THE OCCURRENCE AND MOLECULAR CHARACTERIZATION OF NON-TUBERCULOUS MYCOBACTERIA IN CATTLE, AFRICAN BUFFALO (SYNCERUS CAFFER) AND THEIR ENVIRONMENTS IN SOUTH AFRICA AND GENOMIC CHARACTERIZATION AND PROTEOMIC COMPARISON WITH *MYCOBACTERIUM BOVIS*

BY

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LIST OF ABBREVIATIONS

NTM	Non-tuberculous mycobacteria
BCG	Bacillus Calmette Guérin
TB	Tuberculosis
IFN	Interferon
RD	Region of difference
DIVA	Differentiation of infected and vaccinated animals
ESAT-6	6 kDA early secretory antigenic target
CFP 10	10 kDA culture filtrate protein
BLAST	Basic Local Alignment search tool
PPD	Purified protein derivative
SGM	Slow growing mycobacteria
RGM	Rapidly growing mycobacteria
PCR	Polymerase Chain reaction
ARC-OVI	Agricultural Research Council-Onderstepoort Veterinary Institute
CPGR	Centre for Proteomics and Genomics Research
GRI	Genomics Research Institute

AA	Amino acid(s)
TST	Tuberculin skin test
EM	Environmental mycobacteria
MOTT	Mycobacteria other that tuberculosis
ZN	Ziehl-Neelsen
RFLP	Restriction fragment length polymorphism
MTBC	*Mycobacterium tuberculosis* complex
PRA	PCR restriction analysis
MAC	*Mycobacterium avium* complex
ITS	Internal transcriber subunit
Hsp65	65 kDA heat shock protein
RpoB	RNA polymerase beta subunit
SodA	superoxide dismutase
MAA	*Mycobacterium avium* subsp *avium*
MAP	*Mycobacterium avium* subsp *paratuberculosis*
DGGE	Denaturing gradient gel electrophoresis
TNF	Tumor necrosis factor
IL	Interleukin

CMI	Cell mediated immunity
SIT	Single intradermal test
SICCT	Single intradermal comparative cervical test
CFT	Caudal fold test
FPA	Fluorescence polarization assay
MAPIA	Multiple antigen print immune-assay
CFU	Colony forming units
DNA	Deoxyribo-nucleic acid
HCL	Hydrochloric acid
LJ	Löwenstein-Jensen
PACT	PolymyxinB, AmphotericinB, Carbenicillin and Trimethoprim
RIDOM	Ribosomal Differentiation of Micro-organism
NCBI	National Centre for Biotechnology information
MEGA	Molecular Evolutionary Genetics Analysis
BWA	Burrow's Wheeler Aligner
SPAdes	St. Petersburg genome assembler
QUAST	Quality Assessment Tool for Genome assemblies
CDS	Coding sequences

BRIG	BLAST Ring Image Generator
TCA	Tri-chloro acetic acid
IAA	Iodoacetamide
TEAB	Triethylammonium bicarbonate

SUMMARY

THE OCCURRENCE AND MOLECULAR CHARACTERIZATION OF NON-TUBERCULOUS MYCOBACTERIA IN CATTLE, AFRICAN BUFFALO (*SYNCERUS CAFFER*) AND THEIR ENVIRONMENTS IN SOUTH AFRICA AND GENOMIC CHARACTERIZATION AND PROTEOMIC COMPARISON WITH *MYCOBACTERIUM BOVIS*

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Degree: PhD (Department of Veterinary Tropical Diseases)

The aim of this study was to investigate the diversity and prevalence of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes and their environments in South Africa and the potential of these NTM to elicit cross- reactive immune responses in these animal species which may in turn lead to false diagnosis of bovine tuberculosis. A total of 40 NTM species were identified during a countrywide survey. *Mycobacterium terrae, Mycobacterium nonchromogenicum, Mycobacterium vaccae/ Mycobacterium vanbaalenii* and a group of isolates closely related to *Mycobacterium moriokaense* (*M. moriokaense*-like isolates) were the four most frequently isolated species. Further characterization of *M. moriokaense*- like

isolates revealed two novel NTM species which were named *Mycobacterium malmesburii* sp.nov. and *Mycobacterium komanii* sp.nov. respectively. Genomes of *M. nonchromogenicum, M. malmesburii* sp. nov., *M. komanii* sp. nov., and *M. fortuitum* ATCC 6841 were elucidated and investigated for genes encoding homologues of *M. bovis* predominant immunogenic proteins. These included genes encoding for the Esx family proteins (*esx* genes), *mpb70, mpb63, mpb64, hspX, tpx, Rv1120c, canA* and *dnaK*. The e*sx* gene orthologs encoded in ESX-1 (*esxA* and *esxB*), ESX-3 (*esxH* and *esxG*), *esxR*, and ESX-4 (*esxT* and *esxU*) loci were identified in the NTM genomes while those encoded in ESX-2 locus were absent in all the four NTM genomes and only *esxN* (encoded in the ESX-5 locus) and its homologue, *esxK* were present in *M. nonchromogenicum*. Gene orthologs encoding for MPB70 (*M. malmesburii* sp.nov. and *M. komanii* sp.nov.), DnaK (all four NTM species), CanA (all four NTM species), MPB64 (all four NTM species), Rv1120c (in all four NTM species), TpX, MBP63 and HspX (all in *M. nonchromogenicum* and *M. fortuitum*), were found in the NTM genomes. In contrast orthologs of *mpb83* and *espC* were not detected in any of the four NTM. We could not judge just based on the overall protein sequence homologies of the antigens whether the NTM homologues will give rise to cross-reactive immune responses. We consequently checked the existence in NTM of epitopes shown to be immunogenic in *M. bovis* and *M. tuberculosis.* Amino acid sequence alignment of the EsxA and EsxB of the NTM sequenced in this study as well as *M. smegmatis, M. bovis* and *M. tuberculosis* respectively was done to investigate their similarities at "immunogenic" epitope level. In this analysis, we found that the six bovine T-cell recognized epitopes of *M. bovis* ESAT-6 described by Vordermeier *et al.*, 2003 and 2007 had similarities to those of *M. fortuitum* and *M. nonchromogenicum* (showing sequence similarity of as high as 81.28% and as low as 52.9%). Likewise a certain degree of sequence similarity between the six *M. bovis* CFP 10 immunogenic epitopes and those of the NTM species (highest similarity of 75% observed between all NTM and *M. bovis* and lowest similarity of 50% between *M. komanii* sp.nov, *M. malmesburii* sp.nov and *M. bovis.*) was observed. Still, with sequence homologies of less than 100% between the *M. bovis*

immunogenic epitopes and those of the NTM, it was difficult to unambiguously predict T-cell cross-recognition. Comparison of the EsxR and EsxH amino acid sequences at immunogenic epitope level, revealed higher sequence similarities in the epitopes of NTM and those of *M. bovis* than the predicted protein sequences of EsxA and EsxB. A sequence similarity of 100% was observed between two of the five *M. bovis* immunogenic epitopes of EsxR and those of *M. fortuitum*, *M. malmesburii* sp. nov. and *M. komanii* sp.nov. Full cross- recognition of these NTM EsxR epitopes is therefore highly likely, and may lead to misdiagnosis of bovine Tuberculosis (BTB). The other three EsxR/EsxH epitopes shown to be immunogenic in *M. bovis* also exist in the three NTM showing similarity of as low as 77.7%.

Two immunogenic epitopes of the *M. bovis* MPB70 described by Pollock *et al*., 2004 were also observed in *M. malmesburii* sp.nov. and *M. komanii* sp.nov. predicted MPB70 homologues with sequence similarities of 83.3% and 80% at amino acid level to the *M. bovis* antigen. The occurrence of these immunogenic epitopes in these NTM predicted antigen sequences may, despite sequence differences, lead to cross- reactive immune responses and therefore need to be tested on animal models for their immunogenicity. Their use as markers for BTB diagnosis warrants further investigation taking into consideration pre-exposure of animals to these NTM especially in regions where they are endemic, especially considering the fact that *M. fortuitum* is widely distributed across the globe and has been found to infect cattle and buffalo from different countries.

