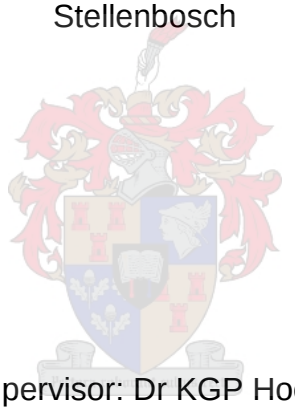
OPTIMISATION AND COMPARISON OF A PHENOTYPIC MALDI-TOF ASSAY WITH MOLECULAR AND PHENOTYPIC METHODS FOR THE RAPID IDENTIFICATION OF SELECTED FUNGAL, NOCARDIA AND NONTUBERCULOUS MYCOBACTERIA.

Wilma Immelman

Thesis presented in partial fulfilment of the requirements for the degree of Masters of Medical Microbiology in the Faculty of Medicine and Health Sciences at the University of Stellenbosch



Supervisor: Dr KGP Hoek
Co-supervisor: Dr Wasserman

March 2020

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ABSTRACT

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) has been utilised in clinical microbiology laboratories for several years, but is mostly used for the rapid and accurate identification of bacteria and yeasts; and to a lesser extent for nontuberculous mycobacteria (NTM), *Nocardia* and moulds.

Due to the variety of methods used for the identification of NTM, *Nocardia* and moulds , the promise of an identification method 'fit for all', as reported in some studies, would have a significant impact on the work flow in a diagnostic laboratory. The MALDI-TOF MS is a relatively low-cost technology with a quick turnaround time following culture. Promising results were reported in various studies and includes identification rates of 87.7% - 99.0% for NTM, 76.0% - 98.0% for *Nocardia* and 66.8% - 94.0% for moulds.

The aim of this study was to compare the identification of selected NTM, *Nocardia* and moulds using MALDI-TOF MS with various phenotypic and molecular methods including routine fungal culture, the Genotype Mycobacterium CM / AS assays, as well as a pan-bacterial and pan-fungal sequencing approach. The study also included a cost and workflow analysis between the different methods employed.

Our study produced identification rates of 21.8% for NTM, 62.5% for *Nocardia* and 38.5% for moulds. A recurring theme for all organism identifications on the Vitek MS was a high rate of "no identifications", despite adequate protein spectral profiles being generated as well as the majority of the organisms being represented in the Vitek MS Knowledge Base Database. Despite significant troubleshooting of the methodology for all organisms, the percentage of successful identifications did not improve. The manufacturer representatives were unable to resolve the issues during the course of this study, suggesting that there may be a software or hardware related problem.

Based on the Vitek MS instrument shortcomings and cost and workflow analysis, we recommend the Mycobacterium CM/AS kit for the speciation of NTMs and the phenotypic identification of moulds. ITS Pan-Fungal sequencing should be used where turnaround time is critical or where culture negative disease is suspected. While the Vitek MS showed promise for *Nocardia* identification, the cost thereof given the large kit size and short stability, makes cost prohibitive. Similarly MLSA analysis provided the most identifications to the species level, but is cost prohibitive. While 16S rRNA sequencing mostly only reported *Nocardia* to the genus level, it remains the only feasible option for *Nocardia* confirmation in the laboratory.

In summary, the Vitek MS requires regular fine-tuning and technical intervention and support. The instrument is perhaps not suited to a high throughput laboratory for the identification of NTMs, *Nocardia* and moulds without increasing it's robustness.

OPSOMMING

MALDI-TOF MS is al vir jare in gebruik in die kliniese mikrobiologie laboratoriums, maar meestal vir die identifikasie van gis en bakterieë; en tot n mindere mate vir die identifikasie van nie tuberkulose Mikobakterieë (NTM), *Nocardia* en skimmel.

As gevolg van verskeie metodes beskikbaar vir die identifikasie van NTM, *Nocardia* en skimmel, die belofte van n metode wat geskik is vir al die bogenoemde organismes soos gerapporteer deur verskeie studies, sal 'n beduidende invloed hê op die werksvloei in 'n diagnostiese laboratorium. Die MALDI-TOF MS is 'n relatiewe laekoste-tegnologie met 'n vinnige omkeertyd. Beloofde resultate is in verskillende studies aangemeld en rapporteer identifikasies van 87,7% - 99.0% vir NTM, 76.0% - 98.0% vir *Nocardia* en 66.8% - 94.0% vir skimmels.

Die doel van die studie was om die identifkasie van geselekteerde NTM, skimmel en *Nocardia* isolate op die MALDI-TOF-MS te vergelyk met verskeie fenotipiese en molekulêre metodes wat insluit die Genotype Mycobacterium CM / AS metodes, asook pan-bakteriële en pan-skimmel DNA volgorde benadering. Die studie sluit ook in n koste en werksvloei analise tussen die verskeie metodes.

Ons studie het identifikasies van 21.8% vir NTM, 62.5% vir *Nocardia* en 38.5% vir skimmel geproduseer. 'n Herhalende tema vir alle organisme-identifikasies op die Vitek MS was 'n hoë mate van "geen identifikasies", ondanks die feit dat voldoende proteïen-spektrale profiele gegenereer is, sowel as die meerderheid van die organismes was verteenwoordig in die Vitek MS databasis. Ondanks beduidende probleemoplossing van die metodologie vir alle organismes, het die persentasie suksesvolle identifikasies nie verbeter nie. Die vervaardiger se verteenwoordigers kon nie die probleme gedurende hierdie studie oplos nie, wat daarop dui dat daar 'n sagteware- of hardeware verwante probleem kan wees.

Op grond van die Vitek MS-instrument tekortkominge en koste- en werkvloei-analise, beveel ons die Mycobacterium CM / AS aan vir die spesifikasie van NTM's en die fenotipiese identifikasie van skimmel. Pan-Fungal-opeenvolging moet gebruik word waar die omkeertyd van kritieke belang is of waar kultuur negatiewe siektes vermoed word. Terwyl die Vitek MS 'n belofte getoon het vir *Nocardia* identifikasie, maak die koste daarvan, gegewe die groot stelgrootte en kort stabiliteit, die metode nie koste-effektief nie. Op dieselfde manier het die MLSA-analise die meeste identifikasies op die spesievlak verskaf, maar dit is nie koste effektief nie. Terwyl 16S rRNA-volgorde meestal slegs *Nocardia* op die genusvlak gerapporteer is, bly dit die enigste haalbare opsie vir bevestiging van *Nocardia* in die laboratorium.

Samevattend benodig die Vitek MS gereelde fyninstellings en tegniese ingryping en ondersteuning. Die instrument is miskien nie geskik vir 'n laboratorium met 'n hoë deurvloei vir die identifisering van NTM's, *Nocardia* en skimmel sonder om die robuustheid daarvan te verbeter nie.

ACKNOWLEDGEMENTS

I would like to thank PathCare management, including Arno Theron (QA Manager) and Stephan Marais (QA Technical supervisor), for providing me this opportunity to further my studies and utilise company facilities in order to do so, I will forever be greatful.

I would like to express my sincere gratitude to my supervisor, Dr K Hoek, and co-supervisor, Dr E Wasserman for the continuous support of my study and research, motivation, enthusiasm, immense knowledge, but most of all for your patience. It did not go unnoticed.

I am also grateful for all the staff in the Microbiology and Molecular departments who provided assistance, moral support and motivation when it was needed. I will truly miss the laughs and chats in the TB laboratory. This really made it a pleasure performing my work in such a friendly welcoming environment.

I would like to thank Petra Raimond and Ilze Uys for their willingness to offer up their time to assist me with the sequencing assays, I will forever be grateful for that. A special thank you to Jaclyn Gerber who was always willing to listen and for assistance whenever it was needed.

To my colleague and friend Daria Prinsloo, you were my rock that kept me steady the whole time and put me back on track when needed, your value during this time cannot be described in words.

I cannot describe the gratitude I feel towards my husband, Johan Immelman, for your support, motivation and strength you provided me with during the last two years. To my child, Wihan Immelman, thank you for allowing me the time needed to complete my studies even though you were too little to really understand, but I treasure the moments when you “studied” with me and highlighted my research papers yellow, that was precious to me. I also want to thank my parents and sisters for supporting and motivating me, especially Aretha vd Merwe. Thank you for always listening, giving advice and calming me down when I felt overwhelmed, it is truly appreciated.

Lastly, but most importantly, I would like to thank God for providing this opportunity, placing the obstacles in my road and providing the means to overcome it. I could not have done this without You carrying me.

LIST OF ABBREVIATIONS

AB	Applied biosystems
AIDS	Acquired immunodeficiency syndrome
ATCC	American type culture collection
BCG	*Mycobacterium bovis* bacille Calmette-Guérin
BD	Beckton Dickinson
bp	Base pair
°C	Degree celcius
CC	Conjugate control
CFU	Colony forming units
CHCA	α-Cyano-4-hydroxycinnamic acid
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DS	Disposable slides
EDTA	Ethylenediaminetetraacetic acid
EQA	External Quality Control
erm	Erythromycin ribosomal methylase
FDA	Food and drug administration
g	g force
GC	Genus control
*gyr*B	β-subunit of the type II DNA topoisomerase
HIV	Human Immunodeficiency virus
HPCSA	Health professions council of South Africa
HPLC	High-performance liquid chromatography
HREC	Health research ethics committee
*hsp*65	65-kDa heat shock protein
IC	Internal control
ID	Identification
ISHAM	International society for human and animal mycology
ITS	Internal transcribed spacer
IVD-CE	In-vitro diagnostic European conformity
KB	Knowledge base
LIS	Laboratory information system

LJ	Lowenstein-Jensen
LSU	Large subunit
M	Molar
MAC	*Mycobacterium avium* complex
MALDI-TOF	Matrix-assisted laser desorption ionisation-time of flight
MCS	Microbiological culture and sensitivity
$MgCl_2$	Magnesium chloride
MGIT	Mycobacterial growth indicator Tube
Min	Minutes
ml	millilitre
MLSA	Multilocus sequence analysis
MS	Mass spectrometry
MTC	*Mycobacterium tuberculosis* complex
m/z	Mass-to-charge ratio
NaOAc	Sodium acetate
NHLS	National health laboratory service
NTC	No template control
NTM	Nontuberculous mycobacteria
PC	Positive control
PCR	Polymerase chain reaction
pmol	Picomole
QC	Quality control
RBT	Round bottomed tube
rcf	Relative centrifugal force
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal database project
RIF	Rifampicin
RNA	Ribonucleic acid
rpm	Revolutions per minute
*rpo*B	RNA polymerase beta subunit
rRNA	Ribosomal ribonucleic acid
SABDRUGS	Sabouraud dextrose agar with cycloheximide
SDC	Sabouraud dextrose agar with chloramphenicol
SDS	Sodium dodecyl sulfate
sec	Seconds
*sec*A1	SecA preprotein translocase
SILVA	from Latin *silva*, forest

SOP	Standard operating procedures
sp.	Species (singular)
spp.	Species (multiple)
SSU	Small subunit of the ribosome
Subsp.	Subspecie
TB	Tuberculosis
TOF/BSA	Time of flight / Bovine serum albumin
VAT	Value added tax
ZN	Ziehl-Neelsen
µl	Microliter

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CHAPTER 1: GENERAL INTRODUCTION

Matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF MS) has been widely implemented in clinical microbiology laboratories for the identification of bacteria and yeasts. The use of this methodology for the rapid identification of nontuberculous mycobacteria (NTMs), *Nocardia* and moulds has been reported, but to a lesser extent. Here we provide a review of the most common laboratory diagnostic techniques to speciate NTMs, *Nocardia* and moulds, as well as discuss the potential of MALDI-TOF MS as a diagnostic tool in the routine laboratory.

1.1 Nontuberculous Mycobacteria

1.1.1 Background

The genus *Mycobacterium* consists of more than 190 species that live in a wide variety of natural environments and are organisms that are responsible for human diseases such as tuberculosis (TB), leprosy, Buruli ulcer, as well as pulmonary nontuberculous disease. While some members of the *Mycobacterium* spp. group are responsible for clinical disease, others are environmental organisms that can be present as commensals or isolated in the laboratory as environmental contaminants (1–3).

Mycobacteria are classified into 2 main groups according to their differences in epidemiology and association with disease: (a) *Mycobacterium tuberculosis* complex (MTC), and (b) NTMs. *M. leprae* and *M. ulcerans* cause distinct diseases, leprosy and Buruli ulcer respectively, and are therefore not included in the category of NTM (4).

While *M. tuberculosis* remains the most clinically significant organism in the genus, there is a steady increase in the number of infections caused by NTMs due to the increase in the number of immunocompromised individuals (5,6).

1.1.2 The pathogen: Nontuberculous Mycobacteria

NTM are important opportunistic pathogens that can be found in an abundance in the environment of which water and soil are natural reservoirs. These organisms have also been isolated from animal, milk and food products. Opportunistic infections caused by NTMs have a tremendous impact on people that are immunocompromised and cause life-threatening infections in acquired immunodeficiency syndrome (AIDS) and transplant patients (7). Transmission of NTMs does not occur from person-to-person and infection is acquired from the environment (8). NTMs have been associated with biofilm formation and their perseverance in these biofilms can cause healthcare-

associated infections. Biofilm formation is the organism's survival response to radical changes in the environment which provides protection against external stressors such as disinfectants and antibiotics (7,9). Organisms residing in biofilms may therefore be more resistant to disinfectants and antibiotics.

Unlike TB, NTM infections is not a notifiable disease (in South Africa), which results in less accurate knowledge of the exact impact NTM infections have on public health (7). However, we do know that the rate of NTM infections is increasing due to the increased number of immunocompromised patients (10).

Over the recent years, the apparent rise of NTM infections and the increased number of recognised novel species may also be due to the availability of advanced genotypic molecular techniques (10). Since 2011, 37 novel species or subspecies have been recognised and a full list is available at http://bacterio.net/mycobacterium.html (last updated in 2017). A select number of the most common and clinically relevant NTMs are discussed below.

NTMs are classified according to the rate of their growth and are divided into slow or fast growers. Fast growing NTMs produce mature colonies on solid medium under ideal conditions in ≤ 7 days, whereas slow growing NTMs require more than 7 days (4,11). Examples (not limited to) of slow growing NTMs include the *M. avium complex* (MAC), *M. kansasii, M. xenopi and M. simiae.* Rapid growers include *M. abscessus, M. fortuitum, M. smegmatis* and *M. chelonae* (8,9).

One of the most common NTM species identified in our setting is *M. avium* complex (MAC) comprising of two species, *M. avium* and *M. intracellulare. M. avium* consists of four subspecies: *M. avium* subsp. *avium*, *M. avium* subsp. *hominissuis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (4).

MAC are slow growing *Mycobacteria* and most commonly isolated from respiratory specimens as they cause pulmonary infections in immunocompetent and immunocompromised individuals. Disseminated disease in immunocompromised patients, especially patients living with HIV/AIDS, may occur (12). In addition to pulmonary infections, *M. avium* can infect the lymph nodes, bones, joints, skin and soft tissue and can spread systemically (13). *M. intracellulare* is primarily a respiratory pathogen and is not a common cause of disseminated disease (4).

The identification of species within the MAC group is crucial to distinguish between chronic pulmonary infection and transient colonisation by different species within this group (4,11,14), as members of the MAC group differ in virulence and ecology. The accurate differentiation between the species would therefore enable better treatment and also increase understanding of the epidemiology (15).

The second most frequently NTM pathogen isolated from clinical samples is *M. abscessus* complex and it represents more than 80.0% of the rapid growing NTMs identified (4). *M. abscessus* complex consists of three subspecies: *M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense*. This complex of mycobacteria is mostly environmental and occurs in soil, water and dust. It is often isolated from respiratory samples taken from patients with cystic fibrosis. In addition to causing pulmonary disease, this group of organisms is also responsible for skin, soft tissue and bone infections (4,11). The differentiation between the subspecies is crucial to optimise treatment as they differ in response to chemotherapeutic agents (16).

Another common fast growing NTM complex is the *M. fortuitum* complex which consists of the following species: *M. fortuitum*, *M. peregrinum*, *M. senegalense*, *M. setense, M. septicum, M. porcinum, M. houstonense, M. boenickei, M. brisbanense* and *M. neworleansense.* Similar to *M. abscessus* complex, this group of organisms can also cause skin, soft tissue and bone disease, but rarely causes pulmonary disease.

Another common NTM isolated in the laboratory is *M. kansasii*, a slow grower, which, if isolated from human specimens, is almost always associated with disease. *M. kansasii* is commonly isolated from municipal water, which can be a reservoir for infection with this organism (4). Infection with *M. kansasii* resembles pulmonary infection with *M. tuberculosis* in that it involves cavitary infiltrates in the upper lobes, but rarely disseminates from the lungs, except in immunocompromised patients. Risk factors for infection include chronic obstructive pulmonary disease (COPD), pneumoconiosis, cancer, alcoholism and HIV/AIDS (4,17).

1.1.3 Laboratory identification methods

It is of critical importance to accurately identify NTM infections so as to establish the clinical relevance of the organism and to assist the clinician in selecting the appropriate treatment options and patient management, avoiding drug over exposure and toxicity (18,19).

For decades, identification and speciation of NTMs relied on multiple biochemical tests and the phenotypic traits of the organism, which includes determination of a pigment with or without exposure to light, growth rate and colony morphology (18,20). Biochemical identification methods (e.g. the niacin accumulation test, nitrate reduction assay and catalase test) are however unable to correctly identify new emerging species (20).

High-performance liquid chromatography (HPLC) has been used to provide a more specific identification and to better discriminate between species, but this method is not suitable for a clinical setting as it is labour intensive and the equipment needed to perform the testing is not readily

available (21). In addition, HPLC requires pure cultures grown on solid media, which delays the turnaround time (19).

The growth of *Mycobacterium* spp. on solid media (e.g. LJ) is regarded as the gold standard and is often used as the reference method for the validation or verification of new diagnostic tests (22). However there has been a move to liquid based culture such as the automated BACTEC Mycobacterial Growth Incubator Tube (MGIT) (Beckton Dickenson, United States) method. While the MGIT liquid culture system has a higher sensitivity and negative predictive value than LJ solid media, the latter has been shown to have better specificity and positive predictive values (22,23). Liquid culture also has a significantly shorter time to positivity than that of solid culture methods (23).

Speciation of *Mycobacterium tuberculosis complex* (MTC) directly from clinical samples can be achieved by identification methods such as GeneXpert MTB/RIF (Cepheid, United States) and Genotype MTBDR*plus* (Hain Lifesciences, Germany) assays, but the identification of NTMs still currently requires a positive culture as there are currently no commercially available NTM identification methods which can be run directly from clinical samples (24). .

PCR based assays allow for the speciation of mycobacterial isolates. DNA sequencing is considered the gold standard (1). Several targets have been shown to be suitable for mycobacterial identification and include the 16S and 23S rRNA genes, the RNA polymerase beta subunit (*rpo*B), *sec*A (Protein translocase subunit) and the 65-kDa heat shock protein (*hsp*65) genes (1,18,25). Sequencing is labour intensive, technically complex and clinical laboratories do not have the resources or specific equipment and expertise to routinely perform these tests (1,25).

More prevalent methods for NTM speciation includes PCR hybridisation-based methods such as the GenoType Mycobacterium CM / AS assays (Hain Lifescience, Germany) which detects 14 of the most common mycobacterial species (CM) and 17 of the less common mycobacterial species (AS) by targeting the 23S rDNA region (21) of all mycobacterial species.
Both GenoType Mycobacterium assays utilise DNA Strip technology (Figure 1-1): The procedure consists of three basic steps: (a) Extraction of DNA from the cultured media, (b) multiplex amplification with biotinylated primers, and (c) reverse hybridisation. After chemical denaturation of the amplification products, the single-stranded amplicons bind to the membrane which is coated with complementary nucleic acids in a process called hybridisation. The combination of buffer composition and a particular temperature ensures the highly specific binding of complementary DNA strands. The sequences of the bacterial species are differentiated by the probes. Alkaline phosphatase is conjugated with streptavidin and binds via the streptavidin moiety to the amplicons' biotin. A substrate is added and the alkaline phosphatase alters it to a dye which is visible as a black

coloured precipitate on the membrane strips. A banding pattern is obtained which is easily interpreted in conjunction with interpretation cards from the supplier.

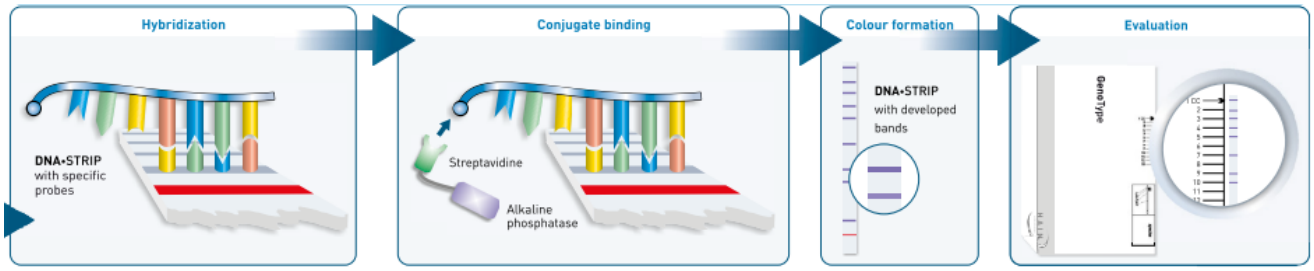

Figure 1-1 Overview of the GenoType Mycobacterium CM / AS Assays technology (Hain-lifescience, Germany)

The speciation of potential pathogens in a clinical laboratory requires identification methods that are rapid, reliable and cost effective. Accurate diagnosis of the etiological agent has a direct impact on patient treatment as the appropriate antimicrobial therapy can be administered earlier (26). MALDI-TOF is a rapid and cost effective system that can be implemented in a clinical laboratory for the identification of nontuberculous mycobacteria and will be discussed below (Section 1.4.4).

1.2 Nocardia

1.2.1 Background

Nocardiosis is a rare opportunistic disease that affects humans as well as animals (27,28). *Nocardia* is a saprophytic environmental organism that occurs in soil, water, dust, air and decaying organic matter (28,29). *Nocardia* belong to the actinomycetes group (Phylum: Actinobacteria, Order: Actinomycetales) of bacteria which are aerobic, non-spore forming, non-capsulated, branching filamentous Gram-positive bacilli which are weakly acid-fast (28,30).

The organism was discovered by Edmond Nocard in 1888 in cattle on an island of West Indies and was thought to be the cause of bovine farcy (28,31). It was first described as a fungus (28) but was reclassified as an aerobic bacterium under the genus *Nocardia* in 1889 (32) and was named *Nocardia farcinica*. A further 5 other species were classified under the genus *Nocardia* by Trevison (31). The first clinical case of human disease caused by this organism was reported in 1890 in a 52-year old glass blower (28).

Nocardia is not part of normal flora but can colonise the airways and is rarely a laboratory contaminant. If this organism is isolated in the laboratory it should be evaluated as a potential pathogen (27). If the clinician suspects *Nocardia* infection, it is advised to inform the testing

laboratory to ensure the necessary steps are taken for the isolation and identification of the causative organism (30).

Nocardia is regarded as an opportunistic pathogen, and most recorded cases are from the immunocompromised patient population in which it can be life-threatening (28). Immunocompetent individuals can also be infected (32), but are less likely to experience dissemination than their immunocompromised counterparts, which are more likely to develop bloodstream infections, require hospitalisation and experience a higher mortality due to *Nocardia* infections (33). Comorbidities which increase the risk of *Nocardia* infection include HIV/AIDS, transplant patients, tuberculosis, alcohol abuse, diabetes mellitus, cirrhosis, neoplastic disease, corticosteroid therapy, connective tissue and lung disorders (28,30,32). Timeous laboratory diagnosis of *Nocardia* infections is important as there are many other clinical conditions that it can mimic (i.e. the clinicians may not suspect *Nocardia* until the laboratory informs them of the culture result), and a delay may result in inappropriate therapy, which can lead to treatment failure and a poor prognosis (30).

There are 92 recognised *Nocardia* spp. that are listed in the “List of Prokaryotic names with Standing Literature” (http://www.bacterio.net/index.html) of which 54 species are considered clinically significant (31). These include *N. abscessus, N. nova* complex*, N. transvalensis* complex*, N. farcinica, N. cyriacigeorgica, N. brasiliensis, N. pseudobrasiliensis and N. otitidiscaviarum* (34). In the past *N. asteroides* was considered to be the most commonly isolated *Nocardia* spp. involved in human disease (28,31). The susceptibility patterns between isolates of *N. asteroides* differs significantly and this gave rise to the grouping of the *N. asteroides* complex into 6 groups depending on their antimicrobial susceptibility pattern: *N. abscessus* , *N. brevicatena / N. paucivorans* , *N. nova* complex , *N. transvalensis* complex , *N. farcinica* , and *N. cyriacigeorgica* . These organisms can not be speciated by phenotypic means, but with the evolution of molecular techniques, including sequence analysis, it is now possible to discriminate between these species and the term *N. asteroides* complex is no longer used. As these species can now be differentiated from each other, *N. asteroides* is now rarely identified from clinical samples and is not the most commonly isolated *Nocardia* spp. anymore (31).

