Matrix Assisted Laser Desorption/Ionisation time of flight mass spectrometry (MALDI-TOF MS) proteomic profiling of *Mycobacteria*

by

Melendhran Pillay

Dissertation presented for the degree of

Master of Medical Science in Pharmaceutical Chemistry

at

University of KwaZulu-Natal
College of Health Sciences
School of Health Sciences

January 2015

Supervisor: Dr Patrick Govender

DECLARATIONS

DECLARATION - PLAGIARISM

I, Mr Melendhran Pillay declare that

1. The research reported in this thesis, except where otherwise indicated, and is my original research.

2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a. Their words have been re-written but the general information attributed to them has been referenced

b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed

_____

SUMMARY

Mycobacterium tuberculosis still remains a major cause of tuberculosis(TB) and death. In addition, nontuberculous mycobacteria (NTM) play a significant role in the aetiology of tuberculosis-like syndromes. A diagnosis of *M. tuberculosis* as the causative agent of pulmonary TB using only clinical symptoms may be inadequate and inaccurate in some cases since *M.bovis*, *M.avium, M.kansasii,* and *M.intracellulare* may all produce a pulmonary disease in humans that may be indistinguishable from that caused by *M.tuberculosis*.

For diagnostic purposes, current mycobacterial identification strategies to the species level primarily rely on phenotypic analysis, complex biochemical tests, cultivation on specific growth media and genotype analysis. However, the main disadvantages that are associated with these techniques include extensive processing times (6-12 weeks to confirm a positive identification) and misidentification. Both factors severely hinder the control and management of diseases associated with mycobacteria, particularly tuberculosis. Apart from an increased patient mortality, interpersonal transmission of mycobacterial infections may increase. This scenario will also result in the prescription of inappropriate anti-tubercular treatment regimes.

The present study assesses the feasibility of using MALDI-TOF MS proteomic profiling as a rapid and precise technology to identify mycobacteria in Kwazulu-Natal, South Africa which is currently a global epicentre of mycobacterial-associated diseases. In this study a modified EFA protein extraction protocol that incorporates an initial cell envelope delipidation step was formulated and shown to produce mass spectra that were unique and highly reproducible. The spectra were used to create an independent main spectral profile reference library (CMEFA-MSP) representing clinically relevant American Type Culture Collection (ATCC) mycobacterial strains. Interestingly, this proof of concept study clearly demonstrates that MALDI-TOF MS-based biotyping of mycobacteria using the CMEFA-MSP reference library correctly identified 11 blind-coded ATCC strains sourced from an autonomous facility to the genus and species level with 100% accuracy.In addition the CMEFA-MSP reference library was employed to differentiate 39 blind-coded clinical mycobacterial isolates. Importantly all CMEFA-derived samples of the 39 clinical mycobacterial isolates displayed log score values of ≥ 2.3 and were correctly identified to the species level. This strongly suggests that MALDI-TOF MS when used in conjunction with the CMEFA sample preparation protocol has potential as a simple and cost-effective alternative for the unambiguous identification of clinically important mycobacteria. Moreover, individual members of the MTBC and NTM groups used in this study were distinguished from each other.

This thesis is dedicated to

My Family

for their unending support and motivation.

BIOGRAPHICAL SKETCH

Melendhran Pillay was brought up in the small suburb of Umhlali. Following his matriculation, he decided to further his studies in Medical Science at the University of KwaZulu-Natal, Howard College Campus. He obtained his Bachelor of Science degree (Honours) majoring inMicrobiology and Molecular Biology. Melendhran has a very intriguing mind and a curious nature hence his interest in research. His passion lies in the understanding of microbes and mechanisms of drug resistance. He currently servesin the field of medical diagnostic research. He has undergone training under Bruker Daltonics approved instructors with respect to the use and maintenance of the MALDI-TOF MS. He has received adequate training with respect to the safety procedures for the safe handling and storage of clinical specimens that presumably contain *Mycobacterium tuberculosis*. He is aims to complete Doctors of Philosophy (PhD) in a related discipline.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude and appreciation to the following people and institutions:

- To **God** for giving me the perseverance but most of all the strength to get through this degree and for enabling a positive attitude in all my endeavors.

- My supervisor **Dr Patrick Govender**, for believing in me and motivating me to be the best that I can be, for his wisdom, understanding, encouragement and guidance throughout my degree and in all aspects of life.

- Professor **KolekaMlisana**, for her motivation and encouragement

- Professor **YacoobCoovaida**, for his motivation and encouragement

- Dr **Manormoney Pillay**, for her motivation and encouragement

- **University of KwaZulu-Natal** for supporting my research study and for providing an environment conducive for me to attain my goals.

- **National Health Laboratory Services, Department of Medical Microbiology, InkosiAlbert Luthuli Central Hospital,** for supporting my research study and for providing anenvironment conducive for me to attain my goals.

- The**Columbia University-Southern African Fogarty AIDS International TrainingandResearch Program (AITRP), Implementation Science Traineeship Programfunded by theUnited States President's Emergency Plan for AIDS Relief (PEPFAR)through theFogarty International Center, National Institutes of Health** for the financial support.

PREFACE

This dissertation is presented as a compilation of five chapters.

Chapter 1 **General Introduction and Project Aims**

Chapter 2 **Literature Review**

Chapter 3 **Research Results I**

Optimization of MALDI-TOF MS sample preparation method

Chapter 4 **Research Results II**

Biotyping of clinical significant mycobacteria using MALDI-TOF MS

Chapter 5 **General Discussion and Conclusion**

CONTENTS

ABBREVIATIONS

AFB	Acid-fast bacilli
AG	arabinogalactan
AIDS	acquired immune deficiency syndrome
AS	Additional species
ATCC	American Type Culture Collection
ATS	American Thoracic Society
CI	Chemical Ionization
CLSI	Clinical Laboratory Standards Institute
CM	Common mycobacteria
CMEFA	Chloroform-methanol ethanol-formic acid
CMI	cell mediated immune response
Da	Daltons
DHBA	dihydroxy-benzoic acid
DST	Drug Susceptibility Testing
EFA	ethanol/formic acid
EFAGB	Ethanol-formic acid-glassbead
EI	Electron Impact
EMB	Ethambutol
ESI	Electronspray
FAB	Fast Atom Bombardment
FDA	Food and Drug Administration
FR-ICR	Fourier Transform Ion Cyclotron Resonance
HCCA	α-cyano-4 hydroxy-cinnamic acid
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
INH	Isoniazid
KAN	Kanamycin
kDa	Kilodaltons

KZN	KwaZulu-Natal
LPA	Line Probe Assay
LRP	luciferase reporter phage
m/z	mass to charge ratio
MAC	*M. avium* complex
MAC	*M. avium-intracellulare*complex
MAC	*Mycobacterium avium* complex
MALDI-TOF-MS	Matrix assisted laser desorption/ ionization time of flight mass spectrometry
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimum Inhibitory Concentration
MODS	microscopic observation drug susceptibility
MOTT	mycobacteria other than tuberculosis
MSP	main spectral profile
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
MTD	M. tuberculosis direct test
NAAs	nucleic acid amplification assays
NK	natural killer
NTM	Nontuberculous mycobacteria
PCA	Principle component analysis
PCR	polymerase chain reaction
PG	peptidoglycan
PZA	Pyrazinamide
RGM	rapid growing mycobacteria
RIF	Rifampicin
SA	South Africa
SM	Streptomycin
SNPS's	single nucleotide polymorphisms
TCH	thiophene-2-carboxylic acid hydrazide
TDM	Trehalose-6,69-dimycolate
TTD	time to detection of growth
turnaround	TAT

WHO	World Health Organisation
XDR-TB	Extensively drug resistant tuberculosis
ZN	ZiehlNeelsen

LIST OF FIGURES

LIST OF TABLES

Chapter 1

INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

Tuberculosis is an ancient infection and is responsible for a major percentage of the mortality and morbidity of HIV/AIDS, and poses a major threat to families and communities [1,2]. The causative agent of this airborne disease is *Mycobacterium tuberculosis* (MTB) and historically has been responsible for more deaths than any other single disease entity. According to the World Health Organisation (WHO), 8.8 million new cases and 1.7 million deaths from tuberculosis occur each year [3-5]. Approximately one third of the world population is latently infected with MTB carrying a 10% lifetime risk of developing the disease[6,7]. Although the prevalence of tuberculosis has declined in industrialized countries, 85% of tuberculosis cases occur in developing countries particularly in 22 high burden countries[8-10]. Sub-Saharan Africa, Eastern Europe and Asia have a high burden of pulmonary tuberculosis and have reported an ever-increasing resistance to antituberculous drugs.

Seventy two countries have reported on the prevalence of multidrug-resistant tuberculosis, a form of tuberculosis resistant to both isoniazid and rifampicin[11,12]. A more deadly form of drug-resistant tuberculosis known as extensively drug resistant tuberculosis (XDR-TB) has emerged and is defined as MDR-TB that is resistant to at least one of the fluoroquinolones (ofloxacin) plus an aminoglycoside/cyclic peptide (kanamycin, capreomycin)[13]. An outbreak of XDR-TB occurred in a rural community in KwaZulu-Natal province in South Africa and was responsible for almost all deaths in HIV positive individuals in less than one month following diagnosis[14]. Of note; KwaZulu-Natal, South Africa has one of the highest rates of HIV and active TB cases in the world [12,15,16].

Moreover an increase in the number of infections caused by the nontuberculous mycobacteria (NTM) has also been observed in the era of the HIV/AIDS epidemic, with a high level of NTM disease occurring in AIDS patients [17-19]. In developed countries nontuberculous mycobacteria (NTM) play a significant role in the aetiology of tuberculosis-like syndromes, especially in HIV-positive patients[20,21]. Of concern is that a diagnosis of *M. tuberculosis* as the causative agent of pulmonary TB using only clinical symptoms may be inadequate and inaccurate in some cases since *M.bovis*, *M.avium, M.kansasii,* and *M.intracellulare*may all produce a pulmonary disease in humans that may be indistinguishable from that caused by *M.tuberculosis*[22-24].

Even though the pulmonary disease caused by all Mycobacterial strains are similar, correct strain identification is crucial since there is a significant difference in the treatment of NTM derived pulmonary infections and tuberculosis caused by *M. tuberculosis.* Although the prevalence of NTM can only be estimated, there is sufficient evidence to show that NTM infection rates and actual disease are increasing [6,17,25,26]. Recent reports also show that there has been a dramatic increase not only in the total number of NTM mycobacterial species but also in the number of clinically significant species[20,27].

Currently, examination of acid-fast bacilli (AFB) by microscopy allows for the rapid diagnosis of tuberculosis. However, one cannot distinguish *M. tuberculosis* from NTM after examination of an AFB smear. It is usually negative in patients with less advanced disease, those with HIV co-infection and patients with extra-pulmonary disease[8,10,28]. Conventional methods for the identification of NTM in a TB laboratory rely on specific phenotypes which include biochemical profiles, pigment production, growth characteristics and colony morphology [2,29,30]. Although mycobacterial cultures are important in differentiating between *M. tuberculosis* and NTM, a limitation exists in that culture results take up to 6-8 weeks after specimen collection[31]. Furthermore, conventional culture methods and biochemical tests are unable to differentiate closely related mycobacterial species. It should also be noted that culturing is not widely available in high prevalence settings and is only available in reference Tuberculosis laboratories.

A number of rapid molecular assays have been introduced to improve the diagnosis of tuberculosis and to provide a presumptive identification prior to culture reports. The Genotype MTBDR Plus (Hain-Lifescience, Germany) and the Gene-Xpert (Cepheid, USA) are some of the more recent commercially available polymerase chain reaction-based approaches introduced for the diagnosis of tuberculosis. These methods cannot be implemented in resource poor settings due to high costs and a lack of technical skills[32], and routine use of these assays in the primary and secondary level of the health care system are thus prevented.

Evolutionary changes of an organism further complicates gene sequencing methods, particularly of the 16S rRNA[24,29,33,34]. Sequencing is dependent on existing predetermined internet databases for organism identification. In order for gene sequence methods to accurately identify mycobacteria, databases need to be specific for high

prevalence settings and contain sequences that represent the local population of mycobacteria. Recent advances in the field of mass spectrometry have made it possible to use matrix–assisted laser desorption/ ionization -time-of- flight- mass spectrometry (MALDI-TOF-MS) to aid in the identification of mycobacterial species by proteomic profiling[35,36]. MALDI-TOF MS is an analytical method that is capable of vaporizing and ionizing biological samples embedded on a UV-absorbing matrix[35,37]. Differentiation of mycobacterial species by MALDI is based on the unique mass spectra obtained from different bacteria due to their different protein make-up, which is defined by their unique cell surface biomarkers[38,39]. Although this application has not been extensively used for the discrimination of mycobacteria, it might be useful for the diagnosis of TB especially in high burden settings.

During the MALDI-TOF MS application process, a matrix containing sample analyte is irradiated by the ultraviolet laser of the MALDI-TOF MS instrument. The matrix then transfers energy to the sample analyte, allowing for ionization and desorption[39,40]. The desorbed ions are accelerated in a flight tube (time of flight) and are analysed according to their mass to charge ratio. The ions are first captured and counted by a detector, and thereafter they are represented as mass-to-charge signals [41]. Spectral fingerprints generatedfrom the mass-to-charge signalsrepresent the molecular composition of a sample and facilitate the species and strain level identification of micro-organisms [20].A wide variety of prokaryotes have been characterized using this approach, including clinically and environmentally relevant Gram positive and negative bacteria. Hettick*et al*., 2004 [35]have shown that mass spectrometry by MALDI is a useful tool in proteomic profiling of various mycobacteria including *M. tuberculosis.*

Currently, there is no consensus on which sample preparation method to use for MALDI-TOF MS since cell deposits, cell lysates, or crude bacterial extracts have been previously recommended. Given the pathogenic nature of mycobacteria, cellular extraction has been a more preferred method option as opposed to whole cell deposits, since this method minimises contact with the infectious organism. The current study is aimed at evaluating a newly developed chloroform-methanol ethanol-formic acid (CMEFA) cellular extraction protocol for the identification and differentiation of clinically significant mycobacterial isolates using MALDI-TOF MS. This is one of few studies aimed at establishing the foundation for the application of mass spectrometry, particularly MALDI-TOF MS in the field of diagnostic mycobacteriology.

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 closely related mycobacteria.

1.3 REFERENCES

1. Sam I-C, Drobniewski F, More P, Kemp M, Brown T (2006) Mycobacterium tuberculosis and rifampin resistance, United Kingdom. Emerging infectious diseases 12: 752.

2. Schlossberg D (2011) Tuberculosis and nontuberculous mycobacterial infections: ASM Press.

3. Aziz MA (2004) Anti-tuberculosis drug resistance in the world: third global report: the WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance, 1999-2002: World Health Organization.

4. Aziz MA, Wright A (2005) The World Health Organization/International Union Against Tuberculosis and Lung Disease Global Project on surveillance for anti-tuberculosis drug resistance: A model for other infectious diseases. Clinical Infectious Diseases 41: S258-S262.

5. Ormerod L (2005) Multidrug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment. British Medical Bulletin 73: 17-24.

6. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Archives of Internal Medicine 163: 1009-1021.

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

8. Campbell IA, Bah-Sow O (2006) Pulmonary tuberculosis: diagnosis and treatment. British Medical Journal 332: 1194.

9. Chaisson RE, Martinson NA (2008) Tuberculosis in Africa—combating an HIV-driven crisis. New England Journal of Medicine 358: 1089-1092.

10. Cole ST, Eisenach KD, McMurray DN, Jacobs Jr W (2005) Tuberculosis and the tubercle bacillus: ASM Press.

11. Albert H, Bwanga F, Mukkada S, Nyesiga B, Ademun J, et al. (2010) Rapid screening of MDR-TB using molecular Line Probe Assay is feasible in Uganda. BMC Infectious Diseases 10: 41.

12. Brust JC, Gandhi NR, Carrara H, Osburn G, Padayatchi N (2010) High Treatment Failure and Default Rates for Patients with MDR TB in KwaZulu-Natal, South Africa, 2000–2003. The International Journal of Tuberculosis and Lung disease 14: 413.

13. De La Salmoniere YG, Li HM, Torrea G, Bunschoten A, Van Embden J, et al. (1997) Evaluation of spoligotyping in a study of the transmission of Mycobacterium tuberculosis. Journal of Clinical Microbiology 35: 2210-2214.

14. Gandhi NR, Andrews JR, Brust J, Montreuil R, Weissman D, et al. (2012) Risk Factors for Mortality among MDR-and XDR-TB Patients in a High HIV-Prevalence Setting. The International Journal of Tuberculosis and Lung Disease 16: 90.

15. Lawn SD, Churchyard G (2009) Epidemiology of HIV-associated tuberculosis Running Head: Epidemiology of TB/HIV. Current Opinion in HIV and AIDS 4: 325.

16. Karim SSA, Churchyard GJ, Karim QA, Lawn SD (2009) HIV infection and tuberculosis in South Africa: an urgent need to escalate the public health response. The Lancet 374: 921-933.

17. Benator DA, Gordin FM. Nontuberculous mycobacteria in patients with human immunodeficiency virus infection; 1996. pp. 285-300.

18. Buijtels PC, van der Sande MA, de Graaff CS, Parkinson S, Verbrugh HA, et al. (2009) Nontuberculous mycobacteria, zambia. Emerging Infectious Diseases 15: 242.

19. Falkinham 3rd J (1996) Epidemiology of infection by nontuberculous mycobacteria. Clinical Microbiology Reviews 9: 177.

20. Buijtels PCAM (2007) Clinical relevance of non-tuberculous mycobacteria in Zambia: Erasmus MC: University Medical Center Rotterdam.

21. Buijtels PCAM, Willemse-Erix H, Petit P, Endtz H, Puppels G, et al. (2008) Rapid identification of mycobacteria by Raman spectroscopy. Journal of Clinical Microbiology 46: 961-965.

22. Devallois A, Goh KS, Rastogi N (1997) Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the hsp65 gene and proposition of an algorithm to differentiate 34 mycobacterial species. Journal of Clinical Microbiology 35: 2969-2973.

23. Reichenbach J, Rosenzweig S, Döffinger R, Dupuis S, Holland SM, et al. (2001) Mycobacterial diseases in primary immunodeficiencies. Current Opinion in Allergy and Clinical Immunology 1: 503-511.

24. Reller LB, Weinstein MP, Hale YM, Pfyffer GE, Salfinger M (2001) Laboratory diagnosis of mycobacterial infections: new tools and lessons learned. Clinical Infectious Diseases 33: 834-846.

25. Corbett EL, CHURCHYARD GJ, Hay M, Herselman P, Clayton T, et al. (1999) The impact of HIV infection on Mycobacterium kansasii disease in South African gold miners. American Journal of Respiratory and Critical Care Medicine 160: 10-14.

26. de Mello KGC, Mello FCQ, Borga L, Rolla V, Duarte RS, et al. (2013) Clinical and Therapeutic Features of Pulmonary Nontuberculous Mycobacterial Disease, Rio de Janeiro, Brazil. Emerging Infectious Diseases 19: 393.

27. Buijtels PC, Petit PL, Verbrugh HA, van Belkum A, van Soolingen D (2005) Isolation of nontuberculous mycobacteria in Zambia: eight case reports. Journal of Clinical Microbiology 43: 6020-6026.

28. Drobniewski F, Caws M, Gibson A, Young D (2003) Modern laboratory diagnosis of tuberculosis. The Lancet Infectious Diseases 3: 141-147.

29. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. Journal of Clinical Microbiology 41: 1010-1015.

30. Winn WC, Koneman EW (2006) Koneman's color atlas and textbook of diagnostic microbiology: Lippincott Williams & Wilkins.

31. 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.

32. Maier T, Klepel S, Renner U, Kostrzewa M (2006) Fast and reliable maldi-tof ms–based microorganism identification. Nature Methods| Application Notes.

33. Kapur V, Li L-L, Iordanescu S, Hamrick MR, Wanger A, et al. (1994) Characterization by automated DNA sequencing of mutations in the gene (rpoB) encoding the RNA polymerase beta subunit in rifampin-resistant Mycobacterium tuberculosis strains from New York City and Texas. Journal of Clinical Microbiology 32: 1095-1098.

34. Ringuet H, Akoua-Koffi C, Honore S, Varnerot A, Vincent V, et al. (1999) hsp65 sequencing for identification of rapidly growing mycobacteria. Journal of Clinical Microbiology 37: 852-857.

35. Hettick JM, Kashon ML, Simpson JP, Siegel PD, Mazurek GH, et al. (2004) Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analytical Chemistry 76: 5769-5776.

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

37. Hettich R, Buchanan M (1991) Matrix-assisted laser desorption Fourier transform mass spectrometry for the structural examination of modified nucleic acid constituents. International Journal of Mass Spectrometry and Ion Processes 111: 365-380.

38. Seibold E, Maier T, Kostrzewa M, Zeman E, Splettstoesser W (2010) Identification of Francisella tularensis by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels. Journal of clinical microbiology 48: 1061-1069.

39. Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, et al. (2010) MALDI-TOF-mass spectrometry applications in clinical microbiology. Future microbiology 5: 1733-1754.

40. Shitikov E, Ilina E, Chernousova L, Borovskaya A, Rukin I, et al. (2012) Mass spectrometry based methods for the discrimination and typing of mycobacteria. Infection, Genetics and Evolution 12: 838-845.

41. 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.

Chapter 2

LITERATURE REVIEW

IDENTIFICATION OF MYCOBACTERIA

2.1 INTRODUCTION

2.1.1 Mycobacterium: The organism

The genus Mycobacterium consists of an ever-expanding family of approximately 100 heterogeneous species of rapid and slow growing gram positive acid fast bacilli [1] belonging to the members of the *Mycobacterium tuberculosis* complex (MTBC) and to the Nontuberculosis family (NTM). There are five members of the tuberculosis complex and at least 80 species of NTM including harmless (non-pathogenic) microbes that live in diverse soil and aqueous environments, opportunistic and pathogenic species that infect humans and animals[2,3]. The MTBC consists of *M.cannetii*, *M.tuberculosis*, *M. africanum*, *M. bovis* and *M.microti*[4-6]. Over the past few decades the number of infections caused by mycobacteria have increased [1] with *M.tuberculosis* being the causative agent of tuberculosis disease. However, after the onset of human immunodeficiency virus (HIV)pandemic and introduction of anti-retroviral and anti-tuberculosis chemoprophylaxis, the incidence of NTM greatly increased with *Mycobacterium avium complex* (MAC) being the most frequent etiologic agent followed by *M. kansasii*, *M.gordonae* and *M.malmoense*[5]. A large number NTM of blood-stream infections were observed in HIV-AIDS patients with a CD4 count of less than 200 [7].

2.1.2 Mycobacteria: Discovery and Classification

The tubercle bacillus was first discovered in 1882 by Robert Koch [7]. Mycobacteria belong to the family Mycobacteriaceae and to one of the mycolic-acid genera within the order Actinomycelates[7,8]. The genus can be divided into MTBC and NTM mycobacteria (Figure 2.1). In 1896, Lehman and Neuman coined the term Mycobacterium which makes it the only genus of the family Mycobacteriaceae[9]. Previously NTM have been referred to by a variety of names, including mycobacteria other than tuberculosis (MOTT), environmental, saprophytic, opportunistic and atypical mycobacteria [10]. Since these species were not typical of *M.tuberculosis,* the term atypical mycobacteria was also applied[7,11]. From an

international perspective, the term NTM had been widely accepted and endorsed by the American Thoracic Society (ATS)[12] and will be used hereon in this review.Timpe and Runyon classified NTM according to their rate of growth, colony morphology and pigmentation in the presence or absence of light[9]. Types I, II and III (Figure 2.1), the 'slow growers'take more than 7 days to cultivateand are further distinguished by colony pigmentation[4,5]. NTMs that produce pigments when exposed to light are called photochromogens (Type I) [5,7,13].Those that produce pigments when grown in the dark are called scotochromogens (Type II) [5].Nonphotochromogens (Type III) are referred to those that are not strongly pigmented [5]. NTMs that grow in less than 7 days are termed “rapid growers” (Type IV) even though they may grow much slower than most bacteria[6].

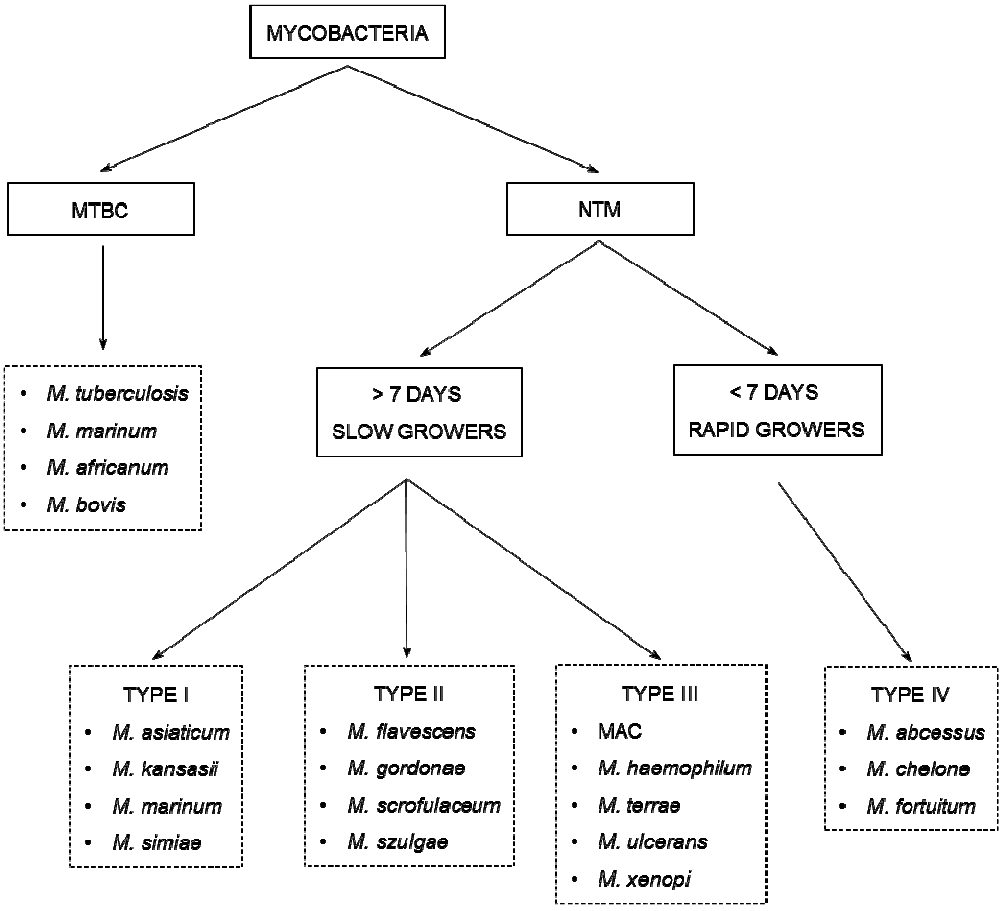

Figure 2.1 Classification of Mycobacteria according to Timpe and Runyon[5].

2.1.3 General Characteristics ofMycobacteria

Mycobacteria are gram positive, aerobic, non-spore forming, non-motile bacteria that may appear as either slightly curved or rod shaped bacilli, 0.2-0, 6 x 1.0-10μm in size[4,14]. They exhibit acid-alcohol fastness, contain genomic DNA with a molecular GC content of 61-71% and display structural similarities of the mycolic acids within their cell walls[9]. These organisms also have a number of characteristic features that distinguish them from other bacteria. These characteristics play a major role in promoting mycobacteria to produce disease and to the pathology of the disease process. In addition they also allow for the control and elimination of the infection and to the diagnosis and treatment of disease [11,14].

2.1.4 Cell Wall and Cell Envelope Structures of Mycobacteria

The cell wall is one of the most important features which differentiate mycobacteria from other bacteria[14-16]. The structural organisation of the cell wall is illustrated in Figure 2.2.It contains N-glycolylmuramic acid instead of the N-acetylmuramic acid[4,14]. In addition to these components, the cell wall is further characterised by high lipid content (60%) complexed to a variety of peptides and polysaccharides[4]. The principal component of these complexes is a long chain α- branched-β-hydroxy fatty acid called mycolic acid which contain up to 90 carbon atoms and are present in all mycobacteria[17]. Unique mycolic acid structures such as monocycloproponated and dicycloproponated α-mycolates, and methoxymycolates present in *M.tuberculosis* are also present in some slow growing NTM species such as *M.gordonae,M.kansasii* and *M.gastri* [11,15].The mycolic acids together with two additional layers namely the peptidoglycan (PG) and arabinogalactan (AG) are covalently linked together and constitute the cell wall of mycobacterium[14,15]. The lipids make the cell surface hydrophobic [14] and as a result will not grow diffusely in liquid media unless surface active reagents are present [11]. In the absence of these surface active agents the mycobacterial cells adhere tightly to each other and form difficult to disperse clumps of cells.

There is no chemical difference between the cell wall structures of the different mycobacterial species [18]. The cross-linked peptidoglycan molecule surrounds the entire bacterial cell and is responsible for the size and shape of the cell [19]. Although the peptidoglycan structure is common to most eubacteria, two types of structures namely the

arabinogalactan and the mycolic acids are covalently linked to the peptidoglycan and are unique to mycobacteria (Figure 2.2) [19]. Another striking feature unique to mycobacteria derived from ultrastructural studies is that the arabinogalactan has unusual components of monosaccharide and linkages and their distal ends are esterified by mycolic acids[17,18].