We could not unambiguously predict expression and T-cell recognition of the NTM protein coding sequence (CDS), we therefore investigated the protein composition of purified protein derivatives (PPDs) prepared from *M. fortuitum* ATCC 6841 strain (PPD-F), *M. nonchromogenicum* (PPD-N), *M. malmesburii* sp. nov. (PPD-M), *M. kansasii* ATCC 12478 (PPD-K) and compared to those of PPD-B and PPD-A used in the commercial immunological assays for TB by mass spectrometry. This analysis revealed the presence of immunogenic proteins shared between PPD-B and NTM PPDs. These include

the CFP 10 (shared with *M. malmesburii* sp. nov.), DnaK (shared with all the NTM PPDs except PPD-M), GroES (shared with all NTM PPDs except PPD-M) and GroEL (shared with all NTM PDs). NTM homologues of other proteins described as immunogenic in *M. bovis*/ *M. tuberculosis* include *M. fortuitum* MPB63 (identified in PPD-F and PPD-N), *M. fortuitum* Ag85C and Ag85A (both identified in PPD-F and PPD-N), *M. kansasii* Ag85B (identified in PPD-K), *M. avium* MPB64 (identified in PPD-A), *M. avium* Ag85A and Ag85B (both identified in PPD-A). The occurrence of these immunogenic proteins in NTM PPDs equates to their expression and potentially could be cross-recognized by T-cells leading to false diagnosis of *M. bovis*. They therefore need to be investigated further in animal experiments.

Since it was apparent that the *esx* genes of the ESX-1 locus genes (*esxA* and *esxB*) occur in non-pathogenic NTM, we set up experiments to screen isolates belonging to different NTM species that are available in our laboratory collection for the presence of *esxA* and *esxB* gene fragments by PCR and sequencing. We showed the presence of *esxA* and *esxB* sequences in *M. fortuitum* ATCC 6841 and *M. mageritense.*

In conclusion, using the combined genomics and proteomics approach, we report the occurrence of NTM homologues of *M. bovis* immuno-dominant antigens. These *M. bovis* antigens have been investigated and applied as markers for bovine (Tuberculosis) B (TB) diagnosis. The acquired knowledge will allow rational design of TB assays using specific markers that are not cross- reactive with NTM species.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Tuberculosis (TB) primarily caused by *Mycobacterium tuberculosis* has been and still is one of the most prevalent infectious causes of death globally, affecting humans of all ages. It typically affects the lungs (pulmonary TB) but can affect other organs as well (extra pulmonary TB). On the other hand, bovine tuberculosis (BTB), primarily caused by *Mycobacterium bovis* is mainly a disease of animals but can be zoonotic (be responsible for the diseases in both humans and animals), resulting in a significant economic loss world-wide (Cosivi *et al.,* 1998). Other mycobacteria of the *Mycobacterium tuberculosis complex* including *M. tuberculosis* can also be zoonotic (Ameni *et al.*, 2013).

Control strategies for human tuberculosis include vaccination, diagnosis and consequently effective treatment which can be achieved if the disease is diagnosed early and accurately (Dye and Floyd 2006). *Mycobacterium bovis* Bacillus Calmette Guérin (BCG) has been widely used for vaccination of humans against TB, but with variable efficacy in different countries (Brandt *et al.*, 2002; Abubakar *et al.*, 2013). The disease in animals is controlled in many countries by the use of test and slaughter strategies which are very costly for developing countries. Treatment is not practical especially in the context of free ranging wildlife and hence has not been attempted because of high costs, lengthy time, larger goal of eliminating the disease and risk of the animals serving as reservoirs if treatment is not effective (OIE Terrestrial Manual 2014; Michel *et al.,* 2006). Therefore effective vaccines and appropriate vaccination strategies are necessary. *M. bovis* BCG vaccine has been tested in different animal species with variable efficacies (Buddle *et al.,* 2013). The availability of the genomes of the tubercle bacilli, allows selection of vaccine candidates and evaluation of vaccination strategies against BTB in different animal species (Buddle *et al.,* 2011). Other mycobacterial strains have been evaluated as vaccine candidates in animals

(Waters *et al.,* 2012). However, with all the efforts of vaccine development in both livestock and wildlife, BCG is still the most promising vaccine (Waters *et al.,* 2012). Until an effective vaccine against BTB is developed, diagnosis is always a cornerstone of control.

The most common method for diagnosis of TB in humans is by sputum smear microscopy in which acid fast bacilli are identified, radiology and clinical signs These methods however, are not able to distinguish the species responsible for the disease, the sputum smear microscopy method merely identifies mycobacteria in general (Cosivi *et al.,* 1998). *Mycobacterium* species identification in both humans and animals is most reliably done by culture based methods followed by molecular identification, which is time consuming as pathogenic mycobacteria may take up to ten weeks to grow and sometimes may fail to do so. Direct PCR or other molecular based tests on clinical specimens are a diagnostic option in some cases (Scherer *et al*., 2011). In addition serological and immunological tests are available for diagnosis of TB in both humans and animals (Ernst *et al.,* 2007; Schiller *et al*., 2010). Bovine TB is often diagnosed in live animals on the basis of the delayed type hypersensitivity reaction elicited by the intradermal injection of biologically active tuberculin/ purified protein derivative (PPD) using the tuberculin skin test (TST) and the Interferon gamma assay (IFNγ). The latter is either used in many countries either in a serial testing regime as a confirmatory test after TST to enhance specificity or in a parallel testing regime to enhance sensitivity of the TST (Schiller *et al*., 2010).

Control of TB in both humans and animals can be hampered by prior exposure to non-tuberculous mycobacteria (NTM) (Farhat *et al.,* 2006; Buddle *et al.,* 2008). NTM, otherwise known as "mycobacteria other than tuberculosis" (MOTT) or environmental mycobacteria (EM) have been isolated from the environment as well as animals and humans. NTM are believed to be natural inhabitants of the environment, even though some species have been isolated from only clinical samples and not the environment (van Ingen *et al.,* 2009). These bacteria have been neglected for many years, probably because their clinical or veterinary significance was always doubtful. NTM are in general of low

virulence and most species are known to rarely or never cause any disease hence they have always been recognized as environmental contaminants or colonizers (Covert *et al.,* 1999). Finding immune compromised hosts, especially with the emergence of the acquired immune deficiency syndrome (AIDS) pandemic, some NTM species are now recognized as potential opportunistic pathogens of humans (Mirsaeidi *et al.,* 2014). Various NTM species have also been implicated in diseases of animals, highlighting the importance of these species as potential animal pathogens (Bercovier and Vincent, 2001). The term 'mycobacteriosis' is used for diseases caused by NTM. Opportunistic mycobacterial diseases have been classified as skin lesions, localized lymphadenitis, TB-like pulmonary lesions and disseminated diseases (Primm *et al.,* 2004). Transmission of NTM is thought to be from the environment as there is no evidence of human to human or animal to animal infection and is supposed to occur by inhalation of aerosols or ingestion of water and soil (van Ingen *et al.,* 2009). NTM cannot be differentiated from the pathogenic turbercle bacilli by acid fast staining/ Ziehl- Neelsen (ZN) staining, likewise members of the tubercle bacilli (known as *Mycobacterium tuberculosis* complex or MTBC) cannot be differentiated from each other by simple ZN staining. Differentiation of all mycobacteria from cultured isolates is often done by either a series of phenotypic tests or molecular based tests like Polymerase Chain Reaction (PCR), restriction fragment length polymorphism (RFLP), and sequencing of the *Mycobacterium* housekeeping genes (Niva *et al.,* 2006; Pontiroli *et al.,* 2013; Bahram *et al.,* 2014).

Some NTM species are believed to elicit cross-reactive immune responses in both humans and animals that interfere with the tuberculin based immunological diagnostic tests for TB, such as the TST and IFNγ assay. Likewise *M. bovis* (BCG), the only approved vaccine against TB in humans is also believed to elicit cross- reactive immune responses that interfere with the tuberculin tests for TB in both humans and animals (Farhat *et al.,* 2006; Buddle *et al.,* 2008). This is attributed to the fact that PPD used in these tests, contains a mixture of somatic and antigenic proteins, some of which are present in pathogenic mycobacteria, BCG and NTM (Thom *et al.,* 2008; Schiller *et al.,* 2010).

These cross-reactive immune responses induced by NTM are believed to also play a role in the reduced efficacy of BCG (Brandt *et al.*, 2002; Buddle *et al.*, 2003; Thom *et al*., 2008). Variation in results of BCG efficacy ranging from non-effective to successful in both human and animal population studies are therefore thought to reflect the differences in population exposure to cross-reacting NTM (Brandt *et al.*, 2002; Buddle *et al.*, 2011).

Continued research on the involvement of NTM in induction of cross reactive immune responses is therefore a priority as these species may play a pivotal role in the control of TB. NTM diversity and prevalence studies may help streamline the investigation of the role played by prevalent NTM species in a particular region in cross-reactive immune responses against TB.

1.2 Literature Review

1.2.1 The genus *Mycobacterium*

The genus *Mycobacterium* belongs to the *Mycobacteriaceae* family, *Actinomycetales* order and *Actinomycetes* class. Mycobacteria are a group of Gram positive bacteria, displaying diverse phenotypes. These are acid fast bacilli with a lipid bilayer. There are more than 150 species and about 13 subspecies of mycobacteria listed in the public database: List of prokaryotic names with outstanding nomenclature (http/ www.bacterio.net).