1.2.2 Nocardiosis

Nocardiosis can present as an acute, subacute or more frequently chronic disease (27,29), involving the skin, lungs and central nervous system (32). The respiratory tract is the main portal of entry (32) and can result in asymptomatic colonisation or progression to the most common manifestation of nocardiosis namely pulmonary disease (35).

Common signs and symptoms of pulmonary nocardiosis may vary but include fever, cough, weight loss, pleural pain, dyspnea and anorexia (28,36). These symptoms are non-specific (28) and cannot be distinguished from patients with pulmonary infections caused by other microbial agents (29). The signs and symptoms may be confused with those of chronic lung infections such as fungal or TB infections (36,37), which consequently may lead to incorrect treatment of the patient with anti-tuberculous drugs (37).

Other clinical manifestations include corneal ulcers, mycetoma and encephalitis (28). Cutaneous nocardiosis in humans results from contact with the bacteria through trauma like cuts or scrapings on the skin, which can result in cellulitis or ulcers (30). There is no evidence of human to human transmission and isolation of these patients is not recommended (27,30).

The prognosis is variable depending on the site and extent of infection and the underlying host factors. The majority of patients (Almost 100.0%) with skin and soft-tissue infections and 90.0% of pulmonary infections can be cured with the appropriate therapy (29). Disseminated nocardiosis can be cured in 63.0% of patients but only 50.0% of patients with brain abscesses will be cured with therapy (29).

Different *Nocardia* spp. vary in their ability to cause infection in humans and in their response to antimicrobial therapy. It is therefore critical to differentiate between the different species as it can have a direct impact on patient treatment and provides important information for epidemiological purposes (37,38). Prolonged treatments of 6 to 12 months are recommended in patients with a severe immunocompromised immune system (32).

1.2.3 Laboratory identification methods

The first step in diagnosing *Nocardia* infection is the microscopic examination and culture of the organism from specimens originating from the site of infection (29,30). The Gram stain and a modified acid-fast stain (Ziehl-Neelsen) is important as it can guide the clinician while waiting for the culture results (27).

Microscopically *Nocardia* spp. can be distinguished from *Mycobacterium* as their morphology differs and *Mycobacterium* do not stain well with Gram or modified acid-fast stains. While *Actinomyces* may have a similar morhpology to *Nocardia*, it is modified acid-fast stain negative. Non-selective media used in the laboratory for the culture of bacteria, fungi and *Mycobacteria* is suitable for the isolation of *Nocardia* spp. (27). Growth of *Nocardia* colonies can appear after 48 hours but is usually present within 3-5 days (29,30). Some species may require growth for up to 3 weeks (27). Plates for routine cultures in many diagnostic laboratories are usually discarded after 48 hours and *Nocardia* can

therefore easily be missed from clinical samples. Therefore, if there is a clinical suspicion of nocardiosis, it is advised to inform the laboratory to prolong incubation of the culture plates (30). However, in samples were mixed flora are present, like sputum, the faster growing bacteria can easily overgrow the *Nocardia* spp. and it may be missed (27).

If growth is successful , species identification can be achieved by biochemical reactions including hydrolysis of adenine, casein, tyrosine, xanthine, and hypoxanthine (27,39) but these are laborious, time-consuming methods (35) which increase turnaround time and are less definitive (37). Furthermore, biochemical methods are insufficient to accurately distinguish between the clinically relevant species (31).

Molecular methods targeting specific *Nocardia* gene regions have been employed for accurate identification of *Nocardia* spp. (40). 16S rRNA sequence analysis was considered the gold standard for definitive *Nocardia* spp. identification (38,40–43). A hypervariable region near the 5' terminus of the 16S rRNA gene exists in all *Nocardia* spp. and this allows for the application of a partial (500 bp) 16S rRNA sequence for the differentiation between the majority of clinically recognised *Nocardia* spp. However, there have been reports that the multiple copies of the 16S rRNA gene in certain *Nocardia* spp. may differ slightly with regards to sequence content (44). This may lead to misidentifications, such as in the case of *N. nova* (38,40,44,45). 16S rRNA sequence analysis can theoretically detect *Nocardia* directly from clinical samples, however, to save costs, the majority of samples are first sent for routine Microbiological, Culture and Sensitivity (MCS) investigations and it is then prefferential to do PCR from the positive culture which would have a higher bacterial load.

Due to the complexities associated with 16S rRNA gene sequencing, multilocus sequence analysis (MLSA) using 16S rRNA, *rpoB (*β-subunit of DNA-dependent RNA polymerase), *erm* (erythromycin ribosomal methylase), *hsp*65 (65-kDa heat shock protein), *gyr*B (β-subunit of the type II DNA topoisomerase) and/or *sec*A1 (SecA preprotein translocase) genes has been proposed as an alternative method able to identify known as well as novel species (37,38,45). The genus *Nocardia* exhibits genetic diversity and MLSA using multiple housekeeping genes can be used for phylogenetic analysis in that sequence clusters represent species clusters (43). In this genus there are distinct species that are closely related based on their gene sequences similarities. An example of such a group is the *N. abscessus* complex which includes *N. abscessus, N. arthritidis, N. asiatica* and *N. beijingensis*.

Sequencing methods such as 16S rRNA and MLSA are expensive and time consuming, and are not available in many routine clinical laboratories. Isolates must often be referred to a reference laboratory with a subsequent delay in the identification of the organism (35,45,46).

1.3 Moulds

1.3.1 Background

Mycology is a specialised discipline involving the study of fungi which includes their taxonomy, genetic and biochemical properties as well as the impact they have on the environment. In the past fungi were not regarded as clinically significant, but today a number of species found in the environment are regarded as important causes of human disease (47), particularly in immunocompromised patients. Fungi are widely distributed on Earth, of which many are free-living in soil, water, air, food and clothing; while others form parasitic or symbiotic relationships with plants or animals (48). Fungi are important to many household and industrial processes such as the production of wine, beer, bread and certain cheeses. It is also considered as a source of food (e.g. mushrooms, morels and truffles) and drugs (e.g. penicillin antibiotic) (49).

In the past two decades there has been an substantial increase in the severity and incidence of opportunistic invasive fungal infections (50). This is once again due to an increase in immunocompromised patients due to transplants, corticosteroid use and HIV/AIDS (51). The alterations in the host caused by immunosuppressive agents and/or serious disease may lead to infections by organisms that are normally considered to be non-pathogenic normal flora . *Aspergillus* is one such mould that can cause opportunistic disease (47). The spores of *Aspergillus* are abundant in the environment (including soil and food) and it is usually considered to be a contaminant (52). Other risk factors include surgical procedures and antibacterial therapy (47).

There are over 200 000 species of fungi but only 100 to 150 of them are considered to be human pathogens. Of these, 25 species account for the majority of human infections. These organisms are mostly saprophytic environmental organisms, living on dead or decaying organic matter. Humans are generally very resistant to fungal infections, except those caused by dimorphic fungi, and become infected by inhaling the spores or due to inoculation during tissue trauma. The capability of these organisms to cause serious disease in immunocompromised individuals means that the laboratory identification procedures must allow for the identification and reporting of a wide range of different fungal organisms (47).

Moulds can be classified into 2 groups depending on hyphae morphology (Figure 1-2).

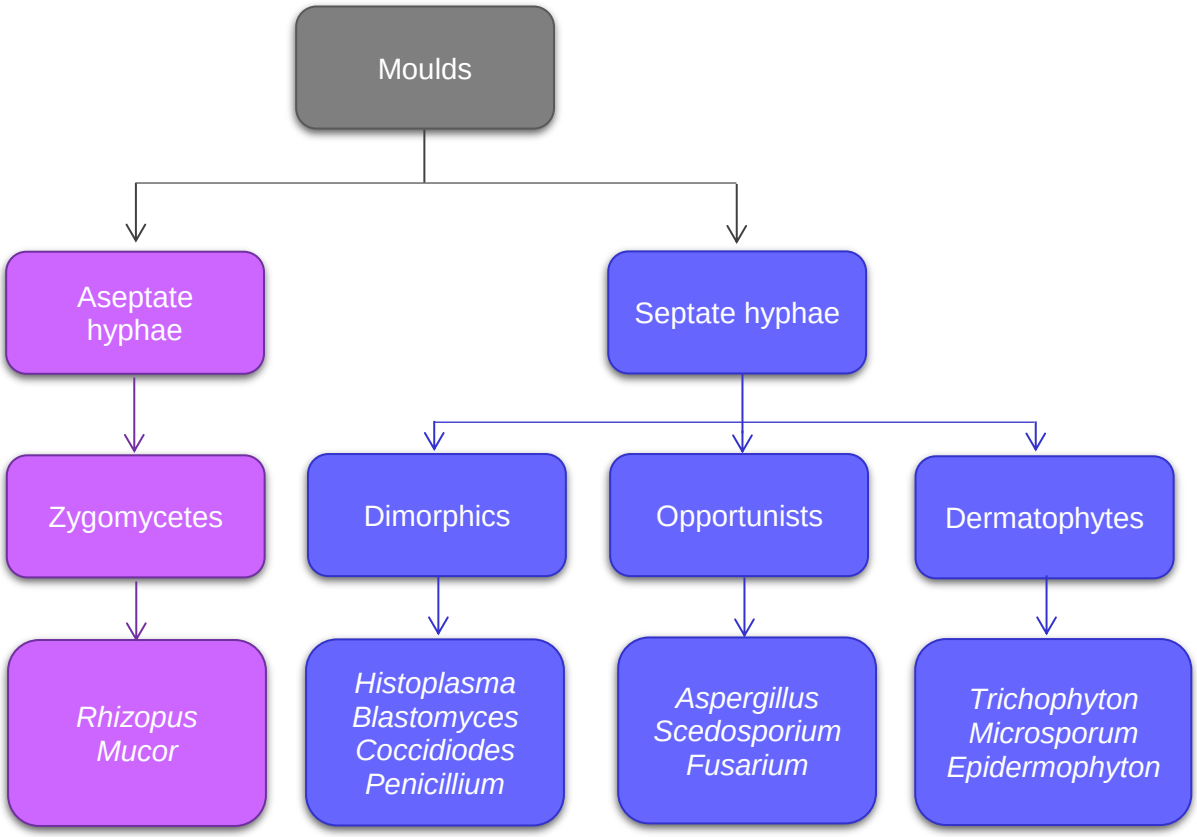

Figure 1-2 Classification of moulds

Adapted from (53,54)

An alternate, but less popular, clinical classification system exists where fungi are grouped according to the type of mycoses (infections caused by moulds) they are involved in, i.e. superficial (cutaneous), subcutaneous, systemic or opportunistic mycoses (47).

1.3.2 Infections caused by moulds

a) Mycoses

- **Superficial (cutaneous) mycoses**

Superficial (cutaneous) mycoses refers to fungal infections of the skin, hair and nails without direct invasion of the deeper tissues (53). This infection is classified according to the site of disease and includes tinea capitis (head), tinea corporis (body), tinea cruris, tinea pedis and tinea barbae. Onychomycosis refers to the infection of nails by a nondermatophyte fungi, although the term is widely used for infection of the nail by any fungal agent. They are seen worldwide and affect approximately 20.0% - 25.0% of the world's population (55). Superficial mycoses also include diseases such as dermatophytosis, candidiasis and pityriasis versicolor (55). The dermatophytes are

responsible for the majority of superficial mycoses (47). This group consists of three genera: *Trichophyton, Microsporum* and *Epidermophyton* (53).

- **Subcutaeous mycoses**

Subcutaneous mycoses include chromoblastomycosis, mycetoma, and phaeohyphomycotic cysts. These fungal infections do not disseminate to distant sites but remain in the subcutaneous tissue (47).

- **Systemic mycoses**

Systemic fungal infections usually involve the lungs and can disseminate extensively and involve any organ system. Fungal genera usually involves in systemic mycoses involve *Blastomyces, Coccidioides, Histoplasma* and *Paracoccidioides* (47).

b) Aspergillus

Aspergillosis is a disease that presents with a variety of clinical manifestations which may include the presence of a fungus ball in the lung or sinus (aspergilloma), asthma, allergic bronchopulmonary aspergillosis, keratitis, chronic pulmonary aspergillosis and otomycosis. Invasive disease is rare, and occurs only in severely immune compromised patients. Of the *Aspergillus* taxon, the species that is most commonly implicated in human disease is *A. fumigatus* followed by *A. flavus* (52).

c) Other moulds

The mucormycetes, hyalohyphomycetes and phaeohyphomycetes are groups of fungi that represent the non-*Aspergillus* filamentous fungi. The most prevalent cause of non-*Aspergillus* mould infections in humans is due to the mucormycetes of which *Rhizopus* is the most common, followed by *Mucor, Rhizomucor* and *Lichtheimia*. The hyalohyphomycetes (hyaline moulds) are fungi with branching septate hyphae but lack pigmentation. Microscopically it is very difficult to differentiate hyalohyphomycetes from *Aspergillus*. The most prevalent genera in this group of fungi are *Fusarium* and *Scedosporium* followed by the less frequently detected fungi such as *Paecilomyces*, *Acremonium*, *Schizophyllum* and *Rasamsonia* (56). The phaeohyphomycetes are also called the dematiaceous fungi due to the dark pigmentation of the colonies which is a result of melanin production. Genera included in this group of fungi include *Alternaria* spp., *Bipolaris* spp., *Wangiella* spp., *Madurella* spp., *Fonsecaea* spp., *Cladophialophora* spp., *Curvularia* spp., *Exophiala* spp. *and Phialophora* spp. (57).

1.3.3 Laboratory identification methods

Diagnosing invasive fungal disease is complicated by the lack of sensitivity and specificity of current laboratory identification methods. Often the result is not obtained in an appropriate turnaround time to make the diagnosis clinically useful. Rapid, accurate species level identification is crucial to identify clinically important isolates for the timely onset of anti-fungal treatment – a critical contributor to patient outcome (50,53,58,59).

For many years the gold standard for diagnosing fungal infections was the culture of the clinical sample which included microscopy and histopathology (50). Identification of filamentous fungi by these methods is reliant on the observation of reproductive structures, which can be subjective (51) and requires highly trained personnel (60). Growth from clinical specimens takes about 3 weeks and once the culture is positive it may take days before an identification can be made (50). This results in a prolonged turnaround time due to extended incubation periods (60). A contributing factor to the extended turnaround time is the identification of unusual or problematic fungi which are usually referred to a reference laboratory for speciation by molecular methods such as DNA sequencing (61). Molecular methods for the identification of filamentous fungi allows the differentiation of several closely related species which are morphologically identical (51), for example *A. lentulus* which is morphologically identified as *A. fumigatus*. Differentiation of this species impacts patient treatment (62).

DNA sequencing methods are highly accurate, but they are expensive and it may take two to three days before the identification results is available (51,59). However they can be performed directly from the clinical sample and are not culture dependent. DNA sequencing is usually confined to reference laboratories and is not widely available in the clinical laboratory setting, which may contribute to an increased turnaround time (51).

The ITS (internal transcribed spacer) region is the most commonly sequenced DNA target for fungal identification and speciation (63,64). The ITS regions are ideal targets for identification of fungal organisms due to their location between the conserved 18S, 5.8S and 28S rRNA gene sequences (64). The ITS regions of the gene are very stable, conserved within species and occur in multi-copies. The latter increases the sensitivity of the assay as compared to targeting single copy regions (65). The target section is located between the 18S of the small subunit (SSU) and the large subunit 28S (LSU) of the ribosome (64). While most fungal species have been identified by targeting the ITS region, some difficulties have been experienced with the identification of *Alternaria, Aspergillus, Cladosporium, Penicillium* and *Fusarium* as this region is not equally variable in these groups of fungi (63).

A future concern is the loss of well-trained, experienced mycology laboratory technologists due to retirement or resignation, resulting in replacement by less experienced staff (47). The spectrum of fungal pathogens identified in the clinical laboratory has undergone major expansion in the last 30 years due to the increase of immunocompromised patients. These patients are susceptible to opportunistic fungal pathogens previously rarely encountered and the identification of these organisms in the laboratory may be a challenging task even for the most qualified, experienced mycologist (50).

The need for an affordable, rapid and accurate method for the identification of fungi to the species level exists, as traditional phenotypic identification methods are time-consuming, lack adequate accuracy, and molecular methods are not readily available in the majority of clinical laboratories (51). The application of MALDI-TOF MS for the routine identification of fungi is a promising method which may fulfil these requirements (66).

1.4 MALDI-TOF MS

1.4.1 Background

Mass spectrometry (MS) was discovered in the early 1900s and was mostly applied in the chemical field. MALDI-TOF followed in the 1980s, and allowed MS to be applied to larger biological molecules such as proteins (26). MALDI-TOF MS has since proven to be a powerful tool for the reliable identification of bacteria and yeast from solid culture media plates in the clinical laboratory (1,67).

1.4.2 Principle

MALDI-TOF technology is a method used to determine the protein composition of an isolate and allows the comparison of the resulting protein spectrum to a commercial database for organism identification. The basic procedure involves mixing a pure culture of the isolated bacterium with a matrix compound and allowing it to dry on a conductive target slide to enable crystallisation of the mixture. The target slide is introduced into a high vacuum environment and the isolate/matrix mixture is subjected to an ultraviolet laser beam which fires brief laser pulses through the sample. The excitation of the matrix causes sublimation from the solid phase to the gaseous phase. Matrix molecules and microorganism proteins are released from the surface of the target slide (desorption) and protons from the matrix are transferred to the proteins which result in a positively charged protein molecule in the gaseous phase (ionisation).

This “cloud of proteins” enters an electrostatic field and with a high voltage supply, the ions are introduced into the high vacuum flight tube where they are separated according to their mass to charge ratio. The quantity of each ion is measured and detection is achieved by a sensor at the end

of the flight tube to create a spectrum that represents the protein constituents of each sample. This is illustrated in Figure 1-3 (68).

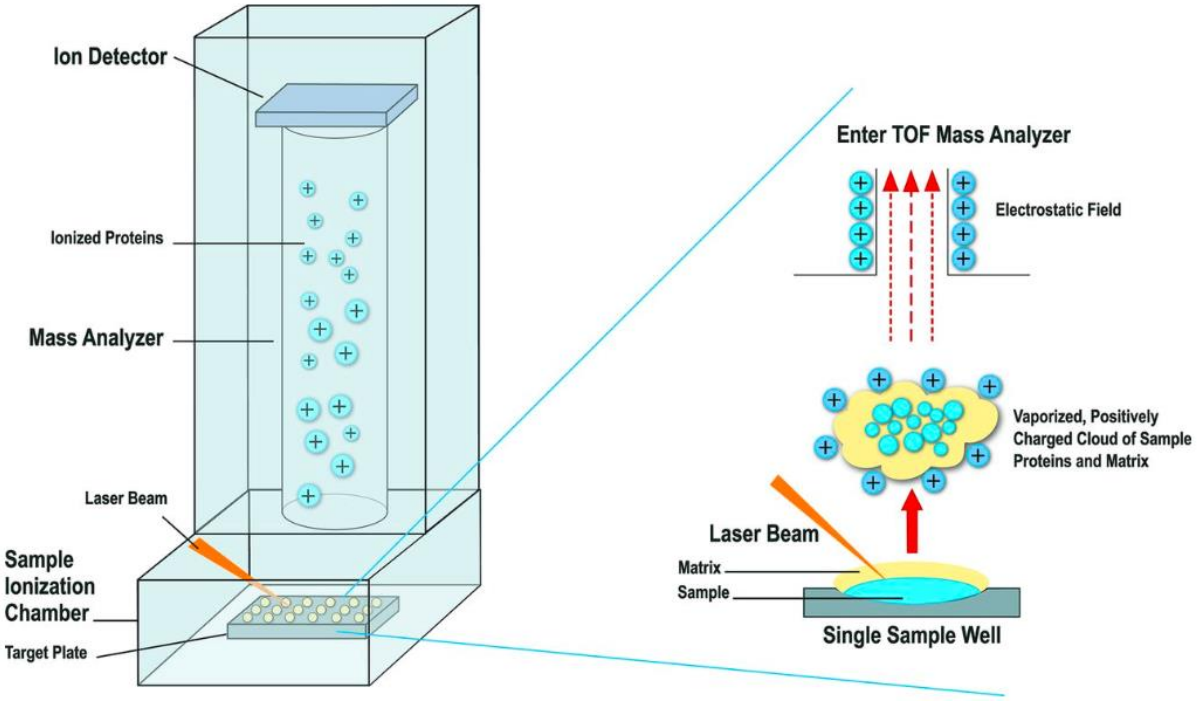

Figure 1-3 Principle of Matrix-assisted laser desorption ionisation-time of flight methodology (68)

The resultant protein profile is considered the fingerprint of the microorganism and the protein spectrum is displayed with the *m/z* (mass-to-charge ratio) values along the x-axis and the intensity of the signal plotted against the y-axis (68) (see Figure 1-4 for an example).

Appendix A contains more details on how the instrument produces and assesses spectral profiles.

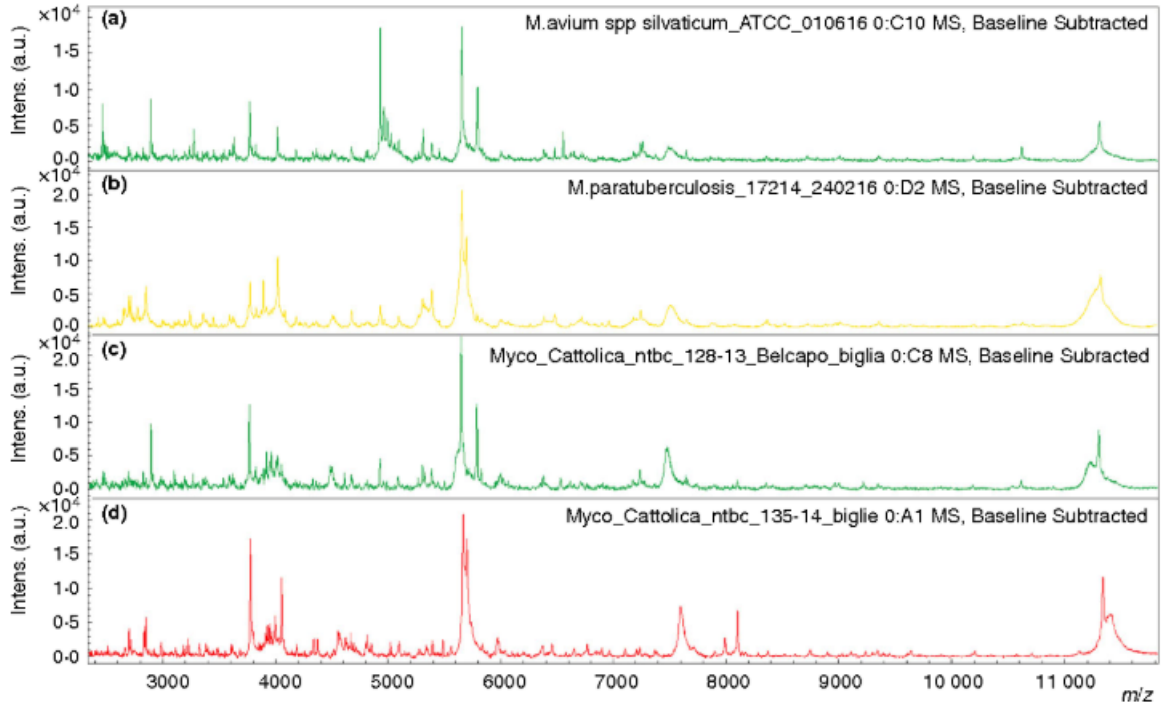

Figure 1-4 Spectral fingerprint from Vitek Mass Spectrometry of members of the M. avium complex (69)

1.4.3 Available MALDI-TOF systems

There are three commercial MALDI-TOF systems currently available (2019) which include the Andromas (Andromas Paris, France), Vitek MS (bioMérieux, France) and the Bruker Biotyper (Bruker Daltonics, Germany) instruments (59).

The Vitek MS and Bruker Biotyper are the only systems currently available in South Africa. Although all of these systems have been evaluated in the past, most published studies refer to the Bruker technique (14,70). The Bruker Biotyper and Vitek MS differ in extraction methods, database construction and evaluation of results (24,59).