The cell wall together with the plasma membrane constitutes the cell envelope of mycobacteria [15] (Figure 2.2). The cell envelope plays an important role by providing osmotic protection, transport of ions and nutrients, mechanical support and protection[18]. Of relevance, the cell envelope not only provides the elements responsible for the physiology of mycobacteria but it also contributes to its disease producing capabilities[15]. It constitutes the primary interface between bacteria and the host, providing the first line of defence against the damaging assaults of the host immune response [14,18].They are able to modify host cell behaviour in favour of the bacteria and resist destructive immune mechanisms [19].The envelope with its complex structural components is able to facilitate survival of mycobacteria within the host macrophage [11,18].

One of the characteristic features is that mycobacteria stain poorly by the Gram staining method. The complex composition of the cell wall with high lipid content cannot readily be stained by the usual aniline dyes. For this reason they are termed “acid fast bacilli” because of their lipid rich cell walls are impermeable to basic dyes unless combined with phenol. Once stained, the cells cannot easily be decolorized with acidified organic solvents [4,11]. This characteristic has been widely harnessed by the ZiehlNeelsen stain technique as a diagnostic tool for the microscopic identification of mycobacteria [3].

2.1.4.1 Trehalose-6,6,9-dimycolate (TDM)

In addition to all of the complex lipid structures mentioned, lipids comprising of glycolipids (wax D fraction), trehalose containing glycolipids (cord factor), glycopeptidolipids and triacylglycerols are loosely bound to the cell wall by hydrophobic forces[20]. These components are extractable by organic solvents. Trehalose-6,69-dimycolate (TDM) also known as the cord factor is a glycosylated form of mycolic acid present in virulent TB strains [19,21].Studies have shown that TDM diffuses into the host cell membranes and damages membrane function. Toxic properties from cord factor play a crucial role in the multiplication of pathogenic mycobacteria and their distribution within the host[19].

2.1.4.2. Wax D fraction

To conclude on the complexity of the cell envelope and its components it is worthy to mention the Wax D fraction, an active glycolipid present in all mycobacterial species[4,11,14]. These glycolipids are responsible for adjuvant activity which causes delayed-type hypersensitivity (DTH) which is due to mycobacterial proteins that cause the destruction of non-activated macrophages and the production of humoral antibodies[21]. The Wax D elicit granulomatous lesions and contribute to organism virulence[14,21].

2.1.4.3 Sulfolipids

These are the third group of mycobacterial lipids that inhibit lysosome fusion to the phagocytic vacuole and suppress the production of superoxide anion, permitting mycobacteria to survive and multiply within the macrophages[5]. Mycobacteria present in developing lesions are able to survive under conditions of low pH, high lactic acid and high CO_2. To date no virulence genes have been defined and disease manifestations results primarily from hypersensitivity to the tuberculoproteins[17]

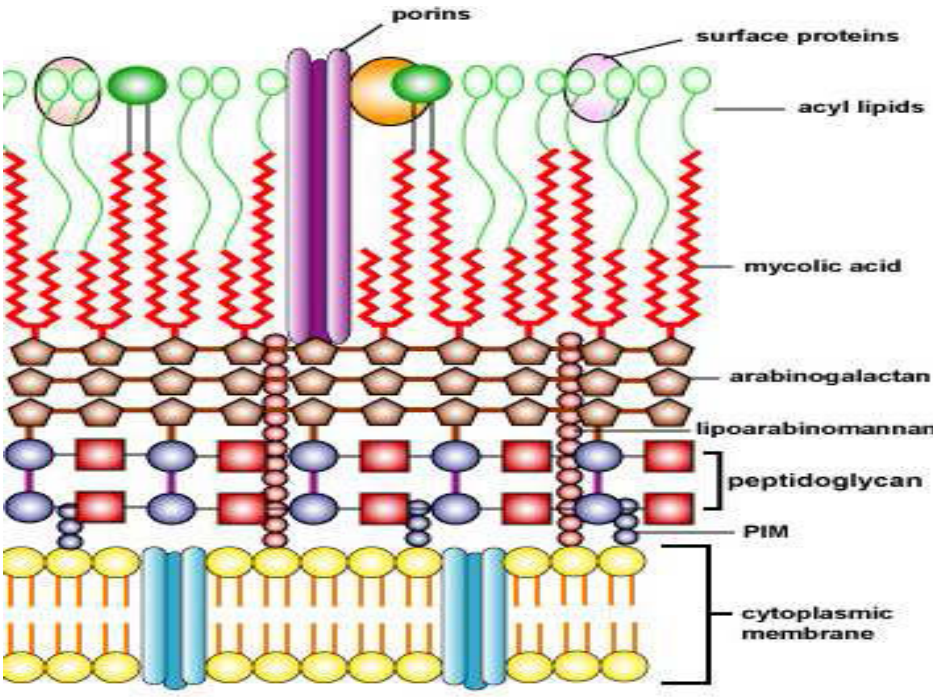

Figure 2.2 General cross sectional area of mycobacterial cell wall/cell envelope structure [22].

2.2 EPIDEMIOLOGY OF TUBERCULOSIS AND NTM INFECTIONS

Globally, the current number of incident Tuberculosis (TB) cases was estimated to be 8.6 million in 2012, with 16.2 million prevalent cases of TB disease and 1.3 million deaths giving an overall case fatality rate of 23% [23]. Among these deaths, an estimated 170 000 were due to MDR-TB[23]. The World Health Organisation (WHO) estimated that approximately one third of the world's population is infected with *M.tuberculosis*(MTB)[23]The global prevalence of MTB infection was estimated to be 32% with 0.18% of the world's population dually infected with MTB and HIV [19]. Global incidence rates increased by 0.4% per year between 1997 and 2000[19] (Figure 2.3).

Sub-Saharan Africa has the highest incidence rate of TB of 300 per 100 000 population (Figure 2.3) South Africa (SA) is one of the twenty-two high-burden countries identified as having more than 80% of the new cases of TB worldwide and is reported to have the third highest TB burden in the world after India and China [19,24]. SA alone has over 2 million HIV-TB co-infected individuals [25,26]. KwaZulu-Natal is the leading province with high rates of TB as well as a high burden of MDR-TB[27]. 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[28,29].Recently, the HIV epidemic has tremendously influenced the epidemiology of NTM disease in the African continent with reports from studies conducted in South Africa reporting an extremely high rate of NTM infections.

Sputum samples obtained from 1196 people in the Zulu community in Natal, South Africa were examined for the presence of mycobacteria by microscopy and culture[30]. *M.tuberculosis* was detected in 9 samples and NTM was detected in 17 samples by culture, translating into prevalence rates of 750 per 100,000 for *M.tuberculosis* and prevalence rates of colonization/infection for NTM of 1,400 per 100,000[12]. Another study was conducted in South Africa on a cohort of HIV-negative gold miners[31]. They were investigated for the presence of mycobacterial disease between 1993 and 1996. The two most common organisms causing infection were *M.kansasii* and *M.scrofulaceum* with NTM rates of 66 and 12 per 100 000 respectively[31]. The overall NTM rate was 101 per 100 000 with MAC making up only 6% of all the isolates[31]. However when another cohort of HIV - positive gold miners were investigated, the NTM rates (per 100 000) were much lower at 37 and 8.8 for *M.kansasii* and *M.scrofulaceum* respectively[26].

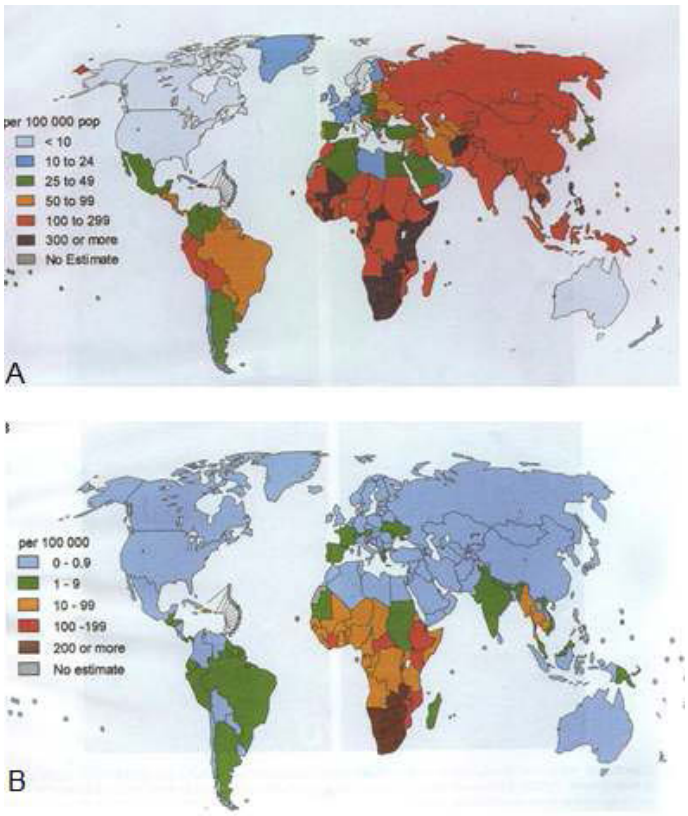

Figure 2.3 Global TB incidence rates (per 100,000 population per year) at the start of 2000. (A) All forms of TB. HIV-related TB (B) [19].

2.3 CLINICAL DISEASES ASSOCIATED WITH MYCOBACTERIA

Members of the *Mycobacteriaceae* family are responsible for a wide range of diseases in humans and lower animals[12]. In humans these diseases, range from superficial self-limited infections of the skin caused by *M. marinum*, to pulmonary disease caused by both *M.tuberculosis* and by NTM[11]. Other infections include lymphatic infections, skin and soft tissue infections, infections of bones and joints and disseminated disease [5,16,32,33].*M.bovis*, *M.avium, M.kansasii,* and *M.intracellulare* all may produce a pulmonary disease in humans that may be indistinguishable from that caused by *M.tuberculosis*(Table2.1)[11]. Furthermore, skin and lymph gland infections caused by

M.tuberculosis can also be produced by other mycobacteria such as *M.scrofulaceum, M.ulcerans* and *M.marinum*.

Table2.1 Clinical Disease associated with Mycobacteria

Mycobacteria	Clinical Disease
M.tuberculosis	pulmonary disease, skin and lymph gland infections
M.bovis	pulmonary disease
M.avium	pulmonary disease
M.kansasii	pulmonary disease
M.intracellulare	pulmonary disease,
M. intracellulare-avium Complex (MAC)	pulmonary disease, disseminated disease, tenosynovitis
M.scrofulaceum	skin and lymph gland infections
M.ulcerans	skin and lymph gland infections
M.marinum	skin and lymph gland infections, tenosynovitis
M. fortuitum	skin and soft tissue infections, osteomyelitis
M. abscessus	skin and soft tissue infections, osteomyelitis

2.4 PATHOGENESIS AND TRANSMISSION

Pathogenicity is defined as the complex of factors required to establish a successful human infection and includes the damage induced by such an infection[34]. The pathogenesis of tuberculosis represents a series of battles between the host and the tubercle bacillus[5]. Host defences include: an activated macrophage i.e., a phagocyte that inhibits an ingested tubercle bacilli and the ability of the host to stop intracellular replication of bacilli in non-activated macrophages[17]. This is achieved through destruction of the macrophages resulting in solid caseous tissue formation[5]. The defences of the bacillus depend on the following:

i) The ability to multiply logarithmically within monocytes that emigrate from the blood stream to tissues at the site of infection.

ii) The ability to multiply extracellularly in liquefied caseous material within cavities[5,11].

In a primary infection (individuals encountering the organism for the first time), *M.tuberculosis* enters the alveoli of exposed humans in an aerosol droplet where its first contact is thought to be with resident macrophages[5,11,17,35]. Infected macrophage cells migrate through the lymphatics to the hylar lymph nodes inducing an immune response dominated by T-helper cells. Inflammation will be present at the site of infection, along lymphatic channels and in the regional lymph nodes[5,17,34]. The defences of the immune system prevent proliferation of the organism and inhibits local spread of the bacilli [17]. Macrophages activated by T cells, kill the bacilli and slow down growth. The cell mediated immune response (CMI) which is detectable 2-8 weeks after an infection, is mediated by Th1 helper cells and promotes protective immunity. However it is also responsible for localised pulmonary disease[5,17,35]. In the hylar lymph nodes, the organism are contained in tubercles which are small granulomas consisting of epithelioid cells, giant cells and lymphocytes. Over time the centres of the tubercle become necrotic and advance to form caseous material[35]. A Ghon complex can be defined as a combination of a single lesion in the lung and caseation in the bronchial lymph node.

Although primary tuberculosis may be mild and asymptomatic, it may still take two courses (Figure 2.4). In healthy individuals the lesions heal spontaneously and become fibrotic or calcified. This can be seen in 90% of the cases. These lesions persist and can be seen after many years in chest x-rays. However in 10% of cases, particularly in immunocompromised individuals, the bacilli may invade the bloodstream and spread to the organs. This may lead to a fatal infection known as disseminated military tuberculosis[17,35]. With time, cellular immunity to the organism develops. Intracellular growth of the organism can be inhibited by the macrophages that have become activated by lymphokines produced by T lymphocytes. In the first two years after an infection, the risk of primary infection developing into clinical tuberculosis is at its highest[36,37].

Reactivation of dormant mycobacteria followed by the development of chronic, necrotic and progressive symptoms characteristic of tuberculosis is defined as secondary tuberculosis. Flare up can be due to impairment of the immune function, chemotherapy, malignancy, extensive corticosteroid therapy, immune- suppression caused by HIV and failure of the T-cell macrophage immune system may render a person vulnerable to reactivation of latent mycobacteria[38,39]. Reinfection with externally acquired tubercle bacilli may lead to the same manifestation [17,35]. Secondary tuberculosis occurs at the apex of the lung due to the greater level of oxygenation for the promotion of growth[17]. Lesions become necrotic,

caseate and merge into larger lesions. Caseous lesions liquefy and contents discharged into the bronchi. Proliferation of the organism occurs in a well aerated cavitary. The disease rapidly progressives when organism are spread to other parts of the lung through the discharge of caseous material[5,17,35].

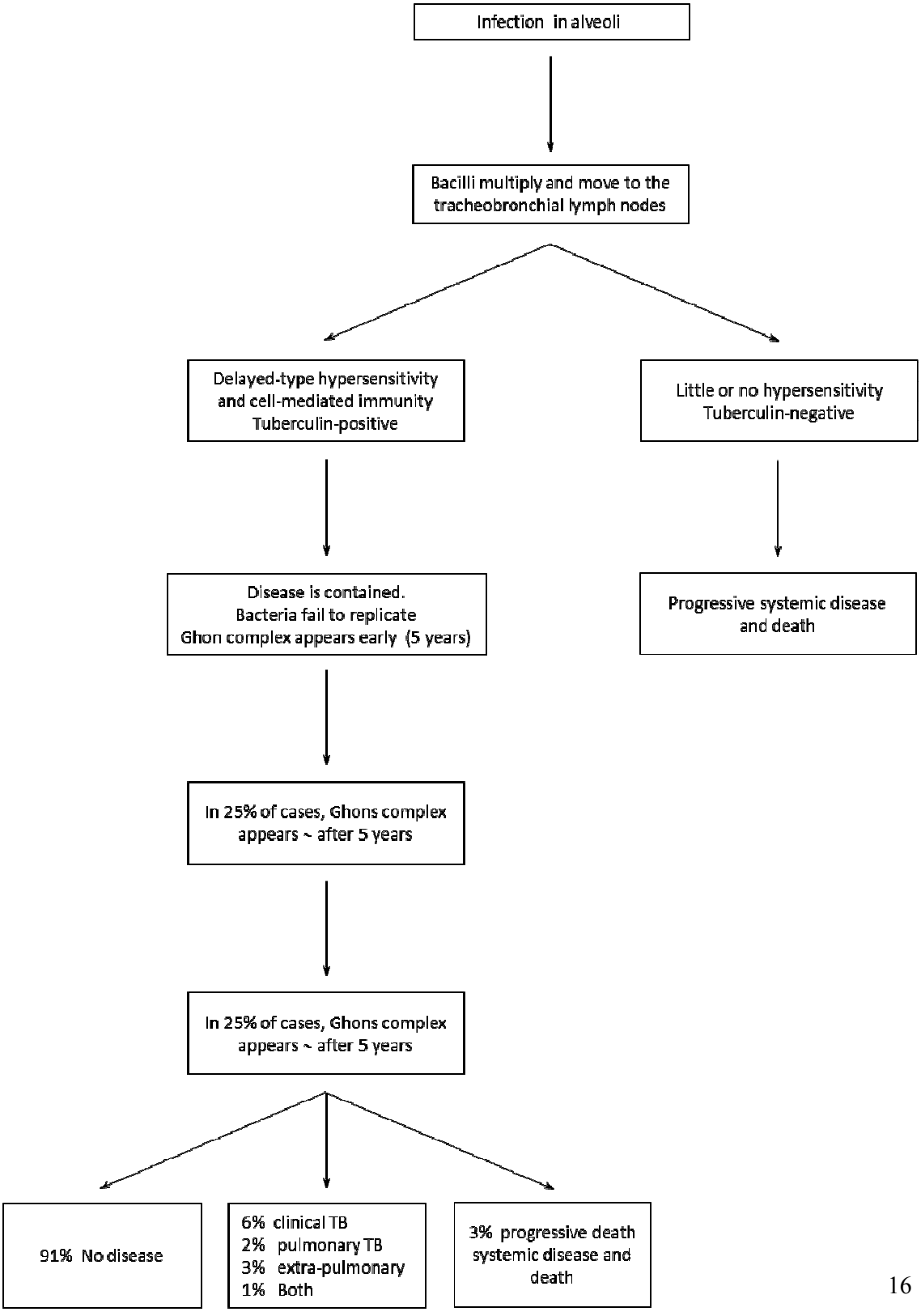

Figure 2.4 Pathogenesis of tuberculosis. Adapted from Mechanisms of Microbial Disease[17].

The route of entry for NTM strains to get into the body includes the gastrointestinal or respiratory mucosal membranes[5,38-40]. However, in HIV-infected patients the gastrointestinal tract serves as an entry point [5,38]. MAC can survive in the normal gastric acidity and are taken up by the enterocytes of the terminal ileum [11]. The ability of MAC to use fibronectin to join bacteria to integrin receptors on mucosal cells is made possible by fibronectin-attachment protein [11]. A change in the MAC phenotype while it is inside the epithelial cell allows it to withstand the antimicrobial properties of the macrophage after exit [5,38]. MAC spreads to the intestinal lymph nodes in acquired immune deficiency syndrome (AIDS) patients and it can disseminate easily if host defences deteriorate [5,36]. NTM that are inhaled from dust or other aerosols are dealt with by the innate host defences mainly by mucociliary clearance and cough [5,35,41]. Excessive secretions in tracheobronchial passages promote bioflim formation which is a characteristic feature of NTM. NTM that spread to the alveolar spaces colonise Type 2 alveolar cells and alveolar macrophages [5,16,17]. NTM are able to survive inside the cells by interfering with fusion of phagosomes and lysosomes. CD4 lymphocytes and natural killer (NK) cells can interact with infected mononuclear cells and kill the bacteria. This is normally observed in immunocompetent individuals.

2.5 TREATMENT OF MYCOBACTERIAL DISEASE

Currently the standard and optimal treatment for tuberculosis disease comprises of an initial intensive phase consisting of isoniazid (H), rifampicin (R), pyrazinamide (Z) and ethambutol for 2 months followed by a continuation phase of H and R for 4 months[42,43]. This preferred regimen forms the basis of the standard treatment of tuberculosis and is referred to by clinicians as 2HZR/4HR[19,42,43]. The success rate of the preferred treatment regimen can be seen in a bacteriologic conversion rate of close to 100% and a relapse rate of approximately 3-5% in patients with drug-susceptible TB.Approximately 10 drugs have been approved by the United States Food and Drug Administration (FDA) for treatment of

tuberculosis. Although fluoroquinolones are used to treat TB caused by drug resistant organisms, these drugs are not approved by the FDA for the treatment of tuberculosis. Table 2.2 shows the first line chemotherapeutic agent used in the treatment of tuberculosis.

Table 2.2 First line chemotherapeutic agents used in treatment of Tuberculosis. Adapted from Handbook of Tuberculosis, Clinics, Diagnostics, Treatment and Epidemiology[43].

Drug	Description	Daily Adult Dosage	Side effects
Isoniazid	Nicotinic acid hydrazide. Bactericidal. Inhibits mycolic acid synthesis most effectively in dividing cells.Hepatically metabolized.	Regular dose:300mg or 5mg/kg. High dose: 900mg or 15mg/kg Administer with pyridoxine 150-300 mg	Hepatitis.l0-20% of patients have elevated transaminasesperipheral neuropathy Less common: fever, GI upset incidence of adverse reactions 5.4%
Rifampicin	Bactericidal, Inhibits protein synthesis by blocking mRNA transcription and synthesis Hepatically metabolized.	600mg or 10 mg/kg	Incidence of adverse reactions: < 4% orange colored bodily secretions common less common: GI upset, hepatitis rare: fever, fatigue, cholestatic jaundice hemolytic anemia, hypersensitivity acute renal failure.
Pyrazinamide	Nicotinamide derivative Bactericidal. Effective in acidic environments e.g.cavitary disease. Hepaticallymetabolized and renally secreted.	15-25mg/kg	Common: hepatotoxicity, hyperuricemia. Less common: GI upset, impaired diabetic control Rare:dysuria, fever, hypersensitivity reaction
Ethambutol	Bacteriostatic. Inhibits lipids and cell wall metabolism.	15-25mg/kg	Incidence of adverse reactions < 2%. Less common: GI upset, headache, malaise, arthralgia. Rare:dizziness, fever, peripheral neuropathy pleuritis, rash

Although approved for use in the treatment of *Mycobacterium avium* complex (MAC) disease in HIV infections, rifabutin and rifapentineare also used for the treatment of TB in patients who are experiencing severe drug interactions or intolerance with rifampicin[5,12,42,44].

Therapy used for the treatment of NTM infections differs significantly from the approved 6 month treatment for MTB. Treatment forNTM is not only species dependent but is also influenced by immune characteristics and clinical manifestations of the infection [12,37,45-47]. Species specific identification is crucial since mycobacteria are intrinsically resistant to most common antibiotics [12]. *M.tuberculosis* is intrinsically resistant to macrolides whilst NTM are resistant to first-line antituberculosis drugs[47]. In addition to the above, extended

therapy with a long duration is always necessary since mycobacteria are slow growing[12,39,48]. In HIV negative adults infectedwith MAC,treatment duration should continue until the patient is culture negative for 10 to 12 months [12,16,49]. Agents with proven clinical significance for MAC treatment include a whole range of antibiotics (Table 2.3).

Table 2.3 Antimicrobial Agents used for the Treatment of MAC Infections[5,12].

Drug	Common Side Effects
Azithromycin	Diarrhoea, nausea, abdominal pain, rash, elevated hepatic transaminases,tinnitus, hearing loss
Clarithromycin	Diarrhoea, nausea, abnormal taste, abdominalpain, dyspepsia, elevated hepatic transaminases,QT prolongation
Ethambutol	Decreased visual acuity
Rifampin	Diarrhoea, nausea, abdominal pain, rash, elevated hepatic transaminases
Rifabutin	Abdominal pain,nausea,rash,arthralgias,uveitis,leukopenia,elevated hepatic transaminases
Amikacin, Streptomycin	Vestibulotoxicity, cochlear toxicity,nephrotoxicity, rash, fever, neuromuscular blockade
Ciprofloxacin, Gatifloxacin, Levofloxacin, Moxifloxacin	Nausea, vomiting, abdominal pain, rash, abnormal liver transaminases, tendonitis, QT prolongation
Clofazimine	Abdominal pain, diarrhoea, nausea,vomiting,skin discoloration, rash, dry skin, conjunctival and corneal pigmentation
Linezolid	Diarrhoea, nausea, headache,myelosuppression,lactic acidosis,optic neuropathy,peripheral neuropathy
Mefloquine	Dizziness,myalgia,nausea,vomiting,headache,rash,hair loss,sleep disorders,psychosis, elevated hepatic transaminases

2.6 MECHANISMS OF DRUG RESISTANCE

Mycobacteria, like most bacteria have developed efficient means to prevent unwanted metabolites from entering the cell[18,50].The presence of a highly hydrophobic nature of the mycobacterial cell envelope can serve as an effective permeability barrier to many antibiotics[18,51-53]. As a result mycobacteria may be intrinsically resistance to important first and second-line antitubercular drugs. Alternatively several strategies have been employed by bacteria to develop resistance to antibiotics. These include target modification, target overexpression, barrier mechanisms, drug inactivating enzymes and inactivation of drug inactivating enzymes[43,50]. Target modifications in mycobacteria, particularly

M.tuberculosis includes mutations in the inhA, *rpoB, rrs, rpsL,emrB* and *gyrA* genes[5,17,18,50]. These mutations are associated with isoniazid (INH), rifampicin (RIF), kanamycin (KAN), Streptomycin (SM), ethambutol (EMB) and fluroquinolone resistance respectively. Inactivation of drug activating enzymes due to mutations in the*katG*, *pncA* and *ethA/etaA* also contribute to INH, PZA and ETH resistance respectively [5,18,52,54].

However, in NTM, the absence of the *erm37* gene allows for a natural susceptibility to macrolide therapy. Despite macrolide susceptibility, pre-exposure to macrolides also induced macrolide resistance due to the induction of related *erm* genes particularly *erm38* and *erm39* in rapidly growing mycobacteria[55,56].

The exposure of mycobacteria to suboptimal concentrations of drugs *in vivo* leads to the selection of drug resistant mutants. Spontaneous generation of mutants resistant to INH and RIF occurs at a frequency of 1:1 million and 1:100 million respectively [5,50]. Ultimately, poor patient compliance to therapy and the use of an inappropriate treatment regimen for the lengthy 6-month duration have contributed to the global emergence of drug-resistant tuberculosis. This poses a major challenge for effective treatment and control.

2.7 LABORATORY DIAGNOSIS OF *MYCOBACTERIA*

Since the emergence of drug resistant tuberculosis in South Africa in 2006, there has been an urgent need for effective tuberculosis treatment programmes through the initiation of cheaper and rapid diagnostic tools. The extremely rapid progression of mycobacterial infections caused by *M. tuberculosis* and MAC in HIV-positive individuals has warranted the development of rapid methods for mycobacterial species identification and drug susceptibility testing[13,19]. Current conventional methods for the identification of mycobacterial isolates to a species level takes approximately 4-6 weeks and consist mainly of microscopy, culture growth characteristics and biochemical tests[42,45]. Although culture remains the “gold standard” for the detection of *M. tuberculosis* in routine diagnostic laboratories in South Africa, culturing methods are time consuming and delays the diagnosis of tuberculosis disease[45]. Further delays can occur when culture based drug susceptibility testing (DST) is used to monitor growing rates of drug resistance. [57]. The use of automated liquid culturing systems such as the Bactec MGIT 960 system (Becton Dickinson, Sparks, Md.) have speeded up the diagnostic process of *M. tuberculosis*[58] by reducing the detection rate to 5-10 days.

The development of new standardised tests for the detection of mycobacteria is imperative since treatment is species dependent. Since traditional identification approaches are unable to identify new emerging species, more robust and flexible diagnostic tests are required. This will also enable scientists and researchers to manage disease epidemics throughout the world[59]. The preceding sections will briefly discuss some of the conventional diagnostics methods employed in a routine diagnostic tuberculosis laboratory

2.7.1 Smear Microscopy

The discovery of the microscopic staining of the tubercle bacilli in 1882 by Robert Koch, has led to this technique becoming the most basic and most common tool used for the phenotypic confirmation of acid fast bacilli (AFB) in biological samples[13]. In resource poor settings with a high burden of TB, microscopy is a widely used diagnostic technique and remains a mainstay both for the diagnosis of TB and for the monitoring of treatment progress. Although this method has a low sensitivity (50-60%), it is inexpensive and simple [13,43,60]. The conventional gram staining methods are ineffective for the staining of mycobacteria since the high lipid content of the mycobacterial cell wall acts as an impermeable barrier for commonly used aniline dyes[20,60]. In order to address the problems with poor retention and adsorption of basic staining dyes, the auramine O and the Ziehl-Neelsen (ZN) stain have been widely adopted for the microscopic detection of mycobacteria[18]. Smears for AFB detection are prepared directly from untreated or concentrate clinical specimens[19].

The affinity of the mycolic acid in the cell walls for the fluorochromes enables the auramine stain to stain the bacilli bright yellow against a dark background[4]. This can be viewed using a fluorescence microscope with 40 X lower magnification (Figure 2.5). Ziehl-Neelsen stained acid-fast bacilli appear pink against a blue background when observed under the light microscope, as shown in Figure 2.5. Results from a smear examination normally includes a measure of quantity, such as actual number of bacilli seen per field[19].A major disadvantage of smear microscopy is that non-viable or dead bacilli will fluoresce when stained with the auramine O stain and dead cells will be stained by the ZN stain. As a result not all smear positive cases are culture positive. In HIV positive individuals false positives due to the presence of NTM can be an issue since smear microscopy cannot speciate[5,18]. Conversely,

HIV-positive patients with an impaired immune response may have reduced rates of cavitation and as a result reduced numbers of mycobacteria may be present in their sputum sample as compared to HIV-negative TB patients. NTMs particularly the rapid growing mycobacteria (RGM), may be more sensitive to the AFB decolourization procedure and may not stain at all with fluorochrome stains[12].