1.2.1.1 Mycobacterial taxonomic classification by phenotype

The early classification of mycobacteria was based on growth rate, pigmentation and clinical significance (Runyon, 1959; Shinnick and Good, 1994). One fundamental taxonomic division was tied to growth rate. Based on this, mycobacteria can be divided into two main groups: the fast/rapid growers (RGM), referring to those mycobacteria that take less than 7 days for colonies to appear on solid medium and the slow growers (SGM) where colonies take more than 7 days to appear on solid medium. Further subdivision of mycobacteria into four Runyon groups was based on pigmentation, where Group 1 consists of photo-chromogens (colonies are capable of producing carotenoid pigment in the presence of light) and Group 2 of scoto-chromogens (colonies produce carotenoid pigment regardless of the light). The third Runyon group consists of species which do not produce any pigmentation, the non-chromogenic mycobacteria. The slow growers were assigned to the three groups. The fourth Runyon group consists of rapid growing non-chromogens and chromogenic species (Runyon, 1959).

Mycobacteria are also classified based on their biochemical and physiological properties, called Adansonian classification. In this classification, for instance, *M. fortuitum*, *M. acapulsensis*, *M. flavescens*, *M. marinum* were grouped together on the basis that they all utilize only a limited number of carbohydrates (Bojalil *et al.*, 1962; Tsukamura *et al.*, 1966).

1.2.1.2 Taxonomic classification based on clinical significance/pathogenicity

Classification of mycobacteria based on clinical significance separated pathogens and non-pathogens. The slow growing mycobacteria consist of many species which are able to cause disease. These include a group of species collectively known as *Mycobacterium tuberculosis* complex (MTBC), *Mycobacterium*

leprae as well as certain species of non-tuberculosis mycobacteria (Rastogi *et al.,* 2001; Gutiérrez *et al.,* 2009)**.** Pathogenic MTBC species include those mycobacteria that cause tuberculosis in humans and different animal species, like *M. bovis, M. tuberculosis, M. africanum, M. cannetii, M. mungi, M. pinnipedii, M. microti, M.caprae, M. orygis, the Dassie bacillus* and *M. suricattae* (Brosch *et al.,* 2002; Smith *et al.,* 2006a; Huard *et al.,* 2006; Alexander *et al.,* 2010; Parsons *et al.,* 2013). The common slow growing pathogenic NTM include species collectively known as *Mycobacterium avium* complex (MAC) that causes diseases in both humans and animals (Rastogi *et al.,* 2001**)**. *Mycobacterium leprae* causes leprosy in humans and has been isolated from wild armadillos in Southern United States, suggesting potential zoonotic nature of this species (Gutiérrez *et al.,* 2009; Truman *et al.,* 2011). Fast growing mycobacteria commonly known as RGM (rapidly growing mycobacteria) are usually non-pathogenic but some are opportunistic pathogens and still belong to the NTM (van Ingen *et al.,* 2009; Parte, 2014). They are commonly encountered in the environment where they are normal inhabitants (van Ingen *et al.,* 2009). The low virulence in most NTM and the lack of human to human or animal to animal transmission are the most important distinguishing features between these species and the MTBC as well as *M. leprae* (De Groote *et al.,* 2006; Gutiérrez *et al.,* 2009; van Ingen *et al.,* 2009).

1.2.1.3 Molecular based and phylogenetic classification

Advancement in molecular biology has led to identification tools for mycobacteria, which give enough information for phylogenetic classification of these species (Fig 1). Mycobacteria are thought to have one or two *rrn* operons per genome. Slow growing pathogenic mycobacteria, like *M. tuberculosis, M. bovis,* and *M. leprae* have single *rrn* operons and most fast growing mycobacteria are reported to have two, with exception of *M. chelonae* and *M. abscessus* which, although they are fast growers, each have single copy of the *rrn* operon (Liesack *et al.,* 1990; Kempsell *et al.,* 1992; Cole *et al.,* 1998; Menendez

et al., 2002). The apparent correlation between the growth rate and the number of *rrn* operons present in a genome provides possible explanation regarding mycobacteria being classified as either fast or slow growers.

Different phylogenies for mycobacteria have been developed using analysis of the 16S rRNA (Cloud *et al.,* 2002; Devulder *et al.,* 2005). The 16S rRNA is encoded by the 16S rDNA sequence that is approximately 1500bp. The 16S rDNA is a highly conserved gene, however, there are sequence variations that are located at specific areas of the gene. The sequence stretches that are common to all mycobacteria, together with the hyper variable regions (region A and region B) that define species specificity make this gene very useful for species identification (Tortoli, 2003). The secondary structure of the 16S rRNA contains helices: 8, 9, 10 and 11 falling within region A, while helix 18 is in region B. The correlation between slow growth and a long helix 18 was first proposed by Stahl and Urbance (1990). Nucleotide insertion (varying in size) in helix 18 occurs with slow growers, while a short helix (no insertion) is associated with rapid growers (Stahl and Urbance, 1990). However this proposed classification was later found contradictive since other slow growing mycobacteria, like *M. simiae, M. intermedium* and *M. triviale,* have a short helix 18 (Menendez *et al.,* 2002).

Identification of the nucleotide sequence in region A of the 16S rRNA is sufficient for speciation (Harmsen *et al.,* 2003). The only drawback with the use of the 16S rRNA for species identification is very high interspecies similarities, ranging from 94%-100% (Adékambi and Drancourt, 2004; Adékambi *et al.,* 2006). Some species have identical 16S rRNA sequences. For instance, members of the *M. tuberculosis* complex have virtually identical 16S rRNA sequences, and cannot be differentiated with this locus. Likewise this gene typing can neither differentiate between members of the MAC nor the *M. fortuitum* complex; *M. kansasii* and *M. gastri, M. vaccae* and *M. vanbaalenii* as well as between other closely related mycobacterial species (Devulder *et al.,* 2005; Adékambi *et al.,* 2006; Gcebe *et al.,* 2013). However, it provides robust phylogenetic trees and enough information for *Mycobacterium* taxonomy

(Devulder *et al.*, 2005). Other molecular targets of diagnostic interest in *Mycobacterium* investigations include the *hsp65* gene encoding the 65-kDA heat shock protein (Telenti *et al.*, 1993). The *hsp65* gene is also highly conserved among *Mycobacterium* species, but contains hyper variable regions at nucleotide positions 624 to 664 and 683 to 725 making this gene useful for identification and taxonomic purposes. PCR restriction analysis (PRA) of the 441bp fragment of the *hsp65* gene commonly known as the Telenti fragment has been used as an identification tool for *Mycobacterium* species (Telenti *et al.*, 1993; Devulder *et al.*, 2005). MTBC except *M. africanum* cannot be differentiated with the use of the *hsp65* sequence analysis (Devulder *et al.*, 2005).

The region between the genes encoding the 16S and the 23S rRNA commonly known as the internal transcriber subunit (ITS) is also of taxonomic interest. Sequence determination as well as the PRA of the ITS were shown to be useful in differentiating most phylogenetically related slow growing *Mycobacterium* species (Roth *et al.*, 1998). However, some mycobacteria like *M. marinum* and *M. ulceris* share the same ITS and cannot be separated by the use of ITS (Roth *et al.*, 1998).

Other loci including, among others, *gyrB* encoding the beta subunit of the DNA gyrase (Kasai *et al.*, 2000), *rpoB* encoding the beta subunit RNA polymerase (Adekambi *et al.*, 2006), *dnaJ* (Takewaki *et al.*, 1993), *dnaK* (Dai *et al.*, 2011), *recA* (Blackwood *et al.*, 2000), *sodA* encoding the superoxide dismutase (Adekambi and Drancout 2004), *ssr*A (Mignard and Flandrois, 2007), *tuf* (Mignard and Flandrois2007) and *secA* (Zelazyn *et al.*, 2005) have been reported as being useful in *Mycobacterium* identification.

The *rpoB* gene contains a single copy and thus problems of sequence ambiguity associated with the use of 16S rDNA have not been experienced. However, the *rpoB* gene sequence is also not able to differentiate members of the MTBC. This gene was only used for predicting rifampicin resistance of *M. tuberculosis* strains until recently, when it was applied for *Mycobacterium* identification (Adekambi *et*

al., 2006). Phylogenetic trees based on the *rpoB* gene are reported to be not as robust as trees based on the *hsp*65 and the 16S rDNA (Devulder *et al.*, 2005).

Primer failure for *sodA* and *dnaJ* has been reported for mycobacterial identification. Both these genes cannot differentiate MTBC (Adekambi and Drancourt 2004; Yamada-Noda *et al.,* 2007).

