)	-
-	835 ±9)	835 ± 9
529(±3)	-	-
-	-	394 ± 7
585(±2)	-	585 ± 2

(±) represent the standard deviation between the same mass signals, these mass to charge signals were derived from sample preparations of clinical *M. tuberculosis* isolates and inter-spectral comparisons of data sets were conducted. Mass to charge values of peptide/proteins that each isolate synthesize.

5.5 DISCUSSION

The recent emergence of drug resistant *M. tuberculosis* (MDR and XDR) isolates has necessitated the development of robust TB identification methods that will aid either in the initial diagnosis or verify the findings of other identification tools, so as to facilitate appropriate healthcare interventions and TB disease management practices that would contribute to the effective treatment of infected individuals and prevent dissemination of these potentially devastating infections to the greater population. *M. tuberculosis* and *M. bovis* strains are members of the MTBC that share 99 % nucleotide similarity with identical 16S rRNA (Brosch, *et al.*, 2002). The MTBC members have the same host selection range and as a result many similar proteins may be expressed for adaptation in a well-oxygenated environment inside the host and for pathogenesis (Maeda, *et al.*, 2001). The results obtained in the present study, using MALDI-TOF MS, illustrate the potential of this technology to effectively discriminate MTBC to a species and strain level.

It has been previously hypothesized that the chemicals do not penetrate cell walls of some mycobacteria for optimum protein extraction and as a result poor spectra are obtained when cells are not pre-treated (Saleeb, *et al*., 2011). To this end, we evaluated three MALDI-TOF MS sample preparation methods (EFA, EFGB and CMEFA) in chapter 3. The CMEFA protein extraction method was selected for this study based on its ability to generate reproducible and consistent spectra over the EFGB and EFA sample preparation methods. Chloroform/methanol (1:1, v/v) was incorporated in the protocol to increase the quality of mass signals through the removal of lipids and physiological salts, both of which are likely to interfere with matrix crystallization and spectral quality (Gustafsson, *et al.*, 2011). In comparison, these contaminants were not removed with EFGB or EFA method, peaks with low intensities and noise signals were generated. Furthermore, due to clumping of mycobacterial cells, EFGB requires dispersion of cells with micropestle before the addition of beads. The chloroform/methanol method transforms the culture into a pellet without application of the micro-pestle and is therefore more cost effective in terms of apparatus required and less tedious when working with large number of samples to obtain high quality of spectra that will lead to correct identification of an organism. From a biosafety perspective, this method also avoids repeated vortexing of samples when compared to EFGB method.

According to PCA data, fully susceptible MTB and MDR-TB isolates are more related to each other and distantly related to XDR-TB isolates. This suggests that susceptible MTB isolates are more likely to mutate to MDR than XDR-TB isolates when patients are not compliant to treatment instructions. Furthermore, dendrograms have clearly discriminated *M. tuberculosis* at the strain level. The extent of differences between TB isolates was further explored using other software. Therefore, different algorithms were employed to measure the extent of differences. It has been previously suggested that MALDI-TOF MS technology can discriminate mycobacteria to a strain level (Hettick, et al., 2006). Unique mass signals of *M. tuberculosis* isolates were investigated using ClinProTools 2.2 software. Pseudo-gel view image of mass signals and intensities was obtained. Background "noise" increased at low mass range (1 to 4 kDa) and it was difficult to identify unique biomarkers since the sensitivity and accuracy of the instrument is lower at this mass range (Eidhammer, *et al.*, 2007).

Relying only on the visual inspection of pseudo-gel view images was not sufficient to explore the unique mass signals between isolates. However, manual comparisons of conserved mass signals between these isolates enabled further strain discrimination. Accuracy of the mass peptide was observed at 500-6000 Da mass area which was also reported by (Eidhammer, *et al.*, 2007). It has been reported that the expression of proteins is influenced at the translational level,

therefore an increase of proteins within the cell is determined by balanced peptide synthesis, posttranslational modification and protein folding as well as degradation by endogenous proteases (Bloom, 1994). It is possible that the four incorrectly identified fully susceptible tuberculosis (TB) isolates had mutations in unknown regions that were not detected with a GenoTypeMTBDRplus assay, but were expressed at protein level which had an influence on the pattern of spectral profile. GenoTypeMTBDRplus assay detect specific mutations in regions covered by the kit, therefore some mutations occurring on the same gene but on different sites may be missed because are not covered by the assay.