In 2017 bioMérieux expanded the In-vitro Diagnostic- European Conformity (IVD-CE) marked VITEK MS® database to include *Mycobacteria*, *Nocardia* and moulds (which includes dermatophytes and dimorphic fungi). This version (v3.2) of the database allows for the identification of 242 new bacterial (including 39 mycobacteria taxa [comprised of 49 total species] and 15 *Nocardia* spp.) and 55 new fungal species. Refer to Appendix B for a list of organisms included in the Vitek MS Knowledge Base (KB) v3.2. With this database update, two reagent kits were introduced: the VITEK MS® Mycobacterium/Nocardia kit (bioMérieux, France, Ref 415659) and the VITEK MS® Mould kit (bioMérieux, France, Ref 415680). Testing is performed from organism growth on solid media. In addition to the VITEK MS® Mycobacterium/Nocardia kit, a Liquid Myco Supplemental kit (bioMérieux, France, Ref 421564) is also available for the identification of *Mycobacteria* from liquid media e.g. MGIT.

1.4.4 MALDI-TOF MS identification of nontuberculous Mycobacterium

The application of MALDI-TOF for the identification of *Mycobacterium* spp. began over a decade ago, but the technique used for the identification of bacteria and yeast involving the direct spotting of the cultured organism onto a MALDI-TOF target slide was not suitable (5,45). The cell wall of *Mycobacteria* contains lipids and peptidoglycans esterified with mycolic acids which creates a hardiness to the cell wall with a low permeability. Due to the cell wall constituents and the pathogenicity of *Mycobacteria*, an inactivation and extraction step is necessary to release the contents within the cells (25,45,71).

Literature review does not provide a clear protocol for the identification of *Mycobacteria* from liquid cultures, but bioMérieux has standardised the procedure by providing a commercial kit for the standardisation of the method.

1.4.5 MALDI-TOF MS identification of Nocardia

Following genomic methods for the identification of *Nocardia* spp., proteomic methods have been evaluated for this purpose (38). Bacteria and yeast can be identified by using the direct spotting technique but this is not applicable for *Nocardia* (45) due to the presence of aliphatic acids in the cell wall which renders a complication for achieving acceptable protein profiles (38). To overcome this burden, mechanical disruption and a protein extraction step is needed when processing *Nocardia* spp. for identification using the MALDI-TOF technology (45).

MALDI-TOF has been in use for many years for the identification of *Nocardia* spp., but there is a significant variability in the performance as well as in test methodologies used (45). Deficiencies in the available reference databases and the non-standardised methodologies may have contributed to this variability in test performance (2) which varies from 41.9% - 90.6% of identification to species level (1,38,72). It is evident from recent research that well-curated and validated databases are needed to account for the variability that is encountered in the protein spectra (45). Another aspect to consider is the complementation of the reference database with “in-house” protein profiles, relying on local epidemiology knowledge, and hence, may assist with the identification to genus level, or even species level (38).

Due to the limited amount of studies available on the Vitek MS for the identification of *Nocardia* spp. additional studies is needed to evaluate the commercial system (31).

1.4.6 MALDI-TOF MS identification of moulds

Fungi are biologically complex organisms and different phenotypes (hyphae and/or conidia) co-exist in the same organism (26), which produces protein spectra that may vary. Substantial spectral varieties have been noted between different stages of fungal growth of the same isolate and between subcultures of the same strain (51). This has led to a slower implementation rate of the MALDI-TOF MS technology for the identification of fungi than for bacterial identification in the clinical laboratory (26).

The cell wall of fungi differs significantly from that of bacteria in that it consists mostly of polysaccharides, including chitin and glycoproteins (51). Identification of filamentous fungi on the MALDI-TOF MS requires additional processing steps to disrupt the cell wall, extract the proteins and to inactivate the organism. This has contributed to the slower implementation rate of this technology into the clinical laboratory (73). There are also aspects of processing moulds for MALDI-TOF MS that may influence the spectrum profile, including different maturation stages of selected colonies, if conidia are present or not; and the presence of melanin in some moulds that may interfere with ionisation (58).

The MALDI-TOF MS instrument has reduced the turnaround time for the identification of moulds although the technology is still dependent on fungal cultures (59). The benefits for using the MALDI-TOF MS for the identification of filamentous fungi is the ability to report to a species level and the identification of isolates that do not produce the morphological structures required for traditional identification methods, for example the sterile moulds (51,74). Furthermore, relying on the protein profiles of a MALDI-TOF MS is more objective than phenotypic methods of mould identifications, which is dependant on the users interpretation skills.

MALDI-TOF MS protein extraction is particularly complex and can result in diverse levels of performance depending on the sample type used for extraction (whole mould versus spores) and if solid or liquid media has been used. All of these result in different spectrums influencing the results and are dependent on the database coverage (74). MALDI-TOF MS is simple to use and for this reason technologists may never develop the skills or lose the ability to visually identify fungi macroscopically and microscopically, which may pose a problem during possible instrument downtime and loss of skilled technologists (24).

1.5 Problem statement

While various laboratory methods exist for the identification of NTMs, *Nocardia* and moulds, most rely on culture of the organism which can lead to significant delays in diagnosis. Average turn-around times for phenotypic culture of *Nocardia*, *Mycobacteria* and moulds are ~2 days, 1 to 6 weeks (slow

and fast-growers) and 1 to 4 weeks respectively. The identification of these pathogens is of clinical importance as it influences the selection of drugs used for treatment of these particular infections. Speciation of these organisms by phenotypic methods requires technically competent staff. Mould identification is particularly challenging as it is based on the subjective morphological characterisation of colonies, hyphae and spores (47,75).

Molecular methods offer an advantage in that they can decrease turn-around times to less than 3 days and are not as subjective as phenotypic classification systems. However, the majority of commercially available molecular assays target a limited range of organisms per assay, depending on the detection method used. DNA sequencing methodologies targeting hypervariable regions unique to bacterial and fungal species allow identification of a broader range of organisms (including potentially novel organisms). However, these methods require expensive equipment and reagents as well as highly skilled staff; and are therefore not commonly performed in smaller clinical laboratories (1,5). Pan-fungal and 16S rRNA (bacterial) sequencing also requires single organism infections as multiple infections will interfere with result interpretation.

Matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS) is a rapid and relatively simple method to perform for the identification of organisms in clinical microbiology laboratories. Although the initial placement of the instrument is expensive, the continuous use with consumables is very cost-effective (5,67). The method is currently used in our setting and has shown potential in publications for use in speciation of NTMs, *Nocardia* and moulds. There is a need to investigate which of these methods would be most suitable to a clinical diagnostic (reference laboratory) setting, taking performance, turn-around time, ease of use and cost analysis into account.

1.6 Aim and objectives

The aim of this study was to compare the Vitek MALDI-TOF MS (bioMérieux, France) to various phenotypic and/or hybridisation and sequencing based molecular assays, in order to identify the most suitable assay to speciate NTM, *Nocardia* and moulds in a reference laboratory setting.

The objectives included:

a) Optimisation of the Vitek MS method for NTM, *Nocardia* and mould identification

b) Comparison of the performance, workflow impact and crude cost of the Vitek MS method to the following **NTM** identification methods:

   i) Genotype Mycobacterium CM/AS assay

c) Comparison of the performance, workflow impact and crude cost of the Vitek MS method to the following **mould** identification methods:

   i) Mycology (Culture and Microscopy)

   ii) ITS Pan-Fungal sequencing

d) Comparison of the performance, workflow impact and crude cost of the Vitek MS method to the following ***Nocardia*** identification methods:

   i) 16S rRNA sequencing

   ii) *Nocardia* specific Multi-Locus Sequence Analysis

1.7 Ethical approval

Ethical approval (Reference #: S18/10/208) was obtained from the Health Research Ethics Committee (HREC) from Stellenbosch. This was a laboratory based study. The organism identifications were performed on clinical isolates derived from routine laboratory procedures or retrospectively. All investigators are healthcare professionals registered with the HPCSA as well as PathCare employees bound by confidentiality agreements. Samples were anonymised for reporting in the thesis, and patient management was not affected by any of the results. Although results were available prior to testing on the Vitek MS, this lack of blinding did not have any affect on the outcome of results as the Vitek MS software provides an objective report and is not interpreted by the user.

CHAPTER 2: IDENTIFICATION OF NONTUBERCULOUS MYCOBACTERIUM

2.1 Introduction

The objective of this chapter was to evaluate the performance of the Vitek MALDI-TOF MS for the identification of NTMs by comparing the results to molecular hybridisation-based techniques such as the Genotype Mycobacterium CM / AS assays.

2.2 Materials and methods

2.2.1 Sample selection

78 NTMs isolated from consecutive clinical samples (January to September of 2019) were identified in the PathCare Reference Laboratory as part of routine clinical care using the Hain Mycobacterium CM and AS kits, according to the manufacturer's instructions.

Briefly, positive MGIT cultures (with positive ZN) were sent for MPT64 antigen (Beckton Dickenson, United States) testing to guide downstream analysis. Positive MGIT cultures presenting with a negative ZN were excluded from the study. MPT64 positive samples (indicative of *M. tuberculosis* complex) were excluded from the study. MPT64 negative samples (suggestive of NTMs) were included in the study for further testing by the Genotype Mycobacterium CM assay which speciates the more common NTMs. Where speciation was not resolved using the CM assay, the Genotype Mycobacterium AS assay was used which targets the less common NTMs.

The current PathCare laboratory workflow (applicable to this study) for the identification of NTMs is visualised in Figure 2-1.

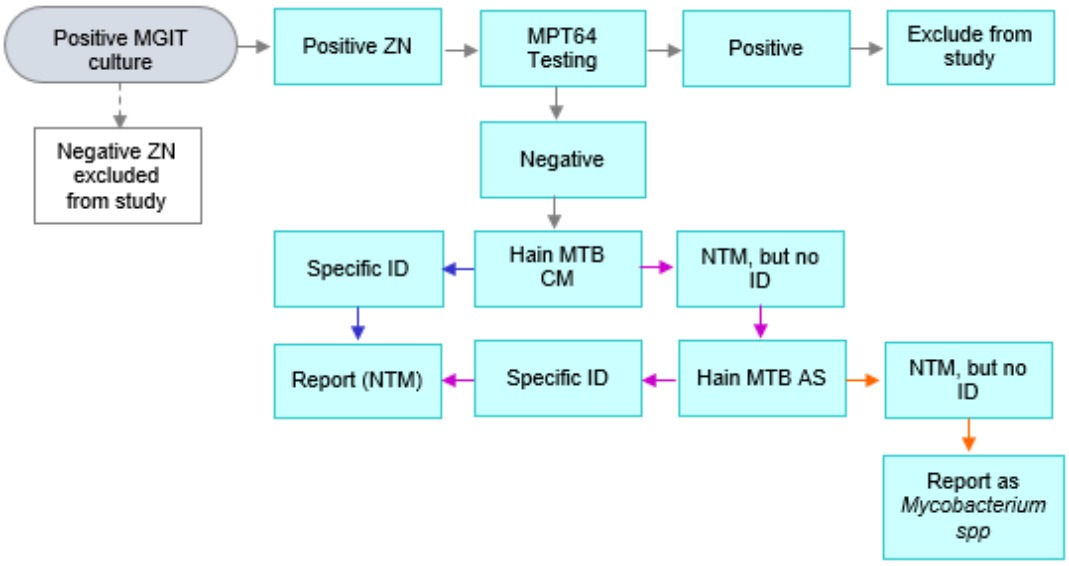

Figure 2-1 PathCare reference lab positive Mycobacteria Growth Indicator Tube workflow

2.2.2 Genotype Mycobacterium CM / AS assay

a) DNA extraction

DNA was extracted using a crude boil method (PathCare validated package insert deviation): the positive MGIT culture tube was vortexed adequately after which 2ml fluid was transferred into a labelled screw cap Eppendorf tube. The tube was centrifuged at 14 000 rpm for 5 min and the supernatant was carefully removed using a sterile pastette without disturbing the pellet. 200µl of PCR grade dH_2O, containing 4 µl Internal Control DNA (IC), was added and the sample was vortexed to resuspend the pellet. The Eppendorf tube was incubated at 100° C in a heating block for 30 minutes to inactivate the organism. After the incubation period, the sample was cooled down for 5 minutes at room temperature and centrifuged at 14 000 rpm for 5 minutes. The supernatant was used as a template for the PCR reaction.

b) DNA amplification

The supplied Hain Mycobacterium Amplification Mixes A and B (AM – A and AM – B) contain all the required biotinylated primers and polymerase for the reaction to produce biotinylated amplification products. The stored Amplification Mixes (-20°) were thawed at room temperature and carefully mixed by pipetting up and down.

The amplification master mix was prepared by adding 10µl of AM-A and 35µl of AM-B to a PCR reaction tube, after which 5µl of sample DNA was added (final reaction volume was 50µl). To prevent cross-contamination, the DNA extraction, preparation of amplification mixes and addition of sample DNA; and the hybridisation step were done in separate rooms.

Thermal cycling was conducted in an Applied Biosystems (AB) Proflex thermal cycler according to the manufacturer's (Hain LifeSciences, Germany) recommended touch-down profile: initial denaturation at 95°C for 15 minutes followed by 10 cycles of denaturation at 95°C for 30 seconds and annealing at 65°C for 2 minutes; and 20 cycles of denaturation at 95°C for 25 seconds, annealing at 50°C for 40 seconds and elongation at 70°C for 40 seconds. The thermocycling was completed with a final extension step at 70°C for 8 minutes (Heating/Ramp rate of ≤2.2°C/sec).

c) Denaturation and hybridisation

The amplification products were chemically denatured on the GT-Blot-48 automated analyser according to the manufacturer's instructions, and the single stranded amplicons underwent reverse hybridisation to specific probes present on the Hain membrane strip, which were subsequently visualised following a colorimetric reaction.

Resultant hybridisation patterns were used to speciate the NTM. Amplification and hybridisation procedures are the same for the Mycobacterium CM and AS assays, therefore amplicons could be used interchangeably with the kit specific hybridisation strips.

d) Quality control

Each Genotype Mycobacterium test strip contains three control probes which are included to monitor the performance and functioning of the contents of the manufacturer's kit. Figure 2-2 indicates the three control areas. The Conjugate Control (CC) indicates the efficiency of binding of the conjugate on the strip and controls for a correct chromogenic reaction. The Internal Control (IC) reveals effective DNA extraction and amplification. The Genus Control (GC) show the presence of a member of the genus *Mycobacterium*.

A negative control was added to each batch of samples run to test for possible contamination. This control sample was processed from the extraction step as per the study samples and was expected to only show hybridisation for the IC and CC zones. If not, the whole run was considered invalid and was repeated. A separate positive control was not included as this was deemed optional by the manufacturer. We deemed the 3 hybridisation strip controls to be a suitable substitute for the positive control as we had already pre-screened MGIT cultures using ZN microscopy and an MPT64 antigen assay.

e) Evaluation and interpretation of results

Each Genotype Mycobacterium test strip contains 17 reaction zones, which included the three control zones: CC,IC and GC.

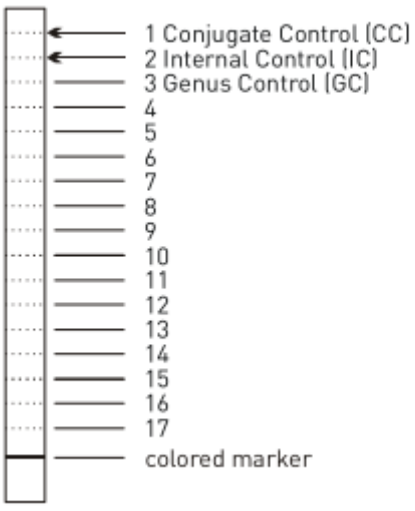

Figure 2-2 Layout of the Genotype Mycobacterium CM / AS test strip (76)

A correctly performed test would result in the binding of a control amplicon to the IC probe. A valid negative result would have a IC and CC positive signal, with no other positive probes/zones.

If the IC band failed to develop in a negative test result, the test result was deemed invalid, either due to inhibition of the amplification or errors made during DNA extraction or amplification.
The signal strength on all the bands may differ and only those bands that showed similar or stronger intensities than the IC were considered. Figure 2-3 and Figure 2-4 represent the result interpretation of the Genotype Mycobacterium CM / AS assays.

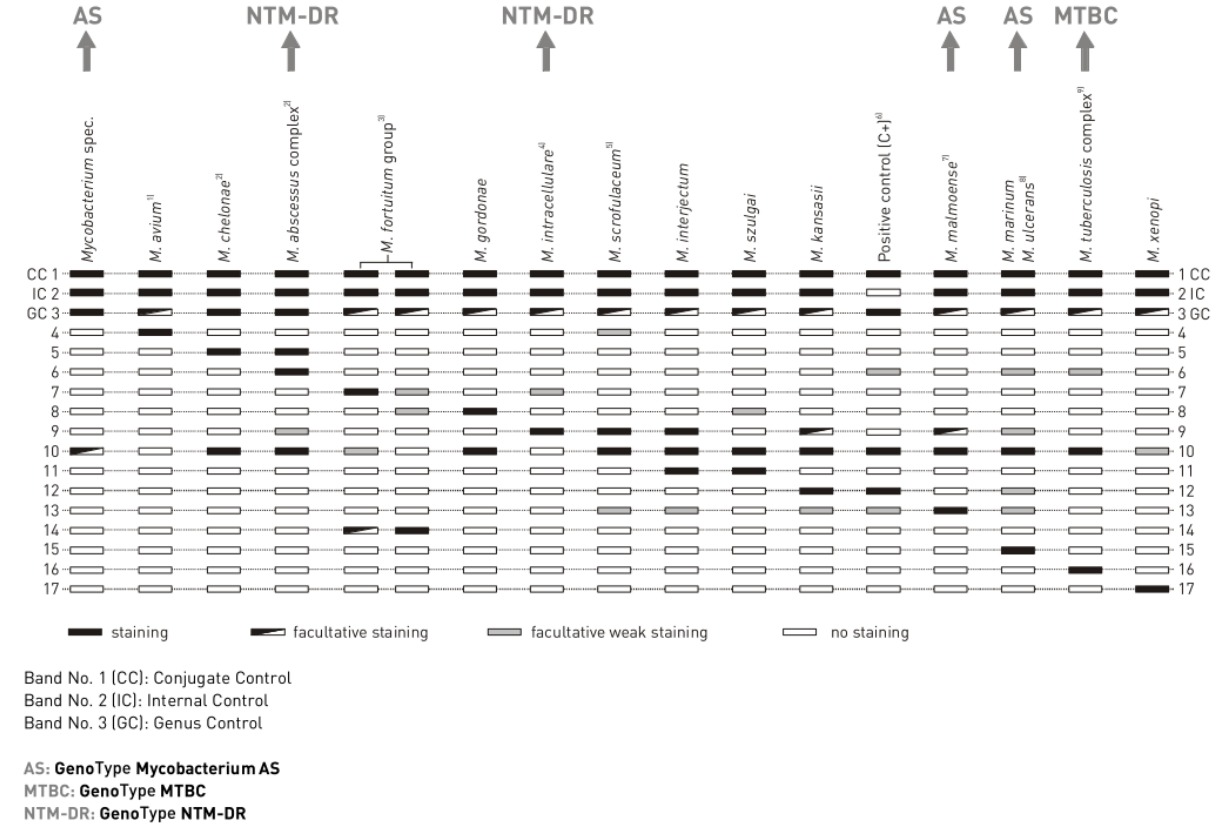

Figure 2-3 Genotype Mycobacterium CM result interpretation chart (76)

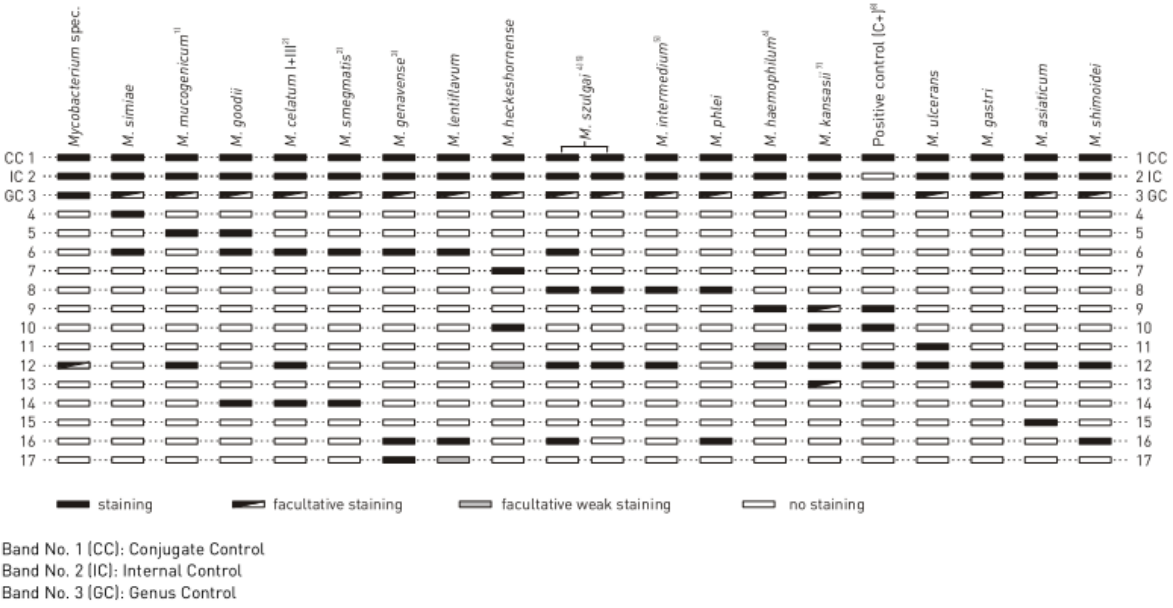

Figure 2-4 Genotype Mycobacterium AS result interpretation chart (77)

2.2.3 Extraction and inactivation protocol for MALDI-TOF MS

The extraction and inactivation of NTMs was done in a Biosafety Level 2 environment according to the manufacturer's instructions using the Vitek MS Mycobacterium/Nocardia kit (Ref: 415659). Once the inactivation step was completed, the rest of the process was conducted outside the Biological Safety Cabinet (BSC).

The process involved a centrifugation step of 3ml MGIT fluid for 10 min at 3000 x g after which the inactivation step was performed by adding 500µl of 70.0% Ethanol to the pellet, transferring the mixture to a supplied tube containing beads and vortexing horizontally on a Genie 2 vortex for 15 min. The inactivation was concluded with an incubation period (tube in vertical position) for 10 min at room temperature. Following centrifugation for 2 min at 14 000 x g (Hermle Microliter Centrifuge Z233 M-2), the extraction was performed by adding 10µl of 10.0% Formic acid and 10µl of 100.0% Acetonitrile to the pellet. The process was concluded with a final centrifugation step for 2 min at 14 000 x g. The DS target slide was prepared by transferring 1 µl of the supernatant in duplicate to the target slide and left at room temperature to air dry. Each dried sample spot was overlaid with 1µl of α-Cyano-4-hydroxycinnamic acid (CHCA) matrix and left to air dry after which the slide was processed on the Vitek MS within 72 hours (as per the manufacturer's instructions). The spectra of the target slides were acquired using the Vitek MS Prep and Acquisition Stations (bioMérieux, France); and Spectra were analysed using Myla software with Knowledge Base (KB) v3.2.0.

As recommended by the manufacturer, the *Escherichia coli* ATCC 8739 strain, used as a calibrator and internal identification control, was inoculated on the calibration spots of each acquisition group. Negative and positive controls were included in each batch of samples prepared. The negative control used consisted of reagents only and the positive controls included *M. smegmatis* ATCC 2415119 or *M. intracellulare* ATCC 13950 grown on Lowenstein-Jensen medium (LJ).

Deviations to the manufacturer's protocol involved centrifugation of 1.5ml MGIT fluid twice in 2 ml Eppendorf tubes, or centrifugation of 3 – 5.5 ml in 50ml conical tubes, rather than the recommended 3ml in a 5ml tube. In addition, all vortex steps were done without the mobio-adapter, due to this device not being available during the course of the study. However, we felt that we were able to simulate a similar vortexing effect by securing the tubes with parafilm horizontally on the platform head.

MGIT tubes were initially processed following storage at room temperature for between 4 – 31 days (Method 1) (23). In an attempt to optimise results we examined the effects of different incubation times as well as initial processing volumes:

- Method 2: MGIT tubes incubated at 37°C between 24-72 hrs post positivity (package insert, bioMérieux, France) and 1.5ml MGIT fluid centrifuged twice in 2 ml Eppendorf tubes.
- Method 3: 3-5 ml MGIT fluid centrifuged in 50 ml conical tube with the same incubation conditions as Method 2
- Method 4: 5.5 ml MGIT fluid centrifuged in 50 ml conical tubes after a 6 day incubation period at 37°C post positivity (5).