The gyrase gene has only been tested in slowly growing mycobacteria, like MTBC and its identification allows differentiation between members of the MTBC but cannot differentiate *M. bovis* and *M. bovis* BCG (Nieman *et al.*, 2000).

The recently identified *dnaK* locus has been tested in both slowly and fast growing species, but only 64 *Mycobacterium* species were included in the study (Dai *et al.*, 2011), and therefore further studies of these loci (*dnaK* and *gyrB)* in other *Mycobacterium* species are still needed.

While the 16S rDNA and *hsp65* are still the most widely used genes for *Mycobacterium* identification and phylogenetic studies, they have limitations as discussed above, as a result the use of multiple gene sequences for taxonomic classification of mycobacteria has been proposed. Phylogenetic trees derived from concatenated sequences of a panel of genes have been explored as a taxonomic model and were shown to classify mycobacterial species accurately (Devulder *et al.,* 2005). Figures 1.1, 1.2a and 1.2b illustrate the phylogenetic classification of mycobacteria based on the 16S rDNA as well as comparing the trees based on *hsp65* and the 16S rDNA sequences respectively. These illustrations show how these gene sequences can be used as a taxonomic tool to separate slow growing mycobacteria (SGM) from rapidly growing mycobacteria (RGM).

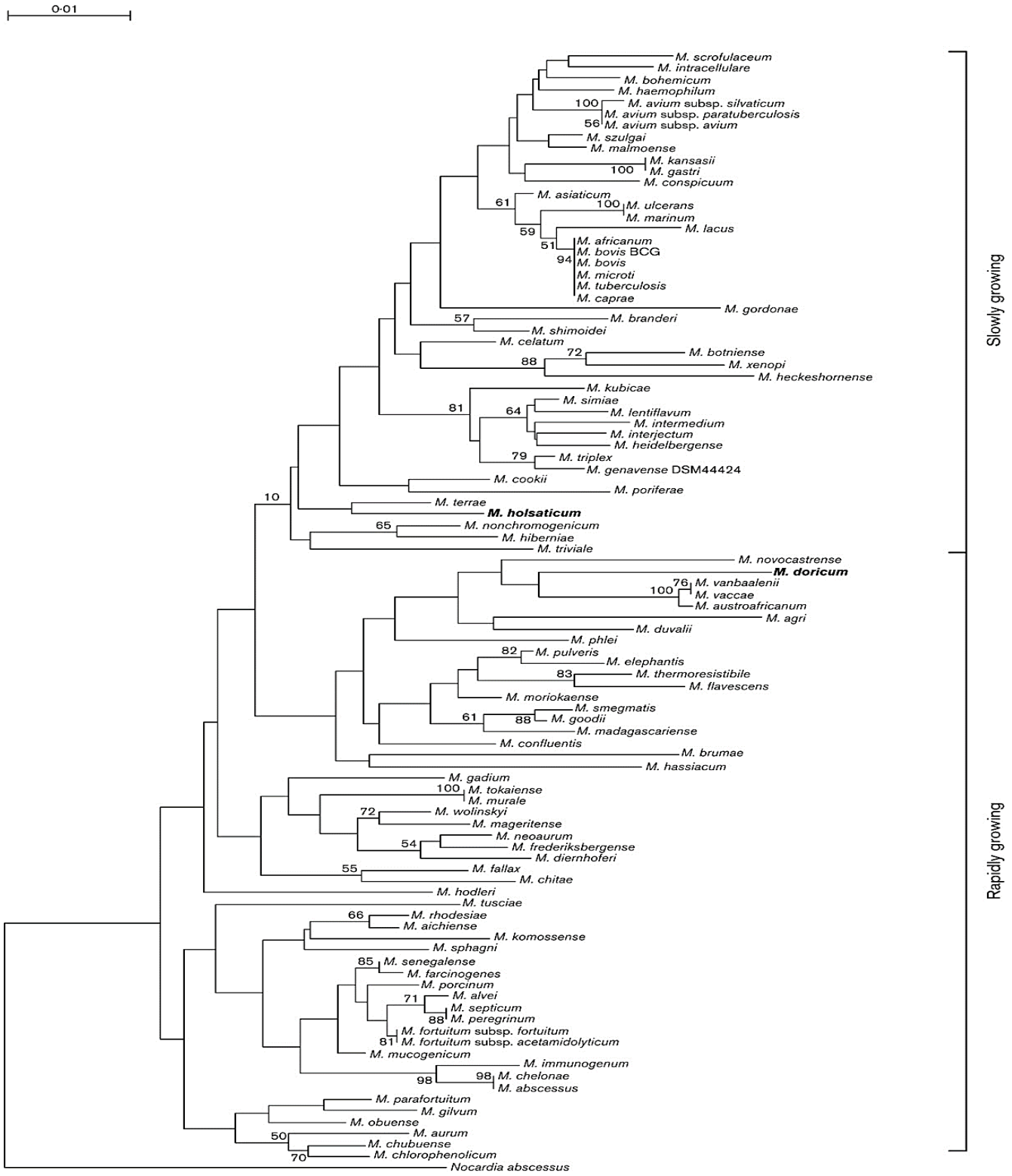

Fig 1.1: Phylogenetic tree of the genus *Mycobacterium* computed from 16S rRNA gene sequences by the neighbor-joining method and Kimura's two-parameter model as the substitution model. The tree includes 97 strains and was rooted using *N. abscessus* DSM 44432(extracted from: Devulder *et al.*, 2005).

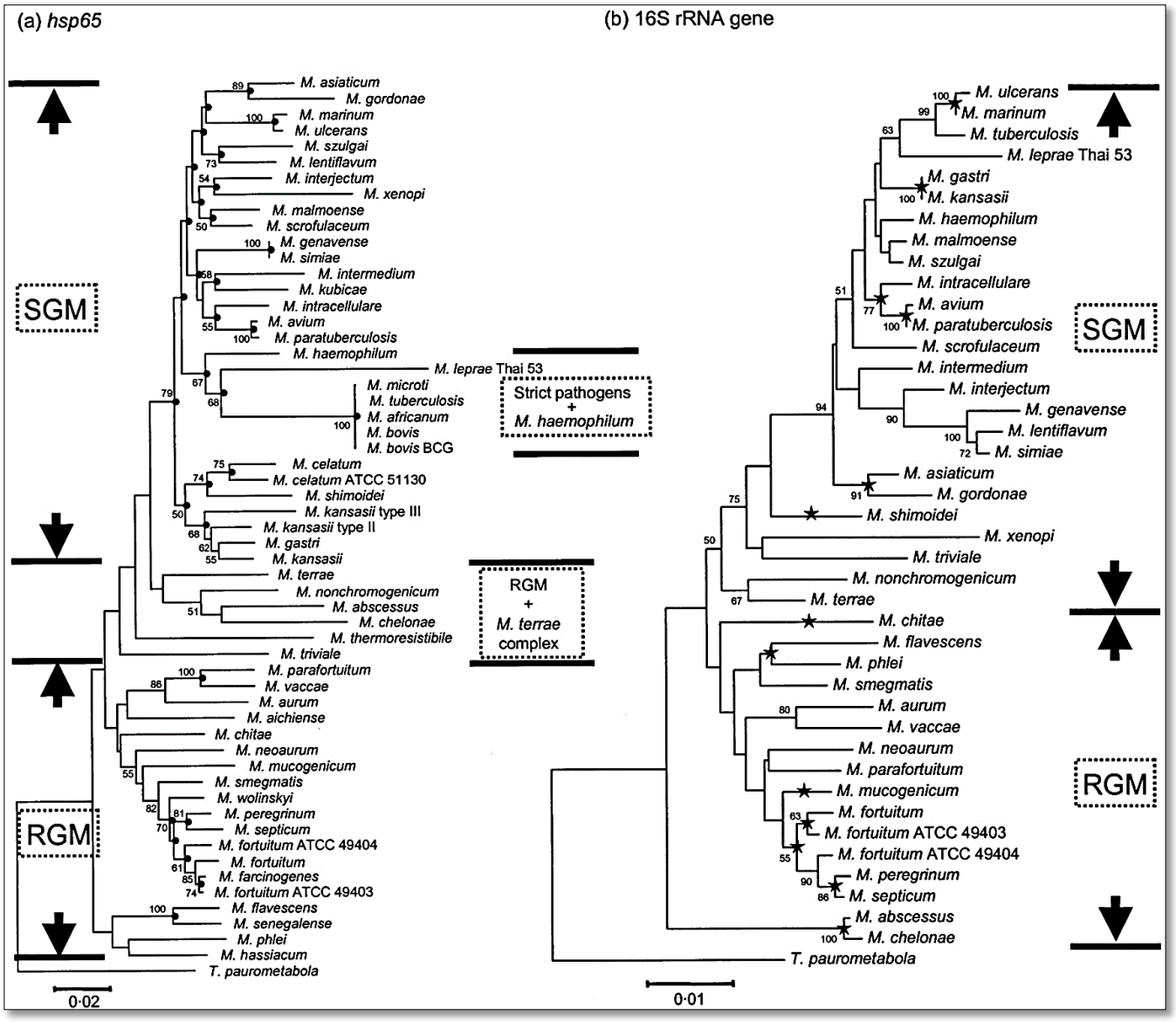

Fig 1.2: Extracted from Kim *et al.*, (2005), illustrating the phylogeny of mycobacteria based on a): the hsp65 and b): the 16S rDNA. The *hsp65* sequence tree was constructed from 56 mycobacterial reference strains but the 16S rDNA tree was constructed from 41 mycobacterial reference strains. Both trees were constructed using the neighbor-joining method.