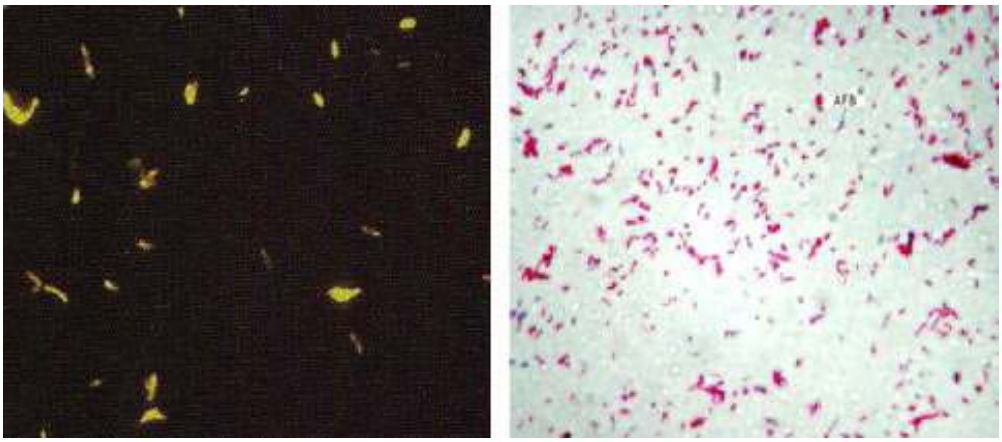

Figure 2.5 Microscopic visualization of stained *Mycobacterium tuberculosis* with Ziehl-Neelsen (*right*) and Auramine O (*left*) staining procedure[4].

2.7.2 Culture

Mycobacterial culture on either solid or liquid media is considered to be the gold standard method for the identification of mycobacteria and is able to distinguish *M. tuberculosis* from some of the NTM[61]. Culture can be used on biopsy material and needle aspirates and unlike smear microscopy it is widely used for the diagnosis of extra-pulmonary or paucibacillary TB[43]. Unlike smear microscopy with a limit of detection of approximately 5 x 10^3 to 1x10^4 bacilli/ml, a few as 10 viable bacilli can be detected by culture[19]. Culture is more demanding in terms of expertise and laboratory infrastructure than smear microscopy and is largely restricted to a reference laboratory. A major limitation of culture is that it is time consuming requiring more than 4 weeks in obtaining a conclusive result from solid media[61].

Solid media can be either agar based or egg-based. Middelbrook 7H10 and Middelbrook 7H11 are the two most commonly used agar based media whilst Lowenstein-Jensen (LJ) is a

commonly used egg-based media[5,19]. These media differ in their chemical composition and formulation. Several commercially available liquid culturing systems namely the Bactec 460 and 960 systems (Becton Dickinson, USA) have been developed to reduce the time to detection of growth (TTD)[19,43]. These systems are sophisticated and are able to detect the presence of actively dividing bacilli by measuring either the excretion or consumption of metabolic products and are not dependent on appearance of visible growth as observed by the solid culture process. Cultures in broth media have a higher yield of mycobacteria and produce more rapid results than those on solid media even thoughthe contamination rate was found to be statistically higher [12,62]. There are several advantages of using solid media. These include the ability to observe colony morphology, growth rates, more than one mycobacterial species (mixed species), and quantitation of the infecting organism[12]. Solid culture method requires a skilled microscopist as in the case of sputum smears. Furthermore, fastidious NTMs such as *M. genavense*, *M. avium* subsp. *paratuberculosis* and *M. ulcerans* require special supplementation for recovery on culture [12].

2.7.3 Biochemical Tests

The preliminary classification of mycobacteria, particularly NTM is achieved using growth rates. NTMs have also been traditionally classified into 3 groups based on the production of pigments. The use of pigment production may not be useful for final species identification; however it may be beneficial for narrowing the possibilities[4,5]. Although growth characteristics are seldom used for the definitive diagnosis of mycobacteria, the presence of pigmentation and smooth colony morphology can exclude the isolate as belonging to the *M. tuberculosis complex*[12,60]. As mentioned previously, *M. tuberculosis* complex form non-pigmented and rough colonies. In addition to culture, various biochemical tests such as niacin accumulation, reduction of nitrates to nitrites, tween 80 hydrolysis, catalase activity, arylsulfatase activity, urease activity, pyrazinamidase, iron uptake and growth inhibition by thiophene-2-carboxylic acid hydrazide are available for mycobacterial species identification. The discrimination of *M. tuberculosis* and *M. bovis* can be achieved by using just four biochemical tests. *M. tuberculosis* is niacin and nitrate positive, resistant to 5ug of thiophene-2-carboxylic acid hydrazide (TCH) per ml incorporated into 7H11 agar and is positive for a pyrazinamidase test[19]. All of these tests are reversed for *M. bovis*. However,

the identification using conventional biochemical analysis is both time consuming and increases the turnaround time, leading to significant delays in diagnosis. Of note, testing for NTM usually requires a series of biochemical tests based on growth rate. These tests can be quiet labour intensive and complicated. It has been shown that isoniazid resistant MTB strains may be negative for both the niacin and nitrate test[19]thus resulting in false negatives. Furthermore MTB may be negative for the pyrazinamidase test particularly in patients who have been treated with pyrazinamide (PZA) and have developed PZA resistance[13,19,43].

2.7.4 Drug Susceptibility Testing (DST)

The rapid detection of drug resistance organisms is crucial for the management of tuberculosis disease. There are several methods for phenotypic drug susceptibility testing. These include the proportion method, the resistance ratio, and the absolute-concentration method[19]. The resistance ratio measures the Minimum Inhibitory Concentration (MIC), the lowest concentration of the drug capable of inhibiting growth of the organism, compared to the MIC of the reference M. tuberculosis 25177 strain[63]. A value of 2 or less derived from the resistance ratio measures indicates susceptible whilst a value of 8 and above indicates resistance. Susceptibility testing using the agar method is robust and can be used to tests both first and second line anti-TB drugs. These methods require an adequate infrastructure andcannot be implemented in resource poor settings.

Current *in vitro* susceptibility methods using rifampicin and ethambutol may not be reliable when used for the testing of MAC. MAC has limited *in vitro* susceptibility and clinical response has been shown to correlate only with macrolides.*M. abscessus* and *M. simiae* have limited in vitro susceptibility and as a result there is limited data for a correlation between the clinical response in the treatment of pulmonary disease by an antimicrobial and in vitro susceptibility testing[12]. According to Clinical Laboratory Standards Institute(CLSI), a broth-based method particularly microdilution and macrodilution methods are recommended for the susceptibility testing of slow growing mycobacteria such as MAC [3,12,64]. These methods are not available in resource poor settings. For less commonly isolated NTMs such as *M. malmoense, M. xenopi,* and *M. terrae*, there is no specific susceptibility method. Antimycobacterial MIC testing for isolates of *M. chelonae* and *M. abscessus* using imipenem may be problematic due to a lack of reproducibility.

2.7.5 Mycobacteriophage-based tests

Mycobacteriophage-based assays are proving to be useful tools for the identification of viable bacilli as well as for antimicrobial susceptibility testing[65,66]. These methods have been incorporated in two methods, the FASTPlaque TB-RIF test (Biotech Laboratories, Ipswich, United Kingdom) and the luciferase reporter phage (LRP)[19]. Both assays are based on the ability of the viable *M tuberculosis* bacilli to protect infecting mycobacteriophages from chemical inactivation and to facilitate the replication of infecting phage[67]. The FASTPlaque TB-RIF test (Figure 2.6) uses a mycobacteriaphage that is added to decontaminated sputum samples or culture suspension[19]. After an overnight incubation, the external phages that did not infect the host cells are destroyed using a virucidal solution. Phages that proliferate within mycobacterial cells are detected when the phage-infected pathogen suspension is mixed with rapidly growing *M. smegmatis*[19]. The phages released from the lysed *M. tuberculosis* infect the *M. smegmatis* cells. They replicate and amplify in the helper cell host (*M. smegmatis*). The progeny phages undergo cycles of infection and replication resulting in lysis of the mycobacteria. This can be seen as clear plaques in a lawn of helper cells. According to Mani *et al.,* 2003, the number of plaques generated is directly proportional to the number of viable *M. tuberculosis* cells that were in the original sample[68].

Rifampicin resistant*M. tuberculosis*strains can be detected within 48 hrs using mycobacteriophage-based assays[69]. The number of plaques in a rifampicin-free control is compared with the number of plaques produced from a sample incubated in the presence of rifampicin. A sensitive strain can be observed when there is an absence of plaques from the rifampicin containing sample. In this case the tubercle bacilli are nonviable and cannot support phage replication. The presence of plaques in the rifampicin-containing sample indicates rifampicin resistant strains that are viable and are able to support phage replication. It several studies, the FASTPlaque TB-RIF assay correlated very well with the BACTEC 460 for the detection of rifampicin susceptibility in *M tuberculosis* cultures. In a study conducted by Eltringham*et al.,*1999, the phage assay was compared to the (Real-Time) RT-PCR for the detection of rifampicin resistance[67]. When compared to the resistance ratio method, the phage assay showed a good concordance of 100%, whilst the RT-PCR showed a sensitivity of 97% (35 of 36) for the diagnosis of rifampicin resistant isolates[5]. Currently there is no platform available for the testing of NTM.

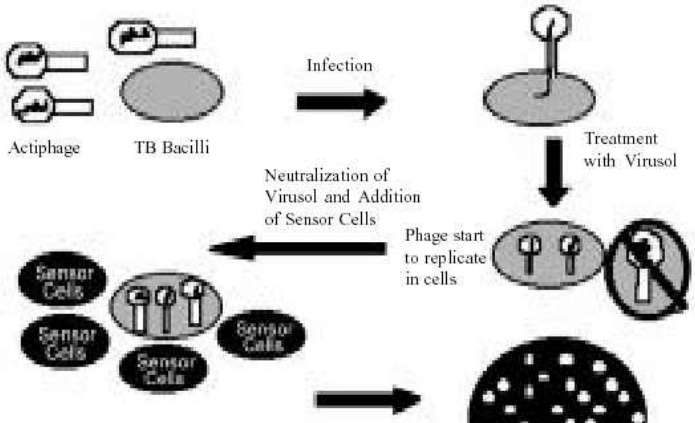

Figure 2.6 FASTPlaque TB-RIF test showing the ability of the viable *M tuberculosis* bacilli to protect infecting mycobacteriophages from chemical inactivation by Virusol treatment and to facilitate the replication of infecting phage[70].

2.7.6 Microplate Assays

Several colorimetric methods have developed for the detection of drug-resistant *M. tuberculosis*. These methods utilize oxidation-reduction indicators for the detection of mycobacterial growth in the presence of antituberculosis drugs[71,72]. Alamar blue, resazurin(Figure 2.7) and tetrazolium bromide are some of the indicators that can be added to microplates containing antibiotics of increasing concentrations and pure cultures of isolates. After 5 to 7 day incubation of the microplate, growths can be recognised as a change in the colour of the indicator due to the reaction of the dye. The minimum inhibitory concentration (MIC) of each drug can be determined by the change of colour in the wells. Since the indicators are added 5-7 days after growth, one of the main concerns with this type of testing is biosafety. The microscopic observation drug susceptibility (MODS) testing is a microplate assay which has less biosafety risk, uses critical drug concentrations and measures growth by microscopic reading[19,72]. According to Palomino *et al*.,2002 the agreement for rifampicin and isoniazid when comparing the MODS assay with the agar proportion method and the alamar blue assay was found to be 100% and 89% respectively[73]. Of note, microplate based assays are limited in use for the testing of NTM.

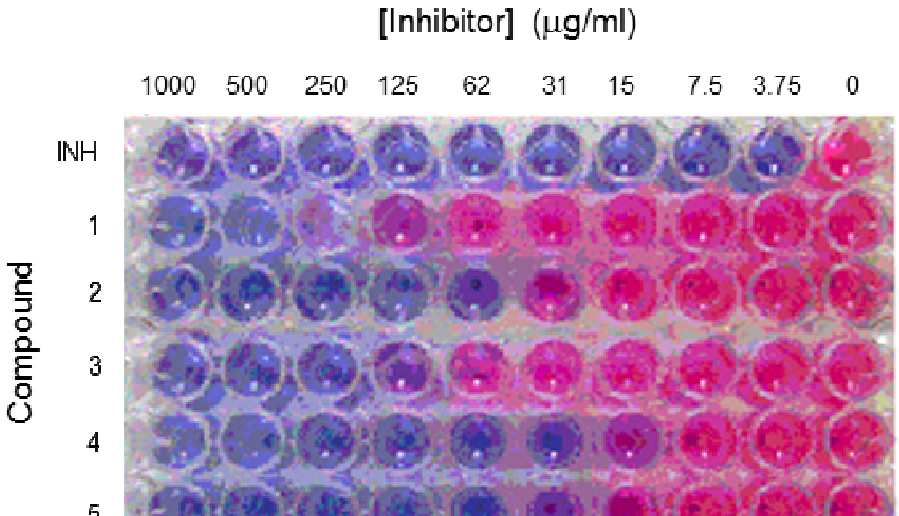

Figure 2.7 Resazurin microplate assay. A change to pink indicates reduction of resazurin and therefore bacterial growth. The MIC is defined as the lowest drug concentration that prevents colour change [73].

2.7.7 Molecular Testing

A number of in-house and commercial available amplification methods have been developed for the detection and identification of mycobacteria directly in the patient's specimen. In the last decade, there have been numerous studies published on the ability of nucleic acid amplification assays (NAAs) to directly detect *M. tuberculosis* in clinical specimens. Of note, the U.S Food and Drug Administration (FDA) have approved some of these commercially available assays for the diagnosis of *M. tuberculosis* complex (MTBC) in clinical specimens. These polymerase chain reaction (PCR) based assays include the amplified *M. tuberculosis* direct test (MTD) (Gen-Probe, Inc., San Diego, California), the AMPLICOR *M. tuberculosis* test (Roche Molecular Systems, Branchburg, N.J) (STC) and the ProbeTec Direct TB which is a strand displacement amplification (SDA) test by Becton-Dickinson[19]. The first molecular tests were introduced in the 1980s for the rapid discrimination of MTBC and NTM (Gen-Probe, San Diego, California, USA) in smear positive sputum samples[12]. The AccuProbe DNA hybridisation assay became available for the identification of most commonly encountered mycobacteria such as MTBC, *M. avium* complex (MAC), *M. avium*, *M. intracellulare*, *M. kansasii* and *M. gordonae*. In KwaZulu-Natal (South Africa), researchers announced the emergence of resistant TB strains not only to isoniazid, and rifampicin but also to at least three classes of second line-drugs[29]. This form of resistant TB became known as extensively drug-resistant tuberculosis (XDR-TB). This has led to an unprecedented level of interest on the development of new molecular tools for rapid diagnosis of *M. tuberculosis*. In 2008, the World Health Organisation approved the Genotype MTBDR Plus assay (Hain-Lifesciences,Nehren, Germany) for the rapid genotypic detection of *M. tuberculosis* and its resistance to isoniazid and rifampicin in smear positive sputum

samples. Subsequent to this, the Gene-xpert (Cepheid, USA) was approved in 2009 for the screening of patients suspected of *M. tuberculosis* disease.

2.7.7.1 Genotype MTBDR Plus, MTBDRsl, CM and AS Assays

A commercially available Genotype MTBDR Plus assay (Hain-Lifesciences, Nehren, Germany) has been developed for the simultaneous and rapid identification of *Mycobacterium tuberculosis* isolates and drug susceptibility testing against rifampicin and isoniazid from smear-positive and smear negative, culture positive clinical specimens. These assays are based on reverse hybridization of a PCR product to a nitrocellulose strip with immobilized probes for different mycobacterial species. The *rpoB, katG,* and *inhA* are the genetic loci targeted in this assay to identify gene mutations. The ability of the assay to determine significant mutations within the *rpoB*gene (coding for the b-subunit of the RNA polymerase) allows for the detection of rifampicin resistance. For high level isoniazid, the katGgene (coding for the catalase peroxidase) is assessed and for testing of low level isoniazid resistance, the promoter region of the *inhA*gene (coding for the NADH enoyl ACP reductase) is assessed for significant mutations[74].

A commercially available Genotype MTBDRsl assay (Hain-Lifesciences, Nehren, Germany) has been developed for the simultaneous rapid identification of *Mycobacterium tuberculosis* isolates and drug susceptibility testing against fluoroquinolones (ofloxacin and moxifloxacin),aminoglycosides/cyclic peptides (capreomycin, viomycin/ kanamycin, amikacin) and ethambutol from smear-positive and smear negative, culture positive clinical specimens. The *gyrA, rrs,* and *embB* are the genetic loci targeted in this assay to identify gene mutations. Resistance to fluoroquinolones, aminoglycosides and ethambutol in *M.tuberculosis*is most frequently associated with mutations in the *gyr*A, *rrs*, and *emb*B genes respectively[75]. Good correlations were observed between the detected mutations and resistance phenotypes. By targeting mutations in codons 90, 91, and 94 in the *gyrA* gene, approximately 70-90% of all fluoroquinolone-resistant strains could be identified[75]. Mutations A1401G, C1402T, and G1484T in the*rrs* gene have been linked to amikacin, capreomycin and kanamycin resistance, with each responsible for a specific resistance pattern[76]. Mutations G1484T and A1401G were found to cause high level resistance to all drugs, whereas C1484T causes resistance to only capreomycin and kanamycin[76]. Mutations at codon *emb*B306 are found in 30% to 68% of ethambutol-resistant clinical strains.

The MTBDRsl strip consists of 3 *gyrA*probes [wild-type 1(WT1), wild-type 2 (WT2), and wild-type 3 (WT3)] which represent the genes encoding amino acids 85 to 97. In addition to this, 6 additional probes (*gyrA*MUT1 A90V, *gyrA* MUT2 S19P, *gyrA* MUT3A D94A, *gyrA* MUT3B D94N/Y, *gyrA* MUT3C D94G,*gyrA*MUT3D D94H) target the most common mutations[76]. Both the wild type (WT) and mutant (MUT) areas on the strip allow for fluroquinolone resistance to be detected. Amikacin/ Kanamycin resistance are detected by the presence of four probes which cover the wild type region of *rrs and* the most common mutations. These are the *rrs*MUT1 and MUT2 which represent the amino acid changes A1401G and G1484T respectively. For ethambutol resistance wild type probe*embB* (WT1) targets the *embB*306 codon, while embB MUT1A and 1B are available to detect nucleotide exchanges ATG-ATA (M306I) and ATG-GTG (M306V). The omission of a signal for the wild type probe and/or the presence of a signal for the mutant probe will indicate the presence of a resistant strain. A negative control containing no DNA template is included with each run.

The simultaneous execution of the GenoType Mycobacterium CM/AS assays (CM, common mycobacteria; AS, additional species), allows for the simultaneous identification of a variety of mycobacterial species by targeting the 23S rRNA gene[77,78]. Relevant *M.tuberculosis* complex can be distinguished from the members of the *M. avium* complex, *M. kansasii*, and *M. chelonae*. The Additional Species (AS) strip allows for the identification of less frequent mycobacteria namely *M. simiae*, *M. mucogenicum*, and *M. celatum.* The combined use of the CM and AS assays allows for the identification of 35 different mycobacterial patterns of which 23 represent single mycobacterial species, 8 patterns represent mixed mycobacterial species and the latter 4 patterns represent *Mycobacterium* species and gram-positive bacteria with a high GC content[77].

Another region of interest is the 16S ribosomal DNA (rDNA) which codes for a 1,500 nucleotide sequence known as the 16S rRNA[12]. This is a highly conserved gene common to all mycobacterial organisms. It consists of a conserved region and regions with nucleotide variations (variable regions). Mycobacterial identification is based on two hypervariable sequences known as regions A and B. The introduction of 16S DNA sequencing has improved on the identification of mycobacteria. Identification not only became less time consuming, but also much more robust. NTM species identification of organisms such as *M. kansasii* and *M. gastri* as well as *M.ulcerans* and *M.marinum* is based on region A. Sequence of region B is only considered when identification based on Region B is not conclusive. For some isolates such as *M.chelonae* and *M.abscessus* differentiation cannot be accomplished within regions A and B even though their sequences vary by 4bp at other 16S rRNA[79].

Overall, several molecular based methods have been proposed for the identification of NTM. These include the DNA probes or Accuprobe (GenProbe, San Diego,CA), sequencing of housekeeping genes, polymerase chain reaction restriction analysis based methods on 16S rRNA, *rpoB, recA*, 16S-23S rRNA internal transcribed spacer*, hsp65* and *tuf*genes[38]. Routine molecular identification of mycobacteria using the above methods is simply based on single genetic traits. *Hsp65* PRA methods have shown to be rapid and reliable for species identification[38]. However with the emergence and divergence of new species and sub-species, 16S rRNA typing may not be able to discriminate species with highly similar 16S rRNA gene sequence. *M. szulgai*and *M.*malmoenseare two distinct specieseven though they differ by two nucleotides. Molecular methods may not allow for an accurate discrimination of closely related species. On the other hand these methods are costly and cannot be implemented in resource poor laboratory settings often encountered in South Africa.

Genotype PCR testing (Hain-Lifesciences, Nehren, Germany) consists of DNA isolation either from cultured material (culture plates/liquid medium) or direct materials (pulmonary, smear-positive, and decontaminated), multiplex amplification with biotinylated primers and reverse line hybridisation[75,77]. The hybridization process consists of chemical denaturation of the amplification products, hybridization of the single-stranded, biotin-labelled amplicons to membrane-bound probes, stringent washing, addition of a streptavidin/alkaline phosphatase (AP) conjugate, and an AP mediated staining reaction. An interpretation template ensures the easy and fast interpretation of the banding pattern obtained.

Hybridization is initiated by denaturing the amplified double stranded DNA into single strands using 20ul of denaturing solution. Single stranded DNA is hybridized to membrane bound probes for 30min at 45^0C using pre-warmed hybridisation solution. Unbound amplicons are removed using a stringent wash solution. Several rinse solutions are used for washing off amplicons at 25^0C. Hybridised amplicons are chromogenically visualised using streptavidin conjugated with an alkaline phosphatase-biotin and a substrate buffer is added. At least two wash steps are required to stop the reaction.

2.7.7.2 Gene-Xpert

With the growing evidence of the MDR-TB epidemic, nucleic acid amplification tests such has the Gene-Xpert have had great promise in TB diagnosis and for the rapid detection of drug resistance.The GeneXpert MTB/RIF assay (Cepheid, USA) is a fully automated, cartridge based, hemi-nested real time-PCR systemand can diagnose *M.tuberculosis* and

rifampicin (RIF) resistance simultaneously using unprocessed clinical specimens[80]. Results are available within 2 hours, regardless of the sputum smear status. The molecular beacons are designed to only hybridize correctly with amplified wild type (RIF sensitive) *rpoB* sequences[81].Mutations in the 81 base pair (bp) region interferes in 2 ways with hybridization of these beacons; there is either delayed onset/partial inhibition or complete suppression of fluorescence in the corresponding molecular beacon.Rifampicin resistance is particularly suited to rapid molecular detection since 95% of all RIF resistant strains have mutations in 81 bp region of the*rpoB* gene which encodes for the active site of bacterial RNA polymerase[81,82].The*rpoB* core region is flanked by MTB specific DNA sequences, thus it is possible to test for MTB and RIF resistance together by targeting a single amplicon generated using PCR technology[83]. It has been noted by previous studies that mutations in the 81 bp region of the *rpoB* gene are "highly" predictive of RIF resistance whereas susceptible isolates almost always have wild type nucleotide sequence[81].RIF resistance is strongly but not invariably indicative of MDR-TB. RIF mono-resistance is rare; 85-90 % of RIF strains are resistant to INH (isoniazid) as well[81].The sensitivity for detection of isoniazid resistance was 85% and 98% for the detection of RIF resistance[81].Thus, the *rpoB* gene is a better molecular target for diagnosing TB and drug resistance. The assay has advantages over the line probe assay in that it is not technologically demanding and it is conducted in a simple almost fully automated cartridge based system. This reduces cross contamination and decreases the risk of false positives as seen in other PCR based technologies. The simplicity for the operator could make the assay feasible to be implemented outside centralized laboratories and this could potentially impact on TB control.

However, recent studies have come to light which questions the reliability of the GeneXpert MTB/RIF assay's diagnostic ability. GeneXpert cannot be used as an MDR diagnostic tool since it is only used in the detection of RIF resistance and there are no means of detecting INH resistance.Line Probe Assay (LPA) has an advantage over the GeneXpert assay in that it detects both RIF and INH gene mutations. Therefore, this assay can accurately diagnose MDR-TB.Discrepancies between the GeneXpert and DST (Drug Susceptibility Testing) results regarding RIF resistance (false resistance) has been previously reported[84].Several studies have detected false positive RIF resistance results (over diagnosis).The presence of any mutation in the 81 bp region will be interpreted as the isolate being RIF resistant, irrespective if there are no sign of phenotypic resistance[84]. Several studies have shown false negative results by the GeneXpert (under diagnosis). Mutations that lie outside of the 81

bp region (5-10%) will not be identified by GeneXpert, leading to the isolate being interpreted as RIF susceptible[84].

2.7.7.3 IS6110-Based Methods

The IS6110 is a transposable element and is the most widely used repeated sequence in strain identification methods. IS6110 is a 1.365-bp insertion sequence (IS) with a defined 28-bp imperfect inverted repeat at the ends and a single *PvuII* restriction site. The IS contains an open reading frame (ORF) that codes for a transposase and is limited to the *M. tuberculosis* complex[19]. It has shown great potential for use in strain differentiation. On *Pvu*II digestion of the chromosomal DNA, the IS6110 element is split into two domains, each attached to their respective adjacent genomic segment [43]. The restricted *Pvu*II DNA is then electrophoretically separated on an agarose gel, Southern transferred to a nylon membrane and hybridised with a labelled probe complementary to the 3'-domain of the IS6110 [19,43]. Autoradiography is used to visualise the banding patterns and measure the number of IS6110 elements in their genome and their distance from their adjacent chromosomal 3' *Pvu*II restriction sites. The results obtained by testing a large number of strains in different settings allows for the understanding of the distribution and spread of *M.tuberculosis* infection during an outbreak. This allows for strains from different geographic areas to be compared and the movement of individual strains to be traced [85]. Such data may provide important insights into the global transmission of tuberculosis and identify strains with particular properties, such as high infectivity, high virulence, and/or multidrug resistance

Although IS6110 genotyping methods shows the greatest discriminatory power and is regarded as the 'gold standard' for *M.tuberculosis* genotyping, it is cumbersome and time-consuming technique, requiring large quantities of good quality DNA. Furthermore the discrimination power of the technique is dependent on the number of IS6110 elements present in the genome of the *M. tuberculosis* isolate. As a result more than six IS6110 elements need to be present within the genome inorder for an epidemiological relationship to be inferred. Classification of strains with fewer copies requires secondary typing methods.

2.7.7.4 Direct-Repeat Locus-Based Methods

Based on the initial genetic analysis of the *M.bovis* BCG (BacilleCalmette-Guerin), it was later shown that the DR locus is a single region of the *M. tuberculosis* chromosome that contains multiple copies of a 36-bp direct repeat region that is reiterated between 10 and 50 times[43] (Figure 7). One DR and its neighbour spacer sequence are termed a direct variable repeat (DVR). Even though the overall arrangement of the spacers in the DR is conserved among strains, polymorphisms in this region arise from deletion/insertion of segments of the DR locus. A copy of the IS6110 is inserted into a specific site in one of the 36-bp repeats in all of the *M.bovis* (BCG) strains, *M.bovis* strains and *M.tuberculosis* strains. Strains of *M. tuberculosis* that lack IS6110 contain the DR locus. Spacer oligonucleotide typing (Spoligotyping) involves amplification of the DR locus by PCR. The specific primers (biotinylated) used are complementary to the DR sequence and directed outwards from the 36-bp repeat, successfully amplifying the variable spacers and repeats present in a particular strain. Following amplification of the DR locus, the labelled PCR products are hybridised to a membrane containing the corresponding covalently bound oligonucleotides of the 43 variable-spacer sequences present in *M.tuberculosis* strain H37Rv and *M.bovis*. Strains share various combinations of the variable spacers and are distinguished on the basis of their positive and negative signals for each spacer[19]. After performing spoligotyping on clinical samples and cultured isolates, results generated that display the absence of 5 spacers (numbers 38 to 43) enables *M.bovis* species to be determined. One of the limitations of spoligotyping is that polymorphisms in the DR locus do not discriminate *M.tuberculosis*strains as well as RFLP of the IS6110. Stains with similar spoligotyping pattern may have different IS6110 patterns[19] . Another limitation of spoligotyping, is that it measures a small number of polymorphisms at a single genetic locus[19].