MALDI-TOF MS has successfully discriminated *M. tuberculosis* to susceptible, MDR and XDR-TB isolates based on their unique protein profiles, and the strain type-signals. The newly developed chloroform-methanol-ethanol-formic acid (CMEFA) method was the most efficient and cost effective by requiring less expensive reagents for MALDI-TOF MS sample preparation protocol for *M. tuberculosis* biotyping purposes. The limitation of this study is that the MALDI-TOF MS instrument does not provide sensors for drug susceptibility after strain type is revealed. The initial information about the strain type is required before database creation for further identification of unknown organisms. For future studies, the MALDI-TOF MS instrument requires incorporation of drug susceptibility sensors so as to aid the medical experts in efficiently deciding on appropriate drug treatment regimens. An expanded database is required to cover a broader spectrum of pathogens that are routinely isolated in clinical laboratories so as to speed up identification process.

5.6 ACKNOWLEDGEMENTS

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5.7 REFERENCES

Bahk YY, Kim SA, Kim J, Euh H, Bai G, Cho S & Kim YS (2004) Antigens secreted from *Mycobacterium tuberculosis*: Identification by proteomics approach and test for diagnostic marker. *Proteomics* **4**: 3299–3307.

Bienvenut WV, Sanchez JC, Karmime A, Rouge V, Rose K, Binz PA & Hochstrasser DF (1999) Toward a clinical molecular scanner for proteome research: Parallel protein chemical processing before and during Western blot. *Analitical Chemistry* **71**: 4800-4807.

Bloom BR (1994) *Tuberculosis: Pathogenesis, protection, and control*. ASM Press.

Blower SM, Small PM & Hopewell PC (1996) Control strategies for tuberculosis epidemics: New models for old problems. *Science* **273**: 497-500.

Bonk T & Humeny A (2001) MALDI-TOF-MS Analysis of Protein and DNA. *Institute of Biochemistry* **7**: 6-12.

Brosch S, Gordon SV, Marmiesse M, *et al.* (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Science* **99**: 3684–3689.

Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, Raviglione MC & Dye C (2003) The growing burden of tuberculosis. *Archives of Internal Medicine* **163**: 1009-1021.

Dasgupta K & Menzies D (2005) Cost-effectiveness of tuberculosis control strategies among immigrants and refugees. *European Respiratory Journal* **25**: 1107–1116.

Eidhammer I, Flikka K, Martens L & Mikalsen S (2007) *Computational methods for mass spectrometry proteomics*. John Wiley & Sons, LTD.

Gandhi NR, Moll A, Sturm AW, *et al.* (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet Infectious Diseases* **368**: 1575–1580.

Gumber S, Taylor DL & Whittington RJ (2007) Protein extraction from *Mycobacterium avium* subsp. paratuberculosis: Comparison of methods for analysis by sodium dodecyl sulphate polyacrylamide gel electrophoresis, native PAGE and surface enhanced laser desorption/ionization- time of flight mass spectrometry. *Journal of Microbiological Methods* **68**: 115-127.

Gustafsson JOR, Oehler MK, Ruszkiewicz A, McColl SR & Hoffmann P (2011) MALDI imaging mass spectrometry (MALDI-IMS)-Application of spatial proteomics for ovarian cancer classification and diagnosis. *International Journal of Molecular Sciences* **12**: 773-794.

Hettick JM, Kashon ML, Slaven JE, *et al.* (2006) Discrimination of intact mycobacteria at the strain level: A combined MALDI-TOF MS and biostatistical analysis. *Proteomics* **6**: 6416 – 6425.