These methods were not run in parallel on all samples, but were done in a sequential fashion as part of a running optimisation of an ideal protocol, therefor, the difference in sample sizes.

2.3 Results and discussion

A total of 78 isolates identified as NTMs by the Genotype Mycobacterium CM / AS assays were extracted and processed for identification on the Vitek MS by either one of the four methods described in section 2.2.3. Table 2-1 lists the organisms tested with each method and the results obtained by the Vitek MS.

Table 2-1 Nontuberculous mycobacteria Vitek Mass Spectrometry identification results

Samples highlighted in blue represent samples that correlated with the laboratory reference method

Samples highlighted in orange represent samples with "no identification" (good spectrum)

Samples highlighted in green represent samples with "no identifications" (bad spectrum)

Samples highlighted in grey represent contaminated samples

Study Key	TB Lab ID - Hain	Duplicates†	Vitek MS	Correlate?	Method
1	*M. abscessus complex*	a-b	*M. abscessus*	Yes	1
2	*M. abscessus complex*	a-b	*M. abscessus*	Yes	1
3	*M. fortuitum*	a-b	*M. fortuitum group*	Yes	1
4	*M. avium*	a-b	*M. avium*	Yes	1
5	*M. kansasii*	a-b	*M. kansasii*	Yes	1
6	*M. gordonae*	a	*M. gordonae*	Yes*	1
		b	No identification		
7	*M. intracellulare*	a	No identification	Yes*	1
		b	*M. intracellulare*		
8	*M. fortuitum*	a	*M. fortuitum group*	Yes*	2
		b	No identification		
		reshoot a-b	Bad spectrum		
9	*M. avium*	a	*M. avium*	Yes*	2
		b	No identification		
10	*M. kansasii*	a	*M. kansasii*	Yes*	2
		b	Background noise		
11	*M. fortuitum*	a-b	Not enough peaks	Yes*	2
		c-d	*M. fortuitum group*		
12	*M. avium*	a	Bad spectrum	Yes*	3
		b	*M. avium*		
13	*M. fortuitum*	a-b	*M. fortuitum group*	Yes	4
14	*M. fortuitum*	a-b	*M. fortuitum group*	Yes	4
15	*M. gordonae*	a-b	*M. gordonae*	Yes	4
		reshoot a-b	Bad spectrum		
16	*M. intracellulare*	a-b	*M. intracellulare*	Yes	4
		reshoot a-b	Bad spectrum		
17	*M. avium*	a	*M. avium*	Yes*	4
		b	No identification		
18	*M. fortuitum*	a-b	No identification	No	1
19	*M. avium*	a-b	No identification	No	1
20	*M. gordonae*	a-b	No identification	No	1
21	*M. kansasii*	a-b	No identification	No	1
22	*M. malmoense*	a-b	No identification	No	1
23	*M. intracellulare*	a-b	No identification	No	1
24	*M. gordonae*	a	No identification	No	1
		b	Bad spectrum		
25	*M. abscessus complex*	a	No identification	No	1

		b	Bad spectrum		
26	*M. malmoense*	a-b	No identification	No	1
		c-d	Bad spectrum		
27	*M. fortuitum*	a-b	No identification	No	2
28	*M. avium*	a-b	No identification	No	2
29	*M. avium*	a-b	No identification	No	2
30	*M. intracellulare*	a-b	No identification	No	2
31	*M. intracellulare*	a-b	No identification	No	2
32	*M. intracellulare*	a-b	No identification	No	2
33	*Mycobacterium spp.*	a-b	No identification	Possible**	2
		reshoot a-b	Background noise		
34	*Mycobacterium spp.*	a-b	No Identification	Possible**	2
35	*Mycobacterium spp.*	a-b	No identification	Possible**	2
36	*Mycobacterium spp.*	a-b	No identification	Possible**	2
37	*M. fortuitum*	a	Bad spectrum	No	2
		b	No identification		
38	*M. avium*	a	Bad spectrum	No	2
		b	No identification		
39	*M. kansasii*	a	Bad spectrum	No	2
		b	No identification		
40	*M. fortuitum*	a-b	No identification	No	3
41	*Mycobacterium spp.*	a-b	No identification	Possible**	3
		reshoot a	Bad spectrum		
		reshoot b	Too many peaks		
42	*M. scrofulaceum*	a-b	No identification	No	3
43	*M. intracellulare*	a-b	No identification	No	4
44	*M. malmoense*	a-b	No identification	No	4
45	*M. intracellulare*	a-b	No identification	No	4
46	*M. intracellulare*	a-b	No identification	No	4
47	*M. intracellulare*	a-b	No identification	No	4
48	*Mycobacterium spp.*	a-b	No identification	Possible**	4
49	*M. intracellulare*	a	No identification	No	4
		b	Bad spectrum		
50	*M. intracellulare*	a	No identification	No	4
		b	Bad spectrum		
51	*M. scrofulaceum*	a	Bad spectrum	No	4
		b	No identification		
		reshoot a-b	Bad spectrum		
52	*Mycobacterium spp.*	a	No identification	Possible**	4
		b	Bad spectrum		
53	*M. kansasii*	a-b	Bad spectrum	No	1
54	*M. kansasii*	a-b	Bad spectrum	No	1
55	*M. intracellulare*	a-b	Bad spectrum	No	1
56	*M. kansasii*	a-d	Bad spectrum	No	1

57	*M. kansasii*	a-d	Bad spectrum	No	1
58	*M. intracellulare*	a-d	Bad spectrum	No	1
59	*M. avium*	a-b	Bad spectrum	No	2
60	*M. avium*	a-b	Bad spectrum	No	2
61	*M. intracellulare*	a-b	Bad spectrum	No	2
62	*M. intracellulare*	a-b	Bad spectrum	No	2
63	*M. kansasii*	a-b	Not enough peaks	No	2
64	*M. intracellulare*	a-b	Background noise	No	2
65	*M. fortuitum*	a	Bad spectrum	No	2
		b	Not enough peaks	No	
66	*M. avium*	a-d	Not enough peaks	No	2
67	*M. intracellulare*	a-d	Bad spectrum	No	2
68	*Mycobacterium spp.*	a-b	Bad spectrum	No	3
69	*Mycobacterium spp.*	a-b	Bad spectrum	No	3
70	*M. avium*	a-b	Bad spectrum	No	3
71	*M. intracellulare*	a-b	Bad spectrum	No	3
72	*Mycobacterium spp.*	a-b	Bad spectrum	No	4
73	*M. gordonae*	a-b	Bad spectrum	No	4
74	*M. intracellulare*	a-b	Bad spectrum	No	4
75	*M. intracellulare*	a-b	Bad spectrum	No	4
76	*M. intracellulare*	a-b	Bad spectrum	No	4
77	*M. intracellulare*	a	*E. faecalis*	No	2
		b	No identification		
78	*M. kansasii*	a	Not enough peaks	No	2
		b	*E. asburiae* *E. cloacae*		
		c-d	No identification		

† a-b duplicate spots from initial run; c-d duplicate spots from repeat run

* Consensus correlates (best result of a repeat / duplicate spot was considered correct)

** Considered a possible correlation as a “no identification” (good spectrum) result is expected in cases where the organisms are represented in the Vitek MS database.

Methods 1 – 4, see section 2.2.3

According to the Mycobacterium CM/AS classification, only 16.7% (13/78) belonged to the fast growing NTM group with the remaining 83.3% (65/78) classified as slow growers.

Of the 78 isolates tested by either one of the 4 methods, 21.8% (17/78, $CI_{95\%}$:12.6% - 31.0%) correlated 100% with the Vitek MS (confidence level of 99.9%). The method that delivered the highest percentage of identifications was Method 1 (7/22, 31.8%) followed by Method 4 (5/20, 25%), Method 2 (4/28, 14.3%) and then Method 3 (1/8, 12.5%). Method 3 performed particularly poorly and we therefore halted any further testing of that method. As we did not perform all 4 methods on each of the 78 isolates, we analysed the data to determine if there was a clustering effect of any of the strains with a particular method. The majority of fast growers were represented in Method 1 and 2

but no possible association to better performance could be made due to low numbers included in the study. There was overrepresentation of *M. intracellulare* in Method 4, which performed poorly as a whole. Many of these isolates resulted in “no identifications” despite being represented in the database.

The low percentage of identifications obtained in this study correspond to previous publications were the MALDI-TOF MS system failed to identify NTMs (78). In contrast, subsequent studies report identification rates ranging from 83.9% to 97.6%. The manufacturer, using what we describe as our Method 2, reported 87.7% (64/73, $CI_{95\%}$: 77.9% - 94.2%) correct identifications, 2.7% (2/73, $CI_{95\%}$: 0.1% - 6.5%) incorrect identifications and 9.6% (7/73, $CI_{95\%}$: 2.8% - 16.3%) “no identifications’ (6,10,79).

Our study results for Method 2 were statistically different to the manufacturer’s results for correct (P = <0.001, 87.7% [bioMérieux] vs 14.3% [our study]) and “no identifications” (P = <0.001, 9.6% [bioMérieux] vs 85.7% [our study]). There was however, no statistical difference between the number of incorrect identifications (P = 0.1411, 2.7% [bioMérieux] vs zero [our study]) obtained.

We do not believe that included strain types were responsible for variations in findings as both studies (manufacturer and ours) consisted predominantly of *M. avium* and *M. intracellulare.*.

There is a possibility of cross-identification between the Vitek MS displayed taxa (organism present in database) and unclaimed taxa (i.e. not present in the database, but may be reported as the corresponding displayed taxa). Table 2-2 lists the cross-identifications which may have affected this study.

Table 2-2 Vitek Mass Spectrometry Nontuberculous mycobacteria possible cross-identifications applicable to this study

Vitek MS Displayed taxa (Reported by Vitek MS)	Possibility of (Unclaimed taxa)
M. abscessus	*M. abscessus* spp. *abscessus* *M. abscessus* spp. *bollettii* *M. abscessus* spp. *massiliense*
M. avium	*M. avium* spp. *avium* *M. avium* spp. *paratuberculosis* *M. avium* spp. *silvaticum* *M. avium* spp. *chimaera*
M. gordonae	*M. paragordonae*
M. intracellulare	*M. chimaera* *M. colombiense* *M. yongonense / marseillense* *M. yongonense / marseillense / vulneris*

Vitek MS Displayed taxa	Possibility of (Displayed taxa)
M. avium	*M. intracellulare*
M. intracellulare	*M. avium*

As seen in Table 2-2 there can be a cross-identification between *M. intracellulare* and *M. avium* but both these organisms belong to the *M. avium complex* (MAC). The virulence of the two species depends on the underlying disease and the presence of co-morbidities e.g. *M. intracellulare* has a higher pathogenecity in cancer patients than *M. avium* (80). Patients with pulmonary disease caused by *M. intracellulare* are less likely to have a clinical relapse or reinfection after antiobiotic treatment than other species in the MAC group. As treatment options are similar for these two organisms, speciation has a prognostic benefit regarding disease severity and recurrence (81,82). Although these cross-identifications can occur it had zero impact on our study as all identifications correlated 100% with the reference method and no cross-identifications occurred.

78.2% (61/78, $CI_{95\%}$: 69.0% - 87.4%) resulted in a “no identification” result (samples 18-78, Table 2-1). This is in contrast to the 9.6% of “no identification” reported by the manufacturer. Of these 61 samples, 60.7% (37/61) showed good quality spectrums, implying that there were more than 30 protein profiles that passed internal quality control (QC) (samples 18-52). These should have resulted in a successful identification if the organism was represented in the database. Interestingly, most of our results showed good quality profiles, but failed to generate an identification despite the expected organisms being represented in the database.

An advantage of the Genotype Mycobacterium CM / AS assays over the Vitek MS is that when a species is not represented in the Vitek MS database, resulting in a “no identification” (good quality spectrum), the CM/AS assay will still allow identification up to the genus level. Ten samples were reported as *Mycobacterium* sp. following the Mycobacterium CM and AS assays as they did not fall into one of the species targeted by the assays. Of these the Vitek MS obtained “no identification” results of which 70.0% (7/10) were due to good quality spectrums. A “no identification” (good spectrum) result may be due to the organism not being represented in the Vitek MS KB, however we were unable to confirm for these 7 cases if this was the reason for the “no identification” result as we were unable to speciate them with the reference method.

MALDI-TOF MS is designed for use on pure cultures and is incapable of distinguishing between individual components of a polymicrobial culture. The presence of more than one *Mycobacterium* spp. can therefore not be excluded, however, this is not the case with the Genotype Mycobacterium CM / AS assays, where you be able to interpret some mixed infections by analysing the mixed

hybridisation patterns. The Vitek MS identification result will depend on the protein concentration which will most likely be dominated by the faster growing organism (21). Two contaminated samples in our study were identified by the Vitek MS as bacteria not belonging to the genus *Mycobacteria* (samples 77-78), both of these cases identified organisms with much faster growth rates than *Mycobacteria*. In contrast, the Genotype Mycobacterium CM / AS assays were not affected (14) as an *Mycobacterium* identification was achieved.

Eight samples (samples 11, 26, 56-58, 66-67, 78; Table 2-1) which initially did not provide an identification were repeated and only 12.5% (1/8) isolate (sample 11) could be resolved (*M. fortuitum*). The no identifications were mainly due to bad spectrums or not enough peaks, while only 1 sample was due to a good quality spectrum (sample 78 which showed a mixed infection).

It was evident in the early stages of the study that suboptimal results were being obtained due to the numerous “no identifications” by the Vitek MS system, despite good quality spectrums. Troubleshooting, in conjunction with bioMérieux South Africa and France, involved confirmation of the correct procedure, including the need for horizontal vortexing (15 min), exact centrifugation times and settings, complete ethanol removal and the spotting of the supernatant and not the pellet onto the target slide. bioMérieux analysed assay run files of “no identification” results and determined that the instrument was not sensitive enough to detect all the protein peaks to make an identification. The extraction method did not appear to be at fault. The instrument was therefore fine-tuned a number of times to improve the sensitivity. This involved adapting instrument settings while running a control strain. The linear detector of the system has a decreased sensitivity with increased use due to the ions impacting the detector. Consequently, fine tuning is required on a regular basis of which the frequency is determined by the throughput of the laboratory as well as the quality of the calibrator selected.

Similar problems were encountered by the routine diagnostic laboratory when testing bacterial and yeast isolates at the time and included extremely slow acquisition and failed calibrations in Myla despite appearing to pass during acquisition. The linear detector was replaced and although there was a slight improvement in the number of identifications obtained after each fine-tuning intervention, the problem was not permanently resolved. Several slides (samples 8, 15, 16, 33, 41, 51) were reshooted on two different Vitek MS instruments (including one at a different pathology practice). Initial identifications (e.g. *M. intracellulare* and *M. gordonae*) and “no identifications” (good spectrums) resulted in bad spectrums with the reshoot. This strongly indicates that even though a bad spectrum result may be resulted on one instrument, that another may be able to report a species due to sufficient protein profiles.

The remaining 39.3% (24/61) “no identification” results (samples 53-66) were due to bad spectrums (i.e. <30 spectral peaks) and were subsequently reported as either (a) bad spectrum during acquisition, (b) too few or (c) too many poor quality peaks or (d) background noise (14) by the Myla software. “Bad spectrum” results may be due to a number of reasons including:

- insufficient growth of the organisms and a subsequent suboptimal limit of protein to be analysed; or conversely,
- overgrowth of the organisms which result in inactivation of the genes encoding protein syntheses, with subsequent lysis of proteins already present in the culture (MGIT cultures only) (Personal communication, bioMérieux).

The Vitek MS KB database was constructed with protein profiles of organisms in the log phase of growth (6). It was therefore essential to test the organisms during this phase. Despite adapting the protocol to include an additional incubation step of 24 to 72 hours at 35 ± 2°C post MGIT incubation, we did not see an improvement in results.
A previous study by Huang et al. showed that there was no association between the duration of culture positivity and the success of rapid growing mycobacteria identification using a time frame of 0 to 6 days, however, the majority of our identifications were for slow-growing mycobacteria. They reported that slow-growing mycobacteria showed an increase in successful identifications with the increased number of incubation days post positivity (21 days) at room temperature. (21). A similar approach was reflected as Method 1 which yielded the most identifications of the 4 methods.

A further attempt to improve assay performance included increasing the biomass (Method 3, section 2.2.3), although it did not lead to an increased assay performance. Some samples contained less biomass than others and did not create an obvious pellet after the initial centrifugation step, especially *M. intracellulare* of which 41.8% (23/55) were included in the slow growing group tested. The identification of fast growers (53.8%) compared to the the slow growers (18.2%) strongly suggests that incubation time and resultant biomass (i.e. pellet size) may be crucial contributors to the success of identification obtained (21).

Garner et al. 2019 showed that an increased biomass led to 100.0% accurate identification rates with no “no identification” results (personal communication, Dr Garner, University of California, Los Angeles). This was achieved by adapting bioMérieux’s protocol (Method 2 of this study) by incubating MGIT tubes 6 days post positivity and processing 6 ml fluid (Method 4 of this study). They tested 51 NTM isolates, of which the majority were slow growers (29 /51, 56.9%) (83), and 22 (22/51, 43.1%) fast growers. Our study included only 13 (13/78, 16.7%) fast growers. The organism mostly isolated in their study belonged to the slow grower *M. simiae* complex (12/51, 23.5%) which was not represented in our study. Other organisms tested which were not represented in our study included

M. chelonae (5/51,9.8%), *M. mucogenicum* complex (10/51, 19.6%) and *M. arupense* (2/51, 3.9%). A limitation of the study is their data did not include *M. intracellulare* which was the most isolated organism in our study. Ten of the 20 (10/20, 50.0%) samples tested following the protocol proposed by Garner et al. were *M. intracellulare* (Method 4). The fact that our study did not replicate the success experienced by Dr Garner and his team could suggest that the root cause of the problems experienced may be far deeper than obtaining sufficient biomass.

Buckwater et al. suggested that interfering substances present in MGIT tubes may have an adverse effect on test results (1). Our results support this as we were able to successfully identify NTM ATCC strains from solid Lowenstein-Jensen media (LJ), but failed to obtain results when tested from liquid MGIT media. Identifications could only be obtained by increasing the processing volume of MGIT cultures to 5 ml, however, we were unable to resolve patient samples in this way. A possible explanation for this is that the QC MGIT tubes lack normal flora and cell debris normally present in patient samples which may have affected identification (6).

However, the performance characteristics for *Mycobacterium* liquid cultures from clinical specimens is documented in the Vitek MS user manual as 87.7% (64/73) with 2.7% (2/73) incorrect identifications and a total of 9.6% (7/73) no identifications.

A limitation of our study was processing samples without the recommended mobio-adapter (Figure 2-5) and it cannot be excluded that it contributed in obtaining less proteins required for an identification. However, we were able to mimic the positioning of samples on the same vortex and obtained satisfactory results when tesing quality control strains.

Figure 2-5 Genie 2 vortex with attached mobio-adapter

Furthermore, centrifuge rotors for 5ml tubes were not available in our setting and samples were processed in eppendorf or conicle tubes. The outcome of the results were not significantly affected by applying different tube sizes. However, we are confident that these deviations did not significantly affect the results of the study.

The poor results (particularly the high rate of “no identifications” in our study and issues with the routine microbiology bacterial identifications) were further investigated by bioMérieux. In an attempt to eliminate all possible factors influencing rolling calibration and poor instrument performance, the

company further suggested to allocate dedicated staff members to apply calibration spots and adapting some of the routine microbiology procedures including: (a) correct maintenance, storage and reculture of the *E.coli* ATCC 8739 strain (Appendix C) used during rolling calibration checks or the manual calibration / fine tuning of the instrument; (b) spot preparation to add matrix to each spot as they are processed, and not per batch, as this would affect crystalisation.

However, despite all interventions, no improvement in the instrument performance was seen. bioMérieux did a TOF/BSA (Bovine serum albumin) test on the two instruments and the problem was suspected to be software related. This finding fits in perfectly with the “no identification” (good spectrum) results obtained in our study as the software was unable to read the protein profiles effectively to supply an identification despite the organisms theoretically being represented in the Vitek MS KB software. The problem was unfortunately not rectified at the time of thesis submission.

2.4 General workflow and cost analysis

The workflow on the Vitek MS for organism identification was examined by calculating the amount of time needed for hands-on and hands-off procedures for the method. Calculations were done using Microsoft Excel,v.1904 (Microsoft, Washington) and included factoring in the number of control samples per batch.

The process was broken down into three steps for which the duration was recorded:

- Sample processing - included the time from the start of processing the isolates until the supernatant was spotted onto the target slide. The time allocated to hands-on procedures was determined as the total sample processing time, less the fixed hands-off time. Hands off time included bead beating, incubation and centrifugation steps.
- Target slide drying - included the time the supernatant was first spotted onto the slide until ready for processing on the Vitek MS (regarded as hands-off procedures).
- Vitek MS - included processing on the Vitek MS until a result was available (regarded as hand-off procedure).

The initial capital cost and associated maintenance contracts for all instrumentation were excluded from calculations. The crude cost analysis was determined by including direct costs such as reagent and consumables (including controls) and excluding indirect costs such as instrument, infrastructure and personnel time. Processing of samples for the various methods was done by skilled technologists or scientists registered with the Health professions council of South Africa (HPCSA). Depending on the complexity of the method or interpretation of the results, the expertise level and associated employment cost varied. A limitation of the costing analysis was that we were only able

to perform a crude costing approach as many of the costing values are subject to PathCare's confidentiality agreements (i.e. negotiated costs with suppliers, etc). Ideally concrete values should be reported, but we felt that for comparative purposes, reporting cost as a proportion of another assay's cost would be sufficient.

2.4.1 NTM workflow (hand-on / hands-off) determination

The current workflow (post-MGIT incubation) in the PathCare TB laboratory is represented in Figure 2-1. Briefly, ZN stains are performed on positive MGIT cultures after which the MPT64 testing is done on all ZN positive MGIT samples. The workflow for MPT64 positive MGIT samples will be excluded as it is not applicable to this study. Genotype Mycobacterium CM testing was done on all ZN positive, MPT64 negative MGIT cultures and results were reported if a definite identification was obtained. If not, the Genotype Mycobacterium AS was performed. If no species was identified, the results were reported as *Mycobacterium* spp. Genotype Mycobacterium CM / AS testing was batched and only done on a Monday, Wednesday and Friday in the laboratory. Unloading of positive MGIT cultures were done on a daily basis except over weekends and public holidays, therefore, the Vitek MS procedure for the identification of NTMs could easily be incorporated into the daily workflow.

The average turnaround time for speciation of an NTM by the PathCare TB laboratory was calculated for the applicable dataset of samples tested from the time the positive MGIT tube was unloaded from the Bactec MGIT 960 instrument, until the result was entered into the Laboratory Information System (LIS). The average turnaround time for reporting results using the Mycobacterium CM and AS assays was calculated as 3 days. One outlier was excluded from the calculation (17 days) as the test was an after-request. Batching samples for the Genotype CM / AS assays led to a longer turnaround time, but a result can be potentially produced within 1 day.

Table 2-3 contains details of the time allocated to hands-on and hands-off procedures for the Vitek MS when processing NTM isolates of up to 6 isolates a batch. The total time taken to process six isolates was just under 3 hours, with only 46 minutes considered as hands-on procedures. This dataset represented data where the target slides had been dried in the BSC. If slides had been left on the open bench to dry (as per the manufacturer's instructions), the total processing time would increase with an average of 18 minutes.

Table 2-3 Workflow of processing Nontuberculous mycobacteria isolates on Vitek Mass Spectrometry (hh:mm) for a batch of 1 to 6 isolates

Procedure	1	2	3	4	5	6
Hands-on	00:08	00:16	00:24	00:32	00:40	00:48
Hands-off	01:27	01:33	01:39	01:45	01:52	01:58
Total	01:35	01:49	02:03	02:18	02:32	02:46

For the Vitek MS, the accuracy of the results obtained was not taken into consideration when estimating turnaround times, but rather the generation of a first valid Vitek MS result. Repeat or alternative testing would have a negative impact on the turnaround time. Not all samples in this study could be repeated due to either insufficient sample volume remaining in the MGIT tube, exceeding the required incubation time period, or preference given to routine laboratory diagnostic procedures. One of the expected benefits of using the Vitek MS for NTM identification was the reduction in turnaround time (5,21), however, in this study, the most successful method (Method 1) involved an extended incubation period of up to 31 days which then does not provide a time saving over the 3 days turnaround time of the Mycobacterium CM/AS assays. Method 4 may therefore be more promising, as the incubation time was only 6 days, which still allowed additional biomass accumulation.