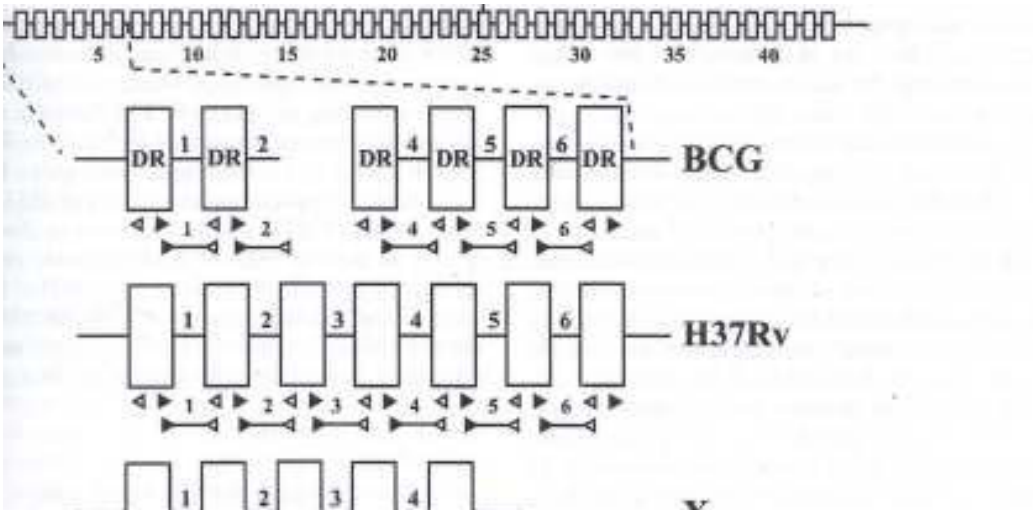

Figure 2.8 The top section shows the 43 DR (rectangles) and spacers (horizontal lines) used in spoligotyping. The bottom section shows the spoligotypes of three strains. DR locus contains 10 to 50 copies of a 36-bp direct repeat, separated by spacer DNA with various sequences, each of which is 37 to 41bp. A copy of the IS6110 is inserted within a 36-bp DR in the middle of the DR locus in most strains. *M.tuberculosis* strains have the same overall arrangement of spacers but differ in terms of the presence or absence of specific spacers [86].

2.8 MASS SPECTROMETRY

For the first time in 1897, a pioneering scientist JJ Thompson was able to measure the mass of an electrically charged particle known as an electron[87]. In his experiment the potential to separate charged particles based on a mass to charge ratio was demonstrated. Since the development of 'Soft Laser Desorption' (SLD) technology in 1987 by Koichi Tanaka and John Bennett, subsequent improvements were made by Franz Hillenkamp and Michael Krans[88] through the introduction of Matrix-Assisted Laser Desorption/Ionization (MALDI) [89]. The mass-to-charge (m/z) ratio of an electrically charged molecular or ion can be measured using a mass spectrometer[90]. The mass unit of measurement was expressed as Daltons (Da) and the magnitude of charge of the electron is indicated by the integer z. Therefore the mass to charge ratio (m/z) represents a Dalton per unit of charge of an ion. The fundamental components of a mass spectrometer consist of the following (Figure 2.8):

a) An ion source

b) A mass analyser

c) A detector

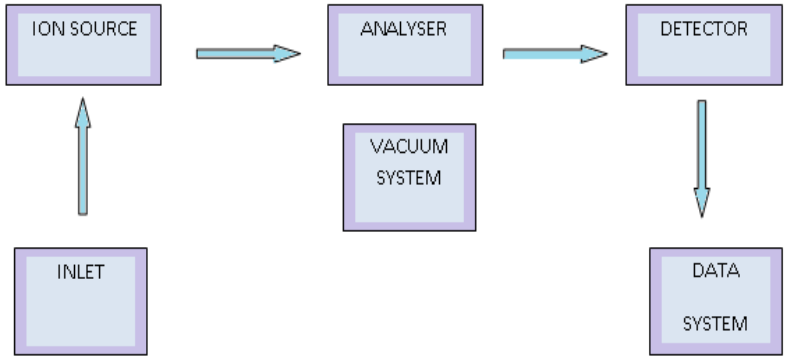

Figure 2.9 Fundamental components of a mass spectrometer[90].

The ion source allows for the vaporisation, ionisation and acceleration of sample analytes. Prior to analyses the samples are converted into the gas phase during the process of vaporisation. In conventional mass spectrometry vaporisation can be achieved through the process of simple heating. Ionisation on the other hand is accomplished by the use of 70eV electrons[90]. Both processes allow for the decomposition of the analyte. The ions are then separated according to their mass-to-charge (m/z) ratio by the mass analyser. Gas phase ions produced in the ion source are directed by either an electric or magnetic field into the mass analyser. As the ions pass through an electromagnetic field, separation to their mass to charge ratio is achieved through the presence of filters. In order to prevent collision or interference of ions with ubiquitous air molecules, ions move through a vacuum of 10^{-4}torr or less[91].

However of concern is that the process of ionization by electrons accelerated through a potential of 70eV leads to possible fragmentation of the ion, leaving virtually little or no trace

of the parent ion[92]. Since mass spectrometry may have be carried out on parent ions rather than daughter ion species, the use of conventional mass spectrometry with excessive fragmentation from a 'hard' ionization process may not be conducive for the analysis of large biological molecules. The availability of a more 'softer' sample vaporisation and ionisation methods present in modern mass spectrometry has allowed for the analysis of compounds of biological origins (parent ions), ones with limited volatility and thermostability[91]. Modern mass spectrometry approaches such as the Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS) provides a non-equilibrium energy transfer that can eject (desorb) samples into the gas phase in the absence of heating and utilise a low energy proton transfer for a 'softer' ionisation[90].

There are various types of ionisation methods available. For a 'softer' method, Matrix Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF MS), Electronspray (ESI) andChemical Ionization (CI) are available for ionisation of relatively small volatile molecules, peptides, proteins and nucleotides[90]. The mass range that can be detected with these approaches include up to 0.5 kDa, 1 kDa and 200 kDa for MALDI-TOF MS, CI and ESI respectively[90]. A Fast Atom Bombardment (FAB) approach also offers a softer ionisation method however ionisation is harder than MALDI-TOF. Electron Impact (EI) offers a hard ionisation method for relatively small volatile molecules, up to 1 kDa. There are also various types of mass analysers that use magnetic/electric fields and dispersion/filtering for the separation of ions according to their mass-to-charge ratio[92].

Commonly used mass spectral analysers include:

a) Magnetic Sector Analysers: Employs magnetic or an electric field to accelerate the charged ions. Ions of larger *m/z* are less deflected than ions of smaller *m/z* values resulting in dispersion of these ions in space. Changes of the magnetic field strength allow for a change in the trajectories of the bundles of ions. Ions separate according to their mass to charge ratio as they bend through the mass analyser [90].

b) Time-of-flight Mass Analysers: Similarly charged ions emitted and accelerated in a brief burst (pulsed) travel with the same kinetic energy through a Time-of-flight tube

and their travelling time is dependent on their different m/z ratios. According to Frieden, *et al*.,2003 the ions separate according to their different molecular weight sizes with the smaller ions (m/z values) arriving first at the detector (83).

c) A quadrupole mass filter: Applying a simultaneous constant direct current (dc) and radio frequency (rf) electric fields, mass sorting based on ion motions is achieved. Any changes in field strength such as potential difference allow ions of different *m/z* to be scanned through the mass analyser and transmitted to the detector [93].

d) Fourier Transform Ion Cyclotron Resonance (FR-ICR) Spectrometer: Molecular weight is measured through the detection of the image current produced by the accelerating ions in a magnetic field.

e) Reflectron mass spectrometry analyser: Mass analyser uses the static electric field to reverse the ions in the opposite direction [93]. The ions are reflected back towards the source thereby increasing the maximum achievable resolving power by a factor of 2 [90].

As mentioned earlier, the third basic component of a mass spectrometry is the detector. The detector precisely measures the current released when an ion passes by or hits a surface. The mass-to-charge ratio of the ions allows for the generation of mass spectra which can be visualised on the computerised system. The various types of detectors used in mass spectrometry include electron multipliers, Faraday cups, ions-to-photon detectors and micro channel plate detector (MCP)[90]. All mass spectrometers are connected to computer based systems for the control, acquisition, storage and presentation of data. Computer-based software is available for the quantitation, spectral processing, interpretation and organism identification using spectral libraries.

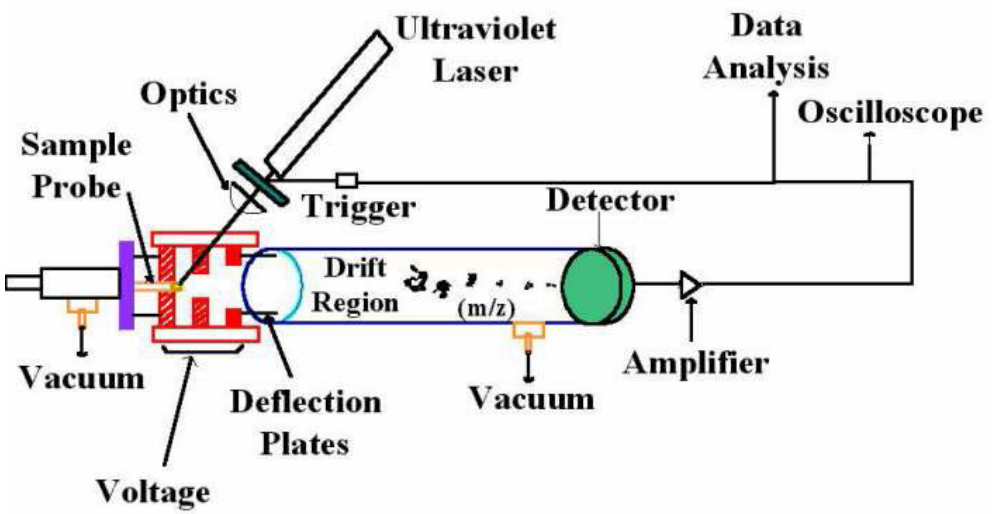

Figure 2.10 Schematic representation of the components of mass spectrometry[90].

In a MALDI-TOF MS system (Figure 2.10) both vaporisation and ionisation can be performed simultaneously in a single step. The use of soft ionisation method offered by MALDI-TOF MS allows for fragile bio-molecules to be analysed without any fragmentation or decomposition of these molecules[90,94]. A soft non-destructive ionisation process prevents fragmentation and the loss of spectra quality primarily due to a decline in resolution quality and complete loss of parent ion[89,95]. The conversion of solid phase sample analytes into gas phase ions allows for a softer non-destructive ionisation with minimal fragmentation[96]. Heat induced sample evaporation or vaporisation typically associated with conventional mass spectrometry differs from modern mass spectrometry. Unlike traditional mass spectrometry systems that utilise Electron Bombardment (EB) and Electron Impact (EI) for ionisation, MALDI-TOF MS on the other hand allows for samples in a high vacuum (10^{-6}torr) to sublime directly into the ion source with minimal heating[90]. Furthermore, ionisation within a MALDI-TOF MS utilises a soft non-destructive ionisation method suitable for the analysis of polar molecules such as proteins and peptides (Figure 2.11) from bacteria and yeast[90]. This is an added advantage over a conventional ionisation approach that can identify smaller organic molecules (<1kDa). MALDI-TOF MS can be utilised directly on bacterial cells for a reliable species-specific identification[90].

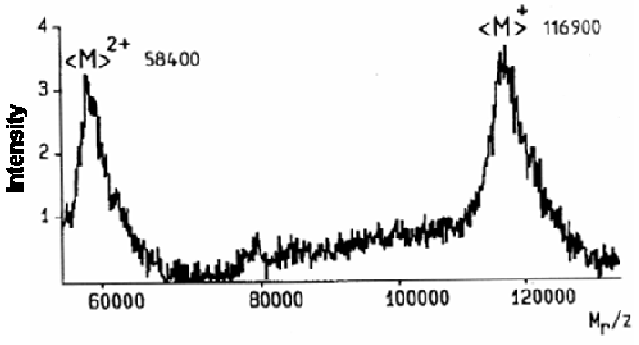

Figure 2.11 The first massspectrum of high molecular weight biomolecules[89].

Prior to sample analysis, the sample analyte is mixed in a matrix solution. The matrix solution is a weak acid with an aromatic ring structure and a carboxylic moiety. It absorbs at a wavelength of the laser resulting in a strong interaction between the matrix solution and the sample analyte. The matrix serves to protect the analyte from excessive energy and it enhances the ion formation of the analyte without reacting with it prior to laser irradiation[90]. Commonly used matrix solutions include 2.5-dihydroxy-benzoic acid (DHBA), sinnapinic acid (SA) and α-cyano-4 hydroxy-cinnamic acid (HCCA). During the sample preparation process, the sample (1ul) is spotted onto the MALDI-TOF ground steel target plate and then allowed to air dry. The spot is then overlaid with approximately 1ul of the matrix solution. The spots are then allowed to air dry and the plate is inserted into the MALDI-TOF ion source. In the ion source, the ions are emitted in a short laser pulse (brief burst). The irradiated becomes rapidly heated and vibrational. The accelerated ions attains a mass dependent velocity and moves from the ion source into the 'drift' or 'field free' tube where there different velocities (based on m/z) differentiate them as they move towards the detector (Figure 2.12)[90]. The laser energy absorbed by the matrix combined with the vibrational energy results in an explosion of the material in a plume just above the irradiated site. A high vacuum allows for desorption in the expanding plume of matrix compound 'carrying' the analyte in the gas phase with minimal heating of the sample. In this manner intact bacterial proteins can be converted into the gas phase without any fragmentation or decomposition that is typical of conventional heat induced evaporation associated with traditional mass spectrometry systems. An acid base-equilibrium allows for protons from the carboxylic acid groups to be attracted to the proteins or polar analytes.

In a linear TOF analyser, in the detector region, the mass-separated ions are counted giving rise to a signal for each mass-to-charge (m/z) that is equivalent to the number of ions present[90]. The TOF reflectron analyser reflects the ions back towards the source increasing the length of time. This approach has shown to increase the resolution power by a factor of 2[90]. When it comes to the identification and characterization of bacteria, the TOF analyser is the more preferred mass analyser. The added advantage includes sensitivity, high mass range and speed. A high sensitivity is due to the ability of the analyser to detect all ions of 'like charges' in a short time. Other analysers such as the ion trap analysers simply discard

ions with non-selected mass-to-charge values resulting in a reduced sensitivity. Ions up to and above 300kDa can be detected allowing for a high mass range. The TOF analyser is able to generate individual spectra in less than a second.

Figure 2.12 Accelerated ions with a mass dependent velocity travel from the ion source into the 'drift' or 'field free' tube. There different velocities (based on m/z) differentiate them as they move towards the detector[90].

2.8.1 MALDI-TOF MS in Bacteriology

Since the development of the MALDI-TOF MS in the 1980's, it has been widely used for the analysis of proteins, nucleic acids, synthetic polymers and sugars[97]. Most of its applications were initially confined to areas of analytical chemistry, biotechnology and pharmaceuticals. Recently, a number of studies have demonstrated the potential of this application in the field of bacterial taxonomy [98,99]and for the analysis of macromolecules from biological origins[97]. When compared to previous mass spectrometry systems, MALDI-TOF MS has shown to have good resolution and mass accuracy for the detection of ions from macromolecules of biological origin in spite of the presence of small amounts of salts and other agents that can interfere with the mass spectrometric analysis[100].

The MALDI-TOF MS approach is currently considered as an attractive tool for the rapid characterisation of bacteria and other microorganisms to the genus and species level. It has recently emerged as a method of choice for the rapid discrimination of microorganisms. It is used for the analyses of proteins of a bacterial cell thus allowing for reliable species-specific

identification. Microbial strain typing is based on the unique proteomic profile of a particular organism by assessing the exact size of important constituents such as peptides and proteins that characterise a bacterial species. The 'soft' ionisation method allows for the desorption of proteins from either whole cells or cellular extracts of bacteria, spores, viruses and fungi allowing for the generation of a proteomic profile of the microorganism of interest. A "Whole cell" analysis involves the analyses of cells that have not been chemically treated or mechanically disrupted for the removal or isolation of cellular components. This form of analysis allows for a rapid taxonomic classification of bacteria and its proven efficiency with reproducible spectral quality has made it an attractive tool in the field of diagnostic microbiology[90,101]. The pre analysis cellular extraction method is a more preferred method when it comes to the analysis of infectious microorganisms such as mycobacteria[102]. It renders the organism non-viable and safe for analysis by the MALDI-TOF MS. The identification of microorganism particularly mycobacteria using mass spectra fingerprints is dependent on the reproducibility of the spectra. Therefore pre analysis sample preparation methods prior to MALDI analysis greatly influences the quality and reproducibility of the spectra. This study is one of few studies undertaken to optimize a sample preparation method for the discrimination of Mycobacteria using MALDI-TOF MS analysis.

2.8.2 MALDI-TOF MS in Mycobacteriology

The use of MALDI-TOF MS in the field of diagnostic mycobacteriology is relatively new. It has been used extensively for the identification of bacteria and fungi from whole cells and cellular extracts[103]. Hettick*et al.,* 2004 conducted one of the first few studies aimed at looking at the identification of a number of unique biomarkers as targets for protein identification by MS/MS experiments[104].

Of relevance is work conducted by Pignone*et al.* looking at 37 mycobacterial strains representing thirteen species and five subspecies that included the MTBCand the MACcomplex, as well as rapid- and slow-growing mycobacteria[1]. This method produced species-specific patterns for all but 1 of the 37 isolates and provided reliable differentiation at the strain level. This study has shown the potential of the MALDI-TOF MS as a rapid and reproducible method for the identification and characterization of *Mycobacterium* species using whole-cells.

To the best of our knowledge, very few studies have been conducted to discriminate *mycobacteria* to a strain level from cellular extracts using MALDI-TOF MS. Of note, the cellular extraction of proteins from pathogenic organisms is imperative so as to prevent disease transmission to the operator. Furthermore, the lack of a local mycobacterial database has been a limiting factor for mycobacterial identification, however; it can be supplemented by increasing the entries of mass spectra in the database. The current study is aimed at optimising a sample preparation protocol for the discrimination of mycobacteria to a species-specific level using MALDI-TOF MS.

2.9 REFERENCES

1. 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.

2. Pfyffer GE, Murray P, Baron E, Jorgensen J, Landry M, et al. (2006) Mycobacterium: general characteristics, laboratory detection, and staining procedures. Manual of Clinical Microbiology: Volume 1: 543-572.

3. Reller LB, Weinstein MP, Hale YM, Pfyffer GE, Salfinger M (2001) Laboratory diagnosis of mycobacterial infections: new tools and lessons learned. Clinical Infectious Diseases 33: 834-846.

4. Winn WC, Koneman EW (2006) Koneman's color atlas and textbook of diagnostic microbiology: Lippincott Williams & Wilkins.

5. Schlossberg D (2011) Tuberculosis and nontuberculous mycobacterial infections: ASM Press.

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

7. Buijtels PCAM (2007) Clinical relevance of non-tuberculous mycobacteria in Zambia: Erasmus MC: University Medical Center Rotterdam.

8. Butler WR, Guthertz LS (2001) Mycolic acid analysis by high-performance liquid chromatography for identification of Mycobacterium species. Clinical Microbiology Reviews 14: 704-726.

9. Shinnick T, Good R (1994) Mycobacterial taxonomy. European Journal of Clinical Microbiology and Infectious Diseases 13: 884-901.

10. Dawson DJ (2000) Mycobacterial terminology. Journal of Clinical Microbiology 38: 3913-3913.

11. Youmans G, Paterson P, Sommers H (1985) The biological and clinical basis of infectious diseases. Saunders, Philadelphia, PA.

12. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, et al. (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine 175: 367-416.

13. Drobniewski F, Caws M, Gibson A, Young D (2003) Modern laboratory diagnosis of tuberculosis. The Lancet Infectious Diseases 3: 141-147.

14. Ryan KJ, Ray CG (2010) Sherris medical microbiology: McGraw Hill Medical New York.

15. Brennan PJ, Nikaido H (1995) The envelope of mycobacteria. Annual Review of Biochemistry 64: 29-63.

16. Gangadharam P. Microbiology of nontuberculosis mycobacteria; 1996. pp. 231-243.

17. Schaechter M, Medoff G, Eisenstein BI (1993) Mechanisms of microbial disease: Lippincott Williams & Wilkins.

18. Kaufmann SH, Britton WJ (2008) Handbook of tuberculosis: Wiley-VCH.

19. Cole ST, Eisenach KD, McMurray DN, Jacobs Jr W (2005) Tuberculosis and the tubercle bacillus: ASM Press.

20. Liu J, Rosenberg EY, Nikaido H (1995) Fluidity of the lipid domain of cell wall from Mycobacterium chelonae. Proceedings of the National Academy of Sciences 92: 11254-11258.

21. Saito R, Tanaka A, Sugiyama K, Azuma I, Yamamura Y (1976) Adjuvant effect of cord factor, a mycobacterial lipid. Infection and immunity 13: 776-781.

22. Kaiser G (2006) The prokaryotic cell: bacteria. Sizes, shapes, and arrangements of bacteria. Doc Kaiser's Microbiology

23. Organization WH (2012) Global tuberculosis report 2012. Geneva, Switzerland: World Moreno S, Jarrin I, Iribarren JA, et al Incidence and risk factors for tuberculosis in HIV-Guyatt GH, Oxman AD, Vist GE, et al, GRADE: an emerging consensus on rating quality of 0 Guyatt GH, Oxman AD, Kunz R, et al, Incorporating considerations of resources use into.

24. Wood R, Lawn SD, Johnstone-Robertson S, Bekker L-G (2011) Tuberculosis control has failed in South Africa: time to reappraise strategy. South African Medical Journal 101: 111-114.

25. Donald PR, Van Helden PD (2009) The global burden of tuberculosis—combating drug resistance in difficult times. New England Journal of Medicine 360: 2393-2395.

26. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Archives of internal medicine 163: 1009-1021.

27. Brust JC, Gandhi NR, Carrara H, Osburn G, Padayatchi N (2010) High Treatment Failure and Default Rates for Patients with MDR TB in KwaZulu-Natal, South Africa, 2000–2003. The International Journal of Tuberculosis and Lung disease 14: 413.

28. Gandhi NR, Andrews JR, Brust J, Montreuil R, Weissman D, et al. (2012) Risk Factors for Mortality among MDR-and XDR-TB Patients in a High HIV-Prevalence Setting. The International Journal of Tuberculosis and Lung Disease 16: 90.

29. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, 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. The Lancet 368: 1575-1580.

30. Steingrube VA, Gibson JL, Brown BA, Zhang Y, Wilson RW, et al. (1995) PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. Journal of Clinical Microbiology 33: 149-153.

31. Corbett EL, CHURCHYARD GJ, Hay M, Herselman P, Clayton T, et al. (1999) The impact of HIV infection on Mycobacterium kansasii disease in South African gold miners. American Journal of Respiratory and Critical Care Medicine 160: 10-14.

32. Collins C, Grange J, Yates M (1984) Mycobacteria in water. Journal of Applied Bacteriology 57: 193-211.

33. Jarzembowski JA, Young MB (2008) Nontuberculous mycobacterial infections. Archives of Pathology & Laboratory Medicine 132: 1333-1341.

34. Wilson BA, Salyers AA, Whitt DD, Winkler ME (2011) Bacterial pathogenesis: a molecular approach: American Society for Microbiology (ASM).

35. Schaechter M, Engleberg NC, DiRita VJ, Dermody TS (2012) Schaechter's mechanisms of microbial disease: Lippincott Williams & Wilkins.

36. Arasteh K, Cordes C, Ewers M, Simon V, Dietz E, et al. (2000) HIV-related nontuberculous mycobacterial infection: incidence, survival analysis and associated risk factors. European Journal of Medical Research 5: 424-430.

37. Brown-Elliott BA, Griffith DE, Wallace Jr RJ (2002) Newly described or emerging human species of nontuberculous mycobacteria. Infectious Disease Clinics of North America 16: 187-220.

38. Chemlal K, Portaels F (2003) Molecular diagnosis of nontuberculous mycobacteria. Current Opinion in Infectious Diseases 16: 77-83.

39. Contreras M, Cheung O, Sanders D, Goldstein R (1988) Pulmonary Infection with Nontuberculous Mycobacteria1, 2.

40. Olivier KN, Weber DJ, Wallace Jr RJ, Faiz AR, Lee J-H, et al. (2003) Nontuberculous mycobacteria: I: multicenter prevalence study in cystic fibrosis. American Journal of Respiratory and Critical Care Medicine 167: 828-834.

41. Buijtels PC, van der Sande MA, de Graaff CS, Parkinson S, Verbrugh HA, et al. (2009) Nontuberculous mycobacteria, zambia. Emerging Infectious Diseases 15: 242.

42. Campbell IA, Bah-Sow O (2006) Pulmonary tuberculosis: diagnosis and treatment. British Medical Journal 332: 1194.

43. Kaufmann SH, Van Helden P, Alcantara F (2008) Handbook of Tuberculosis: Clinics, Diagnostics, Therapy and Epidemiology: Wiley-VCH Weinheim, Germany.

44. Campbell I, Drobniewski F, Novelli V, Ormerod P, Pozniak A (2000) Management of opportunist mycobacterial infections: Joint Tuberculosis Committee guidelines 1999. Thorax 55: 210-218.

45. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. Journal of Clinical Microbiology 41: 1010-1015.

46. Falkinham 3rd J (1996) Epidemiology of infection by nontuberculous mycobacteria. Clinical Microbiology Reviews 9: 177.

47. Doucet-Populaire F, Buriankova K, Weiser J, Pernodet J (2002) Natural and acquired macrolide resistance in mycobacteria. Current Drug Targets-Infectious Disorders 2: 355-370.

48. Brown B, Wallace R, Mandell G, Bennett J, Dolin R (2000) Infections due to nontuberculous mycobacteria. Principles and Practice of Infectious Diseases, 5th ed Phila: Churchill Livingstone: 2630-2636.

49. Imperiale B, Zumárraga M, Gioffré A, Di Giulio B, Cataldi A, et al. (2011) Disease caused by non-tuberculous mycobacteria: diagnostic procedures and treatment evaluation in the North of Buenos Aires Province. Revista Argentina de Microbiología 44: 3-9.

50. Blanchard JS (1996) Molecular mechanisms of drug resistance in Mycobacterium tuberculosis. Annual Review of Biochemistry 65: 215-239.

51. Bastian I, Portaels F (2000) Multidrug-resistant tuberculosis: Springer.

52. Morris S, Bai GH, Suffys P, Portillo-Gomez L, Fairchok M, et al. (1995) Molecular mechanisms of multiple drug resistance in clinical isolates of Mycobacterium tuberculosis. Journal of Infectious Diseases 171: 954-960.

53. Nolan CM, Williams DL, Cave MD, Eisenach KD, El-Hajj H, et al. (1995) Evolution of rifampin resistance in human immunodeficiency virus-associated tuberculosis. American Journal of Respiratory and Critical Care Medicine 152: 1067-1071.

54. Ormerod L (2005) Multidrug-resistant tuberculosis (MDR-TB): epidemiology, prevention and treatment. British Medical Bulletin 73: 17-24.

55. Nash KA (2003) Intrinsic macrolide resistance in Mycobacterium smegmatis is conferred by a novel erm gene, erm (38). Antimicrobial Agents and Chemotherapy 47: 3053-3060.

56. Nash KA, Zhang Y, Brown-Elliott BA, Wallace RJ (2005) Molecular basis of intrinsic macrolide resistance in clinical isolates of Mycobacterium fortuitum. Journal of Antimicrobial Chemotherapy 55: 170-177.

57. 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< i> M. tuberculosis</i>. Journal of Microbiological Methods 88: 122-126.

58. Dowdy DW, Lourenço MC, Cavalcante SC, Saraceni V, King B, et al. (2008) Impact and cost-effectiveness of culture for diagnosis of tuberculosis in HIV-infected Brazilian adults. PLoS One 3: e4057.

59. Warren R, de Kock M, Engelke E, Myburgh R, van Pittius NG, et al. (2006) Safe Mycobacterium tuberculosis DNA extraction method that does not compromise integrity. Journal of Clinical Microbiology 44: 254-256.

60. Ahmad S, Mokaddas E (2009) Recent advances in the diagnosis and treatment of multidrug-resistant tuberculosis. Respiratory Medicine 103: 1777-1790.

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

62. Cruciani M, Scarparo C, Malena M, Bosco O, Serpelloni G, et al. (2004) Meta-analysis of BACTEC MGIT 960 and BACTEC 460 TB, with or without solid media, for detection of mycobacteria. Journal of Clinical Microbiology 42: 2321-2325.

63. Heifets L, Cangelosi G (1999) Drug susceptibility testing of Mycobacterium tuberculosis: a neglected problem at the turn of the century [State of the Art]. The International Journal of Tuberculosis and Lung Disease 3: 564-581.

64. Reller LB, Weinstein MP, Woods GL (2000) Susceptibility testing for mycobacteria. Clinical Infectious Diseases 31: 1209-1215.

65. McNerney R, Traore H (2005) Mycobacteriophage and their application to disease control. Journal of Applied Microbiology 99: 223-233.

66. Gali N, Dominguez J, Blanco S, Prat C, Quesada M, et al. (2003) Utility of an in-house mycobacteriophage-based assay for rapid detection of rifampin resistance in Mycobacterium tuberculosis clinical isolates. Journal of Clinical Microbiology 41: 2647-2649.

67. Eltringham I, Wilson S, Drobniewski F (1999) Evaluation of a bacteriophage-based assay (phage amplified biologically assay) as a rapid screen for resistance to isoniazid, ethambutol, streptomycin, pyrazinamide, and ciprofloxacin among clinical isolates of Mycobacterium tuberculosis. Journal of Clinical Microbiology 37: 3528-3532.

68. Mani C, Selvakumar N, Kumar V, Narayanan S, Narayanan P (2003) Comparison of DNA sequencing, PCR-SSCP and PhaB assays with indirect sensitivity testing for detection of rifampicin resistance in Mycobacterium tuberculosis. The International Journal of Tuberculosis and Lung Disease 7: 652-659.