1.2.2 Species of MTBC and their clinical relevance

The number of members of the MTBC is likely to increase as new genetic differences between strains of the existing members are identified. Many species and subspecies of these tubercle bacilli show a specific host association (Smith *et al.*, 2006b). Despite the difference in host tropisms, MTBC are characterized by a 99.9% or greater similarity at nucleotide level and by virtually identical 16S rRNA sequences (Smith *et al.*, 2006b). In addition to historic taxonomic segregation of the MTBC, based on growth, morphology, physiological and biochemical characteristics and their host range, the elucidation of the complete genome sequences of both *M. tuberculosis* and *M. bovis*, together with comparative genomics between the MTBC members, has led to a better understanding of the genetic variability within this group. The existence of chromosomal deletions (Regions of difference; RD), together with single nucleotide polymorphisms (SNPs), direct repeat region (DR, or spoligotype) and variable number of tandem repeat patterns (VNTR) , allow discrimination between these bacteria (Kamerbeek *et al.*, 1997; Brosch *et al.*, 2002; Mostowy *et al.*, 2002; Smith *et al.*, 2003, Hlokwe *et al.*, 2014). At present, genetic criteria are used routinely to identify MTBC clinical and veterinary isolates to species and sub-species level (Barnes and Cave, 2003; Huard *et al.*, 2006; Warren *et al.*, 2006). Figure 1.3 illustrates how the RD can be used to differentiate MTBC members to species and sub-species level (Gey van Pittius *et al.*, 2012).

Mycobacterium tuberculosis and *Mycobacterium bovis* are the most studied members of the MTBC, probably due to their greatest significance in human and animal diseases as well as their worldwide distribution (Michalak *et al.*, 1998; Ocepek *et al.*, 2005; Ameni *et al.*, 2013). Of the members of the MTBC, *M. bovis* has the broadest host range (Cosivi *et al.*, 1998, Wedlock *et al.*, 2002a). *Mycobacterium*

caprae has been recognized mainly in central Europe, where it has been occasionally isolated from cattle, pigs, red deer, sheep, goats, wild boars a fox and humans (Kubica *et al.*, 2003; Rodriguez *et al.*, 2011). *Mycobacterium mungi* infects banded mongooses (*Mungos mungo*) while *Dassie bacillus* infects rock hyraxes (*Procavia capensis)* and were both isolated in Southern Africa (Parsons *et al.*, 2008; Alexander *et al.*, 2010). *Mycobacterium africanum* is prevalent in West Africa causing up to half of the human tuberculosis infections (de Jong *et al.*, 2010), but has also been reported in pigs and cattle (Afserden and Saxegaard, 1995). *Mycobacterium microti*'s natural hosts are small rodents such as field voles, bank voles, wood mice and shrews. Sporadic cases of infection in other animals like pigs, cattle, cats, dogs and badgers have been reported (van Soolingen *et al.*, 1998). Also cases of human infection by *M. microti* have been reported (Panteix *et al.*, 2010). The natural hosts of *Mycobacterium pinnipedii* are pinniped species, like the fur seals and the most frequently affected being the sea lion (Cousins *et al.*, 2003). The organism is also pathogenic in Guinea pigs, rabbits, humans and tapir (Cousins *et al.*, 2003). *Mycobacterium cannettii* infects humans (van Soolingen *et al.*, 1997). *Mycobacterium orygis* is known to infect antelopes but has also been isolated from humans as well as African buffalo (Smith *et al.*, 2006a; Gey van Pittius *et al.*, 2012). Recently, a new member of the MTBC, named *M. suricattae* was found to occur in meerkats in South Africa (Parsons *et al.*, 2013). *Mycobacterium bovis* is the progenitor of the TB vaccine, *M. bovis* bacillus Calmette-Gúerin (BCG), a strain that was attenuated by serial passaging of *M. bovis* on potato slices soaked in ox bile and glycerol over 13 years. The precise mutations that led to this attenuation are still unknown, although the key deletion of the region of difference (RD1) appears to have played a role (Behr *et al.*, 1999; Pym *et al.*, 2002).

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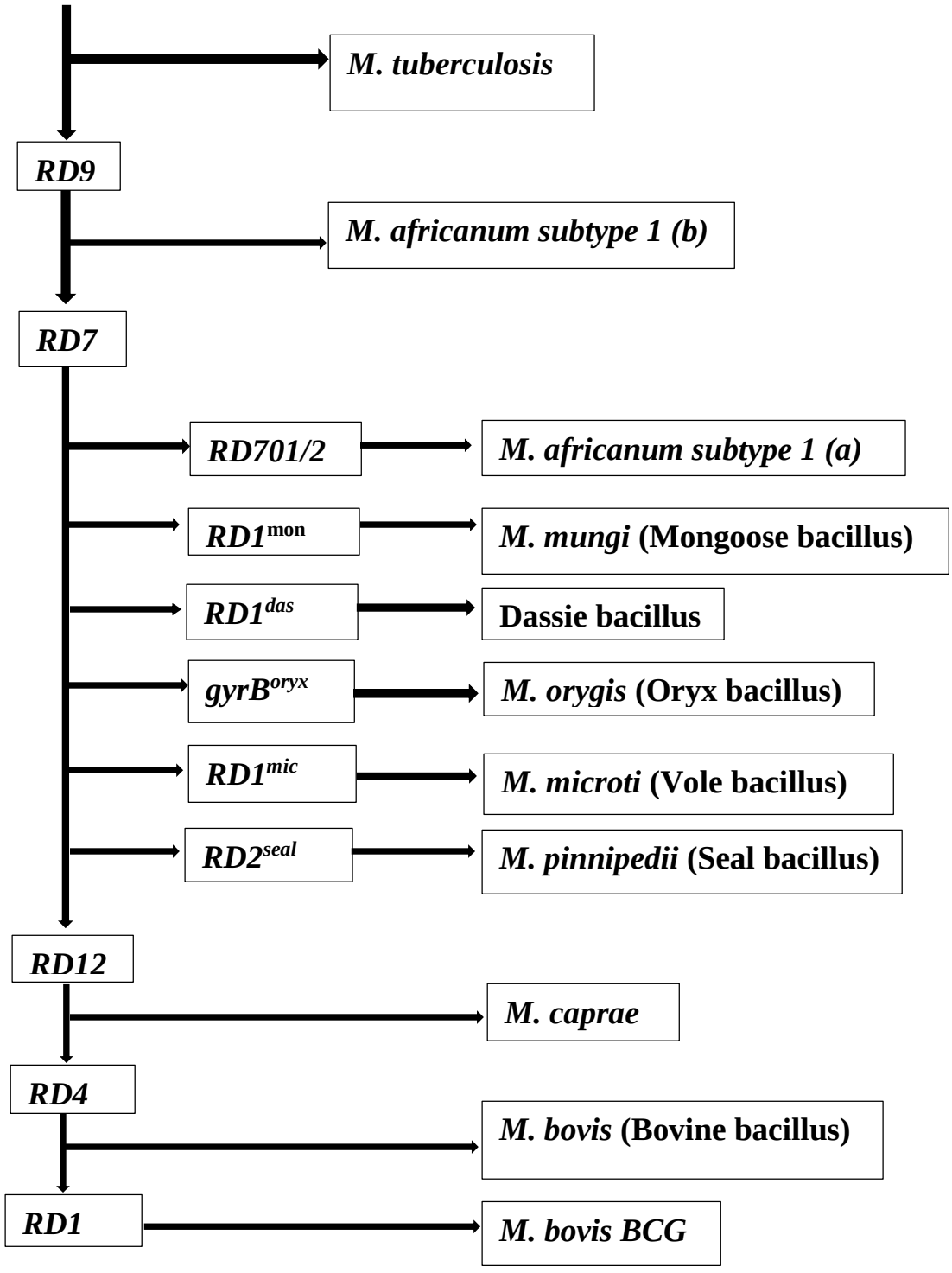

Fig 1.3: The phylogeny of MTBC based on regions of difference (RD) as described by Gey van Pittius *et al.*, 2012.