Hillemann D, Gerdes SR & Richter E (2007) Evaluation of the GenoType MTBDRplus assay for rifampin and isoniazid susceptibility testing of *Mycobacterium tuberculosis* strains and clinical specimens. *Journal of Clinical Microbiology* **45**: 2635-2640.

Huard RC, Lazzarini LCO, Butler WR, Soolingen D & Ho JL (2003) PCR-based method to differentiate the subspecies of the *Mycobacterium tuberculosis* complex on the basis of genomic deletions. *Journal of Clinical Microbiology* **41**: 1637–1650.

Laval F, Laneelle MA, Deon C, Monsarrat B & Daffe M (2001) Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. *Analitical Chemistry* **73**: 4537-4544.

Maciag A, Dainese E, Rodriguez GM, *et al.* (2007) Global Analysis of the *Mycobacterium tuberculosis* Zur (FurB) Regulon. *Journal of Bacteriology* **189**: 730–740.

Maeda MK, Rhee JT & Gingeras TR (2001) Comparing genomes within the species *Mycobacterium tuberculosis*. *Genome Research* **11**: 547-554.

Målen H, Souza GAD, Pathak S, Søfteland T & Wiker HG (2011) Comparison of membrane proteins of *Mycobacterium tuberculosis* H37Rv and H37Ra strains. *BMC Microbiology* **11**: 1-10.

Murase Y, Maeda S, Yamada H, *et al.* (2010) Clonal Expansion of Multidrug-Resistant and Extensively Drug-Resistant Tuberculosis, Japan. *Emerging Infectious Diseases* **16**: 948-954.

Nelson KE & Williams CM (2007) *Infectious Disease Epidemiology: Theory and practice*. Jones and Barlett publishers.

Olano J, Lo´pez B, Reyes A, *et al*. (2007) Mutations in DNA repair genes are associated with the Haarlem lineage of *Mycobacterium tuberculosis* independently of their antibiotic resistance. *Tuberculosis* **87**: 502–508.

Pignone M, Greth KM, Cooper J, Emerson D & Tang J (2006) Identification of mycobacteria by Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry. *Journal Of Clinical Microbiology* **44**: 1963-1970.

Saleeb PG, Drake SK, Murray RP & Zelazyn AM (2011) Identification of mycobacteria in solid-culture media by matrix-mssisted laser desorption ionization-time of flight mass spectrometry. *Journal of Clinical Microbiology* **49**: 1790-1794.

Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R & Kostrzewa M (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS ONE* **3**: 1-10.

Seng P, Drancourt M, Gouriet F, Scola BL, Fournier PE, Rolain JM & Rauolt D (2009) Ongoing revolution in bacteriology: Routine identification of bacteria by Matrix-Assisted laser desorption/Ionization-time of flight mass spectrometry *Clinical Infectious Diseases* **49**: 543 - 551.

Shi L, Zhou R, Liu Z, *et al.* (2008) Transfer of the first arabinofuranose residue to galactan Is essential for *Mycobacterium smegmatis* viability. *Journal of Bacteriology* **190**: 5248–5255.

Shinnick TM & Good RC (2011) Diagnostic mycobacteriology laboratory practices. *Clinical Infectious Diseases* **21**: 291-299.

Streicher EM (2007) Application of Spoligotyping in the Understanding of Mycobacterium tuberculosis Strains in High Incident Communities. Thesis.

Yesilkaya H, Dale JW, Strachan NJC & Forbes KJ (2005) Natural transposon mutagenesis of clinical isolates of *Mycobacterium tuberculosis*: How many genes does a pathogen need? *Journal of Bacteriology* **187**: 6726–6732.