69. Krishnamurthy A, Rodrigues C, Mehta A (2002) Rapid detection of rifampicin resistance in M. tuberculosis by phage assay. Indian Journal of Medical Microbiology 20: 211.

70. Shenai S, Rodrigues C, Mehta A (2002) Evaluation of a new phage amplification technology for rapid diagnosis of tuberculosis. Indian Journal of Medical Microbiology 20: 194.

71. Banfi E, Scialino G, Monti-Bragadin C (2003) Development of a microdilution method to evaluate Mycobacterium tuberculosis drug susceptibility. Journal of Antimicrobial Chemotherapy 52: 796-800.

72. Caviedes L, Delgado J, Gilman RH (2002) Tetrazolium microplate assay as a rapid and inexpensive colorimetric method for determination of antibiotic susceptibility of Mycobacterium tuberculosis. Journal of Clinical Microbiology 40: 1873-1874.

73. Palomino J-C, Martin A, Camacho M, Guerra H, Swings J, et al. (2002) Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance in Mycobacterium tuberculosis. Antimicrobial Agents and Chemotherapy 46: 2720-2722.

74. Barnard M, Albert H, Coetzee G, O'Brien R, Bosman ME (2008) Rapid molecular screening for multidrug-resistant tuberculosis in a high-volume public health laboratory in South Africa. American Journal of Respiratory and Critical Care Medicine 177: 787-792.

75. Hillemann D, Rüsch-Gerdes S, Richter E (2009) Feasibility of the GenoType MTBDRsl assay for fluoroquinolone, amikacin-capreomycin, and ethambutol resistance testing of Mycobacterium tuberculosis strains and clinical specimens. Journal of Clinical Microbiology 47: 1767-1772.

76. Brossier F, Veziris N, Aubry A, Jarlier V, Sougakoff W (2010) Detection by GenoType MTBDRsl test of complex mechanisms of resistance to second-line drugs and ethambutol in multidrug-resistant Mycobacterium tuberculosis complex isolates. Journal of Clinical Microbiology 48: 1683-1689.

77. Richter E, Rüsch-Gerdes S, Hillemann D (2006) Evaluation of the GenoType Mycobacterium assay for identification of mycobacterial species from cultures. Journal of Clinical Microbiology 44: 1769-1775.

78. Russo C, Tortoli E, Menichella D (2006) Evaluation of the new GenoType Mycobacterium assay for identification of mycobacterial species. Journal of Clinical Microbiology 44: 334-339.

79. Kirschner P, Springer B, Vogel U, Meier A, Wrede A, et al. (1993) Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory. Journal of Clinical Microbiology 31: 2882-2889.

80. Hillemann D, Rüsch-Gerdes S, Boehme C, Richter E (2011) Rapid molecular detection of extrapulmonary tuberculosis by the automated GeneXpert MTB/RIF system. Journal of Clinical Microbiology 49: 1202-1205.

81. Lawn SD, Nicol MP (2011) Xpert® MTB/RIF assay: development, evaluation and implementation of a new rapid molecular diagnostic for tuberculosis and rifampicin resistance. Future Microbiology 6: 1067-1082.

82. Hillemann D, Rüsch-Gerdes S, 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.

83. Collins LA, Mirza SP, Kissebah AH, Olivier M (2010) Integrated approach for the comprehensive characterization of lipoproteins from human plasma using FPLC and nano-HPLC-tandem mass spectrometry. Physiological Genomics 40: 208-215.

84. Ioannidis P, Papaventsis D, Karabela S, Nikolaou S, Panagi M, et al. (2011) Cepheid GeneXpert MTB/RIF assay for Mycobacterium tuberculosis detection and rifampin resistance identification in patients with substantial clinical indications of tuberculosis and smear-negative microscopy results. Journal of Clinical Microbiology 49: 3068-3070.

85. Van Embden J, Cave MD, Crawford JT, Dale J, Eisenach K, et al. (1993) Strain identification of Mycobacterium tuberculosis by DNA fingerprinting: recommendations for a standardized methodology. Journal of Clinical Microbiology 31: 406-409.

86. Streicher EM (2007) Application of spoligotyping in the understanding of the dynamics of Mycobacterium tuberculosis strains in high incidence communities: University of Stellenbosch. Faculty of Health Sciences. Dept. of Biomedical Sciences.

87. Tjernberg A (2005) Protein mass spectrometry in the drug discovery process: Institutionen för medicinsk biokemi och biofysik (MBB)/Department of Medical Biochemistry and Biophysics.

88. Cherkaoui A, Hibbs J, Emonet S, Tangomo M, Girard M, et al. (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. Journal of Clinical Microbiology 48: 1169-1175.

89. Hillenkamp F, Karas M, Holtkamp D, Klüsener P (1986) Energy deposition in ultraviolet laser desorption mass spectrometry of biomolecules. International Journal of Mass Spectrometry and Ion Processes 69: 265-276.

90. Liyanage R, Lay Jr JO (2006) An introduction to MALDI-ToF MS: New Jersey: Wiley.

91. Baar BL (2000) Characterisation of bacteria by matrix-assisted laser desorption/ionisation and electrospray mass spectrometry. FEMS Microbiology Reviews 24: 193-219.

92. Claydon MA, Davey SN, Edwards-Jones V, Gordon DB (1996) The rapid identification of intact microorganisms using mass spectrometry. Nature Biotechnology 14: 1584-1586.

93. Willoughby R (2002) A Global View Of Lc/MS: How To Solve Your Most Challenging Analytical Problems Author: Ross Willoughby, Ed Sheehan, Sam Mit.

94. Krishnamurthy T, Ross PL (1996) Rapid Identification of Bacteria by Direct Matrix-assisted Laser Desorption/Ionization Mass Spectrometric Analysis of Whole Cells. Rapid Communications in Mass Spectrometry 10: 1992-1996.

95. Hillenkamp F, Peter-Katalinic J (2013) MALDI MS: a practical guide to instrumentation, methods and applications: John Wiley & Sons.

96. Stump MJ, Fleming RC, Gong W-H, Jaber AJ, Jones JJ, et al. (2002) Matrix-assisted laser desorption mass spectrometry. Applied Spectroscopy Reviews 37: 275-303.

97. 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.

98. Giebel RA, Fredenberg W, Sandrin TR (2008) Characterization of environmental isolates of Enterococcus spp. by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Water Research 42: 931-940.

99. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, et al. (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. Journal of Clinical Microbiology 46: 1946-1954.

100. Ruelle V, Moualij BE, Zorzi W, Ledent P, Pauw ED (2004) Rapid identification of environmental bacterial strains by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Communications in Mass Spectrometry 18: 2013-2019.

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

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

103. 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: An International Journal 17: 31-38.

104. Hettick JM, Kashon ML, Simpson JP, Siegel PD, Mazurek GH, et al. (2004) Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analytical Chemistry 76: 5769-5776.

Chapter 3

RESEARCH RESULTS I

Matrix Assisted Laser Desorption/Ionisation time of flight mass spectrometry (MALDI-TOF MS) proteomic profiling of *Mycobacteria*

Matrix Assisted Laser Desorption/Ionisation time of flight mass spectrometry (MALDI-TOF MS) proteomic profiling of *Mycobacteria*

Melendhran Pillay[1], Siyanda Myende[2], Yacoob Coovadia[3, 4], Koleka Mlisana[3, 4] and

Patrick Govender[2]

Department of Pharmacy[1], Department of Biochemistry[2], Department of Medical Microbiology[3], University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa. Department of Medical Microbiology, National Health Laboratory Service (NHLS)[4], Inkosi Albert Luthuli Central Hospital, 800 Bellair Road, Durban

3.1 ABSTRACT

Up until now proteomic profiling via matrix assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has been used for the identification of variousmicroorganisms (bacteria and yeast) to their genus and specie levels. For this purpose, either whole cells or samples containing proteins (cell surface proteins and ribosomal proteins)extracted from microbes with theethanol/formic acid (EFA) procedurewere used for MALDI-TOF MS analysis.However, unlike other bacteria, a major impediment exists in the efficient extraction of cellular proteins from mycobacteria due to the highly complex lipid structure of their cell wall envelopes. Therefore an EFA-based protein extraction method that uses micro-glass beads (EFAGB) to disrupt the cell envelope has also been suggested for biotyping of mycobacteria. The present study assesses the feasibility of using MALDI-TOF MS proteomic profiling as a rapid and precise technology to identify mycobacteria in Kwazulu-Natal, South Africa which is currently a global epicentre of mycobacterial-associated diseases. However, the data from this locally-based study clearly shows that both the EFA and EFAGB protein extraction methods yielded less than satisfactory proteomic profiles for the purpose of accurate biotyping.As such a modifiedEFA protein extraction protocol that incorporates an initial cell envelope delipidation step was formulated and shown to produce mass spectra that were unique and highly reproducible. The spectrawere used to create an independent main spectral profile reference library (CMEFA-MSP) representing clinically relevant American Type Culture Collection (ATCC) mycobacterial strains. Interestingly, this proof of concept study clearly demonstrates that MALDI-TOF MS-based biotyping of mycobacteria using the CMEFA-MSP reference library correctly identified 11 blind-coded strains sourced from an autonomous facility to the genus and species level with 100% accuracy.

3.2 INTRODUCTION

Microorganisms associated with the bacteria that cause tuberculosis (TB), leprosy and a variety of infections in humans are known as mycobacteria. Mycobacteria comprise of approximately 100 heterogeneous species of rapid and slow growing, acid-fast bacilli (AFB)[1,2] belonging to either the *Mycobacterium tuberculosis complex* (MTBC) or Non-tuberculosis (NTM) family.Most members are harmless microbes, widely distributed in the environment and are normal inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, animals and humans[3]. However there are a number of pathogenic species that infect humans and animals[4]. Over the past few decades the number of human infections caused by mycobacteria has increased[5]. Pulmonary infections, skin and soft tissue infections and disseminated disease are all responsible for significant morbidity in humans [6].

M. tuberculosis is a well-known human pathogen [7] belonging to the MTBC family. It remains the causative agent of the classically defined pulmonary tuberculosis in humans[8]. Other pathogens belonging to this genus include *M. cannetii*, *M. africanum*, *M. bovis* and *M. microti*[7]. A recent world-wide resurgence of *M. tuberculosis* has led to an increase of over 95% tuberculosis cases and deaths occurring among adults in developing countries[9]. Tuberculosis (TB) itself continues to pose a major health risk to young and middle-aged adults and when associated with acquired immuno-deficiency syndrome (AIDS), it kills faster than any other disease. Globally, the current number of incident TB cases was estimated to be 8.6 million in 2012, with 16.2 million prevalent cases of TB disease and 1.3 million deaths giving an overall case fatality rate of 23%[10]. Among these deaths, an estimated 170 000 were due to multidrug-resistant TB (MDR-TB) [9]. South Africa is one of the twenty-two high-burden countries identified as having more than 80% of the new cases of TB worldwide and is reported to have the third highest TB burden in the world after India and China[11]. The HIV/AIDS epidemic has tremendously influenced the global burden of TB, with over 2 million HIV-TB co-infected individuals living in South Africa[12]. KwaZulu-Natal is the leading province with high rates of TB as well as a high burden of multidrug-resistant (MDR-TB) [13,14].

Although the incidence of classical tuberculosis has decreased in developed countries, the occurrence of NTM in pulmonary disease has increased [5]. Notably the HIV/AIDS epidemic has changed the epidemiology of NTM-related disease [14], leaving immunocompromised

individuals vulnerable to opportunistic infections caused by NTM [7]. NTM appears to be increasing worldwide with *M. avium* complex (MAC) being the leading cause of secondary infections in patients infected with HIV[7,12]. Strain types of *M. avium* isolates in AIDS patients were found to be identical to those isolates recovered from patients' drinking water supplies [3]. Since *M. avium* and other NTM are resistant to chlorine and biocide, they are capable of biofilm formation and are able to persist in both natural and municipal water distribution systems [3]. Therefore water is likely the primary source of *M. avium* complex infection in humans with immunosuppression [3]. In first world countries MAC is a common causative agent of opportunistic infection in AIDS patients and represents about 65% of the NTM isolates. The other predominant cause of NTM infections includes *M. fortuitum* complex (19%) and *M. kansasii* (10%) with the remaining 10% being other mycobacterial species. Apart from MAC and other NTM associated infections, *M. marinum* is an emerging pathogen responsible for a variety of cutaneous infections in immunocompromised patients [7].

Conventional laboratory methods for the identification of NTM andMTBCorganisms rely on phenotypic characterization (growth rate, colony morphology, pigment production and staining techniques) and biochemical profiling. However, the main disadvantages associated with these methods are their extensive processing times (6-12 weeks to confirm a positive identification), which are considerably intensive in terms of both labour and cost [7]. Moreover and of particular concern is misidentification when processing large sample volume. According to Cook and co-researchers interspecies homogeneity, intraspecies variations and the existence of uncharacterized species often leads to phenotypic misidentification especially since taxonomic placement is solely dependent on technical personnel. Biochemical algorithms which usually include 15-20 species become too complex when attempting to include the hundreds of currently established species. As a result there is an inherent bias towards the identification of more familiar species of mycobacterium [15].

A drive to unequivocally discriminatemicrobes of both MTBC and NTM families and to speed up their presumptive identification as witnessed the development of a number of molecular biology-based strategies which include the hybridisation of PCR generated amplicons to specific probes and 16SrRNA gene sequencing. These methods have found favour in well-resourced privately- and government-owned pathology laboratories, as they aretechnically demanding and are dependent onexpensivelaboratory consumables and equipment.These methods are proving to be quiet effective for the detection of specific

Mycobacterium species within 3 weeks thus contributing positively to both patient care and prognosis. Unfortunately, only a limited number of mycobacterial species can be identified by these molecular biology-based assays[16].

Given the significant increase in mycobacterial-related infection rates and the limitations of the currently available identification methods described above, the development of a robust MTBC and NTM species differentiation method that is cost effective, simple, rapid and accurate is of paramount importance.A wide variety of prokaryotes (clinically and environmentally relevant Gram positive and negative bacteria) have been characterized using MALDI-TOF MS approach. Recent advances in mass spectrometry haveshown that comparative proteomic profiling can be employed in the identification of mycobacterial species[17,18]. Earlier studies by Hettick and coworkersusing MALDI-TOF MS analysis of whole-cellor acetonitrile/trifluoroacetic acid (ACN/TFA) extracted protein samples demonstrated that that both sample types were capable of resolving the identification of 6 ATCC-typed mycobacterial strains.Thereafter, Pignoneand coworkersusedthe whole-cell sample preparation method and were able to clearly distinguish between 36 strains of the 37 screened ATCC-typed mycobacteria. In a more recent study, undertaken by Saleeb and coworkers, a modified ethanol-formic acid (EFA) protein extractionprotocol using glass beads (EFAGB) was used to construct a mycobacterial mass spectral reference database containing 42 clinically relevant ATCC-typed strains. The database was then used albeit with varied success to biotype 104 mycobacterial clinical isolates. The above studies have useddifferent sample preparation methods for the proteomic profiling of mycobacteria using the MALDI-TOF MS.However, to date there seems to be no consensus on which sample preparation method is preferred in the discrimination of mycobacteria. In this regard, the present study assesses the feasibility of using MALDI-TOF MS proteomic profiling as a rapid and precise technology to identify mycobacteria in Kwazulu-Natal, South Africa which is currently a global epicentre of mycobacterial-associated diseases.

In this proof-of-concept study we present the MALDI-TOF MS-derived proteomic profiling data on mycobacteria employing the EFA, EFAGB and a new organic solvent delipidation-modified EFA (CMEFA)sample preparation protocol.The data from this comparative study seems to suggest that the newly developed CMEFA protocol is most effective in identification of clinically relevant ATCC-typed strains of mycobacteria representing members of the MTBC and NTM families that are prevalent in our locality.

3.3 MATERIALS AND METHODS

3.3.1 Bacterial strains

All mycobacterial species (Table 3.1)used in this study were procured from American Type Culture Collection (ATCC). The ATCC strains were purchased as a KWIK-STIK™ device (Microbiologics) consisting of lyophilised pellets.

Table 3.1 Mycobacterial strains employed in this study.

Mycobacterium	Source
M. *abscessus* ATCC 23040	ATCC
M. *avium* ATCC 700736	ATCC
M. *bovis* ATCC 19210	ATCC
M. *fortuitum* ATCC 23031	ATCC
M. *gordonae* ATCC 23409	ATCC
M. *intracellulare* ATCC 3950	ATCC
M. *kansasii* ATCC 12478	ATCC
M. *marinum* ATCC 927	ATCC
M. *scrofulaceum* ATCC 19981	ATCC
M. *terrae* ATCC 15755	ATCC
M. *tuberculosis* 25177	ATCC

3.3.2 Media Preparationand Cultivation of ATCC strains

The mycobacterial ATCC strains were purchased as lyophilised pellets in a KWIK-STIK™ device (Microbiologics, USA). Mycobacterial pellets contained within the quick stick device was allowed to equilibrate to room and rehydrated according to manufacturer's guidelines. The rehydration process was performed in a Level II Biosafety cabinet, with full personal protective equipment, including a N95 respiratory mask.A swab was heavily saturated with the fluid containing the mycobacterial organism and then transferred to a drug free Middelbrook 7H11 agar plate (Becton Dickinson). The Middelbrook 7H11 agar plate was

inoculated by gently rolling the swab over one-third of the plate. The culture plate was incubated at 37°C for four and twenty one days to cultivate fast (*M. fortuitum)* and slow growing (*M. bovis, M. gordonae* and *M. tuberculosis*) mycobacterial strains, respectively. Once confluent growth was obtained, a single loop-full was transferred into a MicroBankcryovial (Pro-Lab Diagnostics, Canada) and stored at -70°C as per manufacturer's instruction.

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

A disposable 10 µl inoculating loop was used to obtain a single mycobacterial colony grown on Middelbrook 7H11 agar plate. The colony was suspended in 600 µl HPLC grade distilled water contained in a 1.5 ml screw cap "O" ring tube (Sarstedt, Germany). The tube containing the cell suspension was vortexed for approximately 60 s and heat inactivated at 98^{o}C for 30 min in a heating block. The heat inactivated cell suspension was then centrifuged at 13 000 x g for 5 min in a table top centrifuge with an aerosol–tight rotor. 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 cell suspension containing absolute ethanol was vortexed for 2 minat 13 000 x g and the supernatant was discarded using a pipette. An additional centrifugation step was performed to completely remove residual ethanol. After the pellet was air dried at room temperature (RT) for 5 min, depending on the volume of the pellet (ranging from 20-30 µl), 40-80µl of 70% formic acid was added and the tube was thoroughly vortexed for 30 s. An equivalent volume of absolute acetonitrile (HPLC grade) was added to the suspension and homogenized by vortexing for 10 s. The homogenized suspension was centrifuged for 2 min and the supernatant was transferred to a sterile 1.5 ml screw cap "O" ring tube. One microliter of the supernatant from each isolate was spotted onto MTP 384 ground steel target plate (Bruker Daltonics, Germany) and allowed to air-dry (Figure 3.1). One microliter of the Bruker Bacterial Test Standard (BTS) containing an extract of *Escherichia coli* DH5 alpha was spotted onto a separate spot for instrument calibration. The applied samples were air-dried and overlayed with a 1 µl aliquot of freshly prepared saturated matrix solution [10 mg of α-cyano-4-hydroxycinnamic acid (HCCA) dissolved in 1 ml of a solvent mixture containing 50% acetonitrile, 47.5% water and 2.5 % trifluoroacetic acid]. The samples containing matrix were dried for 5 min. The samples on the ground steel target plate were subjected to MALDI-TOFMS analysis.

Figure 3.1 Application of samples to the MALDI-TOF MS ground steel plate.

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

As suggested by Saleeb and coworkers, micro-glass beads was used to disrupt the cell envelope of mycobacteria so as to facilitate the release of cytoplasmic proteins[6]. The EFAGB mycobacterial sample preparation protocol for MALDI-TOF MS analysis was as follows: a 10µl disposable inoculating loop was used to obtain a single mycobacterial colony (ATCC typed strain) from a Middelbrook 7h11 agar plate. The colony was suspended in 600 µl of HPLC grade distilled water contained in a 1.5 ml screw cap "O" ring tube (Sarstedt, Germany). The tube containing the cell suspension was vortexed for approximately 60 s and then heat inactivated at 98ºC for 30 min in a heat block. The heat inactivated cell suspension was then centrifuged at 13 000 x g for 5 min in a table top centrifuge with an aerosol-tight rotor.The cell suspensions were vortexed for one min and heat inactivated at 103°C for 30 min, 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 min. The supernatant was discarded and the pellet was re-suspended in 300 µl of HPLC grade distilled water followed by 900 µl of absolute ethanol (HPLC grade). The cell suspension containing absolute ethanol was vortexed for 2 minat 13 000 x g and the supernatant was discarded using a pipette. An additional centrifugation step was performed to completely remove all residual ethanol. After

the pellet was air dried at room temperature (RT) for 5 minand depending on the volume of the pellet (ranging from 20-30 µl), 40-80 µl of 70% formic acid was added followed by 100 to 200µl of silica glass beads (0.1 mm diameter). The suspension was vortexed for 10 min followed by the addition of absolute acetonitrile (50 µl)and tubes were further vortexed for 10 min. The sample was centrifuged for 2 min at 13 000 x g and the supernatant was utilized for MALDI-TOF MSanalysis. For analysis, 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 freshly prepared saturated HCCAmatrix solution. The matrix coated samples were dried for 5 min before analysis.

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

The newly developed CMEFA protocol was evaluated for its effective delipidation of mycobacterial cells. Lipids and mycolic acids that constitute the mycobacterial cell envelope serve as barriers and hinder the protein extraction process resulting in fewer proteins available for definitive identification. The MALDI-TOF MS CMEFA sample preparation protocol for mycobacteria was as follows: a 10µl disposable inoculating loop was used to obtain a single mycobacterial colony (ATCC typed strain) from a Middelbrook 7h11 agar plate. The colony was suspended in 600 µl of HPLC grade distilled water contained in a 1.5 ml screw cap “O” ring tube (Sarstedt, Germany). The tube containing the cell suspension was vortexed for approximately 60 s and then heat inactivated at 98^{o}C for 30 min. The heat inactivated cell suspension was then centrifuged at 13 000 x g for 5 min in a table top centrifuge with an aerosol-tight rotor. The delipidation process of mycobacterial cells was initiated by the addition of 600 µl of a chloroform/methanol (1/1, v/v) solvent mixture. The cell suspensions were vigorously vortexed for 60 s, centrifuged at 13,000 x g for 5 min and the supernatants discarded. The delipidation treatment was repeated twice more so as to enhance the removal of lipids and mycolic acids. The supernatant was discarded and the pellet was re-suspended in 300 µl of HPLC grade distilled water followed by 900 µl of absolute ethanol (HPLC grade). The cell suspension was vortexed for 2 minat 13 000 x g and the supernatant was carefully removed. An additional centrifugation step was performed to completely remove all residual ethanol. The pellet was air dried at room temperature for 5 min, depending on the volume of the pellet (ranging from 20-30 µl), 40-80 µl of 70% formic acid was added and the suspension was thoroughly vortexed for 30 s. Acetonitrile (50 µl) was added he suspension and tubes were vortexed for another 30 s. The samples were centrifuged

for 2 min at 13 000 x g. A 1µlaliquot of the supernatant was spotted on a MTP 384 ground steel target plate (Bruker Daltonics, Germany). The applied sample was air-dried and subsequently coated with 1 µlof freshly prepared saturated HCCA matrix solution. The extracted samples containing matrix were allowed to air-dry for 5 min before mass spectral analysis.

3.3.6 MALDI-TOF MS instrumentation settings

The spotted MTP 384 ground steel target plate was inserted into the tray of theAutoflex III smartbeam MALDI-TOF MS instrument (Bruker Daltonics, Germany) and processed using the Flex control software. Instrument parameters for ion source 1 and ion source 2 were set at 20 kV and 18.57 kV respectively. The lens was set at 8.5kV and a pulsed ion extraction of 250 ns was used. The protein molecules embedded in the matrix crystals were effectively desorbed and ionized by a smart beam laser pulse with a frequency of 20Hz and accelerated in a high voltage field. Different ions representing intact sample proteins proportional to their mass were detected after a time of- flight resulting in a high resolution mass spectrum of the sample. Spectra were acquired in a linear positive mode with a mass-to-charge (m/z) range of 2to 20kDa.

3.3.7 Mass spectral data analysis and library database creation

Prior to sample analysis, the MALDI-TOF MS instrument was calibrated with the Bruker bacterial test standard (BTS) (Bruker Daltonics, Germany) with a mass range of 3.6 to 17 kDa. The “MBT_FC.par” standard FlexControl method was selected for both analysis and internal calibration of BTS. Using the flex control method “MBT_FC.par”, calibration was performed automatically by adding up 6 times 40 laser shots from different positions of the BTS spot with the maximum deviation below ± 300 ppm as recommended by the manufacturer. Each mycobacterial strain was spotted at 6 positions on the MALDI target plate and measured four times per spot. Thus twenty four spectra were generated. Using replicate cultures, this was repeated on three separate occasions thereby generating 72 spectra per mycobacterial strain. The spectra fulfilled the specifications of Bruker Daltonics for construction of a new main spectral profile (MSP) reference library in that each spectrum must contain a minimum of 25 peaks with a resolution > 400, with a minimum of 20 of the peaks having a resolution > 500.

A main spectral profile (MSP) consisting of a minimum of 24 of the most reproducible spectra were created using the Biotyper 3.0 software individually for all eleven ATCC typed mycobacterial strains to generate a reference MSP database. An MSP is an average "spectrum" or "a reference peak list" which is created by a defined creation method from a number of pre-processed spectra acquired from a known or ATCC typed mycobacterialstrain. For quality assurance, all raw spectra used for MSP creation were processed according to a standardized protocol as advised by Bruker Daltonicsusing theFlexAnalysis 3 software program (Bruker Daltonics, Germany) and utilising the "MBT Process method" for the smoothing and baseline subtraction of raw spectra. The presence of flat-liners, outliers or single spectra with peaks differing from the other spectra was also interrogated using the FlexAnalysis 3 software program. The mass deviation within the spectra was set at not more than 500 ppm (mass window of 3 Dalton for a peak at 6 kDa) therefore any mass deviation above 3 Da, particularly for peaks at mass range between 6-7 kDa were eliminated.

A list of the most significant peaks of a spectrum (*m*/*z* values with a given intensity) was created by performing functions within the FlexAnalysis such as smoothing, normalization, baseline subtraction and peak picking. Biotyper 3.0 software (Bruker Daltonics, Germany) was used to obtain themain spectral patterns (MSP's) of mycobacteria processed in this study using the CMEFAextraction protocol. The newly generated MSP's were used to create a new reference MSP libraryor database named CMEFA-MSP. Due to differences in sample preparation strategies, it must be noted that the CMEFA-MSP library is independent of the Bruker Daltonics main spectral profile database which currently stores reference spectra for more than 3740 microorganisms.Dendrogram analyses of MSP's were processed with the Biotyper 3.0 software.

3.3.8 Principle component analysis (PCA)

In order to assess the complex relationships among the ATCC typed mycobacterial strains, a Principal component analysis (PCA) was generated using ClinProTools 2.2 software (Bruker Daltonics, Germany). Similarities or dissimilarities among mycobacterial strains were determined by the use of PCA plots. ATCC typed mycobacterial strains that cluster together are more similar than those that do not cluster. The PCA was managed by an external integrated MATLAB software tool integrated in ClinProTools to statistically evaluate MALDI-TOF mass spectra.

3.3.9 Biotyping of blind-coded strains using MALDI-TOF MS

To access the accuracy and reliability of the CMEFA-based MALDI-TOF MS strategy in the identification of mycobacteria, eleven typed blind-coded strains were obtained from an independent institution (Medical Microbiology, NHLS, Inkosi Albert Luthuli Central Hospital).Samples for mass spectral analysis of the blind-coded strains were prepared using the CMEFA extraction method. Following acquisition of the mass spectra from blind-coded samples they were processed into their respective MSP's. These were then best matched by Bruker Daltonics Biotyper 3 software to MSP's in the newly created CMEFA-MSP reference library.Biotyping was performed from CMEFA-derived samples of three independent cultures of each blind-coded strain.Interpretation of the profile-matching process for theidentification of mycobacteria was made from log (score) values as shown in Table 3.2.

Table 3.2 Interpretation ofBruker Daltonics identification score values [19].

Range	Description	Colour
2.300 to 3.000	Highly probable species identification	Green
2.000 to 2.299	Secure genus and probable species identification	Green
1.700 to 1.999	Probable species identification	Yellow
0.000 to 1.699	No reliable Identification	Red

3.4 RESULTS

In an attempt to minimize the health risk associated with the handling of pathogenically viable mycobacteria for MALDI-TOF MS analysis, all mycobacterial culture sampleswere heat inactivated prior to their extraction (EFA, CMEFA or EFAGB). The integrity of this inactivation measure was validated asno detectable mycobacterial growth was observed after 6 weeks of incubation at 37°C on Middelbrook 7H11 agar plates.This inactivation procedure effectively rendered the mycobacteria non-viable and non-infectious significantly reducing the risk to the laboratory personnelindicating that the heat inactivation step had rendered the organism nonviable and was hereafter safe for work in an open laboratory [6].