2.4.2 NTM cost determination

The focus of the cost analysis was to determine what the Vitek assay would cost in comparison to the Genotype Mycobacterium AS / CM assays for the identification of NTM. As actual cost reporting is considered sensitive company information, we will report costing in terms of percentage difference to Vitek MS.

The cost analysis was calculated from where the sample was flagged positive on the Bactec MGIT 960 and did not include the culture costs which is the same irrespective of the procedure used for downstream analysis/speciation.

The identification of NTMs for a single sample by Genotypic CM and AS assays was 44.3% and 92.8% respectively, of the cost of the Vitek MS identification. The average of samples processed in a batch for the Vitek MS ranges from 1 to 3 samples per batch. The identification of NTMs for a batch of 3 samples by the Genotype CM and AS assays was 23.5% and 49.1% respectively, of the cost of the Vitek MS identification. The Genotype Mycobacterium CM / AS assays are usually run in combination with Genotype MTBDRplus and MTBDRsl assays which increases the number of samples per batch, using some of the same consumables and reagents which ultimately decrease costs for this assay.

There is also large wastage of Vitek MS reagents as the Mycobacterium/Nocardia kit is supplied for 100 tests and the stability once opened is only 4 weeks.

2.5 Conclusion

While less experienced staff are required to perform the Vitek MS, the prolonged incubation time to optimise biomass doubles the current turnaround time of the Vitek MS method. Although NTM identification by the Vitek MS can easily fit into our current workflow, the reagent wastage due to large NTM/Nocardia kit size does not suit the prevalence of the organisms in our setting. In conclusion, the poor performance of the Vitek MS for the identification of NTMs in our setting (most likely due to instrument issues) as well as the increased turnaround time and cost of NTM identification suggest that the Mycobacterium CM/AS assays remain the methods of choice in our laboratory setting. The opposite may be true for larger regional laboratories such as the National Health Laboratory Service (NHLS) who service a much larger patient population affected by mycobacterial disease. Reagent efficiency can therefore be optimised by processing larger sample batches, which will ultimately decrease cost of the assay.

CHAPTER 3: THE IDENTIFICATION OF NOCARDIA

3.1 Introduction

The objective of this chapter was to evaluate the performance of the Vitek MALDI-TOF MS for the identification of *Nocardia* spp. by comparing the results to 16S rRNA sequencing and Multilocus Sequence Analysis (MLSA).

3.2 Materials and methods

3.2.1 Sample selection

Due to the low prevalence of *Nocardia* infection in our setting, the NHLS Tygerberg Microbiology laboratory assisted in sample collection. A total of eight *Nocardia* isolates were identified for inclusion in the study during the sampling period (July 2018 to August 2019). The isolates were stored on Microbank vials (Pro-lab Diagnostics) at -70°C prior to processing.

Once ready for processing, one or two of the beads were removed from the vial using a sterile nichrome loop and plated onto horse blood agar and incubated aerobically at 35 ± 2°C for 48 hours or until sufficient growth was obtained.

3.2.2 16S rRNA sequencing

Nocardia speciation was done by 16S rRNA Sequencing which targets a 598 base pair (bp) section of the 16S rRNA gene. This gene contains hypervariable regions which can be used for bacterial species identification.

a) DNA extraction

A few colonies (pure growth) were selected from the horse blood agar plates and resuspended in 500µl phosphate buffered saline. 200 µl of each sample was lysed in 180µl of bacterial lysis buffer (Roche, MagNA Pure, Switzerland) containing 20µl of proteinase K on a heating block at 60°C for at least 15 minutes, or until fully lysed, after which the proteinase K was deactivated at 100°C for 10 minutes. The lysate was then extracted on 1 of 2 platforms in our laboratory, including the MagNA Pure Compact or eMag (bioMérieux, France) instruments as per the manufacturer's instructions.

b) Preparation of master mix

The reaction mix was prepared by adding the reagents to a labelled 0.6 ml tube. Table 3-1 contains the volumes and concentration of the reagents used. Sufficient volume of master mix was prepared according to the number of samples and controls (positive control [PC] and no template control [NTC]) to be tested as well as 1 additional sample to control for pipetting errors. The primers used

in the reaction consisted of a previously published forward primer PSL (5'-AGG ATT AGA TAC CCT GGT AGT CCA-3') and reverse primer XB4 (5'-GTG TGT ACA AGG CCC GGG AAC-3') (84).

Table 3-1 Constituents of the 16S Ribosomal Ribonucleic acid sequencing master mix

Reagent	Concentration	Volume per reaction (µl)
KAPA Long Range Buffer	5X	4
$MgCl_2$	25mM	1.4
dNTPs	10mM	0.6
XB4 primer	10 pmol/µl	1
PSL primer	10 pmol/µl	1
PCR grade dH_20	NA	9.9
KAPA Long Range Enzyme	5U/µl	0.1

18 µl of the reaction mix was added to a labelled 0.2 ml thin-walled PCR tube or onto a PCR plate for each sample and control. 2 µl of the template DNA and control DNA was added to the corresponding sample tubes/wells using a pipette and the tubes/wells were sealed. No additional PCR grade dH_20 was added to the NTC tube.

c) Amplification

The 16S target region was amplified using the ABI ProFlex PCR System. Table 3-2 describes the touchdown PCR cycling parameters.

Table 3-2 16S Ribosomal Ribonucleic acid amplification program – ABI ProFlex Polymerase chain reaction system

Temperature	Time	Cycles
94°C	2 min	1
94°C 62°C 72°C	30 sec 30 sec 30 sec	10
94°C 58°C 72°C	30 sec 30 sec 30 sec	20
72°C	2 min	1
4°C	∞ (Hold)	

d) Detection

Amplification products were visualised by gel electrophoresis on a 2.2% Flashgel (Lonza, Switzerland) run for 10 minutes at 120 volts. 2.5 µl of each sample was mixed 1:1 with loading dye

(Bioline loading buffer) and carefully pipetted into each well. A 100bp ladder (Abbott, United States) was also run on the gel to confirm amplicon size. Any samples with positive amplification were prepared for sequencing. A run was deemed valid if the positive control showed an amplification product and the NTC was clear.

e) Sequencing PCR reaction

Dilution of the PCR products was achieved by adding 80 µl of PCR grade water to each well (17.5 µl amplicon remaining) after which mixing was done by pipetting. The reagents (per reaction) were combined as set out in Table 3-3 in a 96 well plate. Separate reactions were prepared for the forward and reverse primers.

Table 3-3 16S Ribosomal Ribonucleic acid sequencing amplification reagents

Reagent	Volume per reaction (µl)
PCR grade dH_20	13
Big Dye Terminator v3.1 (Life Technologies)	2
Primer (1.1 pmol/µl)	3
Diluted PCR product	2

The 96 well plate was sealed and centrifuged briefly to collect the contents at the bottom of the wells. The plate was transferred to the ABI 9700 thermal cycler for amplification. Table 3-4 contains details of the cycling parameters.

Table 3-4 Amplification program – ABI ProFlex Polymerase chain reaction system

Temperature (°C)	Time (seconds)	Number of cycles
96	60	1
96	10	30
55	10	
60	240	
15	∞ (Hold)	

f) DNA purification by ethanol precipitation

In a new 96 well plate, 2µl of sodium acetate (NaOAc) buffer solution (3M, pH 5.2) was added to 10µl of the sequencing product. The plate was centrifuged briefly for 5 seconds at 500 x g to ensure the NaOAc/EDTA (sodium acetate/ethylenediaminetetraacetic acid) buffer was properly combined with the sequencing reactions. 25 µl of 100.0% ethanol (Merck, United States) was added to each sequencing reaction after which the reaction tubes were briefly vortexed. The plate was centrifuged

for 30 minutes at 2 000 x g and the supernatant was removed by gently inverting the tray onto absorbant paper towel. The inverted tray was centrifuged for 10 seconds at 50 – 100 x g to remove all remnants of liquid onto the absorbant paper towel. 50 µl of 80.0% ethanol was then added to each sequencing reaction and centrifuged for 5 minutes at 2 000 x g. The supernatant was removed again by inverting the tray on paper towel and by centrifugation for 10 seconds at 50 – 100 x g.

g) Automated sequence detection on the AB 3500 Genetic Analyser

The sequencing reactions were prepared for loading onto the capillary DNA sequencer by adding 10µl Hi-Di Formamide to each sequencing reaction well. The samples were centrifuged for 10-15 seconds at 900 x g. The sequencing reactions were denatured in a thermal cycler for 2 minutes at 95°C. The 96 well plate was sequenced in-house using the ABI 3500 genetic analyser. Resultant electrophereograms were analysed using BioEdit software and compared to curated online 16S databases (SILVA and / or RDP) for genus and/or species identification (https://www.arb-silva.de/aligner/ and/or http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp). A cut-off value of ≥97.0% homology for genus level only and ≥99.0% for species level identification were used.

3.2.3 Extraction protocol for MALDI-TOF MS

Fresh *Nocardia* cultures were revived twice from Microbank storage vials and were prepared by adding one loopful of cultured organism (using a 1µl loop or cytology brush) to 500µl of 70.0% ethanol in the supplied bead containing tube. The remainder of processing was done as per section 2.2.3 from cell disruption on the Vortex 2 Genie.

3.2.4 Nocardia multi-locus sequencing analysis

MLSA was performed on 7 of the 8 clinical isolates included in the study according to a previously published protocol (43). One isolate was excluded due to repeated failure to amplify all MLSA targets. The researchers described a five-locus MLSA (*gyrB-16S-secA1-hsp65-rpoB*) method, but they acknowledged that the method may be too laborious, time-consuming and expensive in a routine clinical diagnostic setting. They demonstrated similar results (98.5% correlation) when using only 3 of the loci (*gyrB-16S-secA1*). We therefore performed the three-locus MLSA according to the published protocol with the following deviations: samples were extracted on the eMAG instrument and we used OneTaq Hot Start DNA polymerase (New England Biolabs, United States).

a) DNA extraction

DNA was extracted as per section 3.2.2.

b) Primers

Forward and reverse primers (Inqaba Biotechnical Industries) for the three selected targets (*16S, gyrB* and *SecA1*) were used as previously described (43)(Table 3-5). The additional primers listed for the *gyrB* and *SecA* were used in one sample where sequencing with the main primers was unsuccessful.

Table 3-5 Polymerase chain reaction primers for Nocardia Multilocus sequence analysis

	Forward Primer (5' – 3')		Reverse Primer (5' – 3')	
Target Region	**Primer name (position)**	**Sequence**	**Primer name (position)**	**Sequence**
16S bp 31 - 492	E8F (bp 11-30)	AGAGTTTGAT CCTGGCTCAG	534r (bp 493-509)	ATTACCGCG GCTGCTGG
*gyr*B bp 1031 - 1511	Noc-gyrB-F (bp 972-992) Noc-gyrB-F3 (bp 1011-1030)	CTTCGCCAAC ACCATCAACAC CGAGGAGGGC TTCCGCGCGG	Noc-gyrB-R (bp 1563-1582) Noc-gyrB-R3 (bp 1512–1532)	TGATGATCGA CTGGACCTCG ATCGACTGGA CCTCGTTGTTC
*sec*A1 bp 431 - 875	secA1-F47 (bp 413-430) secA1-F47 (bp 413–430)	GCGACGCCG AGTGGATGG GCGACGCCG AGTGGATGG	secA1-ConR2 (bp 876-896) secA1-ConR (bp 913–933)	TTGGCCTTGAT GGCGTTGTTC GCGGACGATG TAGTCCTTGTC

c) Preparation of master mix

The amplification master mix was prepared by adding the reagents to a labelled 0.6 ml tube. Table 3-6 contains the volumes and concentration of the reagents used. Sufficient volume of master mix was prepared according to the number of samples and controls (NTC) to be tested as well as 1 additional sample to control for pipetting errors. All reagents were pulse spun prior to making up the reagent mix. No positive control was added as all isolates were confirmed *Nocardia* spp. formerly identified by the 16S sequencing method.

Table 3-6 Constituents of the Nocardia Multilocus sequence analysis master mix

Components	Concentration	Volume per reaction (µl)
Standard Reaction Buffer	1X	5
dNTPs	200µM	0.5
Forward Primer	0.2µM	0.5
Reverse primer	0.2µM	0.5
OneTaq Hot Start DNA Polymerase	1.25 units/50µl	0.125
Template DNA		5
Nuclease-free water		13.375
Total		25

20 µl of the reaction mix was added to a labelled 0.2 ml thin-walled PCR tube or PCR plate for each sample and control. 5 µl of the template DNA was added to the corresponding sample tubes/wells using a pipette, and the tubes were sealed. The well plate was spun for 15 seconds at 900 x g.

d) Amplification

The MLSA target regions were amplified on the ABI ProFlex PCR system as previously described (43). Briefly, following an initial denaturation step at 98°C for 30 seconds, the samples underwent 35 cycles of denaturation for 5 seconds at 98°C, 5 seconds primer annealing at 56°C (16S), 60°C (*gyrB*) or 67°C (*SecA1*), respectively; and 20 seconds elongation at 72°C. This was followed by a final elongation step at 72°C for 1 minute and a hold at 4°C.

Suboptimal results were obtained for *gyrB* and *SecA1.* Further optimisation was done by gradient PCR to determine the optimum annealing temperature for the PCR reactions. The temperature ranges tested (in increments of 1°C), are shown in Table 3-7. The number of cycles was also increased from 35 to 45.

Table 3-7 Optimising annealing temperatures for gyrB and secA primer sets

	Annealing temperatures	
Sample no	***gyr*B**	***sec*A1**
1	54°C	61°C
2	55°C	62°C
3	56°C	63°C
4	57°C	64°C
5	58°C	65°C
6	59°C	66°C

e) Detection

Amplification products were visualised by gel electrophoresis as per section 3.2.2 d). A 20ng ladder (New England Biolabs, United States, item # NEB N3231S) was also included on the gel. A run was deemed valid if the NTC was clear.

f) Sequencing PCR reaction

Subsequent samples with positive amplification were prepared for sequencing as per section 3.2.2 using the forward and reverse primers for the 16S, *gyrB* and *secA1* targets. Resultant electrophereograms were analysed using BioEdit software. Primer sequences were trimmed to yield

fragments of 462 bp for 16S, 482 bp for *gyrB* and 445 bp for *secA1*. The sequence fragments were concatenated in the order of *gyrB-16S-secA* to obtain a sequence of 1389 bp.

g) Evolutionary analysis by Maximum Likelihood method

The concatenated sequences were used for phylogenetic analysis using MEGA X (v10.0.5)(https://www.megasoftware.net/home). Briefly we applied the Maximum-Likelihood algorithm based on the General Time Reversible model with 1000 bootstrap replication. The tree with the highest likelihood was constructed and a bootstrap value of ≥70.0% was deemed acceptable. Reference strains were sourced from Genbank and includes *N. asiatica, N. beijingensis, N. abscessus, N. cyriacigeorgica, N. brasilensis, N. farcinica, N. asteroides, N. neocaledoniensis, N. otitidiscaviarum* and *N. terpinica.*

3.3 Results and discussion

3.3.1 16S rRNA Sequencing and Vitek MS

All 8 *Nocardia* spp. isolates were extracted twice and tested in duplicate on the Vitek MS (Table 3-8).

Table 3-8 Results of Nocardia isolates on the Vitek Mass Spectrometry

Sample Number	16S Sequencing Identification	Duplicate*	Vitek MS Identification
N1-a	*Nocardia* spp.	a-b	No identification
		c-d	*N. neocaledoniensis*†
N1-b	*Nocardia* spp.	a-b	*N. neocaledoniensis*
N2-a	*Nocardia* spp.	a-b	*N. cyriacigeorgica*
N2-b	*Nocardia* spp.	a-b	*N. cyriacigeorgica*
N3-a	*N. otitidiscaviarum*	a-b	*N. otitidiscaviarum*
N3-b	*N. otitidiscaviarum*	a-b	*N. otitidiscaviarum*
N4-a	*Nocardia* spp.	a-d	No identification
N4-b	*Nocardia* spp.	a-b	No identification
N5-a	*N. otitidiscaviarum*	a-b	Bad spectrum
		c-d	*N. otitidiscaviarum*†
N5-b	*N. otitidiscaviarum*	a-b	*N. otitidiscaviarum*
N6-a	*N. cyriacigeorgica*	a-b	Bad spectrum
		c-d	*N. otitidiscaviarum*
N6-b	*N. cyriacigeorgica*	a-b	*N. otitidiscaviarum*
N7-a	*N. farcinica*	a-b	Bad spectrum
		c-d	No identification†
N7-b	*N. farcinica*	a-b	No identification
N8-a	*N. terpenica*	a-b	No identification
N8-b	*N. terpenica*	a-b	No identification

* a-b duplicate spots from initial run; c-d duplicate spots from repeat run

† Consensus correlates (best result of a repeat / duplicate spot was considered correct)

Five of the 8 isolates tested (5/8, 62.5%) correlated with the Vitek MS to the genus level (N1-N3, N5-N6). Of these, the Vitek MS was able to identifiy *Nocardia neocaledoniensis* (N1) and *Nocardia cyriacigeorgica* (N2) to the species level where the 16S rRNA sequencing was only able to confirm genus.

Of the 5 isolates speciated by 16S rRNA sequencing, 40% (2/5) correlated with the Vitek MS with confidence levels of 99.9% (N3, N5). One isolate (1/5, 20.0%) did not correlate with the Vitek MS on species level (N6). According to the manufacturer there is a possibility of cross-identification between some of the displayed taxa as well as unclaimed taxa (Table 3-9). *N. cyriacigeorgica* is not listed. However, as we see in Table 3-10, the Vitek MS identification was most likely the correct identification for this isolate as it correlated with MLSA.

Table 3-9 Vitek Mass Spectrometry Nocardia Cross-identification with unclaimed taxa applicable to this study

Vitek MS Displayed taxa Reported by Vitek MS	Possibility of Unclaimed taxa
Nocardia farcinica	*Nocardia higoensis* *Nocardia shimofusensis*
Vitek MS Displayed taxa	**Possibility of Claimed taxa**
Nocardia neocaledoniensis	*Nocardia asteroides*

Of the 8 isolates, 5 (62.5%) isolates were repeated after the initial run due to “no identifications” obtained (N1, N4-N7). 60% (3/5) of the “no identifications” were resolved with an identification (N1, N5-N6), but the remaining 40% (2/5) samples resulted in “no identifications” (good quality spectrums) (N4, N7).The overall no identifiction rate was 37.5% (3/8). A “no identification” (good quality spectrum) may be due to the presence of an organism not represented in the reference database. However the species identified by 16S rRNA sequencing as well as during the repeated Vitek MS run suggest that all of these organisms were represented in the Vitek MS database.
However, as described for NTMs (section 2.3), we encountered numberous issues with and discrepancies between different Vitek MS instruments. It therefore cannot be excluded that the “no identifications” obtained were due to the suboptimal performance of the instruments.

The Vitek MS performed more accurately for the identification of *Nocardia* (62.5%) than for NTMs (21.8%, section 2.3). The improved performance of the *Nocardia* assay could be due to the fact that *Nocardia* is processed from solid cultures where NTM were processed from liquid cultures. The biomass available for spectrum acquisition on the system is therefore much more than what is

available in a liquid culture and may be a contributor to the improved performance from solid cultures (1). However, the 62.5% accurate identification obtained for *Nocardia* is much lower than what was claimed by the manufacturer (97.9% total correct genus identification). Where they report incorrect identifications (0.8%) and only 1.3% “no identifications”, our values were higher (20.0% and 37.5%, respectively). Our study results were statistically different to the manufacturer’s results for correct identifications (*P* = <0.001, 97.9% [bioMérieux] vs 62.5% [our study]), “no identifications” (*P* = <0.001, 1.3% [bioMérieux] vs 37.5% [our study]) and the number of incorrect identifications (*P* = <0.001, 0.8% [bioMérieux] vs 20.0% [our study]) obtained.

The results obtained on the Vitek MS for the identification of *Nocardia* range from 83.0% to 98.0%, however, most of these studies aimed to develop and validate a comprehensive spectral database and was published by the research and development team of bioMérieux (2,35,85). A multicenter study published in 2018 showed 76.0% (236/312) correct identification to species level and an additional 14.0% (44/312) to the complex level. 3.0% (10/236) of the *Nocardia* isolates were misidentified and 7.0% (22/236) were not identified. This multicenter study was performed as part of the Food and Drug Administration (FDA) trial of the Vitek MS 3.0 system (45). We acknowledge that the low number of isolates tested (n=8) in our study may contribute to these inflated values, but as discussed above, are of the opinion that suboptimal instrument performance was responsible for the poor performance of the assay in our setting.

The main hurdle for the implementation of the MALDI-TOF technology for the identification of *Nocardia* spp. in a clinical laboratory is the difficulty of obtaining *Nocardia* strains for the validation of the instrument prior to patient use. The restricted availability of reference data sets of those species not frequently isolated in the clinical laboratory and the absence of or the availability of only a small number of isolates of a specific species in the reference database, may be the cause of no identification results obtained by MALDI-TOF (86).

3.3.2 MLSA

MLSA was optimised through gradient PCR (section 3.2.4 d) and the annealing temperature selected for the *gyrB* primer set was 57°C and 65°C for the secA1 primer set. 7 of the 8 samples were successfully analysed, see Figure 3-1 as an example of the electrophoresis of the optimised PCR product for *secA1*.

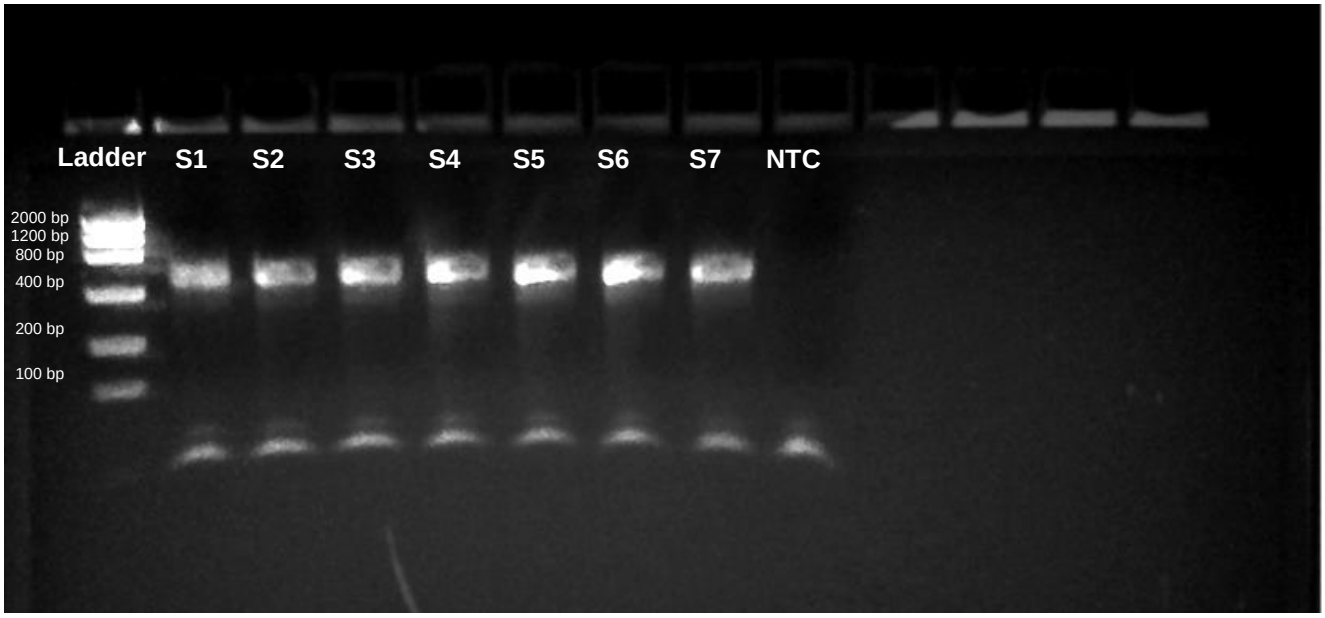

Figure 3-1 Electrophoresis of optimised Ribosomal Ribonucleic acid products of secA1

Phylogenetic analysis obtained from the 7 clinical isolates revealed several clusters with bootstrap threshold values of > 70.0% (Figure 3-2). MLSA confirmed the identification of all 5 *Nocardia* spp. identified to species level by the Vitek MS (Table 3-10). Two previously unidentified species (by Vitek MS) were clustered with *N. asiatica* which is represented in the Vitek MS KB database. The Vitek MS and MLSA results correlated better than either method with the 16S rRNA sequencing method. The latter was unable to speciate 3 of the isolates and provided alternative ID's for 2 of the samples (Samples 6 and 7).