Chapter 6

GENERAL DISCUSSION AND CONCLUSION

6.1 GENERAL DISCUSSION AND CONCLUSION

Approximately one third of the world population is latently infected by tuberculosis (TB). Eight million cases of TB have been recorded every year and two million deaths per year have been reported (He & Zahrt, 2005). Furthermore, the emergence of drug-resistant TB strains in patients as a result of treatment non-compliance, treatment incompletion and the use of inappropriate treatment, has led to an increased incidence of TB caused by multidrug-resistant (MDR) and extensively drug resistant tuberculosis (XDR) TB strain (Brosch, *et al.*, 2002). Current delays in the diagnosis of *Mycobacterium tuberculosis* are impeded by the slow growth of the organism on the agar culture plates. Therefore, rapid detection of infectious TB is essential to offer immediate and appropriate treatment (Brosch, *et al.*, 2002).

Conventional TB diagnostic methods which are dependent on biochemical tests, growth pattern and morphological characteristic of an organism are currently used for detection of *M. tuberculosis*. Unfortunately, these methods proved to be laborious, error prone and time consuming; therefore, cannot be implemented for urgent medical situations. Polymerase chain reaction (PCR)-based approaches have higher resolution power, however these methods are costly for routine diagnosis, as a result cannot be implemented in poor resource setting (Maier & Markus, 2007). Furthermore, these approaches have limited number of species specific probes for known organisms; therefore cannot be used for classification of unknown bacterial sample origin (Sauer, *et al.*, 2008).

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a potentially promising approach for rapid, reliable and cheap diagnosis of TB. MALDI-TOF MS has been extensively used for research purposes. Recently, it has been implemented for diagnostic purposes (Saleeb, *et al.*, 2011). MALDI mass spectrometry analysis has been successfully employed for discrimination of yeast and bacteria in the clinical laboratories (Saleeb, *et al.*, 2011). Nevertheless, there has been limited studies reported the use of MALDI-TOF MS for *Mycobacterium tuberculosis* strain discrimination based on their unique protein fingerprints. Conventional ethanol/formic acid method does not yield reproducible results for mycobacterial analysis. It has been suggested that some mycobacterial species have unique cell wall composition; therefore chemicals do not penetrate their cell walls

for optimum protein extraction, as a result poor spectra are obtained (Saleeb, *et al.*, 2011). When chloroform/methanol based-method was compared to glass bead-based method, the chloroform/methanol method showed high spectral quality due to its ability to remove high content of lipids on mycobacterial cell wall. Based on the results of this study, it shows that MALDI-TOF MS can be a useful tool for rapid discrimination of challenging TB strains in clinical laboratories.

The initial information about the strain type is required before database is created for further identification of unknown organisms. For future approaches, the MALDI-TOF MS instrument requires incorporation of drug susceptibility sensors so as to aid the medical experts in drug therapy selection for patients. Expanded database is required to cover a broader spectrum of pathogens that are routinely isolated in clinical laboratories to speed up identification process. Results obtained in this study suggest that MALDI-TOF MS can be incorporated in routine settings for diagnosis of tuberculosis. The initial acquisition cost of this facility is extremely expensive, however; routine maintenance and reagent costs are economical. It is also notable that a far lower degree of technical expertise is required in the sample preparation and analysis for this technology. As such it can be of high value to both routine and reference laboratories given the highly variable infection manifestations of *M. tuberculosis*.

6.2 REFERENCES

Brosch S, Gordon S V, Marmiesse M, *et al.* (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Science* **99**: 3684–3689.

He H & Zahrt T C (2005) Identification and characterization of a regulatory sequence recognized by *Mycobacterium tuberculosis* persistence regulator MprA. *Journal of Bacteriology* **187**: 202–212.

Maier T & Markus K (2007) Fast and reliable MALDI-TOF MS-based microorganism identification. *Chemistry Today* **25**: 68-71.

Saleeb P G, Drake S K, Murray R P & Zelazyn A M (2011) Identification of mycobacteria in solid-culture media by matrix-mssisted laser desorption ionization-time of tight mass spectrometry. *Journal of Clinical Microbiology* **49**: 1790-1794.

Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R & Kostrzewa M (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS ONE* **3**: 1-10.