The MALDI-TOF MS analysisof samples generated using the EFAprotocolyieldedmass spectral profiles with no discernible peaks and noresolution (results not shown, similar to Figure 3.2 C) that could not be employed in differentiatingmycobacterial species.In viewthis

major failing the EFA extraction method was not considered for further use. The EFAGB protocol which was modified by Saleeb and co-researchers to include mechanical disruption of mycobacterial cell envelopeglycopeptidolipids (GPL) with micro-glass beads [6], yielded protein mass spectral profiles that were characterised by drastically reduced peaknumbers with low intensities. This was especially evident for majority of the ATCC-typed mycobacterial strains (*M. fortuitum, M. abscessus, M. gordonae* and *M. bovis)*when compared to that of *M. tuberculosis* (Figure 3.2). The presence of smaller proteins with a mass/charge (m/*z*) ratio of <3.5 kDa was detected in most instances and little or no diagnostic ions were detected in in the higher mass/charge range. Given the limitation of the EFAGB method to yield samples that display consistent and abundant mass peaks throughout the range (2-10 kDa) its further employment was discontinued in this study.

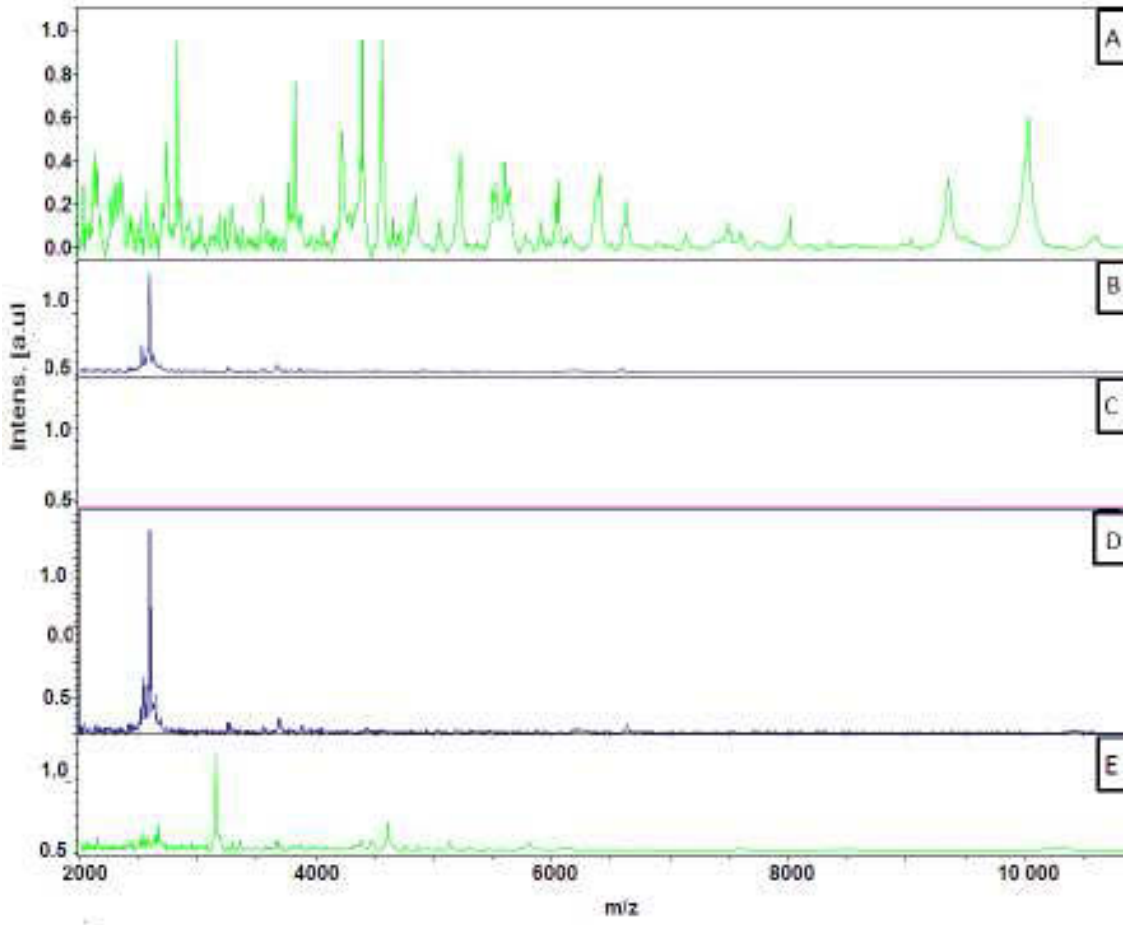

Figure 3.2 Mass spectra of *M. tuberculosis* ATCC 25177 (A), *M. bovis* ATCC 19210 (B), *M. gordonae* ATCC 23409 (C), *M. abscessus* ATCC 23040 (D) and *M. fortuitum* ATCC 23031 (E) generated using the EFGB protein extraction method. The mass spectra with the exception (A) displayed very low numbers of mass peak signals with no peaks detected for (C).

In comparison to the EFA and EFAGB protocols, the delipidation step of mycobacterial cell walls incorporated into the newly developed CMEFA protocol yielded spectra with strong signals and abundant diagnostic ions across the entire mass/charge range (Figure 3.3).The enhanced mass spectral signatures were characterized by high peak intensities and a relative abundance of peaks with low signal-to-noise ratios. These spectra are reflective of samplescontaining higher protein content.Overall in comparison to spectra obtained using the EFA and EFAGB methods, a significantly increased number of diagnostic ions in the mass/charge range of 2 to 10 kDa were observed for CMEFA-derived samples.

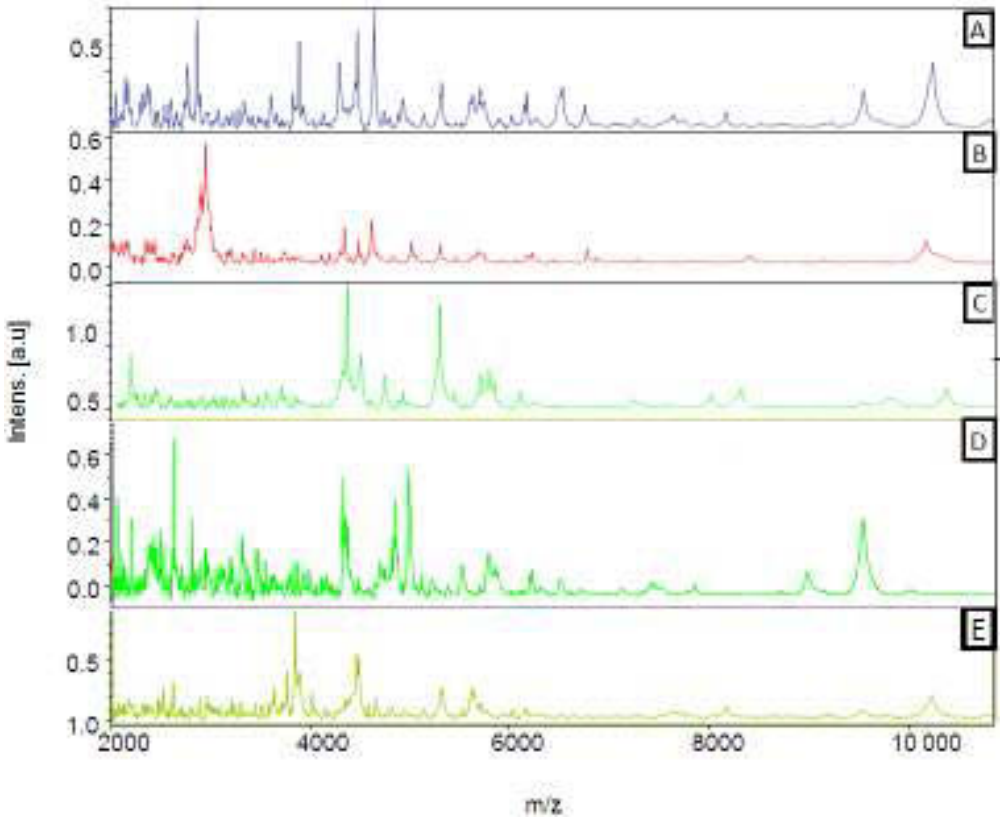

Figure 3.3 Mass spectra of *M. tuberculosis* ATCC 25177 (A), *M. bovis* ATCC 19210 (B), *M. gordonae* ATCC 23409 (C), *M. abscessus* ATCC 23040 (D) and *M. fortuitum* ATCC 23031 (E) samples obtained when using the CMEFA protein extraction protocol. The mass spectral signatures produced strong signals and adequate diagnostic ions in the mass/charge range of 2 to 10 kDa.

To exclude for any interference from reagents of the CMEFA method on mass spectra, negative controls consisting of a culture-free chloroform/methanol (1:1, v/v) and distilled

water were included. The MALDI-TOF MS analyses of chloroform/methanol and distilledwater revealed no diagnostic ions (results not shown). In view of the above the CMEFA sample preparation protocol was deemed the method of choice for all further sample preparations.

A set of mass spectral patterns for each ATCC-typed mycobacterial strain using the CMEFA protocol was further analysed using the MALDI- Biotyper 3.0 software to create their main spectral profiles (MSP). Using default analysis parameters as assigned by Biotypersoftware (distance measure, correlation; linkage, average), the differentiation and clustering of closely related mycobacteria wereascertained from a dendrogram (Figure 3.4). Notably, pathogenic members of MTBC (red) were differentiated and correctly clustered away from members ofNTMmycobacteria. Interestingly, mycobacteria of the *M. avium-intracellulare* complex(MAC) clustered separately indicating that the spectra of the species are more similar to one another than spectra of other mycobacterial species. A similar scenario prevails for organisms of the MTBC which are *M. bovis* and *M. tuberculosis.*

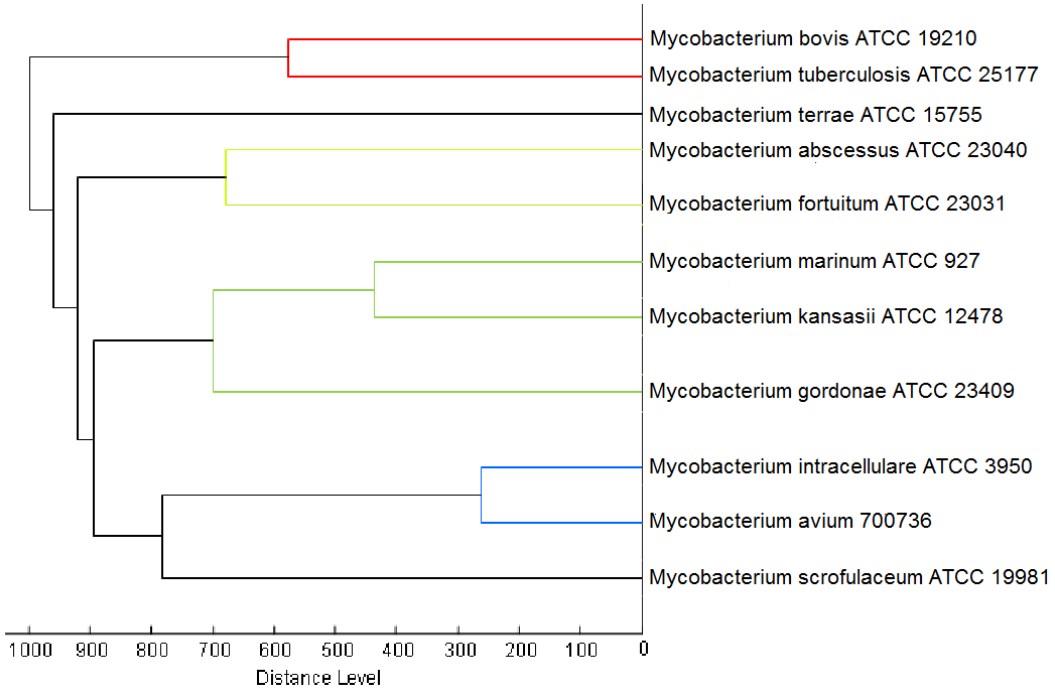

Figure 3.4 Dendrogram created from hierarchical cluster analysis of the main spectral profiles (MSP) of mycobacteria using the CMEFA sample preparation protocol.

The results of the MALDI-TOF MS-based identification of blind-coded mycobacterial strains are presented in Table 3.3. These log score values were interpreted in accordance to stipulations by Bruker Daltonics (Table 3.2). InterestinglyCMEFA-prepared samples of theeleven blind-coded mycobacterial strains displayed of log score values ≥ 2.3 that placed them in the highestdescriptive category were identified correctly to the genus and species level. Of significance, the twoMTBC-related strains (M. *tuberculosis* andM. *bovis)*were unambiguously differentiated from the other nine NTM species. In this proof of concept study, MALDI-TOF MS-based biotyping correctly identified blind-coded strains to the genus and species level with 100% accuracy.

Table 3.3 MALDI-TOF MS based identification of blind-coded ATCC-typed mycobacterial strains through pattern matching algorithms using Bruker Daltonics Biotyper 3.0 software.

Sample	Best matched strain	Overall Score Values	Identification
1	M. *fortuitum* ATCC23031	2.44 ± 0.02	Yes
2	M. *kansasii* ATCC 12478	2.40 ± 0.09	Yes
3	M. scrofulaceum ATCC 19981	2.35 ± 0.10	Yes
4	M. *terrae* ATCC 15755	2.39 ± 0.05	Yes
5	M. *abscessus* ATCC 23040	2.42 ± 0.02	Yes
6	M. *marinum* ATCC 927	2.42 ± 0.02	Yes
7	M. *tuberculosis* ATCC 25177	2.44 ± 0.13	Yes
8	M. *bovis* ATCC 19210	2.44 ± 0.07	Yes
9	M. *intracellulare* ATCC 3950	2.40 ± 0.11	Yes
10	M. *gordonae* ATCC 23409	2.41 ± 0.02	Yes
11	M. *avium* ATCC 700736	2.40 ± 0.01	Yes

Biotyping was performed from CMEFA-derived samples of three independent cultures of blind-coded strains.

To further characterisethe recently data-based main spectral profilesof CMEFA-extracted MALDI-TOF MS samples, the MSP's of three ATCC typed mycobacterial strains were subjected to three dimensional principal component analyses (PCA).The mass spectra of *M. tuberculosis*, *M. bovis*and *M. intracellulare* were non-overlapping and reside in distinct spatial arrangementsto one another (Figure 3.5). Both MTBC members*M. tuberculosis* and *M. bovis* clustered closer together on the same plane away from the NTMstrain*M. intracellulare*.

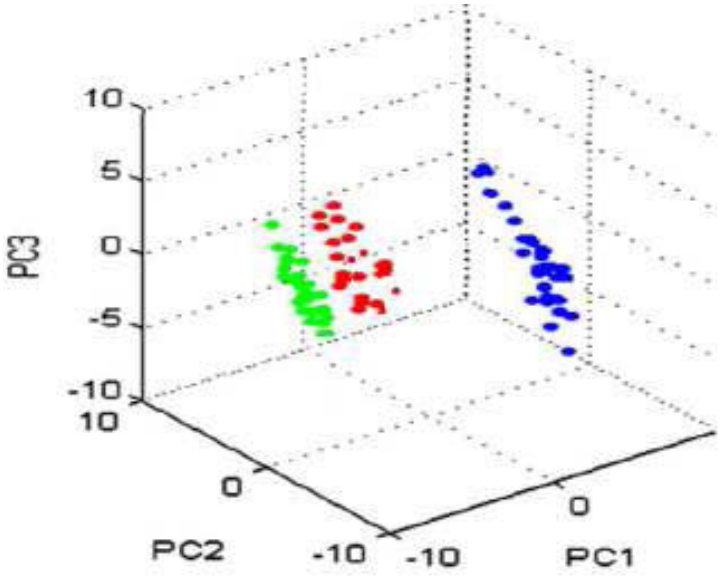

Figure 3.5 Three dimensional principal component analysis (PCA) derived from main spectral profiles (MSP's) of *M. tuberculosis* ATCC 25177 (green), *M. bovis* ATCC 19210 (red) and *M. intracellulare* ATCC 3950 (blue).

3.5 DISCUSSION

For diagnostic purposes, current mycobacterial identification strategies to the species level primarily rely on phenotypic analysis, complex biochemical tests, cultivation on specific growth media and genotype analysis [7].However, the main disadvantages that are associated with these techniques include extensive processing times (6-12 weeks to confirm a positive identification) and misidentification. Both factors severely hinder the control and management of diseases associated with mycobacteria, particularly tuberculosis.Apart from an increased patient mortality, interpersonal transmission of mycobacterial infections may increase. This scenario will also result in the prescription of inappropriate anti-tubercular treatment regimes.

In view of the above, innovative research has touted MALDI-TOF mass spectrometry as a promising candidate for the efficient strain typing of mycobacteria. Two types of sample preparation protocols for MALDI-TOF MS microbial biotyping are recommended by Bruker Daltonics, either the direct transfer whole cell method or an organic solvent protein extraction technique (EFA). To date, research studies have used both methods with no apparent preference [17,18]. Unlike other bacteria, mycobacteria are intrinsically resistant to conventional inactivation methods such as incubation in ethanol or in a saturated matrix solution. However, given the potential pathogenicity of mycobacteria, the use of cellular extracts instead of direct transfer whole cell analysis is a more preferred option.

In a recent study, Saleeb and coworkers [6] modified the EFA method to include cellular disruption by glass beads (EFAGB) for MALDI-TOF MS-based biotyping of mycobacterial strains.It was hypothesized that mechanical agitation with zirconia-silica beads would break mycobacterial cell envelopes and release proteins needed for the accurate identification by MALDI-TOF MS [6]. The EFAGB protein extraction procedure was used to construct a mycobacterial mass spectral reference database (NIH database) containing 42 clinically relevant ATCC-typed strains. The reference database was used to differentiate 104 mycobacterial clinical isolates and accurate identification was made with log score valuesranging from 1.8 to ≥ 2.

In this proof of concept study, samples were extracted using the EFA and EFAGB methods from mycobacterial strains and their proteomic profiles were characterised using MALDI-TOF MS. The mycobacteria included both rapid and slow growing strains representing eleven ATCC-typed strains from the two major mycobacterialfamilies: MBTC and NTM that are clinically relevant to high incidence mycobacterial infections in Kwazulu-Natal, South Africa. The data in this study clearly illustrates that EFA-prepared samples yieldedpoor mass spectral profiles with limited or no diagnostic ions. Surprisingly, the EFAGB processed samples in our setting also yielded mass spectra that were characterised by drastically reduced peak numbers with low intensities. As a result they were deemed inadequate for definitive mycobacterial strain identification.

It has been established that microbe identification and discrimination is impossible when a limitednumber of diagnostic peak signals are obtained because strain differences are likely due to differences in the relative abundance of shared mass to charge *(m/z)* values of ions

[7,17]. In addition it has been previously suggested that precise bacterial taxonomic prediction based on proteomic profiles generated by MALDI-TOF MS analysis is dependent on the relative abundance of cellular proteins (cell surface proteins and ribosomal proteins) in cell sample extracts [20]. Thus it may be tentatively suggested that the EFA and EFAGB methods do not sufficiently disrupt the highly complex waxy cell envelopes of mycobacteria which are predominantly composed of mycolic acid containing glycopeptidolipids (GPL). The ability of to prevent the penetration of chemicals during the protein extraction process is supportive of this notion[21]. It has also been reported that these lipids bind to proteins and hinder theextraction and solubilisation of proteins that are critical to the generation of adequate positively charged ions necessary for MALDI-TOF MS analyses [22,23].

Given this backdrop, the present study developed a protein extraction protocol that wouldsupport unequivocal strain discrimination of the otherwise difficult to identify mycobacteria. In this regard, an enzymatic-basedmycobacterial cell wall disruption strategy was deemed unsustainable due to increased processing times and its cost intensive natureespecially when processing high sample volumes.Organic solvents such as phenol, chloroform and methanol have also been employed as mycobacterial cell wall disruption agents [1,24]. Bearing in-mind our region processes large sample sizes, the relatively low-cost of organic solventsseemed an attractive alternative. Moreover andbased on the intrinsic character of lipids having a high affinity for nonpolar solvents it has been suggested that extraction with chloroform will increasethe fraction of membrane-associated proteins for MALDI-TOF MS analysis [25].In view of this, the present study modified the EFA protocol by incorporation a cell envelopedelipidation pre-treatment procedure with anequimixture containing chloroform and methanol.

The data from this study clearly demonstrates that the newly developed organic solvent-based CMEFA protein extraction method is highly efficient in producing mycobacterial cellular protein samples for MALDI-TOF MSanalysis to generate high resolution MSP's. In comparison to the EFAGB the CMEFA protocol is more cost-effective and simpler to perform as the chloroform-methanolequimixture facilitates disaggregation of mycobacterial cell clumps and thereby avoids use of cost-consuming sterile micropestles (~1 $ per sample).In addition, the newly created independent CMEFA-MSP reference library

containing MSP's of eleven ATCC-typed mycobacterial strains was used in the undisputable differentiation of blind-coded mycobacterial strains sourced from an independent research facility. Furthermore CMEFA-prepared samples of the eleven blind-coded mycobacterial strains displayed of log score values ≥ 2.3 which placed them in the highest descriptive category afforded by Bruker Daltonics thereby unambiguously identifying them to the genus and species level.

To date, genomic sequencing platforms and many polymerase reaction assays that employ synonymous single nucleotide polymorphisms (sSNPS's) are unable clearly discriminate between*M. bovis* and *M. tuberculosis*ATCC-typed strains which share 99.5% similarity at the genetic level. As such these methods are currently restricted to resolvingthese organisms to theMTBC family [26]. A notable milestone of this study is that *M. tuberculosis* and *M. bovis* strains were individually resolvedbased on their unique CMEFA-derived cellular proteomic profiles.This lends credence to the proposal that genomic differences between *M. bovis* and *M. tuberculosis* most probably could be distinguished at the protein level[26].In a similar scenario, the data clearly demonstrates the ability of the CMEFA protein extraction strategy to facilitate species level discriminationof NTM microbes from each other and from individual MTBC organisms. Although the data presented herein strongly suggests that CMEFA-based MALDI-TOF MS analysis should be the most preferred method for mycobacterial biotyping its true potential remains to be assessed with the creation of a complete mycobacterial strain MSP reference library to screen and identify clinical mycobacterial isolates. In addition CMEFA-based MALDI-TOF MS biotyping should be validated using a multi-laboratory approach.

3.6 ACKNOWLEDGEMENTS

This work was made possible through support from the Department of Microbiology, National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital and the Department of Biochemistry, University of Kwa-Zulu Natal. Melendhran Pillay was supported by the Columbia University-Southern African Fogarty AIDS International Training and Research Program (AITRP), Implementation Science Traineeship Program funded by the

United States President's Emergency Plan for AIDS Relief (PEPFAR) through the Fogarty International Centre, National Institutes of Health (grant # D43TW00231)".

3.7 REFERENCES

1. Reller LB, Weinstein MP, Hale YM, Pfyffer GE, Salfinger M (2001) Laboratory diagnosis of mycobacterial infections: new tools and lessons learned. Clinical Infectious Diseases 33: 834-846.

2. Pfyffer GE, Murray P, Baron E, Jorgensen J, Landry M, et al. (2006) Mycobacterium: general characteristics, laboratory detection, and staining procedures. Manual of Clinical Microbiology: Volume 1: 543-572.

3. Primm TP, Lucero CA, Falkinham JO (2004) Health impacts of environmental mycobacteria. Clinical Microbiology Reviews 17: 98-106.

4. Ringuet H, Akoua-Koffi C, Honore S, Varnerot A, Vincent V, et al. (1999) hsp65 sequencing for identification of rapidly growing mycobacteria. Journal of Clinical Microbiology 37: 852-857.

5. Falkinham 3rd J (1996) Epidemiology of infection by nontuberculous mycobacteria. Clinical Microbiology Reviews 9: 177.

6. Saleeb PG, Drake SK, Murray PR, Zelazny AM (2011) Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Journal of Clinical Microbiology 49: 1790-1794.

7. 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.

8. Buijtels PC, van der Sande MA, de Graaff CS, Parkinson S, Verbrugh HA, et al. (2009) Nontuberculous mycobacteria, zambia. Emerging Infectious Diseases 15: 242.

9. Organization WH (2012) Global tuberculosis report 2012. Geneva, Switzerland: World Moreno S, Jarrin I, Iribarren JA, et al Incidence and risk factors for tuberculosis in HIV-Guyatt GH, Oxman AD, Vist GE, et al, GRADE: an emerging consensus on rating quality of 0 Guyatt GH, Oxman AD, Kunz R, et al, Incorporating considerations of resources use into.

10. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Archives of Internal Medicine 163: 1009-1021.

11. Wood R, Lawn SD, Johnstone-Robertson S, Bekker L-G (2011) Tuberculosis control has failed in South Africa: time to reappraise strategy. South African Medical Journal 101: 111-114.

12. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, et al. (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine 175: 367-416.

13. Organization WH (2012) Global tuberculosis report 2012, World Health Organisation.

14. Kana B, Churchyard G (2013) Tuberculosis: The global killer. South African Journal of Science 109: 01-02.

15. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. Journal of Clinical Microbiology 41: 1010-1015.

16. Ryan KJ, Ray CG (2010) Sherris medical microbiology: McGraw Hill Medical New York.

17. Hettick JM, Kashon ML, Simpson JP, Siegel PD, Mazurek GH, et al. (2004) Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analytical Chemistry 76: 5769-5776.

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

19. Bruker (2007) Bruker BioTyper User Manual. Version 2.0.

20. Jarman KH, Cebula ST, Saenz AJ, Petersen CE, Valentine NB, et al. (2000) An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry 72: 1217-1223.

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

22. Collins LA, Mirza SP, Kissebah AH, Olivier M (2010) Integrated approach for the comprehensive characterization of lipoproteins from human plasma using FPLC and nano-HPLC-tandem mass spectrometry. Physiological Genomics 40: 208-215.

23. Narayan M, Mirza SP, Twining SS (2011) Identification of phosphorylation sites on extracellular corneal epithelial cell maspin. Proteomics 11: 1382-1390.

24. Streicher EM (2007) Application of spoligotyping in the understanding of the dynamics of Mycobacterium tuberculosis strains in high incidence communities: University of Stellenbosch. Faculty of Health Sciences. Dept. of Biomedical Sciences.

25. Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, et al. (2010) MALDI-TOF-mass spectrometry applications in clinical microbiology. Future microbiology 5: 1733-1754.

26. Gutacker MM, Smoot JC, Migliaccio CAL, Ricklefs SM, Hua S, et al. (2002) Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: resolution of genetic relationships among closely related microbial strains. Genetics 162: 1533-1543.

Chapter 4

RESEARCH RESULTS II

Matrix Assisted Laser Desorption/Ionisation time of flight mass spectrometry (MALDI-TOF MS) proteomic profiling of clinically significant *Mycobacteria*

Matrix Assisted Laser Desorption/Ionisation time of flight mass spectrometry (MALDI-TOF MS) proteomic profiling of clinically significant *Mycobacteria*

Melendhran Pillay[1], Siyanda Myende[2], Yacoob Coovadia[3, 4], Koleka Mlisana[3, 4] and Patrick Govender[2]

Department of Pharmacy[1], Department of Biochemistry[2], Department of Medical Microbiology[3], University of KwaZulu-Natal, Private Bag X54001, Durban, 4000, South Africa. Department of Medical Microbiology, National Health Laboratory Service (NHLS)[4], Inkosi Albert Luthuli Central Hospital, 800 Bellair Road, Durban

4.1 ABSTRACT

The identification of mycobacteria to the genus and species level in most diagnostic pathology laboratoriesprimarily relies on phenotypic and biochemical characterization methods. More recently, commercially available molecular biology-based genotyping methodsare being used for this purpose. However, these well-established protocols are labour intensive, require considerable technical expertise and sometimes yield unacceptable misidentifications. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been reported as areliable, economicaland highly efficientmethod for both bacterial and yeast strain identification. To date, it has been used to a limited extent to discriminate the mycobacteria which are notoriously challenging to type to the species level. In this study, anovel chloroform-methanol-ethanol-formic acid (CMEFA) sample preparation protocol is used for the extraction of cellular proteins from clinical mycobacterial isolates for MALDI-TOF MS analysis. The data of this study, clearlydemonstrates that CMEFA-prepared samples of clinical isolates gave highly reproducible and distinctive mass spectral profiles. A main spectral profile (MSP) reference database representing ATCC-typed mycobacteria (CMEFA-MSP) was employed to identify39 blind-coded clinical mycobacterial isolates. Importantly all CMEFA-derived samples of the 39clinical isolates displayed log score values of ≥2.3 and were correctly identified to the species level. This strongly suggests that MALDI-TOF MS when used in conjunction with the CMEFA sample preparation protocol has potential as a simpleand cost-effective alternative for the unambiguousidentification of clinically important mycobacteria.

4.2 INTRODUCTION

Members of the *Mycobacteriaceae* family are responsible for a wide range of diseases in humans and lower animals[1]. In humans, these diseases range from superficial infections of the skin caused by *M. marinum*[2-4], to pulmonary disease caused by both MTBand NTM. Tuberculosis (TB) still remains a disease of great antiquity and was one of the first diseases for which the World Health Organization (WHO) declared a global public health emergency in 1993[3]. Presently, it is estimated that one-third of the world's population is infected with MTB[5]. An estimated 8 to 9 million new cases occur each year, with 2 to 3 million deaths[5,6]. Pulmonary TB is characterized by prolonged cough, hemoptysis, chest pain and dyspnea whilst symptoms of systemic and disseminated disease include fever, malaise, weight loss, weakness and night sweats[7-9].