1.2.3 Bovine tuberculosis in Africa

BTB has been reported in both wildlife and domestic animals in different countries of Africa. According to a review by De Garine-Wichatitsky *et al.*, 2013, during the period 1996-2011 the majority of African countries (38/54) reported BTB in livestock (infection or clinical disease) to the Office International ses Epizooties (OIE), with an additional four countries reporting suspected cases, while only four countries that implemented general or targeted surveillance reported the absence of the disease. During the same period, BTB (clinical cases) in wildlife were confirmed in only 6/54 countries, all located in Southern and Eastern African countries. Additional five countries reported suspected cases, while 11 countries that implemented targeted or general surveillance of the disease reported absence of the disease, and 33 African countries indicated unavailability of data (De Garine-Wichatitsky *et al.*, 2013). In Southern Africa, BTB caused by *M. bovis* has been confirmed in free ranging wildlife in countries like Zambia, South Africa and Zimbabwe. In South Africa, *M. bovis* has been detected in nyala, black rhino, leopard, warthog, wild dog, lion, hyena, kudu, cheetah, baboon, wildebeest, honey badger, genet, bushbuck, eland and impala (Hlokwe *et al.*, 2014; Hlokwe unpublished) while *M. tuberculosis* has been isolated from cattle, non-human primates and an elephant (Hlokwe *et al.*, unpublished). Other species of the MTBC as discussed in section 1.2.2, including *M. orygis, Dassie bacillus, M. suricattae* and *M. mungi* have also been isolated from different wildlife species in Southern Africa. BTB has been confirmed in antelopes (*Kobus leche kafuensis)* in Zambia, and in buffalo in Zimbabwe and presence of infection by *M. bovis* has been suspected in African buffalo in Botswana and more recently in buffalo and cattle in Mozambique (De Garine-Wichatitsky *et al.*, 2013). In East Africa, BTB has been confirmed in cattle and several wildlife from Ethiopia, Uganda and Tanzania (Cleaveland *et al.*, 2005; 2007; Olaya *et al.*, 2007; 2008; Durnez *et al.*, 2009; Tschopp *et al.*, 2010). BTB in buffalo and baboon has also been confirmed

in Kenya (Tarara *et al.,* 1985; De Garine-Wichatitsky *et al.,* 2013). In West Africa, suspected cases of BTB in cattle of Niger, Ghana, Mali, and Togo were reported (Bonsu *et al.,* 2000; Muller *et al.,* 2008; Boukary *et al.,* 2011; De Garine-Wichatitsky *et al.,* 2013). In central African countries, no reports of BTB in wildlife have been published, but cases of BTB from cattle in Chad were published (De Garine - Wichatitsky *et al.,* 2013). Figure 1.4 illustrates the distribution of bovine tuberculosis in Africa during 1996-2011 (De Garine-Wichatitsky *et al.,* 2013).

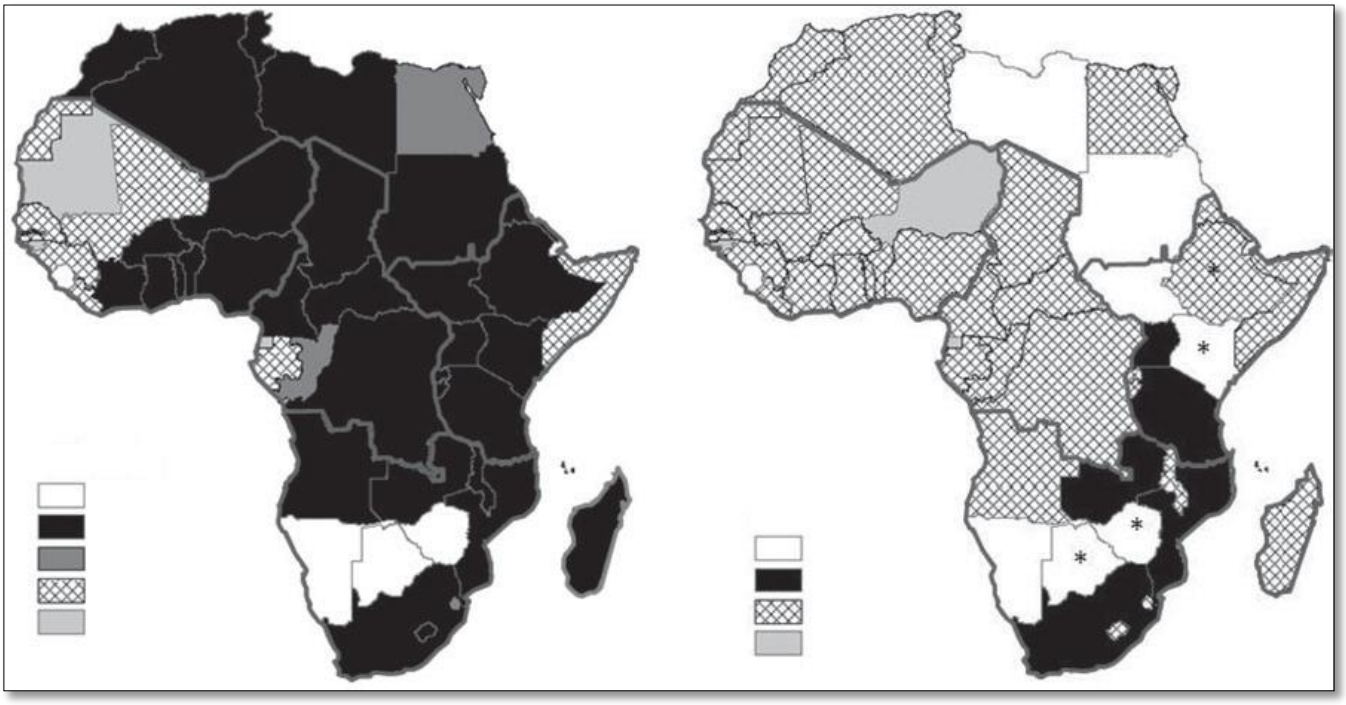

Fig: 1.4: Distribution map of bovine tuberculosis in Africa during 1996–2011. The figure was extracted from De Garine –Wichatistsky *et al.,* 2013. (*a*) Cattle status at country level; (*b*) wildlife status at country level. Asterisk (*) indicates countries (i.e. Botswana, Ethiopia, Kenya, Zimbabwe) where suspected and confirmed cases have been detected but not yet reported to OIE.

1.2.4 NTM and their relevance in human and animal infections

For more than 80 years after its discovery by Robert Koch, *Mycobacterium tuberculosis* was believed to be the only clinically significant *Mycobacterium* species. Other acid fast bacilli isolated in humans, animals and the environment were usually dismissed as saprophytes of little consequence (Covert *et al.,*

1999). It was not until the 1950s that changes in attitude towards NTM occurred when these species were cultured from pathological material under circumstances that led to the belief that they might be of clinical or veterinary significance (Covert *et al.*, 1999). There is convincing evidence that the environment is the source of NTM even though other NTM species have only been isolated from humans and animals, and not the environment (van Ingen *et al.*, 2009). NTM include those mycobacteria that are not part of the MTBC, but exclude *Mycobacterium leprae*, and these include both slow growing (SGM) and rapidly growing mycobacteria (RGM). Because of the ubiquity of NTM, human infections have been reported from most geographic areas in the world. NTM species distribution in humans is thought to be regional (Martin–Casabona *et al.*, 2004; van Ingen *et al.*, 2009). It has also been suggested that the differences in species distribution in the environment, humans and animals may partly determine the frequency and manifestations of NTM disease in each geographical location (Hoefsloot *et al.*, 2013). In recent years there has been a rise in the number of reported infections by non-tuberculous mycobacteria, mainly in immune compromised individuals (Hoefsloot *et al.*, 2013). Likewise cases of nosocomial as well as iatrogenic transmission of NTM, leading to either harmless colonisation or harmful infections have been observed (Phillips and Reyn, 2001). Certain species of NTM like *M. kansasii*, *M. xenopi*, *M. chelonae*, *M. fortuitum* and *M. gordonae* have been implicated in cases of nosocomial and iatrogenic infections (Sniadack *et al.*, 1993, Phillips and Reyn, 2001, Brown Elliot and Wallace, 2002). The reason for the apparent rise of the observed mycobacterial infections is the increasing awareness of these mycobacteria as opportunistic or potential pathogens of humans and animals and improvements in methods of their detection (van Ingen *et al.*, 2009). Among NTM, the RGM species have recently gained increasing attention because of their extensive resistance to a number of antibacterial drugs and their association with specific disease types in specific patient categories (Sexton and Harrison, 2008). Twenty nine new NTM species were isolated from both humans and animals between the years 2003 to 2006 as reviewed by Tortoli, (2006).