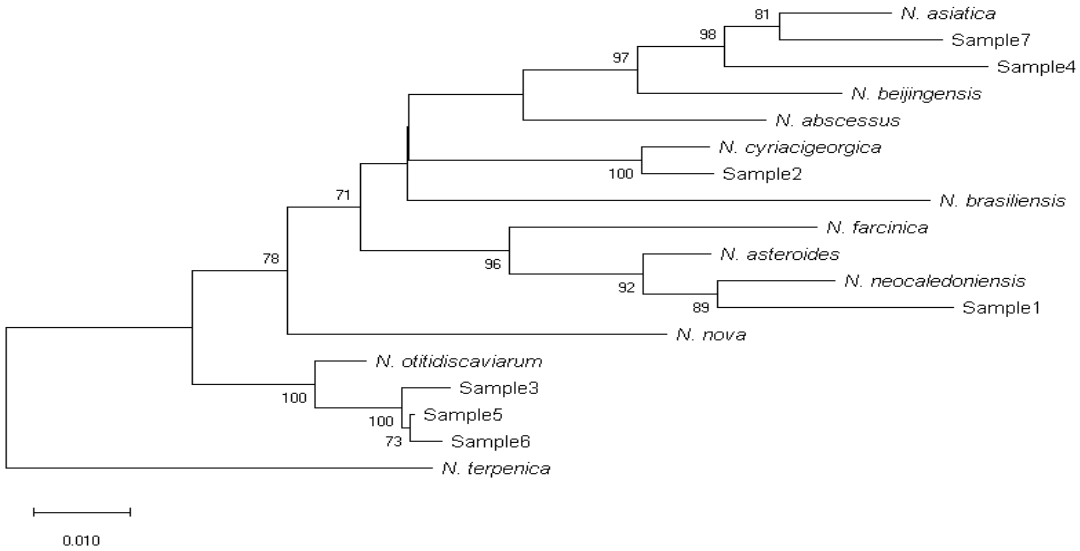

Figure 3-2 Phylogenetic tree based on the concatenated gyrB-16S-secA-sequences

Table 3-10 Comparison of Nocardia identification between 16S rRNA, Vitek MS and MLSA

Sample	16S rRNA	Vitek MS	MLSA
1	*Nocardia* spp.	*N. neocaledoniensis*	*N. neocaledoniensis*
2	*Nocardia* spp.	*N. cyriacigeorgica*	*N. cyriacigeorgica*
3	*N. otidiscaviarum*	*N. otidiscaviarum*	*N. otidiscaviarum*
4	*Nocardia* spp.	No identification	*N. asiatica*
5	*N. otidiscaviarum*	*N. otidiscaviarum*	*N. otidiscaviarum*
6	*N. cyriacigeorgica*	*N. otidiscaviarum*	*N. otidiscaviarum*
7	*N. farcinica*	No identification	*N. asiatica*

Results from MLSA confirmed the superior performance of the Vitek MS compared to the 16S rRNA sequencing for the identification of *Nocardia.* Limitations of 16S rRNA sequencing is that often it is unable to differentiate between closely related species due to insufficient interspecies polymorphisms within the 16S rRNA gene sequences (for *Nocardia* in particular). Incorrect identifications can be obtained due to multiple, although different, copies of the 16S rRNA gene present in certain species such as *N. nova* (87).

While MLSA provided the most identifications, it is a labour intensive and costly exercise which is not feasible as a routine test in our setting.

3.4 General Workflow and Cost analysis

General workflow and cost determinations were done as per section 2.4 .

3.4.1 Nocardia workflow (hand-on / hands-off) determination

Current workflow for *Nocardia* identification involves the phenotypic identification of *Nocardia* spp. and subsequent confirmation with *16S rRNA* sequencing in the molecular laboratory as described in section 3.2.2.

The average turnaround time for speciation of *Nocardia* by the molecular laboratory was calculated for the applicable dataset from culture positive until the result was entered into the Laboratory Information System (LIS). The average turnaround time for reporting results using the 16S rRNA sequencing was calculated as 5 days.

The actual turnaround time for processing on the Vitek MS could not be calculated as the isolates selected for the study were retrieved from Microbank storage at -70°C. Hypothetically, after phenotypic identification, results can be available in 1 day if an Vitek MS is available on site or 2

days if send to a reference laboratory. For this dataset processing *Nocardia* isolates on the Vitek MS reduced the turnaround time by 3 to 4 days depending on the availability of a Vitek MS on site.
For Vitek MS analysis, pure growth of the suspected organism was required and if sufficient biomass could not be obtained due to the presence of mixed organisms, the need to subculture the primary plates would result in a delay of up to 3 days depending on the organism growth.

This highlights the benefit of the availability of a Vitek MS for the identification of *Nocardia* isolates which reduces the turnaround time in comparison to 16S rRNA sequencing which is usually only available in a reference laboratory (1,86).

The workflow on the Vitek MS for *Nocardia* identification was further examined by calculating the amount of time needed for hands-on and hands-off procedures for the method.
Table 3-11 contains details of the time allocated to hands-on and hands-off procedures for the Vitek MS when processing *Nocardia* isolates. The total time it will take to process six isolates on the Vitek MS is approximately four hours, but only 41 minutes is allocated to hands-on procedures.

Table 3-11 Workflow of processing Nocardia isolates on Vitek Mass Spectrometry (hh:mm) for a batch of 1 to 6 isolates

Procedure	1	2	3	4	5	6
Hands-on	00:06	00:13	00:20	00:27	00:34	00:41
Hands-off	02:58	03:02	03:07	03:11	03:15	03:20
Total	03:05	03:16	03:27	03:39	03:50	04:01

The isolation frequency of *Nocardia* spp. was extremely low in our setting and isolates will most likely be processed one at a time which will take just over 3 hours to complete, with only 6 minutes hands-on time.

3.4.2 Nocardia cost determination

The focus of the crude cost analysis was to determine the cost of the Vitek MS procedure in comparison to the 16S rRNA sequencing assay for the identification of *Nocardia* isolates. We reported costing in terms of percentage difference to the Vitek MS.
As with NTM cost determination, we excluded any capital, maintenance and staffing costs from the calculations, but included reagents and consumable costs. The level of staff required and relative hands-on time was discussed.

The identification of *Nocardia* for a single sample by the Vitek MS was 90.6% of the cost of 16S rRNA sequencing. Batches run for 16S rRNA sequencing includes other specimens which will

increase the number of samples per batch, using the same consumables and reagents which ultimately decrease costs for this assay.

As mentioned in Section 2.4.2, the Vitek MS kit is supplied for 100 tests and the stability once opened is only 4 weeks. Although the kit reagents are shared with NTM testing, there will be wastage of reagents due to the combined number of test requests for these assays. However, the Vitek MS requires less experienced staff than the sequencing assay which positively impacts the cost of the Vitek MS assay.

3.5 Conclusion

In conclusion, this study demonstrated that the Vitek MS has potential to replace 16S rRNA sequencing analysis for speciation of *Nocardia.* However, given the requirement to purchase a large Nocardia/Mycobacterium kit , with a limited shelf life, and the poor performance of the method for *Mycobacteria,* most of the kit would expire before possible use. The cost of the assay would therefore become prohibitive due to reagent wastage. Should the issues with the Vitek MS instrument be resolved and the suppliers provide smaller kit sizes, we recommend a larger scale study to fully evaluate the Vitek MS method for *Nocardia* identification in comparison to MLSA, which appears to be superior to 16S rRNA in our setting.

CHAPTER 4: THE IDENTIFICATION OF MOULDS

4.1 Introduction

The objective of this chapter was to evaluate the performance of the Vitek MALDI-TOF MS for the identification of moulds by comparing the results to traditional phenotypic identification and ITS Pan-Fungal sequencing methods.

4.2 Materials and methods

4.2.1 Sample selection

Moulds are frequently isolated in the clinical microbiology laboratory and we were therefore able to include a large selection and a variety of species during the sample collection period (January 2019 to July 2019). Consecutive sample isolates were included, regardless if previosuly isolated from the same patient.

A total of 156 mould isolates were included in the study. This included 140 clinical isolates with confirmed fungal infections by routine MCS at the PathCare Microbiology Reference Laboratory and the NHLS Tygerberg microbiology laboratory. The average age of the mould growth included in the study was between 4 to 5 weeks.

16 proficiency testing samples (NHLS Mycology Mould Survey) from 2017 to 2019 were also included in the study. These were stored in the original airtight vials at room temperature and were revived when ready to be processed.

4.2.2 Mycology – culture and microscopy

All samples were cultured onto Sabouraud Dextrose Agar with chloramphenicol (SDC) and Sabouraud Dextrose agar with cycloheximide (SABDRUGS) plates and incubated aerobically at 25°C – 30°C until sufficient growth was obtained. Reading and interpretation of the fungal culture plates was done on a weekly basis according to the PathCare SOP with the assistance of competent laboratory technologists. Positive cultures of fungal growth were routinely identified by making a wet preparation using the lactophenol cotton blue stain to identify the unique fungal features/structures and by consulting Microbiology reference books. The phenotypic characteristics noted included the size, colour and texture of the fungal colony, pigmentation and the reverse colour of the colony. Where NHLS Mycology Mould Survey samples were cultured, plates were checked for purity prior to analysis. Identification results obtained were compared to the expected results of the proficiency samples.

4.2.3 ITS Pan-Fungal sequencing

a) DNA extraction

DNA was extracted from selected mould cultures using the MagNA Pure Compact or eMag instruments as per manufacturer's specifications with modified pre-processing as per section 3.2.2 a).

b) Preparation of master mix

The reaction mix containing previously published primers (88) was prepared by adding the reagents to a labelled 0.6 ml tube. Table 4-1 contains the volumes and concentrations of reagents used. Sufficient volume of master mix was prepared according to the number of samples and controls (PC and NTC) to be tested as well as one additional sample to control for pipetting errors.

Table 4-1 Constituents of the Pan-Fungal sequencing master mix

Reagent	Volume per reaction (µl)
ITS1F primer (10 pmol/µl)	1.25
ITS2 primer (10 pmol/µl)	1.25
PCR grade dH20	2.5
KAPA 2G Robust Taq	10

15 µl of the reaction mix was added to a labelled 0.2 ml thin walled PCR tube or PCR plate for each sample and control. 5 µl of the template DNA and control DNA was added to the corresponding sample tubes/wells using a pipette and the tubes/wells were sealed. 5 µl of the positive control DNA was added to the positive control tube and was sealed. No additional PCR grade dH_20 water was added to the NTC tube.

c) Amplification

The ITS target region was amplified on the ABI ProFlex PCR System. Table 4-2 describe the details of the cycling parameters.

Table 4-2 Internal transcribed spacer Amplification Program – ABI ProFlex Polymerase chain reaction system

Temperature	Time	Cycles
94°C	2 min	1
94°C 62°C 72°C	30 sec 30 sec 30 sec	10
94°C 58°C 72°C	30 sec 30 sec 30 sec	30
72°C	2 min	1
4°C	∞ (Hold)	

Detection of amplification products, sequencing PCR reaction, DNA purification and automated detection of sequences was done as per section 3.2.2 (c) to (g).

Resultant electrophereograms were analysed using BioEdit software and compared to the curated online ISHAM-ITS (http://its.mycologylab.org/BioloMICSSequences.aspx?expandparm=f&file=ALL) and UNITE databases (https://unite.ut.ee/analysis.php) for species identification. Cut-off values of ≥97.0% homology for genus level only and ≥99.0% for species level identification were used.

4.2.4 Extraction protocol for MALDI-TOF MS

Numerous extraction protocols have been described for successful fungal protein extraction, however, 3 of the studies reviewed used a similar protocol (endorsed by bioMéreiux) that showed promise and this was therefore used in this study (60,89,90).

The inactivation step involved adding 900 µL of 70.0% ethanol to the provided 2 ml round bottomed tube after which the mould was transferred from the culture plate to the ethanol containing tube using a cotton swab dampened with sterile water. The tube was centrifuged (Hermle Microliter Centrifuge Z233 M-2) at 10 000 x g for 2 min to create a pellet after which all ethanol was removed from the tube. Extraction was achieved by adding 40 µl of 70.0% Formic acid and 40 µl of 100.0% Acetonitrile after which the tube was centrifuged for 2 minutes at 10 000 x g. The remainder of the protocol was followed as per section 2.2.3.

4.3 Study results and discussion

A total of 156 mould isolates were extracted and tested on the Vitek MS for identification. Table 4-3 contains details of the species isolated and evaluated against phenotypic/EQA identification (n=156) and ITS Pan- Fungal sequencing (n=26).

Table 4-3 Results of mould isolates on the Vitek Mass Spectrometry
Samples highlighted in blue represent correlation to the laboratory reference method.
Samples highlighted in green represent samples with "no identification".
Samples highlighted grey represents samples where the genus correlates with the reference method but differs with speciation
Samples highlighted in orange represent samples that did not correlate with the laboratory reference method.

Key	Fungal MCS	ITS Pan-Fungal sequencing[#]	Duplicate*	Vitek MS Identification (% Confidence level)
1	*Acremonium* spp.[2]	-	a-b	*A. sclerotigenum*
2†	*Alternaria* spp.	-	a-b	Bad spectrum
			c-d	*A. alternata***
3	*Aspergillus* spp.	-	a-b	*A. fumigatus*
4	*Aspergillus* spp.	*A. flavus.*	a-b	*A. flavus/oryzae*
5	*Aspergillus* spp.	*Aspergillus* spp.	a-b	No identification
			c-d	*A. fumigatus***
6	*Aspergillus* spp.	-	a-b	*A. fumigatus*
7	*Aspergillus* spp.	-	a-b	*A. fumigatus*
8	*Aspergillus* spp.	-	a-b	*A. fumigatus*
9	*Aspergillus* spp.	-	a-b	*A. fumigatus*
10	*Aspergillus* spp.	-	a	*A. flavus/oryzae***
			b	No identification
11	*Aspergillus* spp.	-	a-b	*A. fumigatus***
12	*Aspergillus* spp.	-	a	No identification
			b	*A. niger complex***
13	*A. fumigatus*	*A. fumigatus*	a-b	*A. fumigatus*
14	*A. fumigatus*	*A. fumigatus*	a-b	*A. fumigatus*
15†	*A. fumigatus*	-	a-b	*A. fumigatus*
16†	*A. flavus*	-	a-b	*A. flavus/oryzae*
17	*A. niger*	-	a-b	*A. niger*
18	*Bipolaris* spp.[1]	-	a	Bad spectrum
			b	*E. rostratum* ‡**
19†	*Curvularia* spp.[3]	-	a-c	Bad spectrum
			d	*Curvularia lunata***
20	*E. floccossum*	*E. floccossum*	a-b	*E. floccossum*
21	*Fusarium* spp.		a-b	*F. solani complex*
22	*Fusarium* spp.	-	a	*F. solani complex***

			b	*Bad spectrum*
23	*Fusarium* spp.	-	a-b	*F. solani complex*
24	*Fusarium* spp.	-	a-b	*F. solani complex*
25	*Fusarium* spp.	-	a-b	*F. solani complex*
26	*Geotrichum* spp.[4]	-	a	*G. candidum/klebahnii***
			b	No identification
27	*L. corymbifera*	*L. corymbifera*	a-b	*L. corymbifera*
28	*Mucor* spp.[4]	-	a-b	*M. circinelloides*
29	*Mucor* spp.[4]	*Mucor circelloides*	a-b	*M. circinelloides* (50.0%)**
				F. oxysporum complex (50.0%)
			c-d	Bad spectrum
30	*M. canis*	-	a-b	*M. canis*
31	*M. canis*	-	a-b	*M. canis*
32	*M. fulvum*	*M. fulvum*	a	No identification
			b	*M. fulvum***
33†	*Scedosporium* spp.[3]	-	a-b	*P. boydii* ‡
34	*Trichophyton* spp.	*T. rubrum*	a	No identification
			b	*T. rubrum***
35	*T. rubrum*	-	a	*C. ishiwadae* (22.7%)
				T. rubrum (25.7%)
				A. sydowii (25.7%)
				E. floccossum (25.7%)
			b	*T. rubrum***
36	*T. rubrum*	-	a-b	*T. rubrum*
37	*T. rubrum*	-	a-b	*T. rubrum*
38	*T. rubrum*	*T. rubrum*	a-b	*T. rubrum*
39	*T. rubrum*	-	a-b	*T. rubrum*
40	*T. rubrum*	-	a-b	*T. rubrum*
41	*T. rubrum*	-	a	No identification
			b	*T. rubrum***
42	*T. rubrum*	-	a-b	*T. rubrum*
43	*T. rubrum*	-	a-b	*T. rubrum*
44	*T. rubrum*	-	a-b	*T. rubrum*
45	*T. rubrum*	-	a-b	*T. rubrum*
46	*T. rubrum*	-	a-b	*T. rubrum*
47	*T. rubrum*	-	a	*T. rubrum***
			b	Too many peaks
48	*T. rubrum*	-	a-b	*T. rubrum*
49	*T. rubrum*	-	a-b	*T. rubrum*
50	*T. rubrum*	-	a-b	Bad spectrum
			c-d	*T. rubrum***
51	*T. rubrum*	-	a-b	*T. rubrum*
52	*T. rubrum*	-	a-b	Bad spectrum
			c-d	*T. rubrum***
53	*T. rubrum*	-	a	*T rubrum*

			b	*T violaceum*
			c-d	*T rubrum***
54	*T. rubrum*	-	a-b	*T. rubrum*
55	*T. violaceum*	-	a-b	*T. violaceum*
56	*T. violaceum*	-	a-b	*T. violaceum*
57	*T. violaceum*	-	a-b	*T. violaceum*
58†	*T. tonsurans*	-	a-b	*T. tonsurans*
59	*T. tonsurans*	*T. tonsurans*	a-b	*T. tonsurans*
60	*T. verrucosum*	*T. verrucosum*	a-b	*T. verrucosum*
61	*Acremonium* spp.[2]	-	a-b	No identification
62	*Acremonium* spp.[2]	-	a-b	No identification
63	*Acremonium* spp.[2]	-	a-b	No identification
64	*Acremonium* spp.[2]	-	a-b	No identification
65	*Acremonium* spp.[2]	-	a-b	No identification
66	*Acremonium* spp.[2]	-	a-b	No identification
67	*Acremonium* spp.[2]	*Fusarium* spp.	a-b	No identification
68	*Aspergillus* spp.	*A. versicolor*	a-b	No identification
			c	Too many peaks
			d	Background noise
69	*Aspergillus* spp.	*A. giganteus*[1]	a-d	No identification
70	*Aspergillus* spp.	-	a-b	No identification
71†	*A. clavatus* [1]	-	a-b	No identification
72	*Bipolaris* spp.[1]	-	a-b	No identification
73	*C. bertholletiae*[1]	*C. bertholletiae*[1]	a-b	No identification
74	*Drechslera* spp.[1]	-	a-b	No identification
75	*Fusarium* spp.	-	a	Bad spectrum
			b	No identification
76	*Fusarium* spp.	-	a-b	No identification
77	*Fusarium* spp.	-	a-b	No identification
78†	*Gliocladium* spp.[1]	-	a-b	No identification
79†	*Mucor* spp.[4]	-	a-b	No identification
80	*Penicillium* spp.	-	a-b	No identification
81	*Rhizopus* spp.	-	a-b	No identification
82	*Rhizopus* spp.	-	a	No identification
			b	Too many peaks
83	*Rhizopus* spp.[3]	Unidentified	a-b	No identification
84†	*S. racemosum*[1]	-	a-b	No identification
85	*T. rubrum*	-	a-b	No identification
86	*T. rubrum*	-	a-b	No identification
87	*T. rubrum*	-	a-b	No identification
88	*T. rubrum*	-	a-b	No identification
89	*T. rubrum*	-	a	No identification
			b	Bad spectrum
90	*T. rubrum*	-	a-b	No identification
91	*T. rubrum*	-	a	No identification

			b	Bad spectrum
92	*T. rubrum*	-	a-b	No identification
93	*T. rubrum*	-	a-b	Bad spectrum
			c-d	No identification
94	*T. rubrum*	-	a-d	No identification
95	*T. mentagrophytes*	-	a-b	No identification
96	*T. mentagrophytes*	-	a-b	No identification
97	*T. mentagrophytes*	-	a-b	No identification
98	*Aspergillus spp.*	-	a-b	Bad spectrum
99	*Aspergillus spp.*	-	a-b	Bad spectrum
100	*A. niger*	-	a-b	Bad spectrum
101	*Bipolaris* spp.[1]	-	a-b	Bad spectrum
102	*Bipolaris* spp.[1]	-	a-b	Bad spectrum
103	*Cladosporium* spp.[2]	-	a-b	Bad spectrum
104	*F. dimerum*	*F. dimerum*	a-b	Bad spectrum
105	*Geotrichum* spp.[4]	-	a-b	Bad spectrum
106	*M. canis*	-	a-b	Bad spectrum
107†	*M. canis*	-	a-d	Bad spectrum
108	*Mucor* spp.[4]	-	a-b	Bad spectrum
109	*T. rubrum*	-	a-b	Bad spectrum
110	*T. mentagrophytes*	-	a-b	Bad spectrum
111	*T. mentagrophytes*	-	a-b	Bad spectrum
112	*T. violaceum*	-	a-c	Bad spectrum
			d	Background noise
113†	*A. niger*	-	a-b	Bad spectrum
			c-d	*A. terreus complex*
114	*T. rubrum*	-	a-b	*T. interdigitale*
115	*T. rubrum*	-	a	*T. interdigitale*
			b	Bad spectrum
116	*T. rubrum*	-	a	*T. interdigitale*
			b	No identification
117	*T. rubrum*	-	a	*T violaceum*
			b	Too many peaks
118	*T. rubrum*	-	a	*T. interdigitale*
			b	Too many peaks
119	*T. rubrum*	-	a-b	*T. interdigitale*
120	*Trichophyton* spp.	*T. mentagrophytes*	a-b	*T. interdigitale*
121	*T. mentagrophytes*	-	a-b	*T. interdigitale*
122	*T. mentagrophytes*	-	a-b	*T. interdigitale*
123	*T. mentagrophytes*	-	a-b	*T. interdigitale*
124	*T. mentagrophytes*	-	a-b	*T. interdigitale*
125	*T. mentagrophytes*	-	a-b	*T. interdigitale*
126	*T. mentagrophytes*	-	a-b	*T. interdigitale*
127	*T. mentagrophytes*	-	a-b	*T. interdigitale*
128	*T. mentagrophytes*	-	a	No identification

			b	*T. interdigitale*
129	*T. mentagrophytes*	-	a-b	*T. interdigitale*
130	*T. mentagrophytes*	-	a, c	No identification
			b	*T. erinacei*
			d	*A. benhamiae*
131	*T. mentagrophytes*	-	a-b	*T. interdigitale*
132	*T. mentagrophytes*	-	a-b	*T. interdigitale*
133	*T. mentagrophytes*	-	a-b	*T. interdigitale*
134	*T. mentagrophytes*	-	a-b	*T. interdigitale*
135	*T. mentagrophytes*	-	a-b	*T. interdigitale*
136	*T. mentagrophytes*	-	a-b	*T. interdigitale*
137	*Acremonium* spp.[2]	-	a-b	*P. lilacinum*
138	*Acremonium* spp.[2]	-	a	*T. violaceum*
			b	*T. rubrum*
139	*Acremonium* spp.[2]	-	a	*F. oxysporum complex*
			b	Bad spectrum
140	*Acremonium* spp.[2]	-	a-b	*F. oxysporum complex*
141	*Acremonium* spp.[2]	-	a-b	*F. oxysporum complex*
142	*Aspergillus* spp.	-	a	No identification
			b	*S. apiospermum*
143†	*Aureobasidium* spp.[1]		a-d	*A. flavus/oryzae*
144	*Curvularia* spp.[3]	*C. lunata*	a-d	*A. fumigatus*
145	*E. floccossum*	-	a	*C. albicans* (49.8%)
				P. vermiculatum (50.1%)
			b	*P. vermiculatum* (99.9%)
146†	*E. floccosum*	-	a-b	Bad spectrum
			c-d	*A. flavus/oryzae*
147	*F. oxysporum*	*F. oxysporum*	a-b	No identification
			c-d	*A. fumigatus*
148	*M. audouinii*	*M. audouinii*	a-d	*A. fumigatus*
149†	*M. auddouinii*	-	a-b	*A. fumigatus*
			c-d	
150	*Penicillium* spp.	*P. crysogenum*	a-b	No identification
			c-d	*A. fumigatus*
151	*S. prolificans*	*S. prolificans*	a,c	No identification
			b	*F. solani complex*
			d	Bad spectrum
152	*Trichoderma* spp.	*T. melanomagnum*[1]	a-d	*A. fumigatus*
153	*T. rubrum*	-	a	Bad spectrum
			b	*P. lilacinum*
154	*T. rubrum*	-	a-b	No identification
			c-d	*A. flavus/oryzae*
155	*T. rubrum*	*P. lilacinum*	a	*P. lilacinum* (99.9%)
			b	*F. oxysporum complex* (20.4%)
				P. lilacinum (20.9%)

				T. interdigitale (20.9%)
				A. fumigatus (20.6%)
			c-d	*P. lilacinum* (99.9%)
156	*T. verrucosum*	-	a-d	*A. flavus/oryzae*

* a-b duplicate spots from initial run; c-d duplicate spots from repeat run

**Consensus correlates (best result of a repeat / duplicate spot was considered correct)

† Proficiency Testing Samples

‡Teleomorph/synonym identified, correlated to genus level (samples 18 & 33)

#identification results obtained from ISHAM or UNITE database up to species level.