Awidespread occurrence of NTM-related diseasesdueto *Mycobacterium avium* Complex (MAC), *M. kansasii* and other NTM'shave been observed in the period of HIV-AIDS[3,10,11]. During this period, a salient observation was seenin patients who had CD4 counts of less than 50/ml presenting withbloodstream associated MAC infections. Of note, NTM's particularly *M.avium, M.kansasii,* and *M.intracellulare*produce pulmonary disease in humans that may be indistinguishable from that caused by the members of the MTBC (*M.tuberculosis* and *M.bovis)*[3]. There is a significant difference in the treatment of NTM relatedpulmonary infections and tuberculosis caused by MTB. As a result, a species-level identification of mycobacteriais necessary for the selection of appropriate antimicrobial therapy[12,13].

A microscopic evaluation of an auramine stain, although widely used in low-income countries for the diagnosis of TB, isunable to confirm the presence of viable mycobacteria[14]. An over-diagnosis of a treatment failure may possibly be the final outcome after performing a sputum smear microscopic on a sputum sample received from a patient on treatment[14]. Routinely employed laboratory methods for the species identification of mycobacteria such as growth rate, biochemical tests, analysis of antibiotic resistance patterns and HPLC (high pressure liquid chromatography) analysis of fatty acid constituents of the

bacterial cell wall are time-consuming, requiring as long as 6-12 weeks to confirm a positive identification[3,15,16]. According to Buchan *et al.*, HPLC is insufficient to accurately differentiate between closely related species such as *M. chelonae/M. abscessus*, *M. avium/M. intracellulare*, and species within the *M. mucogenicum* group, which may be important to patient care decisions [12]. In addition to the above, mycobacterial isolates from patients with chronic infections often lose their characteristic phenotypes, thus hindering the performance of several conventional diagnostic methods[3].

In most pathology TB laboratories in South Africa, commercially available Genotypic PCR-based identification assays coupled with reverse line hybridization are widely used for thedetection ofpolymorphisms within the 16S or 23S rRNA. These methods have been widely utilised for the species-specific identification and differentiation of more than 30 clinically relevant mycobacterial species[13,17] and when coupled with other diagnostic modalities have been shown to greatly reduce the diagnostic turnaround (TAT) to a week. However limitations such as misidentification, inability to discriminate closely related members of the MTBC as well as the emergence of new species, limits these diagnostic assays to only detecting a few mycobacteria[13,18,19].A more expensive approach involving sequencing of the 16S rRNA has been widely accepted as the reference method for species identification[20]. However, the analysis of sequence data can be quite cumbersome and the retrieval of valid identification results requires the use of a comprehensive quality-controlled database[21]. As a result many researchers are looking for a cost effective, rapid and reproducible method to differentiate between a wider range of mycobacterial species.

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been recently recognized for its ability to identify bacteria to a species level through the analysis of the protein composition of a bacterial cell [17,22]. The principle of MALDI-TOF MS for the identification of bacterial species is based on the ability of the assay to measure the exact sizes of peptides and proteins through the generation of a unique mass spectral profile[17,23]. Various factors including a low reproducibility of the results, variations in the sample preparation protocol and a limited availability of reference data have

made it impossible to routinely utilize the application of MALDI-TOF MS for the speciation of mycobacteria in a clinical laboratory[21]. Furthermore, the applications of MALDI-TOF MS for the discrimination of clinically relevant mycobacteriausing a reference library database have been documented in a limited number of studies[12,19,21,24,25]. Currently there is no preference on which sample preparation method to use since whole-cell deposits, cell lysates, or crude bacterial extracts have been previously recommended. Given the pathogenic nature of mycobacteria, cellular extraction has been a more preferred method option as opposed to whole cell deposits since this method minimises contact with the infectious organism. Thus far and to the best of our knowledge a studydemonstrating the application of MALDI-TOF MS for the identification of clinically relevant mycobacterial isolates in KwaZulu-Natal, South Africa has not yet been conducted. Therefore the present study is the first of the relatively few studies presented to evaluate a newly developed chloroform-methanol-ethanol-formic acid (CMEFA) cellular extraction protocol for the identification and differentiation of clinically significant mycobacterial isolates using MALDI-TOF MS. The MALDI-TOF MS bioinformatics and statistical algorithms such as pattern-matching algorithm, cluster analysis, and principal component analysis (PCA)were also evaluated during the identification and discrimination of clinical mycobacterial isolates.The reference spectral database library (CMEFA-MSP, Chapter 3) waschallenged using blind-coded clinically relevant mycobacterial isolates sourced from an independent facility.

4.3 MATERIALS AND METHODS

4.3.1 Mycobacterial strains and clinical isolates

Eight different mycobacterial species consisting of American Type Culture Collection (ATCC) strains and clinical isolates were used in this study (Table 4.1). The ATCC strains were purchased as a KWIK-STIK™ device (Microbiologics,USA) consisting of lyophilised pellets. The clinical isolates were well characterised using the Genotype CM (Common

Mycobacteria), AS (Additional Species) and MTBC (*Mycobacterium tuberculosis Complex*) assays (Hain-Lifescience, Nehren Germany).

Table 4.1 Mycobacterial ATCC-typed strains and clinical isolates employed in this study.

Mycobacteria	No. of Clinical Isolates	Source
M. abscessus ATCC 23040		ATCC
M. abscessus	8	Clinical isolates
M. avium	4	Clinical isolates
M. bovis		ATCC
*M. fortuitum*ATCC 23031		ATCC
M. fortuitum	15	Clinical isolates
*M. gordonae*ATCC 23409		ATCC
M. gordonae	4	Clinical isolates
*M. intracellulare*ATCC 3950		ATCC
M. intracellulare	3	Clinical isolates
M. marinum ATCC 927		ATCC
M. marinum	2	Clinical isolates
*M. scrofulaceum*ATCC 19981		ATCC
M. scrofulaceum	1	Clinical isolates
M. tuberculosis ATCC 25177		ATCC
M. tuberculosis	2	Clinical isolate

4.3.2 Media and Culture Preparation of ATCC strains

The mycobacterial ATCC strains were purchased as lyophilised pellets in the form of a KWIK-STIK™ (Microbiologics,USA) device packaged within a pouch. Mycobacterial pellets contained within the quick stick device was allowed to equilibrate to room and

rehydrated according to manufacturer's guidelines. The rehydration process was performed in a Level II Biosafety cabinet, with full personal protective equipment, including a N95 respiratory mask. A swab was heavily saturated with the fluid containing the mycobacterial organism and then transferred to a drug free Middelbrook 7H11 agar plate (Becton Dickinson,USA).The Middelbrook 7H11 agar plate was inoculated by gently rolling the swab over one-third of the plate. The culture plate was incubated at 37°C for four and twenty one days to cultivate fast (*M. fortuitum)* and slow growing (*M. gordonae*and *M. tuberculosis*) mycobacterial strains respectively. Once confluent growth was obtained, a single loop-full was transferred into a MicroBankcryovial (Pro-Lab Diagnostics, Canada) and stored at -70°C as per manufacturer's instruction.

4.3.3 Acquisition andGenotypic-based Identification of Clinical Isolates

The following aspects relating to the collection, cultivation and identification of the 39 clinical isolates of mycobacteria used in this study were performed by the NHLS (National Health Laboratory Service) TB reference pathology laboratory based at Inkosi Albert Luthuli Central Hospital (IALCH). The isolates are presented in Table 4.1 and it must be highlighted that this screening process did not source and identify clinical isolates representing *M. bovis, M. kansasii and M. terrae*. This is indeed not surprising due to the extremely infrequent incidence of *M. bovis,M.kansasii*and *M. terrae*related mycobacteriosis in the Durban metropolis of KwaZulu-Natal.

4.3.3.1 Collection and culturing of clinical isolates of mycobacteria

A large number of sputum samples representing putative clinical mycobacterial infections were collected from different regional hospitals of Durban, South Africa. Sputum samples (1 ml) were decontaminated with an equal volume N-acetyl-L-cysteine-sodium hydroxide (NALC-NaOH). All putative mycobacterial specimen manipulations were performed in a biological safety cabinet class-II equipped with high-efficiency particulate air filters (HEPA). The samples were allowed to stand at room temperature for 15-20 min. Sodium phosphate buffer (pH 6.8; 48ml) was added to samples and centrifuged at 3,000 x g for 15 min. The sediment was retained with approximately 3 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 [26].

Mycobacterial growth indicator tubes (MGIT) supplemented with a BBL™ MGIT™ OADC enrichment and BBL™ MGIT™ PANTA™ antibiotic mixture (Becton Dickinson, USA) were inoculated with decontaminated sputum sediments and incubated in the Bactec 960 mycobacterial detection system (Becton Dickinson, USA). Only MGIT-positive cultures were further assessed for the presence of an acid fast bacilli (AFB) using a ZiehlNeelsen (ZN) stain. All AFB positive MGIT cultures were then sub-cultured onto drug free solid Middelbrook 7H11 agar and incubated at 37°C. Genotypic-based identification of isolates was performed on cultures that displayed confluent growth between 4 and 21 days.

4.3.3.2 Genotypic-based screening of clinical isolates of mycobacteria

Speciation of isolates was performed according to manufacturer's instructions using the Genotype CM (Common Mycobacteria), AS (Additional Species) and MTBC assays (Hain-Lifescience GmbH, Nehren Germany). This technique uses DNA strip technology based on the reverse hybridization of PCR products to their complementary probes and targets the 23S rRNA gene region for the simultaneous detection and identification of mycobacteria. Subspecies of the *M.tuberculosis* complex (MTBC) were easily discriminated using the primer nucleotide mix (PNM) provided with the Genotype MTBC assay. The PNM mix targets the 23S ribosomal DNA fragment, detects*gyrB*DNA sequence polymorphisms and the RD1 deletion of *M.bovis* BCG for an accurate speciation[17]. The protocol consisted of DNA extraction, PCR amplification, reverse hybridization of the amplified PCR products to species-specific oligonucleotide probes (probe sequences immobilized on nitrocellulose strips) followed by chemical detection.

4.3.3.2.1 Isolation of genomic DNA

A single colony of culture isolate was harvested using a disposable 10 µl inoculating loop on Middlebrook 7H11 agar plate and suspended in 300µl of molecular grade distilled water contained within a 1.5 ml screw cap "O" ring tube. The tubes were vortexed for approximately 10 s and the mycobacterial cells were pelleted by centrifuging at 10 800 x g for 15 min in a table-top centrifuge with an aerosol-tight rotor. The supernatant was discarded and the pellet resuspended in 100µl of molecular grade distilled water. The suspension was incubated for 30 min at 98°C in a heat block to allow for heat inactivation and cell lysis. This process was followed by sonication in an ultrasonic water bath for 15 min at room

temperature (RT). After a centrifugation at 10800 x g for 5 min, free DNA present in the supernatant was used as a DNA template (5µl) for PCR amplification.

4.3.3.2.2 PCR amplification

DNA was amplified using species specific optimized primers provided with the Genotype CM, AS and MTBC assay. The PCR amplification was performed in a 50µl reaction mixture consisting of 5µl DNA template, 35µl primer- nucleotide mix (PNM), 5µl PCR buffer, 2µl of a 2.5mM $MgCl_2$, 0.2µl of a 1.25 U hot start*Taq* DNA Polymerase (Qiagen, Hilden, Germany) and 3µl distilled water. Amplification was performed in an ABI 2700 thermocylcer (Applied Biosystems, USA) under the following PCR cycling conditions: denaturation at 95°C for 15 min; 10 cycles of denaturation at 95°C for 30s and elongation at 58°C for 120s; an additional 20 cycles of denaturation at 95°C for 25s; annealing at 53°C for 40s and elongation at 70°C for 40s and a final extension of 70°C for 8min.

4.3.3.3 DNA Hybridization

The hybridization of PCR-generated fragments to probes was performed in a semi-automated washing and shaking device(Twincubator, Hain-Lifesciences, Nehren, Germany). Hybridization was initiated by denaturing 20µlof the amplified double stranded DNAinto single strands using 20µl of denaturing (DEN) solution. Single stranded DNA was hybridized to membrane bound probes for 30min at 45°C using a pre-warmed hybridisation solution. All steps were carried out in a plastic hybridisation plate with the DNA samples treated in separate wells. The unbound amplicons were removed using a stringent wash solution. Several rinse solutions were used for washing off amplicons at 25°C. Hybridised amplicons containing biotin were chromogenically visualised using streptavidin conjugated with an alkaline phosphatase. A substrate buffer was also added to generate the formation of a colourful band. At least two wash steps were performed to stop the reaction, allowing for the strips to be air dried and interpreted. Once the test strips were attached to a Genotype template, the development of a conjugate, universal and genus control bands were carefully assessed for each isolate. Reactions with banding patterns whose intensities were as strong as or stronger than the universal control line were interpreted. Specific banding patterns composed of clear cut hybridisation and colorimetric staining signals on a nitrocellulose

membrane strip are shown in Figure 4.1. Strain identification was performed according to the manufacturer's guideline(Figure 4.2 and 4.3) which stipulatesthetheoretical banding patterns of different mycobacterial species.

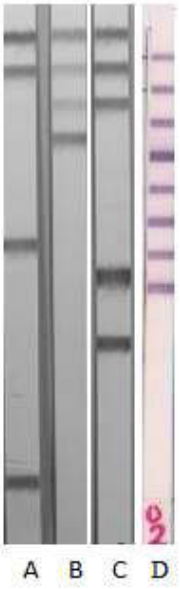

Figure 4.1 Genotype CM assay (Strips A, B and C). *M.fortuitum* (StripA, bands 7 and 14), *M.avium* (StripB, band 4) and *M.gordonae* (StripC, bands 8 and 10).Genotype MTBC assay (Lane D) showing*M.tuberculosis* (bands 1-8).

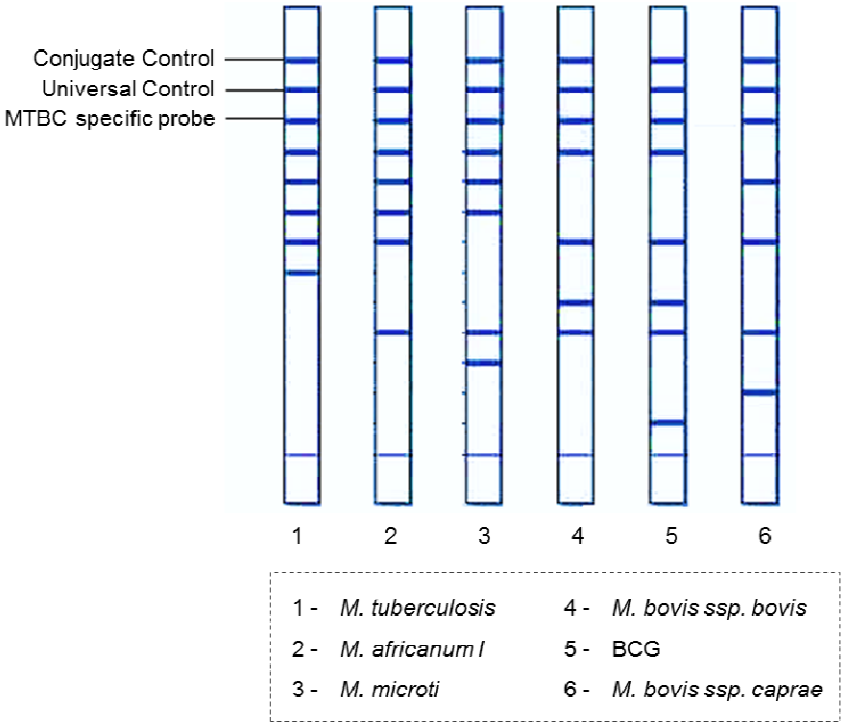

Figure 4.2 Interpretation chart used for the discrimination of the members of the *M. tuberculosis* complex.

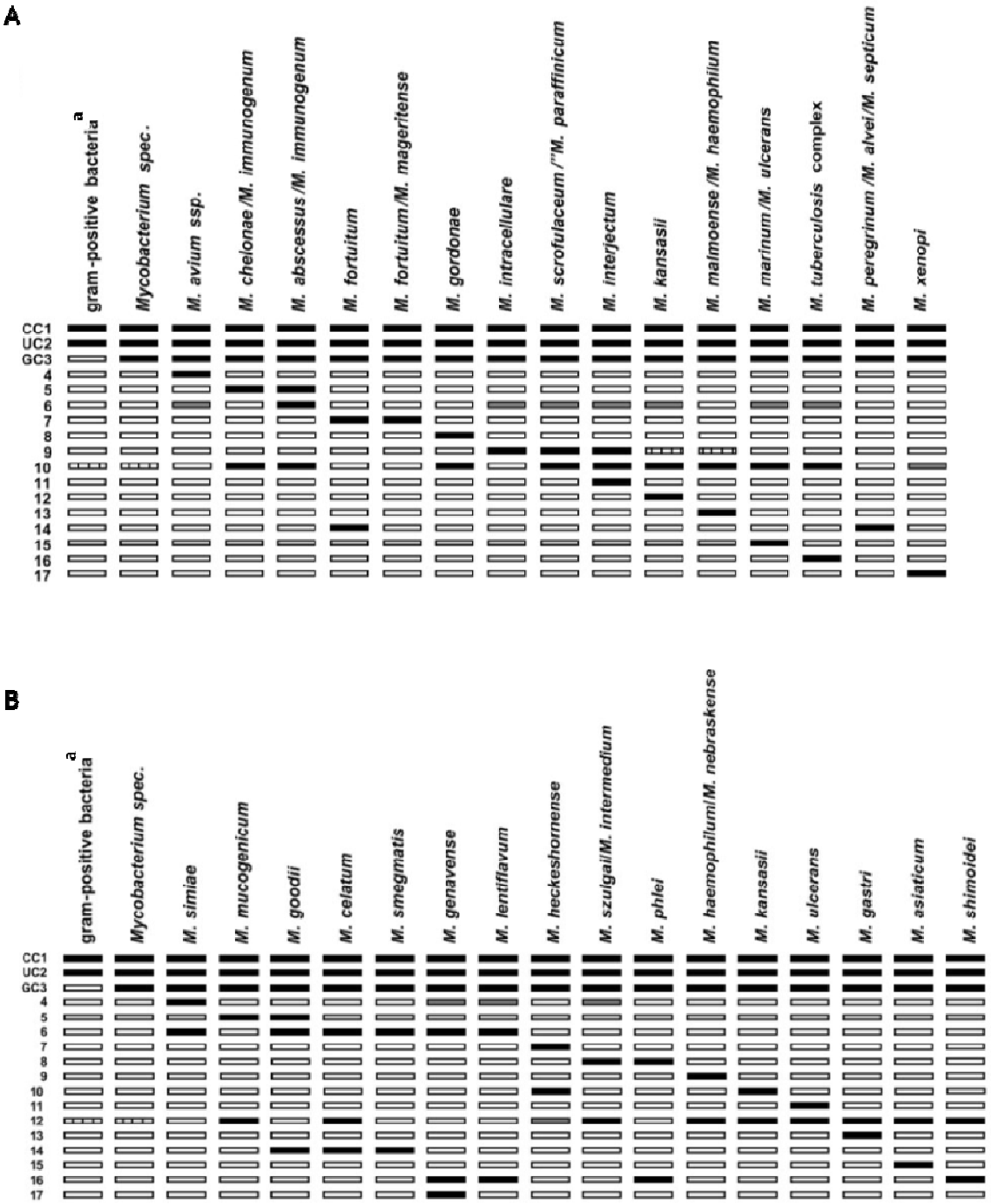

Figure 4.3 Interpretation chart for the Genotype CM (A) and AS (B) assays. Bands from top to bottom are as follows: 1, conjugate control (CC); 2, universal control (UC); 3, genus control (GC); 4 to 17, species-specific probes for the identification of individual and combined mycobacterial species. Gram-positive bacteria with a high G+C content in their DNA are indicated by the superscript "a". The filled boxes represent staining, striped boxes represent facultative staining; grey boxes represent facultative weak staining and blank boxes represent no staining (Hain-LifeSciences, Nehren, Germany).

4.3.4 MALDI-TOF MS Proteomic Profiling of Clinical Isolates

4.3.4.1 Chloroform-methanol ethanol-formic acid (CMEFA) extraction protocol

The newly developed CMEFA protocol was evaluated for its effective delipidation of mycobacterial cells. Lipids and mycolic acids that constitute the mycobacterial cell envelope serve as barriers and hinder the protein extraction process resulting in fewer proteins being available for accurate identification by MALDI- TOF MS. The CMEFA sample preparation protocol for mycobacteria was as follows: a 10µl disposable inoculating loop was used to obtain a single mycobacterial colony from a Middelbrook 7H11 agar plate containing growths of either an ATCC mycobacterial strain or a clinical isolate. The colony was suspended in 600 µl of HPLC grade distilled water contained in a 1.5 ml screw cap "O" ring tube (Sarstedt, Germany). The tube containing the cell suspension was vortexed for approximately 60 s and then heat inactivated at 98^{o}C for 30 min. The heat inactivated cell suspension was then centrifuged at 13 000 x g for 5 min in a table top centrifuge with an aerosol-tight. The delipidation process of mycobacterial cells was initiated by the addition of 600 µl of a chloroform/methanol (1/1, v/v) solvent mixture. The cell suspensions were vigorously vortexed for 60 s, centrifuged at 13,000 x g for 5 min and the supernatants discarded. The delipidation treatment was repeated twice more so as to enhance the removal of lipids and mycolic acids. The supernatant was discarded and the pellet was re-suspended in 300 µl of HPLC grade distilled water followed by 900 µl of absolute ethanol (HPLC grade). The cell suspension was vortexed for 2 minat 13 000 x g and the supernatant was carefully removed. An additional centrifugation step was performed to completely remove all residual ethanol. The pellet was air dried at room temperature for 5 min, depending on the volume of the pellet (ranging from 20-30 µl), 40-80 µl of 70% formic acid was added and the suspension was thoroughly vortexed for 30 s. Acetonitrile (50 µl) was added to the suspension and tubes were vortexed for another 30 s. The samples were centrifuged for 2 min at 13 000 x g. A 1µl aliquot of the supernatant was spotted on a MTP 384 ground steel target

plate (Bruker Daltonics, Germany). The applied sample was air-dried and subsequently coated with 1 µl of freshly prepared saturated HCCA matrix solution. The extracted samples containing matrix were allowed to air-dry for 5 min before mass spectral analysis**.**

4.3.4.2 MALDI-TOF MS instrumentation

The spotted MTP 384 ground steel target plate was inserted into the tray of theAutoflex III smartbeam MALDI-TOF MS instrument (Bruker Daltonics, Germany) and processed using the Flex control software. Instrument parameters for ion source 1 and ion source 2 were set at 20 kV and 18.57 kV respectively. The lens was set at 8.5kV and a pulsed ion extraction of 250 ns was used. The protein molecules embedded in the matrix crystals were effectively desorbed and ionized by a smart beam laser pulse with a frequency of 20Hz and accelerated in a high voltage field. Different ions representing intact sample proteins proportional to their mass were detected after a time of-flight resulting in a high resolution mass spectrum of the sample. Spectra were acquired in a linear positive mode with a mass-to-charge (m/z) range of 2to 20 kDa.

4.3.4.3 Acquisition of MALDI-TOF MS data

Prior to sample analysis, the MALDI-TOF MS instrument was calibrated with the Bruker bacterial test standard (BTS) (Bruker Daltonics, Germany) with a mass range of 3.6 to 17 kDa. The "MBT_FC.par" standard FlexControl method was selected for both analysis and internal calibration of BTS. Using the flex control method "MBT_FC.par", calibration was performed automatically by adding up 6 times 40 laser shots from different positions of the BTS spot with the maximum deviation below ± 300 ppm as recommended by the manufacturer. Each mycobacterial ATCC-typed strainor clinical isolate was spotted at 6 positions on the MALDI target plate and measured four times per spot. Thus twenty four spectra weregenerated using replicate cultures; this was repeated on three separate occasions thereby generating 72 spectra per mycobacterial sample.The spectra fulfilled the specifications of Bruker Daltonics for construction of a main spectral profile (MSP) in that each spectrum must contain a minimum of 25 peaks with a resolution > 400, with a minimum of 20 of the peaks having a resolution > 500.A main spectral profile (MSP) consisting of a minimum of 24 of the most reproducible spectra were individuallycreated using the Biotyper 3.0 software for all mycobacterial samples.

For quality assurance, all raw spectra used for MSP creation were processed according to a standardized protocol as advised by Bruker Daltonicsusing theFlexAnalysis 3 software program (Bruker Daltonics, Germany) and utilising the "MBT Process method" for the smoothing and baseline subtraction of raw spectra. The presence of flat-liners, outliers or single spectra with peaks differing from the other spectra was also interrogated using the FlexAnalysis 3 software program. The mass deviation within the spectra was set at not more than 500 ppm (mass window of 3 Dalton for a peak at 6 kDa) therefore any mass deviation above 3 Da, particularly for peaks at mass range between 6-7 kDa were eliminated. A list of the most significant peaks of a spectrum (*m*/*z* values with a given intensity) was created by performing functions within the FlexAnalysis such as smoothing, normalization, baseline subtraction and peak picking. Biotyper 3.0 software (Bruker Daltonics, Germany) was used to obtain themain spectral patterns (MSP's) of mycobacteria processed in this study using the CMEFAextraction protocol.

4.3.4.4 Principle component analysis (PCA)

Principle component analysis (PCA) was employed to statistically evaluate MALDI-TOF mass spectra. PCA is a broadly used mathematical technique for reducing the dimensionality of multivariate data whilst preserving most of the variance. PCA was performed using ClinProTools software 2.2 (Bruker Daltonics, Germany). The PCA is managed by an external MATLAB software tool, which is integrated in ClinProTools. Prior to PCA analysis spectra were processedinto their MSP's as described above. Complex relationships between samples of mycobacterial isolates were explored and illustrated with the use of a PCA plot. This model provides insights into similarities or dissimilarities between samplesof mycobacterial isolates. For example samples that cluster closer together are more similar than samples that are further apart.

4.3.4.5 Biotyping of blind-coded isolates using MALDI-TOF MS

To access the accuracy and reliability of the CMEFA-based MALDI-TOF MS strategy in the identification of mycobacteria, 39blind-coded mycobacterial isolates were obtained from an independent institution (Medical Microbiology, NHLS, Inkosi Albert Luthuli Central Hospital).Samples for mass spectral analysis of the blind-coded isolates were prepared using the CMEFA extraction method. Following acquisition of the mass spectra from blind-coded samples they were processed into their respective MSP's. An MSP was then identified or speciated by Bruker Daltonics Biotyper 3 software by comparison to those contained within

the CMEFA-MSP reference library of ATCC-typed mycobacterial strains (Chapter 3).Biotyping was performed from CMEFA-derived samples of three independent cultures of each blind-coded isolate. The results from the profile-matching process were expressed as log (score) values and ranged from 0 to 3 as shown in Table 4.2.

Table 4.2 Interpretation of Bruker Daltonics identification score values [27].

Range	Description	Colour
2.300 to 3.000	Highly probable species identification	Green
2.000 to 2.299	Secure genus and probable species identification	Green
1.700 to 1.999	Probable species identification	Yellow
0.000 to 1.699	No reliable Identification	Red

4.4 RESULTS

The mass spectral fingerprints generated from CMEFA extracted proteinsamples of clinical mycobacterial isolates were defined by strong signals and abundant diagnostic ions across the entire mass/charge range (Figure 4.5). The mass spectral signatures of individual isolates were characterized by high peak intensities and a relative abundance of peaks with low signal-to-noise ratios.

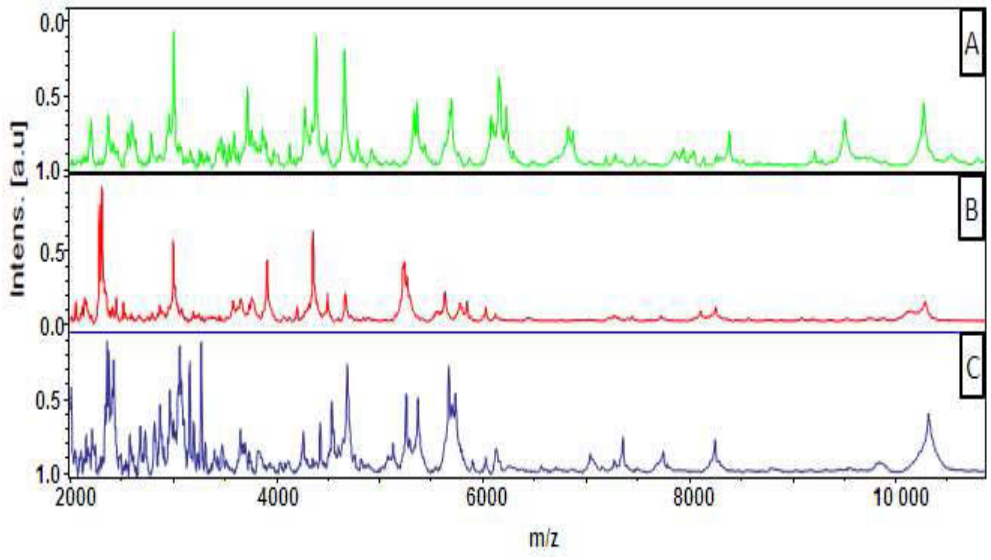

Figure 4.4 Mass spectra of clinical mycobacterialisolates generated with MALDI-TOF MS using CMEFA protein extraction method. (A) *M. abscessus*, (B) *M. fortuitum* and (C) *M. marinum.*

To further characterise the main spectral profiles of CMEFA-extracted samples, the MSP's of five clinical isolates were subjected to three dimensional principal component analyses (PCA) [Figure 4.5]. Replicate spectra from closely related species formed discrete clusters with little variation.Of importance, the MSP's of MTB and NTM isolates reside in distinct spatial arrangementsto one another that were non-overlapping (Figure 4.5). The MTB isolate clustered away from the clinically significant NTM isolates. Of relevance is that *M. avium* and *M. intracellulare,* although closely related at a biological level, clustered on different planesemphasising their dissimilarity.