By far the most studied slowly growing opportunistic NTM pathogens of both animals and humans are the members of the *Mycobacterium avium* complex (MAC) consisting of closely related species: *M. avium* subsp *avium, M. avium* subsp *paratuberculosis, M. avium* subsp *hominissuis, M. intracellulare, M. sylvalticum, M. colombiense, M. bouchedurhonense, M. timonense, M. chimaera, M. arosiense, and M. marseillense* (Cayrou *et al.*, 2010).

Other SGM as well as RGM species have been reported to cases diseases in humans and the most common are *M. kansasii, M. marinum, M. haemophilum, M. szulgai, M. fortuitum, M. chelonae, M. wolinskyi, M. scrofulaceum, M. abscessus, M. xenopi* and *M. genavense* (Troesch *et al.*, 1999, Martin-Casabona *et al.*, 2004). *M. kansasii* is one of the first NTM pathogens shown to be responsible for pulmonary diseases in humans and together with MAC they infect many sites of the body but primarily cause pulmonary and cervical lymphadenitis (Buhler and Pollock, 1953*). M. ulcerans*, *M. marinum* and *M. haemophilum* are the primary causes of localized skin and soft tissue lesions (Horsburgh, 1991; Portaels, 1995). Pulmonary presentation caused by *Mycobacterium szulgai* is the most common although extra-pulmonary and disseminated diseases have been reported (Tortoli *et al.*, 1998; van Ingen *et al.*, 2008). The *M. fortuitum* and *M. chelonae* are associated with infections of injection sites or wound sites in hospitals (Brown –Elliot and Wallace, 2002). *M. fortuitum* was isolated from a suspect cases of pulmonary tuberculosis in Zambia (Malama *et al.*, 2014). *Mycobacterium wolinskyi* is most commonly associated with post traumatic wound infections. A few cases of *M. wolinskyi* including bone infections, hip prosthesis, and bacteremia have been reported (Brown *et al*., 1999; Pulcini *et al*., 2006; Chen *et al*., 2008). Infections of the cervical lymph node by *M. scrofulaceum* in children are common, and far less frequently observed clinical manifestations in adults include pulmonary diseases and disseminated diseases in immune compromised patients (Wolinsky, 1995; Marrazi *et al.*, 2009). Pulmonary cases due to *M. abscessus* and *M. xenopi* have been reported (Martin –Casabona *et al.*, 2004; De Groote and Huitt, 2006; Park *et al.*, 2007).

A number of NTM species have also been reported to cause diseases in animals. Like in humans, among the slowly growing NTM species, diseases caused by MAC in animals have received the most attention. Avian TB caused by *Mycobacterium avium* subspecies *avium* (MAA) is a serious disease of animals. Birds are particularly susceptible to MAA, often leading to fatal organ tuberculosis (Dvorska *et al.,* 2003; Thegerstrom *et al.,* 2005). Avian TB in birds may cause serious economic losses including the death of rare and endangered species (Marco *et al.,* 2000). *Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes Johne's disease in livestock and occurs worldwide. *M. avium* subsp *hominissuis* has also been isolated in different species but most often in pigs (Pavlik *et al.,* 2003) and less frequently in non-vertebrates (Fisher *et al.,* 2001, 2003 a; b; 2004 a; b), wild boar (Machackova *et al.* 2003) and wild ruminants (Machackova *et al.,* 2004). MAC strains have also been isolated from horses and cattle (Pavlik *et al.,* 2005). *M. intracellulare, M. chimaera* and *M. colombiense* have been isolated from African rodents and insectivores in Tanzania (Durnez *et al.,* 2008; 2011). *M. kansasii* has been detected in wild animals in Africa, Europe, Asia and America. It has been isolated from cattle, pigs and a goat (Bercovier and Vincent, 2001). *Mycobacterium simiae* has been isolated from monkeys and ornamental fish (Bercovier and Vincent, 2001). *M. xenopi* is principally a pathogen of pigs but was originally isolated from a toad and later more frequently from pigs (Bercovier and Vincent, 2001). *M. marinum* is reported to be a pathogen of fish but they may rarely infect domestic pigs, cattle and wildlife (Bercovier and Vincent, 2001). *M. chelonae* has been isolated from cats, dogs, turtles, and cattle (reviewed by Bercovier and Vincent, 2001). *M. scrofulaceum* has been isolated worldwide and frequently from lymph nodes of cattle, buffaloes, deer, swine, feral pigs, patas monkeys, and fish (Bercovier and Vincent, 2001). *M. szulgai* has been detected in cattle in Tanzania (Durnez *et al.,* 2009). Among RGM, the most common animal opportunistic pathogens are *M. fortuitum, M. porcicum, M. farcinogens, M. senegalense,* and *M. genavense. M. fortuitum* has been isolated worldwide from wild animals including African buffaloes,

reptiles, amphibians, seals, cattle and invertebrates (Bercovier and Vincent, 2001, Michel *et al.,* 2011, Malama *et al.,* 2014). In the review by Bercovier and Vincent (2001), *M. porcicum* was reported to have been isolated in Japan from lymph nodes of pigs with tuberculosis-like lymphadenitis. In this review, *M. farcinogenes* and *M. senegalense* were reported to be responsible for bovine farcy, pathology found in Africa and *M. genavense* is recognized as the most frequent etiological agent of avian mycobacteriosis in pet birds. Rare animal NTM pathogens include *M. smegmatis* which has been isolated from diseased cats, dogs and more frequently from cattle (Bercovier and Vincent, 2001) and *Mycobacterium goodii* which was reported to have caused infection in a hyena in South Africa as well as in African rodents in Tanzania (van Helden *et al.,* 2009; Durnez *et al.,* 2011).

1.2.5 NTM in the environment and factors associated with their diversity and distribution

NTM are ubiquitous in nature and can be found in a variety of ecosystems. The environment is of interest as a source of NTM infection and exposure. NTM have commonly been found in soil (Covert *et al.,* 1999) and water (Collins *et al.,* 1984; Shitaye *et al.,* 2009). They have also been isolated from biofilms (September *et al.,* 2004), dust (Kamala *et al.,* 1994) and animal feeds (Pavlik *et al.,* 2007). There is still a debate of whether soil or water is the natural habitat of NTM (van Ingen *et al*., 2009, Botha *et al.,* 2013). The distribution and prevalence of NTM in the environment is likely to be influenced by human and animal activities (Portaels, 1995). Most NTM diversity studies in the environment have concentrated on NTM distribution in both human engineered water systems as well as natural water systems like rivers and lakes. Soil sampling is less frequently done than water sampling, probably because of technical difficulties in isolation due to high levels of contamination that hampers culture (van Ingen *et al.,* 2009). A review of 24 studies on NTM isolation and diversity mainly in natural and man-made water systems, soil, dust and biofilms in the habitats of humans and animals worldwide is summarized in table 1.1. These

studies employed different methods for assessing *Mycobacterium* diversity in different geographic areas at different environmental conditions. In this review *Mycobacterium fortuitum* appears to be the most frequently isolated NTM in 14/24 studies, followed by *Mycobacterium gordonae* (10/24) and MAC (4/24). Unlike with human and animal samples, identification of mycobacteria from environmental samples may be difficult due to the complexity of the environment and contamination of samples with other micro-organisms. In addition, there may be other factors that influence the distribution of NTM in different environments. Factors determining the survival and growth of NTM in the environment are still largely unknown. However, several factors like chemical properties of soil and water, seasonal and temperature differences as well as the methods used for assessing *Mycobacterium* diversity and distribution have been investigated.

Seasonal differences

Parashar *et al.*, (2009) observed seasonal difference in the isolation rate and diversity of *Mycobacterium* species between winter, rainy and summer seasons, with higher isolation rates and more variable *Mycobacterium* species in winter than in summer and rainy season. Similarly, Chilima *et al.,* (2006) noted a higher *Mycobacterium* recovery rate during the dry season than the wet season. The authors also noted geographic variations between the different regions and *Mycobacterium* counts during the wet and the dry seasons.

Temperature

The association of NTM presence with high temperatures was reported (Harvelaar *et al.*, 1985; Kirschner *et al.*, 1992). To the contrary, other studies associated NTM presence with low temperatures in water and

soil (Kamala *et al.,* 1994; Bland *et al.,* 2005; Chilima *et al.,* 2006), or did not observe effect of temperature on NTM counts (Le Dantec, 2002a). The effect of temperature on *Mycobacterium* count is still a controversial issue probably because survival of mycobacteria in different temperatures differs from species to species, may be influenced by moisture and linked to survival of other bacteria at different temperatures that can hamper the detection of NTM (Livanainen *et al.,* 1997).