- Test not done

[1] Isolates not represented in the Vitek MS KB

[2] Only 1 species in the Vitek MS KB

[3] Represented by 2 species in the Vitek MS KB

[4] Represented by 3 species in the Vitek MS KB

Of the 156 isolates tested, 38.5% (60/156, $CI_{95\%}$: 30.8% - 46.1%) correlated with the Vitek MS (samples 1-60). Of the 156 isolates, 65.4% (102/156) of isolates were identified by phenotypic identification and/or ITS Pan-Fungal sequencing to the species level, of which 38.2% (39/102) correleated with the Vitek MS (samples 4,13-17, 20, 27, 29-32, 34-60). In addition, the Vitek MS was able to identify 21 isolates up to species level where phenotypic and ITS Pan-Fungal identification failed to do so (samples 1-3, 5-12, 18-19, 21-26, 28, 33). A limitation of the study was that the reference methods did not identify all organisms to the species level, but only to the genus level. Where organisms identified by the reference methods to the genus level correspondedto the Vitek MS (bioMérieux, France) genus level, it was considered a 100.0% correlation regardless of additional species identification by the Vitek MS (bioMérieux, France). In this study, the Vitek MS (bioMérieux, France) was able to provide more species identifications which highlights the fact that fungal MCS is an imperfect gold standard.

Of the 156 isolates, 33.3% (52/156, $CI_{95\%}$: 25.9% - 40.7%) resulted in a “no identification” with 71.2% (37/52) as good quality spectrums and 28.8% (15/52) as bad spectrums, too many peaks or background noise (samples 61 -112). Twelfth (12/156, 7.7%) isolates were not represented in the Vitek MS database. Seven (7/12, 58.3%) of these resulted in a “no identification” result (good spectrum) which is expected when the organism is not in the database and can be considered a possible correlation, although it contributed to the high “no identification” percentage. 28.2% (44/156, $CI_{95\%}$: 21.1% - 35.3%) isolates did not correlate with the phenotypic and/or ITS Pan-Fungal sequence identification (samples 113-156). 54.5% (24/44, $CI_{95\%}$: 39.8% - 69.3%) of these correlated to genus level only with the laboratory reference method (samples 113 – 136). Only 2 of these was not represented in the database, namely *Aureobasidium* spp. and *T. melanomagnum*.

All single identifications were reported by the Vitek MS with a high confidence level of >90.0%, except for one *T. interdigitale* (63.6%) but this is still regarded as a high confidence level (>60.0%) for species level identification. Split identifications (where the Vitek MS was unable to propose only 1 result) were obtained for 4 isolates (samples 29, 35, 145, 155). Two of these samples which were phenotypically identified (samples 29 & 155) were resolved with ITS Pan-Fungal sequencing as *M. circinelloides* (sample 29) and *P. lilacinum* (sample 155).

The 38.5% accurate identification obtained is much lower than what was claimed by the manufacturer which achieved an 92.7% total correct identification. Where they report incorrect identifications (0.9%) and 6.4% “no identifications”, our values were higher (28.2% and 33.3%, respectively). Our study results were statistically different to the manufacturer’s results for correct identifications (*P* = <0.001, 92.7% [bioMérieux] vs 38.5% [our study]), “no identifications” (*P* = <0.001, 6.4% [bioMérieux]vs 33.3% [our study]) and the number of incorrect identifications (*P* = <0.001, 0.9% [bioMérieux] vs 28.2% [our study]) obtained.

Rychert et al. also reported increased accurate identifications to species level (91.0%) and an addditional 2.0% at genus level. They achieved 6.0% “no identifications” and 1.0 % incorrect identifications. Staff members involved in their study received training after which they were required to undergo a competency test prior to processing study samples (90). This highlights the complexity of processing mould isolates for identification by MALDI-TOF MS and the requirement for an extensive training protocol and standardisation in performing the assay within in the laboratory. In contrast to the study results achieved by bioMérieux and Rychert et al., a study published by Mcmullen et al., achieved poorer results. 76.8% of their mould isolates were correctly identified with 0.9% incorrect identification. In their study 22.3% remained unidentified of which the majority of those molds (97.0%) were not represented in the database (60).

In total 26 (26/156) isolates were repeated due to incorrect or “no identification” results (samples 2, 5, 19, 29, 50, 52-53, 68-69, 93-94, 112-113, 130, 143-144, 146-152, 154-156). Six (6/26, 23.1%) samples were resolved on repeat analysis (samples 2, 5, 19, 51, 53, 54). This highlights the issues encountered with the reliability and repeatability of the Vitek MS instrument evident in the *Nocardia* processing (section 3.3).

The majority of moulds tested belonged to the dermatophyte group (n=81) which comprised of the following genera: *Microsporum* (n=7), *Epidermophyton* (n=3) and *Trichophyton* (71).

Of the 71 *Trichophyton* spp. tested 27 (27/71, 38.0%, $CI_{95\%}$: 26.7% - 49.3%) correlated with the Vitek MS (samples 34-60). 17 (17/71, 23.9%, $CI_{95\%}$: 14.0% - 33.9%) isolates resulted in a “no identification” with 13 (13/17, 76.5%) as good quality spectrums (samples 85-97) and 4 (4/17, 23.5%) as bad

spectrums or background noise (samples 109-112). Twenty-seven (27/71,38.0%, $CI_{95\%}$: 26.7% - 49.3%) phenotypically identified isolates did not correlate with the Vitek MS (samples 114-136, 153-156). The Vitek MS reported a split identification on 1 of these isolates (sample 155) and was resolved with ITS-Pan-Fungal sequencing which correlated with the Vitek MS.

The high percentage (23.9%) of "no identification" results obtained in the *Trichophyton* group results were mostly due to good quality spectrums (76.5%) of which 76.9% (10/13) were identified as *T. rubrum* by phenotypic identification (samples 85-94). Phenotypic identification is very subjective and requires expertise and it can be hypothesised that the organisms may not be represented in the database yet and that the phenotypic identification was incorrect to species level. More likely, the high percentage of "no identification" (good spectrum) were due to the suboptimal performance of the instrument as encountered with NTM processing (section 2.3).

Twenty-three (23/71, 32.4%, $CI_{95\%}$: 21.5% - 43.3%) *Trichophyton* isolates did not corelate to species level with the Vitek MS (samples 114-136) of which 17 (17/23, 73.9%) were *T. mentagrophytes,* the remaining 6 (6/23, 26.1%) were *T. rubrum*, see Table 4-4.

Table 4-4 Discordant Trichophyton spp. identified on Vitek Mass Spectrometry (species level)

Phenotypic identification	n	Vitek MS identification
T. mentagrophytes	16	*T. interdigitale*
	1	*T. erinacei*
T. rubrum	5	*T. interdigitale*
	1	*T. violaceum*

This failure of accurate species identification for the *Trichophyton* isolates may be due to the possibility of cross-identification as indicated by the manufacturer, see Table 4-5.

Table 4-5 Vitek Mass Spectrometry possible cross-identification between Trichophyton *displayed taxa applicable to this study*

Displayed Taxon (Reported by Vitek MS)	Possibility of :
T. interdigitale	*T. mentagrophytes* *T. tonsurans* *T. verrucosum*
T. rubrum	*T. violaceum*
T. erinacei	*T. verrucosum*

Most of the cross-identifications were displayed as *T. interdigitale.* As per manufacturer, 27 out of the 30 (90.0%) *Trichophyton mentagrophytes* tested by them were cross-identified as *T. interdigitale*. This was also reported by Sanguinetti et al. where the mass spectral profiles of *T. mentagrophytes* and *T. interdigitale* were almost indistinguishable from each other (59). Strains in the *T. mentagrophytes* complex, of which *T. interdigitale* is a member of, are difficult to distinguish with the traditional identification techniques as they are very closely related (53,59). It cannot therefore be excluded that the *T. mentagrophytes* identified by phenotypic means, was indeed not accurately identified. *T. violaceum* can be cross-identified on the Vitek MS as a *T. rubrum* as was the case in our study for sample 117. In our study 5 *T. rubrum* isolates (samples 114-116, 118-119) were identified as *T. interdigitale*, and 1 T. mentagrophytes as T. erinacei (sample 130) but this cross-identification was not indicated as a possibility by the manufacturer.The cross-identifications in the *Trichophyton* group should not have a direct impact on patient management as the treatment of onchomycosis caused by these organisms is independent of the causative agents in respect to dermatophyte, yeasts and moulds (91).

The second largest mould group tested was the *Aspergillus* group which comprised of 24 isolates (samples 3-17, 68-71, 98-100, 113, 142). This group of moulds showed a better performance in comparison to the dermatophyte group with 15 (15/24, 62.5%, $CI_{95\%}$: 43.1% - 81.9%) isolates that correlated with the Vitek MS (samples 3 -17). In addition, the Vitek MS was able to identify 9 isolates up to species level where phenotypic identification and/or ITS Pan-Fungal identification failed to do so (samples 3, 5-12). Seven (7/24, 29.2%, $CI_{95\%}$: 11.0% - 47.4%) isolates resulted in a “no identification” with 4 (4/7, 57.1%) as good quality spectrums (samples 68-71) and 3 (3/7, 42.9%) as bad spectrums. (samples 98-100). *A. clavatus* was not represented in the database but as the Vitek MS generated a “no identification” (good spectrum) result which can be viewed as correct. Two (2/24, 8.3%, $CI_{95\%}$: 0.1% - 19.4%) phenotypically identified isolate (sample 142) did not correlate with the Vitek MS result of *S. apiospermum*.

Of a total of 28.2% (44/156, $CI_{95\%}$: 21.1% - 35.3%) for the whole dataset that did not correlate with the Vitek MS, 22.7% (10/44) were identified as a member of the *Aspergillus* group with *A. flavus / oryzae* and *A. fumigatus* identified as the main pathogens (samples 143-144, 146-150, 152, 154, 156). The majority of *Aspergillus* identifications that did not correlate with the Vitek MS were identified from isolates retrieved from storage either from proficiency testing samples or stored Tygerberg NHLS samples. At the time when these samples were plated out for processing on the Vitek MS, they were considered pure cultures. These isolates were incubated untill good growth were obtained which were between 1 to 2 weeks before analysis which is less than the average of 4 weeks obtained for clinical samples. There is the possibility that the storage conditions prior to retrieving as well as the shorter incubation period may have had an influence on the protein profile

of the organisms which were less mature than if grown for 4 weeks . The Vitek MS database has been constructed including the different protein profiles of the organisms as incubation time progress (90), but as *Aspergillus* is a known laboratory contaminant, it is more likely the cause of the discordant results.According to the manufacturer there is a possibility of cross-identification between the Vitek MS displayed taxon and unclaimed taxon. The only one applicable to the *Aspergillus* group was *A. nominus* (unclaimed taxon) which can be reported as *A. flavus / oryzae.*

The performance of the assay for the remaining dermatophyte genera (*Microsporum* and *Epidermophyton*) was also not optimal. 40.0% (4/10) were correctly identified to the genus and species level (samples 20, 30-32). Two of the 3 (2/3, 66.7%) *E. floccosum* and both (2/2, 100%) *M. audouinii* isolates were incorrectly identified by the Vitek MS (145-146, 148-149) and 2 (2/4, 50%) of the *M. canis* isolates could also not be identified due to bad quality spectrums (samples 106-107).

Several factors can influence the protein spectrum of organisms, including agar contamination, maturation stage of selected colonies and the presence or absence of conidia or melanin in some moulds which may affect ionisation (58). The majority of the previously stored samples (EQA and NHLS samples) were sub-cultured again and processed on a specific Vitek MS, which required a hardware change during the study (linear detector), however, there was no improvement in the results.

Three *Acremonium* spp. identified phenotypically by PathCare Microbiology department were identified as *F. oxysporum* complex by the Vitek MS. *Fusarium* spp. is one of the most challenging genera to identify via traditional macroscopy and microscopy examinations due to its high phenotypic variability and the division between sexual and asexual stage taxonomy. It is commonly identified as *Acremonium* spp. as they do look very similar microscopically, therefore, there is a strong possibility that the *Acremonium* spp. identified by the laboratory was indeed *Fusarium* spp. which correlated with the Vitek MS (https://mycology.adelaide.edu.au/descriptions/hyphomycetes/acremonium/; https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/acremonium). This hypothesis was strengthened by ITS Pan-Fungal sequencing of one of the phenotypically identified *Acremoniom* spp, which confirmed that the isolate was *Fusarium* spp (sample 67).

Conidial melanin pigment present in highly pigmented moulds such as *A. niger* and *Fusarium* spp. inhibit ionisation and could have contributed to the lower identification rates obtained by these organisms (58). Five of the 10 (5/10, 50.0%) *Fusarium* spp. tested, were identified by the Vitek MS to genus level and only 1 of 3 (1/3, 33.3%) *A. niger* isolates tested was identified to specie levels (samples 17, 21-25, 75-77, 100, 104, 113, 147).

A major limitation of this study was the suboptimal performance of the instruments as previously discussed (section 2.3). Several slides were reshooted on another available Vitek MS in the Microbiology department as well as on a Vitek MS in another pathology group to compare results, but no improvements were seen. Another major limitation of this study is that not all phenotypic identification results could be subjected to ITS Pan-Fungal sequencing due to high cost implications and phenotypic identification of moulds is an imperfect reference standard.

For many decades direct microscopy and fungal culture from clinical samples has been the gold standard in the identification of fungal species but requires highly skilled and trained staff usually only found in reference laboratories (50). Molecular methods (e.g. sequencing) have been used successfully but the methods are expensive, require specialised equipment and expert staff and is usually only available in reference laboratories (59). The application of MALDI-TOF MS for the identification of moulds can result in the loss of skills and expertise to visually identify fungi microscopically and macroscopically due to the simplicity of the extraction and identification procedure on the Vitek MS (24), although the advantage is that it can be employed in peripheral laboratories where there is a lack of expertise.

4.4 General workflow and cost Analysis

General workflow and cost determinations were done as per section 2.4.

4.4.1 Moulds workflow (hand-on / hands-off) determination

The average turnaround time for identification of moulds by the PathCare Microbiology laboratory by routine phenotypic methods was calculated for the applicable dataset from the time the original sample was received in the laboratory, until the result was entered into the Laboratory Information System (LIS) which was on average 3 weeks and 1 day. The disadvantage of phenotypic and Vitek MS identification of moulds is that both require growth on solid media prior to processing, resulting in the same turnaround time for the Vitek MS as for phenotypic identification. Good growth of approximately 4 weeks is required prior to processing on the Vitek MS. ITS Pan-Fungal sequencing can be performed directly from clinical samples and is routinely run in batches twice a week. The average turnaround time in our laboratory for ITS Pan-Fungal sequencing from clinical samples is therefore 7 days.

The workflow on the Vitek MS for the identification of moulds was further examined by calculating the amount of time needed for hands-on and hands-off procedures for the method.
Table 4-6 contains details of the time allocated to hand-on and hands-off procedures for the Vitek MS when processing mould isolates up to 6 isolates a batch. The total time it will take to process 6

isolates is just over 2 hours, but only 20 minutes is allocated to hands-on procedures. The remaining 01:46 is time allocated to hands-off procedures.

Table 4-6 Workflow of processing mould isolates on Vitek Mass Spectrometry (hh:mm) for a batch of 1 to 6 isolates

Procedure	1	2	3	4	5	6
Hands-on	00:03	00:06	00:10	00:13	00:17	00:20
Hands-off	01:29	01:32	01:36	01:39	01:43	01:46
Total	01:32	01:39	01:46	01:53	02:00	02:07

The timespan to identify a single mould isolate on the Vitek MS was estimated as 1 hours and 32 minutes of which 3 minutes was allocated to hands-on procedures.

The phenotypic identification by lactophenol cotton blue stain took far less time than the Vitek MS for a single mould isolate to generate a result. Although the total timespan of mould identification on the Vitek MS is far more to generate a final result, the hand-on procedures might be the same as for the phenotypic identification depending on the experience and expertise of the technologist. The reporting of the result to the client will not be delayed by days but rather hours if the Vitek MS is used.

Smaller laboratories can benefit by implementing MALDI-TOF MS for mould identification as the isolates can be processed in-house and not be referred to a reference laboratory which may have a positive impact on the turnaround time for mould identification in a peripheral laboratory.

4.4.2 Moulds cost determination

The focus of the crude cost analysis is to determine what the Vitek MS assay will cost in comparison to ITS Pan-Fungal sequencing assay and traditional phenotypic identification of mould isolates.

We reported costing in terms of percentage difference to the Vitek MS.
As with NTM cost determination, we excluded any capital, maintenance and staffing costs from the calculations, but included reagents and consumable costs. The level of staff required and relative hands-on time was discussed.

The identification of a single mould by the Vitek MS is 22.0% of the cost of ITS Pan-Fungal sequencing, which is the most expensive method. Phenotypic identification is only 0.3% of the cost of sequencing and 1.3% of the Vitek MS identification.
Cost analysis done by Dr Barker from ARUP laboratories also found that the implementation of MALDI-TOF MS for mould identifications did not benefit the reference laboratory where highly skilled

personnel are employed (74). Although Vitek MS identification is considerably more cost effective than ITS Pan-Fungal sequencing the opposite is true when compared to phenotypic identification which is the primary identification method used in our setting. In contrast to the NTM and *Nocardia* testing, there were minimal wastage of reagents as sample volume was much higher than the other two groups of organisms tested and reagents were used optimally before the reagent open vial stability expired.

4.5 Conclusion

In conclusion, this study demonstrated that the Vitek MS is not a suitable replacement for phenotypic identification in our setting due to the high failure rate and higher cost of the method. However, should issues with the instrumentation be overcome, there is the potential to revisit implementation of the assay should their be a shortage of staff experienced in phenotypic speciation methods. Furthermore, ITS Pan-Fungal sequencing is more expensive than the Vitek MS and routine microscopy, it may have benefits where a reduced turn around time is essential or where there is culture negative disease as it can be performed directly from the clinical sample.

CHAPTER 5: GENERAL CONCLUSION

Several studies have been conducted on the use of MALDI-TOF MS for the identification of NTM, *Nocardia* spp. and moulds but the majority of studies concentrated on the Bruker technology and the construction of laboratory-developed databases to supplement the commercial database available from the manufacturer. These laboratory-developed databases improved the performance of the MALDI-TOF assay when used in conjunction with the commercial database available from the manufacturer. A disadvantage of this is that these databases are laboratory specific and not available to use by other laboratories. This is in contrast to sequence alignment databases for bacteria and fungi such as Genbank, ISHAM and SINA.

In our setting, the results obtained for the identification of NTM, *Nocardia* and moulds on the Vitek MS v3.2 KB did not match the performance characteristics claimed by the manufacturer or previously published studies. Although certain issues were highlighted during our study which included inferior quality spots and incorrect handling of the calibration *E.coli* strain, we were unable to resolve the problem. The high rate of “no identifications” experienced in our study were not due to organisms not being in the database, but due to suspected software and/or hardware issues on an instrument which requires regular intervention and fine tuning. A high throughput laboratory requires a number of back up systems in the event of instrument problems or failures. We currently have 2 Vitek MS instruments, however both delivered suboptimal results.

We therefore recommend that the Mycobacterium CM/AS assays remain the methods of choice in our laboratory setting. The *Nocardia* identification on the Vitek MS showed potential with a higher identification rate but is not cost effective due to the low isolation frequency in our setting. The performance of MLSA was superior to 16S rRNA sequencing and this may show potential for implementation, but is costly. We therefore suggest investigating an alternative single gene target for *Nocardia* speciation following culture which may be more cost effective. The poor performance in our setting for mould identification (most likely due to instrument issues) by the Vitek MS suggest that phenotypic identification remains the most promising speciation technique, with ITS Pan-Fungal sequencing as an alternative for culture negative samples, or where turn-around time is critical.

A major limitation of the study is the continued issues experienced with the Vitek MS instrument and continuous trouble-shooting of the “no identification” results. Following testing by the manufacturer, we suspect that there is a calibration or software/hardware issue which could not be resolved. We are concerned that the fragility of the instrument may be a hinderance to the use of the method in our high throughput environment. As the first such study conducted in South Africa, the Vitek MS database was challenged with locally circulating strains as opposed to those prevalent in the manufacturer’s research and development sites. This may have lead to a the decreased sensitivity of the assay in our setting.

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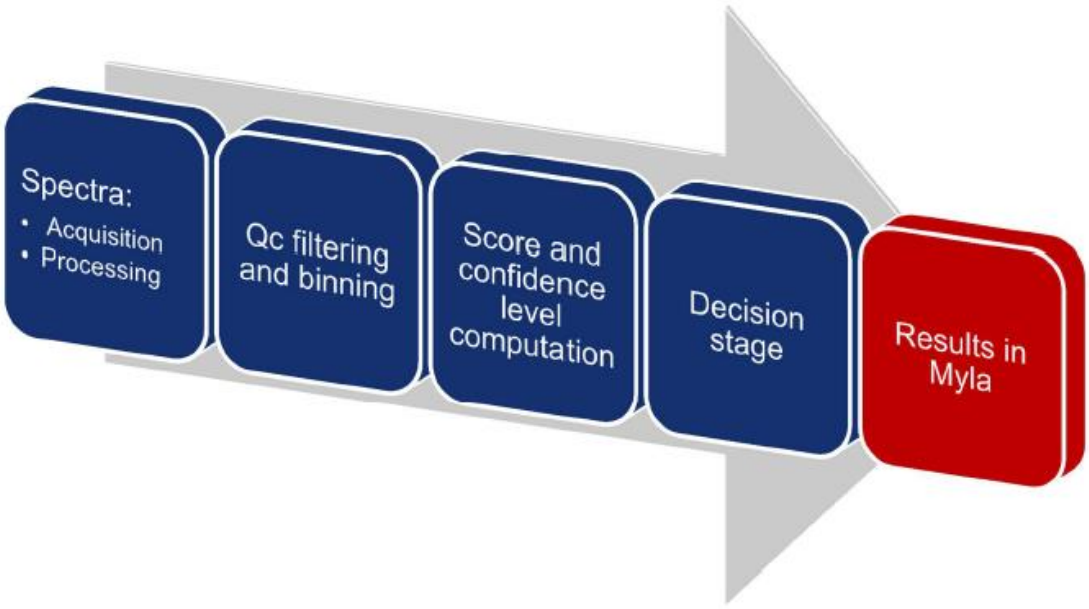
APPENDIX

Appendix A: Vitek MS Technology

The Vitek MS uses a 5-step identification algorithm to obtain an identification result (92).



Step 1 - Spectra (Acquisition)

The acquisition station software and sample programming will not be discussed in detail but relevant information will be included to demonstrate how results are displayed while acquisition is in progress and how to interpret those results from the software screen display.

The software displays four target slides with sample and calibration spots on the screen before slides are loaded and scanned for acquisition. Once the slides have been loaded onto the instrument, the instrument will prepare itself for the acquisition and display the programmed sample and calibration spots in blue as illustrated in Figure A-1(93).