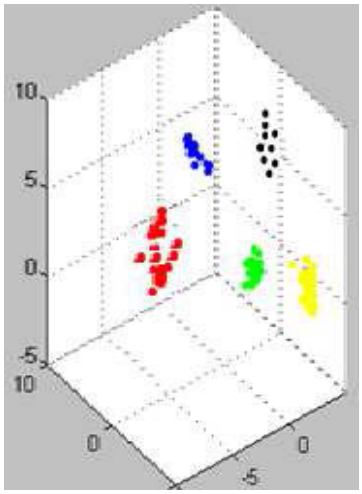

Figure 4.5 Three dimensional principal component analysis (PCA) derived from main spectral patterns ofmycobacterial clinical isolates.*M. tuberculosis*(red), *M. marinum*(blue), *M. intracellulare*(green), *M. avium* (yellow) and *M. scrofulaceum* (black).

The results of the MALDI-TOF MS-based identification of blind-coded clinical mycobacterial isolates are presented in Table 4.3. Interestingly all of the39blind-coded mycobacterial isolateswhen best matched by Bruker Daltonics Biotyper 3 software to MSP's of the CMEFA-MSP reference librarydisplayedlog score values ≥ 2.3 and were identified

correctly to the genus and species level (Table 4.3). TheM. *tuberculosis*isolate was unambiguously differentiated from theNTM isolates. In this study, MALDI-TOF MS-based biotyping correctly identified blind-coded mycobacterial isolates to the genus and species level with 100% accuracy.

Table 4.3 MALDI-TOF MS-based identification of blind-coded mycobacterial isolates.

Sample	Number of isolates	Best match	Score Values	Identification
1a to 1c	3	*M. intracellulare*	> 2.3	Yes
2a to 2d	4	*M. avium*	≥ 2.3	Yes
3a to 3o	15	*M. fortuitum*	> 2.3	Yes
4a to 4d	4	*M. gordonae*	≥ 2.3	Yes
5a & 5b	2	*M. marinum*	> 2.3	Yes
6	1	*M. scrofulaceum*	≥ 2.3	Yes
7a to 7h	8	*M. abscessus*	> 2.3	Yes
8a & 8b	2	*M. tuberculosis*	> 2.3	Yes

A set of mass CMEFA -derived spectral patterns for each ATCC-typed mycobacterial strain and clinical isolate were further analysed using the Biotyper 3.0 software to create a main spectral profile (MSP).A dendrogram was created using default analysis parameters as assigned by Biotyper software (distance measure, correlation; linkage, average) to access the differentiation and clustering of these mycobacteria (Figure 4.6). Notably, *M. tuberculosis* and *M. bovis* organisms from MTBC wereclearly differentiated from each other. In addition the MTBC-related organisms (ATCC and clinical isolates) correctly clustered away from NTM-related mycobacteria.

The MSP's of NTM-related mycobacteria fell into two major cladeswith *M. avium*,*M.intracellulare, M. scrofulaceum*,*M. marinum*and*M. gordonae* in one clad whilst the other contained *M. fortuitum* and *M. abscessus.* It was also noted that each of the NTM

family members formed individually discreet clusters. The latter phylogeny is indicative of high levels of spectral similarities in terms ofthe location and intensity of diagnostic ions.Due to negligible differences in distance, the proteomic profiles of the 15 clinical isolates of *M. fortuitum* seem to be highly conserved whilst there appears to be greater variation with other NTM-related mycobacteria. It should be noted that *M. abscessus,* like *M. fortuitum*is a rapidly growing mycobacteria (RGM) and forms part of the *M. fortuitum*complex. However, although *M. abscessus* shared the same clade as *M. fortuitum*, the formation of discrete clusters has allowed for a phylogenetic discrimination of these two closely related organisms.

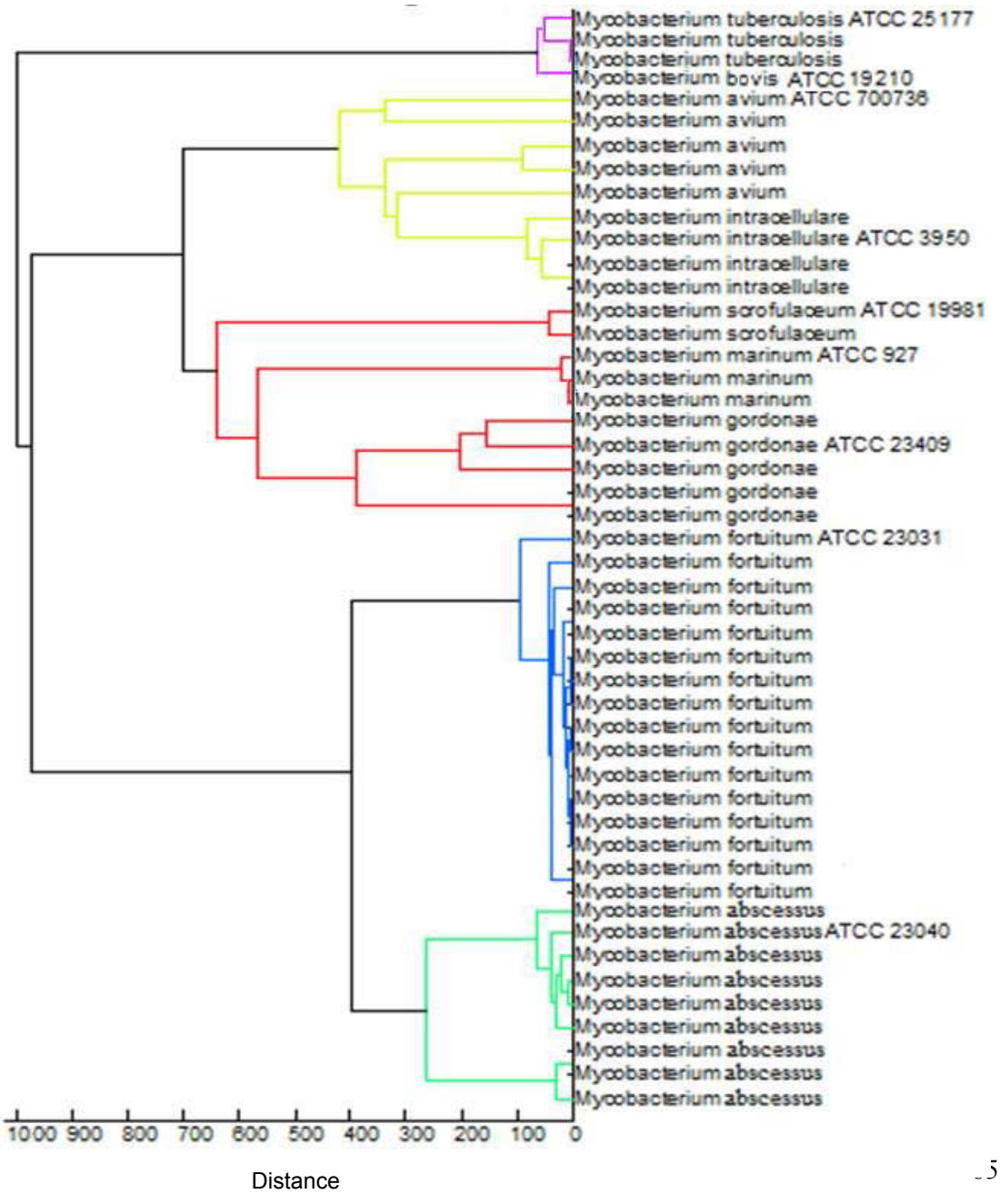

Figure 4.6 Dendrogram created from hierarchical cluster analysis of the main spectral profiles (MSP) of ATCC-typed strains and clinical isolates of mycobacteria using the CMEFA sample preparation protocol.

4.5 DISCUSSION

This is the first study that has generated a localMALDI-TOF main spectral profile (MSP) database for the explicit purpose of biotypingclinically significant mycobacterial isolatesusing CMEFA sample preparation protocol.The diagnosis of MTB as the causative agent of pulmonary TB using only clinical symptoms may be inadequate and inaccurate since*M.bovis*, *M.avium, M.kansasii,* and *M.intracellulare*all produce pulmonary disease in humans that may be indistinguishable from that caused by MTB[4,28].In this regard, the potential of the CMEFAsample preparation protocol was evaluated for a rapid and reliable discrimination of clinical relevant mycobacterial isolates using MALDI-TOF MS.

The data from this present study clearly demonstrates that the newly developed organic solvent-based CMEFA protein extraction method which is cost-effective and simple to perform is highly efficient in producing mycobacterial cellular protein samples for MALDI-TOF MS analysis to generate high resolution MSP's. In addition, the independent CMEFA-MSP-reference library containing MSP's of ATCC-typed strains were used in the undisputable differentiation of blind-coded clinical mycobacterial isolates sourced from an independent research facility. All 39 blind-coded clinical mycobacterial isolates representing eight different mycobacteria were identified with 100% accuracyto the species level(log score values $\geq$ 2.3).Members of the MTBC family (*M. tuberculosis and M. bovis*) were unambiguously differentiated from each other and also from mycobacteria representing NTMclinical isolates.Although our study shares similar findings to that of Saleeb and co-workers in terms of differentiation of clinical relevant mycobacterial isolates, it should be highlighted that the previous study madeidentifications with lower log score values ranging from 1.8 to $\geq$ 2 which are only reflective of probable species only as per Bruker Daltonic. In this respect it seems that the CMEFA-based MALDI-TOF MS mycobacterial biotyping strategy employed in this study is superior to EFAGB-based strategy used by Saleeb*et al.* which did not clearly resolve or distinguish between MTBC-related mycobacteria [25].

According to Gutacker*et al.,* genomic differences can be seen at the protein level[29]. Thus it can be suggested that the unambiguous classification of the clinical mycobacterial isolates employed in this study were achievedthrough therecognition of differences in the proteomicprofilesof clinical isolates. It has also been reported that the proteomic profiling of intact mycobacteria using MALDI-TOF MS analysis is based on conserved ribosomal proteins thatare abundantly expressed within the cells[25,30,31]. In this study, cluster analysis based on a matrix of pair wise correlation values of all clinical mycobacterial MSP'sresulted in two separate clusters at the species level, one for MTBC members and the other including all clinical isolates of the NTM. Within the second cluster, NTM isolates formed discrete clusters with biologically similar isolates clustering closer together.Of significance, is thatthe closely related organisms*M. abscessus*and *M. fortuitum (M. fortuitum*complex*)* shared the same clade and formed discrete clustersresulting in phylogenetic discrimination of these two organisms.Similar findings were observed for *M. avium* and *M.intracellulare* isolates.In this respect it seems that the CMEFA-based MALDI-TOF MS mycobacterial biotyping strategy employed in this study is superior to EFAGB-based strategy used by Saleeb*et al.* which did not clearly resolve or distinguishsimilar NTM-related mycobacteria[25].

The use of the newly developed CMEFA protein extraction protocolseems to havefacilitated the removal of unwanted lipids and physiological salts, both of which are likely to interfere with matrix crystallization and spectral quality[32]thereby increasing the quality of the mass signals. Overall in comparison to the spectra obtained by Saleeb and co-researches, a significantly increased number of diagnostic ions in the mass/charge range of 2 to 10 kDa were observed for CMEFA-derived samples and are most probably reflective of higher protein content samples.

This study joins the relatively few studies available describing a specific protein extraction protocol that is useful for all types of mycobacteria. Although the newly developed reference library was not as comprehensive as those described in previous studies [12,19,21,24,25], the potential of the library was observed for the unequivocal discrimination of clinically relevant mycobacterial isolates frequently associated with disease in our local setting. In addition to the above, a platform is now available for the addition of other species of mycobacteria into

the database as needs arise. This is an important milestone since the number of species within the genus *Mycobacterium* is ever expanding hence diagnostic laboratories need to continuously employ strategies to identify new emerging mycobacterial pathogens. Although the CMEFA-based MALDI-TOF MS mycobacterial biotyping strategy remains to be ratified in other independent laboratories, this study demonstrates its viable potential as a simple and cost effective diagnostic tool that providesrapid results (approximately 2 hours). Moreover the system lends itself to automation thereby permitting high throughput workflow.

4.6 ACKNOWLEDGEMENTS

This work was made possible through support from the Department of Microbiology, National Health Laboratory Services, Inkosi Albert Luthuli Central Hospital and the Department of Biochemistry, University of Kwa-Zulu Natal. Melendhran Pillay was supported by the Columbia University-Southern African Fogarty AIDS International Training and Research Program (AITRP), Implementation Science Traineeship Program funded by the United States President's Emergency Plan for AIDS Relief (PEPFAR) through the Fogarty International Centre, National Institutes of Health (grant # D43TW00231)".

4.7 REFERENCES

1. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, et al. (2007) An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. American Journal of Respiratory and Critical Care Medicine 175: 367-416.

2. Karyadi E, Schultink W, Nelwan RH, Gross R, Amin Z, et al. (2000) Poor micronutrient status of active pulmonary tuberculosis patients in Indonesia. The Journal of Nutrition 130: 2953-2958.

3. Schlossberg D (2011) Tuberculosis and nontuberculous mycobacterial infections: ASM Press.

4. Youmans G, Paterson P, Sommers H (1985) The biological and clinical basis of infectious diseases. Saunders, Philadelphia, PA.

5. Organization WH (2013) Global tuberculosis report 2013: World Health Organization.

6. Aziz MA (2004) Anti-tuberculosis drug resistance in the world: third global report: the WHO/IUATLD Global Project on Anti-tuberculosis Drug Resistance Surveillance, 1999-2002: World Health Organization.

7. Buijtels PCAM (2007) Clinical relevance of non-tuberculous mycobacteria in Zambia: Erasmus MC: University Medical Center Rotterdam.

8. Katoch V (2004) Infections due to non-tuberculous mycobacteria (NTM). Indian Journal of Medical Research 120: 290-304.

9. Ryan KJ, Ray CG (2010) Sherris medical microbiology: McGraw Hill Medical New York.

10. Corbett EL, CHURCHYARD GJ, Hay M, Herselman P, Clayton T, et al. (1999) The impact of HIV infection on Mycobacterium kansasii disease in South African gold miners. American Journal of Respiratory and Critical Care Medicine 160: 10-14.

11. Miguez-Burbano MJ, Shor-Posner G, Hadrigan S (2005) Non-tuberculous mycobacteria in HIV-infected patients: geographic, behavioural, and immunological factors. The Lancet infectious diseases 5: 394-395.

12. Buchan BW, Riebe KM, Timke M, Kostrzewa M, Ledeboer NA (2014) Comparison of MALDI-TOF MS with HPLC and nucleic acid sequencing for the identification of Mycobacterium species in cultures using solid medium and broth. American Journal of Clinical Pathology 141: 25-34.

13. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. Journal of Clinical Microbiology 41: 1010-1015.

14. Salim H, Aung K, Hossain M, Van Deun A (2006) Early and rapid microscopy-based diagnosis of true treatment failure and MDR-TB. The International Journal of Tuberculosis and Lung Disease 10: 1248-1254.

15. Cole ST, Eisenach KD, McMurray DN, Jacobs Jr W (2005) Tuberculosis and the tubercle bacillus: ASM Press.

16. Reller LB, Weinstein MP, Hale YM, Pfyffer GE, Salfinger M (2001) Laboratory diagnosis of mycobacterial infections: new tools and lessons learned. Clinical Infectious Diseases 33: 834-846.

17. Mellmann A, Cloud J, Maier T, Keckevoet U, Ramminger I, et al. (2008) Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. Journal of Clinical Microbiology 46: 1946-1954.

18. Imperiale B, Zumárraga M, Gioffré A, Di Giulio B, Cataldi A, et al. (2011) Disease caused by non-tuberculous mycobacteria: diagnostic procedures and treatment evaluation in the North of Buenos Aires Province. Revista Argentina de Microbiología 44: 3-9.

19. 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.

20. Torkko P, Suutari M, Suomalainen S, Paulin L, Larsson L, et al. (1998) Separation among species of Mycobacterium terrae complex by lipid analyses: comparison with biochemical tests and 16S rRNA sequencing. Journal of clinical microbiology 36: 499-505.

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

22. de Mello KGC, Mello FCQ, Borga L, Rolla V, Duarte RS, et al. (2013) Clinical and Therapeutic Features of Pulmonary Nontuberculous Mycobacterial Disease, Rio de Janeiro, Brazil. Emerging Infectious Diseases 19: 393.

23. Seibold E, Maier T, Kostrzewa M, Zeman E, Splettstoesser W (2010) Identification of Francisella tularensis by whole-cell matrix-assisted laser desorption ionization-time of flight mass spectrometry: fast, reliable, robust, and cost-effective differentiation on species and subspecies levels. Journal of clinical microbiology 48: 1061-1069.

24. Hettick JM, Kashon ML, Simpson JP, Siegel PD, Mazurek GH, et al. (2004) Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analytical Chemistry 76: 5769-5776.

25. Saleeb PG, Drake SK, Murray PR, Zelazny AM (2011) Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Journal of Clinical Microbiology 49: 1790-1794.

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

27. Bruker (2007) Bruker BioTyper User Manual. Version 2.0.

28. Jarzembowski JA, Young MB (2008) Nontuberculous mycobacterial infections. Archives of Pathology & Laboratory Medicine 132: 1333-1341.

29. Gutacker MM, Smoot JC, Migliaccio CAL, Ricklefs SM, Hua S, et al. (2002) Genome-wide analysis of synonymous single nucleotide polymorphisms in Mycobacterium tuberculosis complex organisms: resolution of genetic relationships among closely related microbial strains. Genetics 162: 1533-1543.

30. Jarman KH, Cebula ST, Saenz AJ, Petersen CE, Valentine NB, et al. (2000) An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Analytical Chemistry 72: 1217-1223.

31. Maier T, Klepel S, Renner U, Kostrzewa M (2006) Fast and reliable maldi-tof ms–based microorganism identification. Nature Methods| Application Notes.

32. Gustafsson JO, 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.

Chapter 5

GENERAL DISCUSSION AND CONCLUSION

5.1 GENERAL DISCUSSION AND CONCLUSION

Mycobacterium tuberculosis remains the causative agent of tuberculosis (TB) disease worldwide with Southern Africa being the most affected sub-Saharan country. Tuberculosis disease is found to be the leading cause of death in people living with HIV and AIDS, and in a high HIV prevalence setting such as South Africa, TB is often the first sign of HIV infection[1,2]. In 2006, an outbreak of a deadly and almost incurable form of TB was reported in a rural community of KwaZulu-Natal province in South Africa[1]. The deadly strain of TB known as extensively drug-resistant TB (XDR-TB) occurred among HIV-infected people[1] and was found to be resistant to both first and second-line anti-tuberculosis drugs. Fifty two of the 53 patients diagnosed with the disease at the time of the outbreak, died on average within 16 days of diagnosis[1].

As much as the emergence of multi drug resistant (MDR) and extensive drug resistant (XDR) strains of MTB have contributed to the burden of tuberculosis disease, the apparent increase in the number of infections caused by the NTM have also been observed in the era of the HIV/AIDS epidemic, with a high level of interest of NTM disease occurring in AIDS patients[3-5]. Of concern is that in developed countries NTM play a significant role in the aetiology of tuberculosis-like syndromes, especially in HIV-positive patients[3,6,7].Of note the prevalence of pulmonary NTM in developing countries remains unknown and may be underestimated [22]due to a lack of routine culture and identification methods. Even though the prevalence of NTM can only be estimated, there is sufficient evidence to show that NTM infection rates and actual disease are increasing [3].

Recently there has been a dramatic increase not only in the total number of NTM mycobacterial species but also in the number of clinically significant species [23].Prevalence rates of NTM colonization and infection have been reported in two epidemiological studies conducted in South Africa. The prevalence rates were found to be 1,400 and 6,700 per 100,000, respectively when results of sputum cultures from South African native people were analysed [7-10]. Anotherstudy conducted on South African gold miners showed the annual rates of NTM infection to be 101 per 100 000 with *M. kansasii*(66/100,000) and *M. scrofulaceum*(12/100,000) being the two most common organisms isolated [3,5,11-13].

Conventional laboratory methods for the identification of NTM and MTBC organisms rely on phenotypic characterization (growth rate, colony morphology, pigment production and staining techniques) and biochemical profiling. However, the main disadvantages associated with these methods are their extensive processing times (6-12 weeks to confirm a positive identification), which are considerably intensive in terms of both labour and cost [7]. A drive to unequivocally discriminate microbes of both MTBC and NTM families and to speed up their presumptive identification as witnessed the development of a number of molecular biology-based strategies which include the hybridisation of PCR generated amplicons to specific probes and 16SrRNA gene sequencing. These methods have found favour in well-resourced privately- and government-owned pathology laboratories, as they are technically demanding and are dependent on expensive laboratory consumables and equipment. These methods are proving to be quiet effective for the detection of specific Mycobacterium species within 3 weeks thus contributing positively to both patient care and prognosis. Unfortunately, only a limited number of mycobacterial species can be identified by these molecular biology-based assays [14].

Recent advances in the field of mass spectrometry have made it possible to use matrix–assisted laser desorption/ ionization time-of- flight mass spectrometry (MALDI-TOF-MS) to aid in the identification of mycobacterial species by proteomic profiling[15,16].A wide variety of prokaryotes have been characterized using this approach, including clinically and environmentally relevant Gram positive and negative bacteria. However a limited number of studies have shown the potential of MALDI-TOF MSfor the proteomic profiling of various mycobacteria including *M. tuberculosis*[15-21]. To date there seems to be no consensus on which sample preparation method is preferred in the discrimination of mycobacteria. In this regard, the present study assessed the feasibility of using MALDI-TOF MS proteomic profiling as a rapid and precise technology to identify clinically-relevant mycobacteria in Kwazulu-Natal, South Africa. Eleven different mycobacterial species consisting of American Type Culture Collection (ATCC) strains representing four major groups: the MBTC, the MAC, the rapid, and the slow growing mycobacteria were employed in the proteomic profiling of mycobacteria by MALDI-TOF MS using a CMEFA sample preparation protocol.

Our data clearly illustrated that the standard EFAsample preparation method although used for other genera of bacteria was inefficient for the isolation of proteins from ATCC-typed mycobacterial strains resulting in poor mass peak intensities for organism identification.It

was hypothesized that mechanical agitation with micro-glass beads employed in the EFAGB sample preparation method would break mycobacterial cell envelopes and release proteins needed for the accurate identification by MALDI-TOF MS [6]. However, ATCC typed mycobacterial strains processed by the EFAGB method yielded protein mass spectral profiles with low intensities and noise peaks,precluding organism identification. This was not unexpected since strain identification and discrimination which are likely due to differences in the relative abundance of shared mass to charge*(m/z)*values of ions from different organisms would be impossible when few peaks are obtained[19,22].

Thus far, the data obtained in the present study has shown that when using the newly developed CMEFA sample preparation protocol coupled with HCCA (α-cyano-4-hydroxycinnamic acid) as the MALDI matrix, highly reproducible and distinctive mass spectral fingerprints of ATCC typed mycobacterial strains were obtained. Thecreation of anindependent CMEFA-MSP reference library containing the MSP's of ATCC-typed stains was used for the explicit purpose of biotyping clinically relevant mycobacterial isolates.All 39 blind-coded clinical mycobacterial isolates representing eight different species were accurately identified (log scores >2.3) to both the genus and species levels. Cluster analysis based on a matrix of pair wise correlation values of all mycobacterial spectra produced two separate clusters at the species level, one for the MTBand the other including all isolates of the NTM. Within the second cluster, NTM isolates of the respective species formed discrete clusters with biologically similar isolates clustering closer together.

Based on results obtained in this study, it can be seen that MALDI-TOF MS is an ideal biotyping tool for the identification of mycobacteria, as it allows for the testing of a large number of isolates, provides rapid results (approximately 2 hours), requires easy and relatively inexpensive procedures, and permits automation. This is thus the first study to establish the potential of MALDI-TOF MS as a rapid biotyping tool for the identification of clinically significant mycobacteria using a CMEFA sample extraction protocol. The initial acquisition cost of this facility is extremely expensive, however; routine maintenance and reagent consumables are economical. It is also noted that a far lower degree of technical expertise is required in the sample preparation and analysis for this technology. It can thus be of high value to both routine and reference laboratories given the highly variable infection manifestations of mycobacteria.

The performance of MALDI-TOF MS as a powerful biotyping tool for the identification of mycobacteria can only improve as more spectra of appropriate reference strains are added to the newly created independent database. A realistic expectation is that a well-established database that is clinically validated will contribute immensely to the diagnosis of tuberculosis and other mycobacteriosis. In future, this technology has potential to greatly assist in the early detection of emerging pathogens particularly amongst the NTM.

5.2 REFERENCES

1. Gandhi NR, Moll A, Sturm AW, Pawinski R, Govender T, 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. The Lancet 368: 1575-1580.

2. Gandhi NR, Andrews JR, Brust J, Montreuil R, Weissman D, et al. (2012) Risk Factors for Mortality among MDR-and XDR-TB Patients in a High HIV-Prevalence Setting. The International Journal of Tuberculosis and Lung Disease 16: 90.

3. Buijtels PCAM (2007) Clinical relevance of non-tuberculous mycobacteria in Zambia: Erasmus MC: University Medical Center Rotterdam.

4. Cook VJ, Turenne CY, Wolfe J, Pauls R, Kabani A (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of nontuberculous mycobacteria: cost analysis. Journal of Clinical Microbiology 41: 1010-1015.

5. Corbett EL, Watt CJ, Walker N, Maher D, Williams BG, et al. (2003) The growing burden of tuberculosis: global trends and interactions with the HIV epidemic. Archives of Internal Medicine 163: 1009-1021.

6. Buijtels PC, Petit PL, Verbrugh HA, van Belkum A, van Soolingen D (2005) Isolation of nontuberculous mycobacteria in Zambia: eight case reports. Journal of Clinical Microbiology 43: 6020-6026.

7. Buijtels PC, van der Sande MA, de Graaff CS, Parkinson S, Verbrugh HA, et al. (2009) Nontuberculous mycobacteria, zambia. Emerging Infectious Diseases 15: 242.

8. Katoch V (2004) Infections due to non-tuberculous mycobacteria (NTM). Indian Journal of Medical Research 120: 290-304.

9. Corbett EL, CHURCHYARD GJ, Hay M, Herselman P, Clayton T, et al. (1999) The impact of HIV infection on Mycobacterium kansasii disease in South African gold miners. American Journal of Respiratory and Critical Care Medicine 160: 10-14.

10. Wallace RJ, Swenson JM, Silcox VA, Good RC, Tschen JA, et al. (1983) Spectrum of disease due to rapidly growing mycobacteria. Review of Infectious Diseases 5: 657-679.

11. Buijtels PCAM, Willemse-Erix H, Petit P, Endtz H, Puppels G, et al. (2008) Rapid identification of mycobacteria by Raman spectroscopy. Journal of Clinical Microbiology 46: 961-965.

12. Arend S, Cerda de Palou E, De Haas P, Janssen R, Hoeve M, et al. (2004) Pneumonia caused by Mycobacterium kansasii in a series of patients without recognised immune defect. Clinical Microbiology and Infection 10: 738-748.

13. Contreras M, Cheung O, Sanders D, Goldstein R (1988) Pulmonary Infection with Nontuberculous Mycobacteria1, 2.

14. Ryan KJ, Ray CG (2010) Sherris medical microbiology: McGraw Hill Medical New York.

15. Hettick JM, Kashon ML, Simpson JP, Siegel PD, Mazurek GH, et al. (2004) Proteomic profiling of intact mycobacteria by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Analytical Chemistry 76: 5769-5776.

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

17. Buchan BW, Riebe KM, Timke M, Kostrzewa M, Ledeboer NA (2014) Comparison of MALDI-TOF MS with HPLC and nucleic acid sequencing for the identification of Mycobacterium species in cultures using solid medium and broth. American Journal of Clinical Pathology 141: 25-34.

18. Cherkaoui A, Hibbs J, Emonet S, Tangomo M, Girard M, et al. (2010) Comparison of two matrix-assisted laser desorption ionization-time of flight mass spectrometry methods with conventional phenotypic identification for routine identification of bacteria to the species level. Journal of Clinical Microbiology 48: 1169-1175.

19. 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.

20. Saleeb PG, Drake SK, Murray PR, Zelazny AM (2011) Identification of mycobacteria in solid-culture media by matrix-assisted laser desorption ionization–time of flight mass spectrometry. Journal of Clinical Microbiology 49: 1790-1794.

21. El Khéchine A, Couderc C, Flaudrops C, Raoult D, Drancourt M (2011) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry identification of mycobacteria in routine clinical practice. PLoS One 6: e24720.

22. Maquelin K, Dijkshoorn L, van der Reijden TJ, Puppels GJ (2006) Rapid epidemiological analysis of Acinetobacter strains by Raman spectroscopy. Journal of Microbiological Methods 64: 126-131.