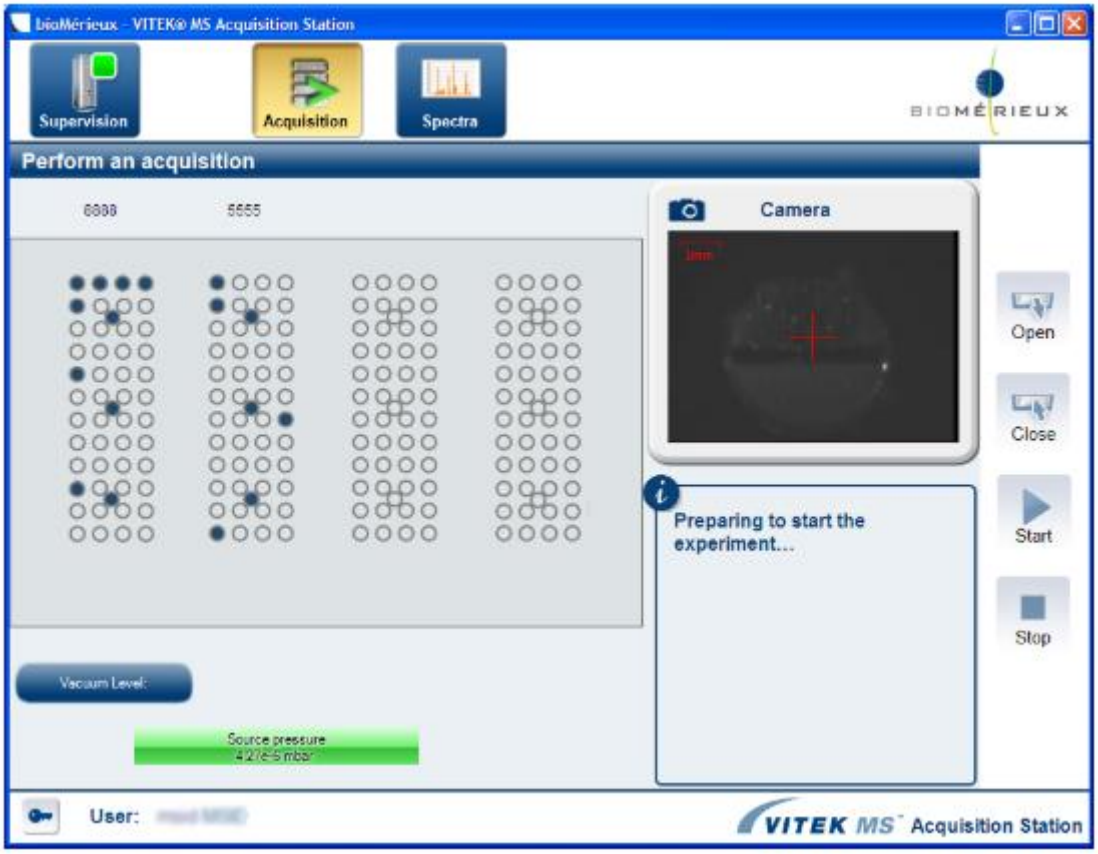


Figure A-1 VITEK Mass Spectrometry acquisition station screen displaying programmed sample spots (93)

The instrument will automatically initiate acquisition once the operating pressure has been reached and first starts with the calibration spot in an acquisition group. The calibration needs to pass before the first sample spot will be analysed. Once all the sample spots in an acquisition group have been analysed, the calibration spot will be tested again as an internal check before proceeding to the second acquisition group.

When acquisition is taking place, the instrument will fire the laser onto the inoculated spot on the target slide. The instrument will fire on set points on the target slide, which are called raster points. The laser will hit each raster point 5 times creating 1 profile. The target is to obtain 100 good profiles, but a minimum of 30 good profiles is still acceptable and can result in an identification. Each profile must pass specific quality control criteria. If acceptable protein profiles are not obtained during the firing of a specific raster point, the instrument will move on to the next raster point until 100 good profiles have been obtained or alternatively all raster points have been exhausted. If a spot has failed identification (e.g. sample spot bad acquisition) then it implies that either a) all raster points have been exhausted, b) there was either not enough peak data or c) when the peak data was analysed the criteria for a “good spot” was not met. If the peak criteria are not met, a spot will not pass for identification. We can refer to this as an internal QC check (93)(Personal communication (bioMérieux).

The software displays the particular spectrum and the number of profiles that passes while it is in the process of firing the laser onto the slide (93).

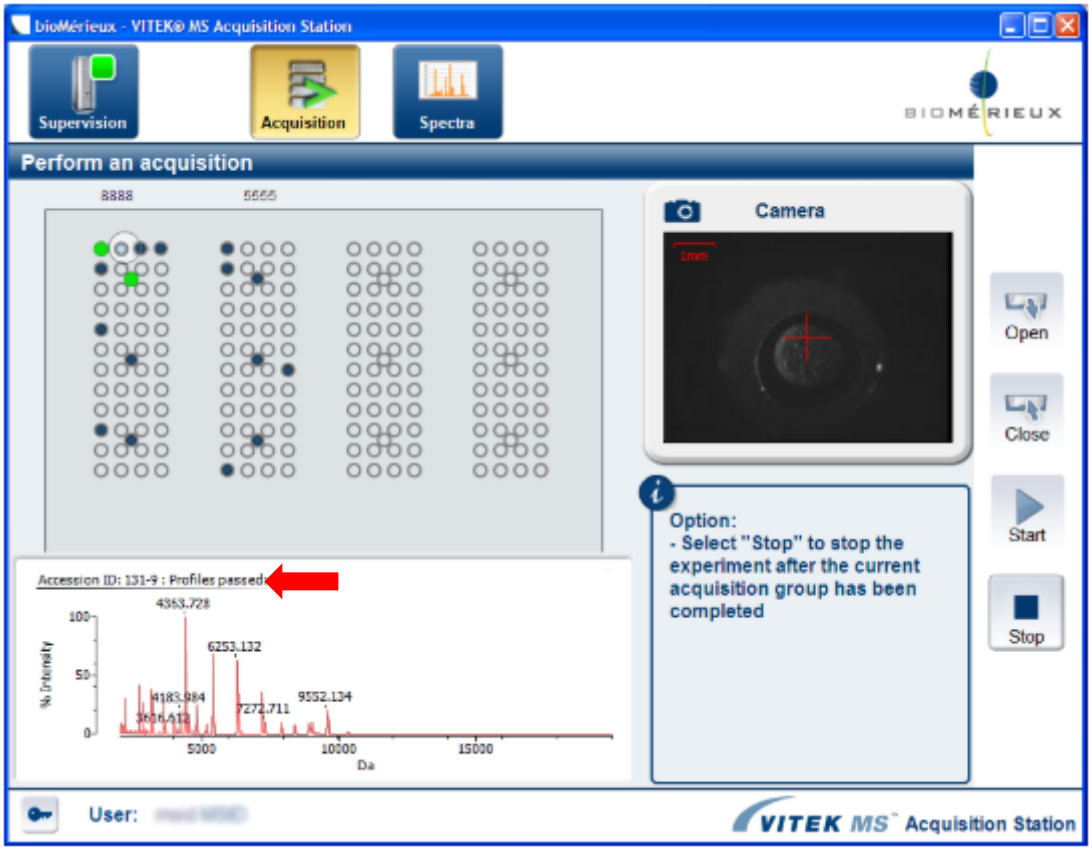


Figure A-2 VITEK Mass Spectrometry acquisition station screen (93)

An instrument will determine the average of all the spectra collected for the sample (raw spectrum), removing the background noise and smoothing results in an averaged processed spectrum. At this stage, peak detection takes place and lists of peaks are created according to the mass and intensity of the peaks obtained. Figure A-3 is a schematic presentation of this process which can be viewed in the spectra feature on the instrument's acquisition station.

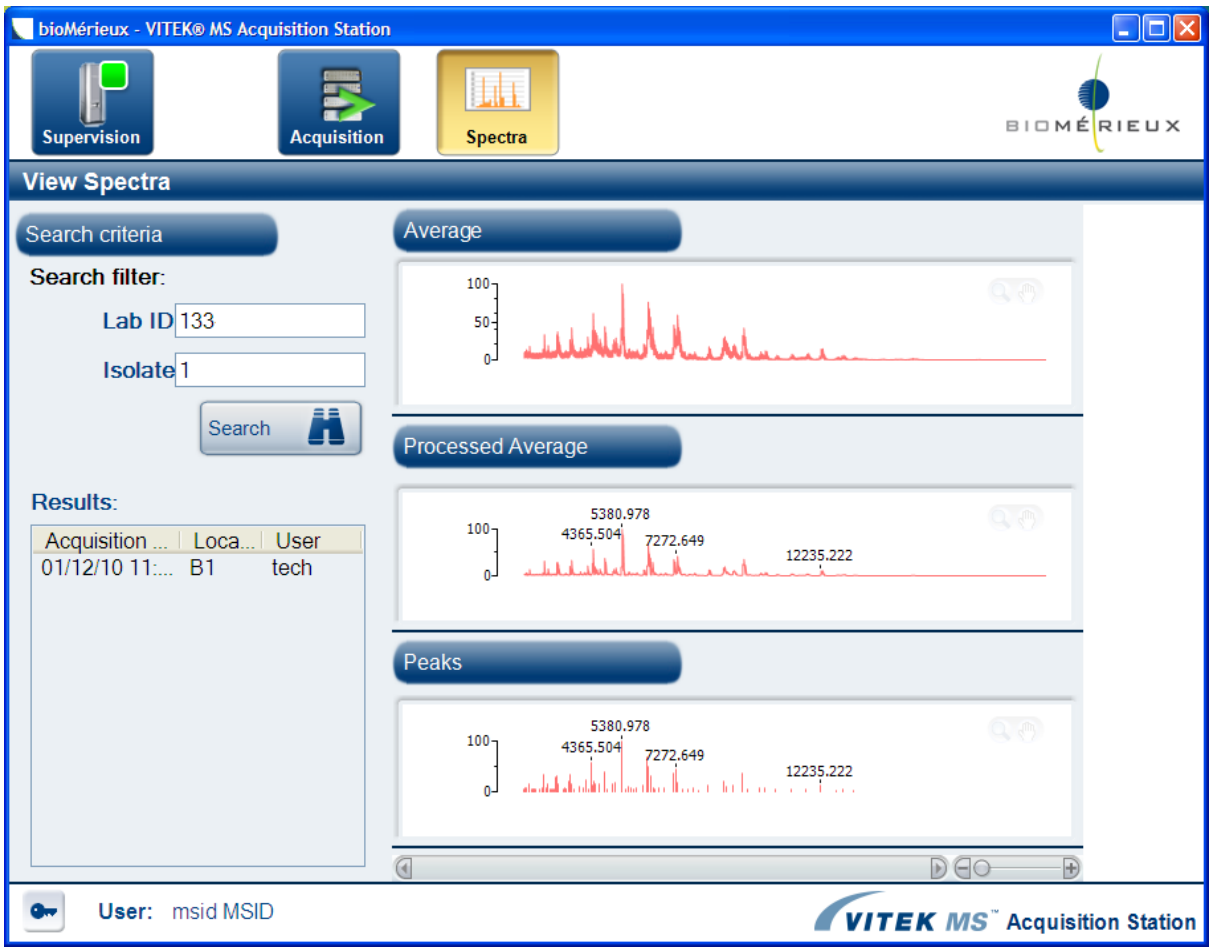


Figure A-3 Spectra obtained and processed during the acquisition of a sample (94)

There are various spot colours that can be displayed depending on the status of the spot's acquisition as seen in Table A-1 and Figure A-2 (93,94).

Table A-1 displaying various colour spots as displayed on acquisition screen (93)

Graphic presentation	Spot Colour	Description
	Grey	Spot with no sample.
	Dark grey	Spot acquired during previous acquisition.
	Dark Blue	Spot waiting for acquisition.
	Light blue	Spot where spectrum is being acquired.
	Green with green border	Spot where spectrum has been acquired and passed quality checks. Submitted to the Analysis Server/ MYLA®.

	Green with red border	Spot where spectrum has been acquired and passed quality checks. Not submitted to the Analysis Server/ MYLA® (communication error). Submission to the Analysis Server/MYLA® will be automatically retried.
	Red with green border	Spot where spectrum has been acquired and failed quality checks. Resulting spectrum peak list is however submitted to the Analysis Server/MYLA® which may or may not provide an identification.
	Red with red border	Spot where spectrum has been acquired and failed quality checks. Not submitted to the Analysis Server/ MYLA® (communication error). Submission to the Analysis Server/MYLA® will be automatically retried.
	Light yellow	Spot selected for re-acquisition

Step 2 – QC filtering and binning

Bin matrixing is a methodology that is proprietary to bioMérieux and the Vitek. The simplest way to describe this is by looking at two genetically similar organisms: coagulase-negative *Staphylococcus* and *S. aureus*. The peaks generated by these two organisms are sufficiently similar for the Vitek MS to call it a *Staphylococcus* but small variations that exists between the two species allows for the accurate differentiation at species level (83). Bin matrixing involves the instrument evaluating each peak of the spectrum to determine if its presence or absence is related to species identification. The pre-processed spectrum is divided into 1300 predefined intervals and these are called the “bins”. Only the peak with the highest intensity is retained in each bin, and the other peaks are discarded. This algorithm transforms the list of peaks of variable intensity into a list of bins with the corresponding intensity. Each peak is then weighted based on its specificity at the genus level and species level. This reduction in data allows memory gain and a more rapid calculation time, but the disadvantage is that it prevents the creation of in-house laboratory developed databases (95).

It is also at this stage that QC filtering is taking place which must pass to enable the deliverance of an identification result. Figure A-4 is a simplified example of a binned peak list.

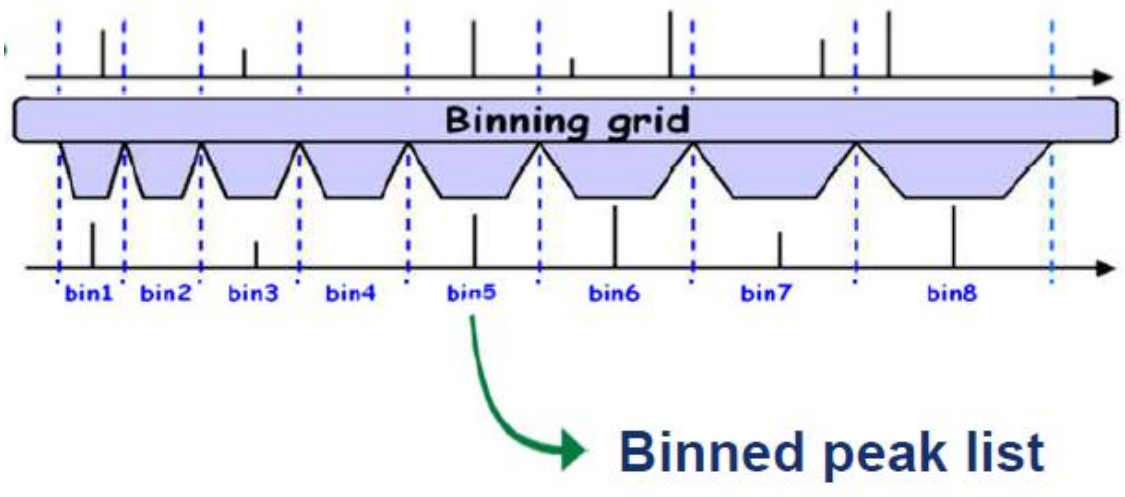
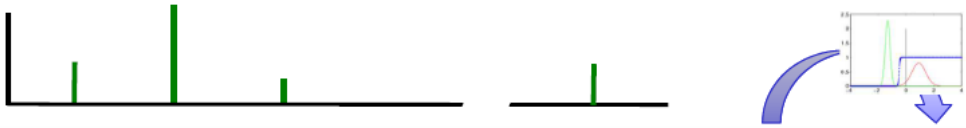


Figure A-4 Binned peak list (bioMérieux)

Step 3 – Score and confidence level computation

The binned peak list is sent to the MS-ID server, Myla, where the sample binned peak list is compared to the selected database classes. The MS-ID server calculates 756 scores and probabilities for the bacterial protocol or 157 scores and probabilities for the Fungal protocol. Figure A-5 is an example of score and confidence level computation (92).



	Bin 1	Bin 2	Bin 3	Bin 4	…	Bin 1300	Score	Probability
Citrobacter freundii	0,134	- 2,561	1,886	- 2,612	…	0,157	1,812	99,90%
⋮	⋮	⋮	⋮	⋮	…	⋮	⋮	⋮
Morganella morgani	- 0,888	- 3,246	1,116	0,751	…	- 2,564	1,432	65%
⋮	⋮	⋮	⋮	⋮	…	⋮	⋮	⋮
Proteus mirabilis	- 0,826	- 2,272	0,022	- 0,16	…	1,854	1,064	75%
⋮	⋮	⋮	⋮	⋮	…	⋮	⋮	⋮

Figure A-5 Score and confidence level computation (bioMérieux)

Step 4 – Decision stage

Zero to 4 classes will be retained dependent on 5 parameters (92):

- Score thresholds: > -0.4
- Score tolerance*: ± 0.5
- Probability thresholds: >60.0%
- Probability tolerance*: 0.3 / 30.0%
- Maximum number of low discriminations: 4

*eliminates classes with lower score or probability as it is too far from the best.

Step 5 – Results in Myla

Identification results generated by the Vitek MS may fall within one of three categories (93):

- A single high confidence identification
- A split identification
- No identification

The Vitek MS reports a confidence level along with the results. A single high confidence identification is one significant organism or organism group identified with a confidence value between 60.0%-99.9 (%). A split identification will result in low discrimination and happens when no more than four organisms are identified as possibilities in which the sum of the confidence levels will be equal to 100. No identification results may be due to more than four organisms or organism groups being identified; a list of possible organisms is displayed and the sum of the confidence values will be less than 100 or when no match is found in the database, the organism is also considered as not identified (93).

The following symbols are also displayed as seen in Figure A-6:

- High confidence results
- Low discrimination results
- No Identification

Figure A-6 Example of Vitek Mass Spectrometry result screen (93)

The probable causes of split identifications and identification failures are displayed in Table A-2.

Table A-2 Possible causes of absent or unexpected identifications (68)

Poor Quality or No Mass Spectrum: No identification obtained
• Matrix was not added before analysis. • Too much or too little biomass was used in analysis. • Biomass and/or matrix do not cover the entire target spot. • Isolate is mucoid; too much capsular material was transferred to target slide. • Organism type is not responsive to the direct colony transfer technique, extraction is needed. • Organism biomass is contaminated with agar or primary specimen. • Culture is mixed, organism not properly isolated. • Reagents have expired / evaporated / crystallised or were incorrectly prepared. • Organism is too old or too young. • Organism was refrigerated before analysis. • Scratches or dents are on the target slide or plate. • Laser intensity is inadequate. • Detector needs maintenance or replacement. • Uneven target spot application.
Good Quality Mass Spectrum: Unexpected identification, Split identification or No identification
• The sample was transferred to the wrong spot on the target slide (inaccurate sample tracking) • Sample cross-contamination is present (this may occur from sloppy spotting, using a single pipette to apply the matrix to multiple spots, or from inadequate cleaning of reusable slides). • The culture is mixed (the microorganism not properly isolated before analysis). • Reagents are contaminated with microorganisms. • Organism is not represented in the database.

Appendix B: List of NTM, *Nocardia* spp. and moulds included in KB 3.2 (79)

Displayed identification	
NTM	
Mycobacterium abscessus	*Mycobacterium gordonae*
Mycobacterium agri	*Mycobacterium haemophilum*
Mycobacterium arupense	*Mycobacterium immunogenum*
Mycobacterium asiaticum	*Mycobacterium intracellulare*
Mycobacterium aurum	*Mycobacterium kansasii*
Mycobacterium avium	*Mycobacterium kubicae*
Mycobacterium brisbanense	*Mycobacterium lentiflavum*
Mycobacterium celatum	*Mycobacterium mageritense*
Mycobacterium chelonae	*Mycobacterium malmoense*
Mycobacterium cosmeticum	*Mycobacterium marinum*
Mycobacterium flavescens	*Mycobacterium mucogenicum*
Mycobacterium fortuitum group	*Mycobacterium nebraskense*
Mycobacterium alvei	*Mycobacterium neoaurum*
Mycobacterium farcinogenes	*Mycobacterium paraffinicum*
Mycobacterium fortuitum	*Mycobacterium phlei*
Mycobacterium fortuitum ssp *fortuitum*	*Mycobacterium scrofulaceum*
Mycobacterium houstonense	*Mycobacterium shimoidei*
Mycobacterium peregrinum	*Mycobacterium simiae*
Mycobacterium porcinum	*Mycobacterium smegmatis*
Mycobacterium senegalense	*Mycobacterium szulgai*
Mycobacterium gastri	*Mycobacterium triplex*
Mycobacterium genavense	*Mycobacterium vaccae*
Mycobacterium goodii	*Mycobacterium xenopi*
NOCARDIA	
Nocardia abscessus	*Nocardia neocaledoniensis*
Nocardia africana/nova	*Nocardia otitidiscaviarum*
Nocardia asiatica	*Nocardia paucivorans*
Nocardia asteroides	*Nocardia pseudobrasiliensis*
Nocardia beijingensis	*Nocardia transvalensis*

Nocardia carnea	*Nocardia veterana*
Nocardia cyriacigeorgica	*Nocardia wallacei*
Nocardia farcinica	
MOULDS	
Acremonium polychromum	*Lecythophora hoffmannii*
Acremonium sclerotigenum	*Lichtheimia corymbifera*
Alternaria alternata	***Metarhizium anisopliae var anisopliae***
Arthroderma benhamiae	*Microsporum audouinii*
Aspergillus brasiliensis	*Microsporum canis*
Aspergillus calidoustus/ustus	*Microsporum fulvum*
Aspergillus candidus	*Microsporum gypseum*
Aspergillus flavus/oryzae	*Microsporum persicolor*
Aspergillus fumigatus	*Microsporum praecox*
Aspergillus glaucus	***Mucor circinelloides***
Aspergillus lentulus	***Mucor circinelloides***
Aspergillus nidulans	***Mucor circinelloides* f.sp *circinelloides***
Aspergillus niger complex	*Mucor lanceolatus*
Aspergillus niger	*Mucor racemosus* complex
Aspergillus tubingensis	*Mucor velutinosus*
Aspergillus ochraceus/westerdijkiae	***Myrmecridium schulzeri***
Aspergillus sydowii	***Ochroconis humicola***
Aspergillus tamarii	***Oxyporus corticola***
Aspergillus terreus complex	***Paecilomyces fulvus***
Aspergillus thermomutatus	*Paecilomyces variotii* complex
Aspergillus unguis	*Paracoccidioides brasiliensis*
Aspergillus versicolor	***Penicillium brevicompactum***
Aureobasidium pullulans	***Penicillium brevicompactum***
Beauveria bassiana	***Penicillium brevicompactum* ssp *biourgeianum***
Bjerkandera adusta	*Penicillium camemberti*
Blastomyces dermatitidis	*Penicillium chrysogenum*
Chaetomium globosum	*Penicillium citrinum*
Cladophialophora bantiana	***Penicillium decumbens***
Cladosporium cladosporioides complex	*Penicillium expansum*
Cladosporium sphaerospermum	***Penicillium glabrum***

Coccidioides immitis/posadasii	*Penicillium italicum*
Curvularia hawaiiensis	*Penicillium pinophilum/aculeatum*
Curvularia lunata	*Penicillium roqueforti*
Curvularia spicifera	*Penicillium vermiculatum*
Epicoccum nigrum	***Phialemonium obovatum***
Epidermophyton floccosum	*Pseudallescheria boydii*
Eutypella scoparia	*Purpureocillium lilacinum*
Exophiala dermatitidis	*Rasamsonia argillacea* complex
***Exophiala phaeromuriformis* complex**	*Rhizopus arrhizus* complex
Exophiala spinifera	*Rhizopus microsporus* complex
Exophiala xenobiotica	*Sarocladium kiliense*
Exserohilum rostratum	*Scedosporium apiospermum*
Fusarium chlamydosporum complex	*Scedosporium prolificans*
Fusarium dimerum	*Sporobolomyces salmonicolor*
Fusarium oxysporum complex	***Trametes lactinea***
Fusarium oxysporum	***Trametes lactinea***
Fusarium oxysporum f.sp aechmeae	***Trichoderma ghanense***
Fusarium oxysporum f.sp cyclaminis	***Trichoderma longibrachiatum***
Fusarium proliferatum	*Trichophyton equinum*
Fusarium solani complex	*Trichophyton erinacei*
Fusarium thapsinum	*Trichophyton interdigitale*
Fusarium tricinctum complex	*Trichophyton mentagrophytes*
Fusarium verticillioides	*Trichophyton rubrum*
Geotrichum candidum/klebahnii	*Trichophyton schoenleinii*
Geotrichum fermentans	*Trichophyton terrestre*
Histoplasma capsulatum	*Trichophyton tonsurans*
Irpex lacteus	*Trichophyton verrucosum*
Lecythophora fasciculata	*Trichophyton violaceum*

Bold text: Organisms new in KB v3.2

Grey text: Organisms not displayed by Vitek MS but is part of the complex / group

Appendix C: Maintenance of ATCC 8739 E. coli strain (96)