Chemical treatment

The presence of many NTM species in municipal drinking water supplies has been associated with their innate chlorine and biocide resistance (Falkinham *et al.,* 2001; Bland *et al.,* 2005; Steed and Falkinham, 2006). Le Dantec *et al.,* 2002a have shown that resistance of different *Mycobacterium* species to free chlorine is dependent on the concentration as well as other factors like temperature and pH. For instance, *M. gordonae* and *M. aurum* were shown to be more susceptible to chlorine than *M. fortuitum* and *M. chelonae*. However, even the most susceptible *Mycobacterium* species are more resistant to chlorine than *E. coli* (Le Dantec *et al.,* 2002a). Chlorine resistance was also reported to be dependent on the type of matrix in which the cells are grown (Steed and Falkinham, 2006). It was shown that *M. avium* and *M. intracellulare* grown in biofilm were more resistant to chlorine than cells grown in solution (Steed and Falkinham, 2006).

pH

The association of the acidity of the environment with *Mycobacterium* survival has been noted. Several studies have suggested that an increase in acidity increases the count of mycobacteria and decreases the counts of activity of other heterophobic bacteria (Brooks *et al.,* 1984). However, other studies have noted a positive correlation between the *Mycobacterium* counts and the number of heterotrophic bacteria

(Livanainen *et al.,* 1997). Humic and fulvic acids have been shown to positively favor growth of *Mycobacterium avium* (Kirtchner *et al.,* 1999). Mycobacteria have been isolated from acidic soil and waters (Brooks *et al.,* 1984; Livanainen *et al.,* 1993; 1999; Niva *et al.,* 2006). Contrarily, other studies noted no significant correlation between pH and environmental *Mycobacterium* counts (Bland *et al.,* 2005; Chilima *et al.,* 2006).

Ability of mycobacteria to form biofilm

The ability of certain *Mycobacterium* species to form biofilms has been reported as a survival strategy. MAC, in particular *M. avium* and *M. intracellulare* (Steed and Falkinham, 2006), *M. kansassii*, *M. flavescens* , *M. fortuitum* and *M. chelonae* (Schulze-Röbbecke *et al.,* 1992; Hall-Stoodley and Lappin-Scott, 1998), *M. gordonae, M. gilvum, M. abscessus* (September *et al.,* 2004), *M. terrae* and *M. non chromogenicum* (Schulze-Röbbecke *et al.,* 1992)are capable of forming biofilms and hence they can persist in flowing systems like water distribution systems and rivers, although they grow slowly. Organic substances such as plastic and rubber were reported to enable a large number of mycobacteria to form biofilm than inorganic substances such as copper and glass (Schulze-Röbbecke *et al.,* 1992).

Different methods for identification of mycobacteria in the environment

Methods for isolation and identification of NTM species from environmental samples may affect reported NTM distribution and diversity. Currently there is no standard method for *Mycobacterium* identification from environmental samples. Traditional culture methods for *Mycobacterium* isolation employing different decontaminating agents at different concentrations as well as different growth media and incubation temperatures and periods have been investigated (Neumann *et al.,* 1997; Parashar *et al.,* 2004;

Thomson *et al.*, 2013). Decontamination is employed in order to kill a maximum of non-mycobacterial contaminants like other water borne or soil borne bacteria and fungi and a minimum mycobacteria. Others have employed the use of direct polymerase chain reaction (PCR), cloning, followed by sequencing of the target gene and others have used denaturing gradient gel electrophoresis (DGGE) and pyro sequencing (Niva *et al.*, 2006; Pontiroli *et al.*, 2013). *Mycobacterium* identification from cultured isolates is either done by phenotypic methods including demonstration of acid fast bacilli by Ziehl Neelsen staining followed by microscopy and biochemical tests (Bahram *et al.*, 2014). Molecular based identification methods like PCR and sequencing of Mycobacterium housekeeping genes has proven to perform more accurately than phenotypic tests (Le Dantec *et al.*, 2002b). Even better, combined molecular approaches have been shown to enable more accurate diagnosis of mycobacteria from environmental samples (Pontiroli *et al.*, 2013). The use of these molecular approaches, whether directly or on culture isolates, has provided an important contribution to the understanding of *Mycobacterium* taxonomy and diversity**.** However, use of different *Mycobacterium* isolation and identification methods from environmental samples in different studies may have led to selection of certain mycobacteria over the others, resulting in different diversities reported. In addition, NTM diversity and distribution studies in the environment in different countries have only focused on certain regions, and no proper country wide surveys have been conducted. Hence NTM distribution in the environment is still poorly understood.

Chapter One

Table 1.1: Summary of NTM diversity in environmental samples based on 24 studies published between the years 1994 and 2013 in different countries: 1. Kamala *et al.*, 1994 (India); 2. Thomson *et al.*, 2013 (Australia), 3 Chilima *et al.*, 2006 (Malawi); 4 Torvinen *et al.*, 2004 (Finland), 5 De Groote *et al.*, 2006 (USA); 6 Le Dantec *et al.*, 2002 (France), 7 Covert *et al.*, 1999 (USA); 8 Parashar *et al.*, 2009 (India); 9 Beran *et al.*, 2006 (Czech Republic); 10 Kankya *et al.*, 2011 (Uganda); 11 Rahbar *et al.*, 2010 (Iran); 12 Shitaye *et al.*, 2009 (Czech republic); 13 September *et al.*,2004 (South Africa); 14 Neumann *et al.*, 1997 (Germany), 15 Narang *et al.*, 2009 (India); 16 Bland *et al.*, 2005 (USA), 17 Bahram *et al.*, 2014 (Iran); 18 Pavlik *et al.*, 2007 (Czech republic), 19 Wang *et al.*, 2006 (Japan), 20 Pontiroli *et al.*, 2013 (UK/ Ethiopia), 21 Michel *et al.*, 2007 (S.A), 22 Niva *et al.*, 2006 (Finland); 23. Leoni *et al.*, 1999 (Italy), 24. Portaels, 1995.

Most frequently isolated NTM species in the study	**Study References**	**Sample type**	**Study setting, locale or country**	**Method used for assessing diversity**	**Other *Mycobacterium* spp isolated in the study**
M. fortuitum, M. smegmatis,	Bahram *et al.*, 2012	water	Different (SDW), Iran	Culture, biochemical tests	*M. chelonae like, M. terrae complex, M. gordonae and M. mucogenicum, M. avium complex, M. phlei, M.xenopi, M. fallax and M. flavescens*
M. kansasii, M. gordonae, M. mucogenicum	Thomson *et al.*, 2013	Water	Water distribution system (SDW) Brisbane Australia	Culture, PCR-sequencing	*M. abscessus, M. angelicum/szulgai, M. arupense, M. austroafricanum, M. bolletii/M. massiliense, M. chelonae, M. cookie, M. cosmeticum, M. diernhoferi, M. farcinogenes, M. flavescens, M. fluoranthenivorans, M. fortuitum complex, M. gadium, M. gilvum, M. gordonae, M. interjectum, M. intracellulare, M. kansasii, M. lentiflavum, M. mageritense, M. moriokaense, M. mucogenicum, M. poriforae, M. rhodesiae, M. sengalense* *M. simiae, M. species* NFI, *M. szulgai, M. terrae, M. tilburgii, M. triplex, M. wolinsky*

M. moriokaense, M. colombiense, M. riyadhense	Pontiroli *et al.*, 2013	Soil	Private land Ethiopia and UK	Direct PCR-DGGE, pyro sequencing	*M. chlorophenolicum, M. sp. GR* 2009-164; *M. neglectum* ; *M. vaccae; M. holsaticum, Mycobacterium sp*. T126 and *Mycobacterium sp.* DCY42, *M. gordonae, M. aemonae, M. asiaticum, M. malmoense* , *M. sp.* NLA000202017, and *M. angelicum*
M. fortuitum-peregrinum complex, MAC, M. gordonae, M. non chromogenicum	Kankya *et al.*, 2011	Soil, water, animal feces.	Pastoral community households, Central Uganda	Culture, PCR-sequencing	*M. arupense, M. senuense, M. terrae, M. hibernaiae, M. engbackii, M. kubicae, M. senuense, M. simiae, M. chubuense, M. vanbaalenii, M. parafortuitum*
M. fortuitum, M. peregrinum, M. flavescens	Rahbar *et al.*, 2010	Soil and natural man-made water	Soil, ponds, rivers, brooks, North west Iran	Culture and biochemical tests	*M. chelonae, M. mucogenicum, M. thermoresistible, M. abscessus, M. neoaurum, M. smegmatis*
M. chelonae, M. kansasii, M. intermedium	Shitaye *et al.*, 2009	Mixed samples	Czech republic	Culture on liquid medium, biochemical , *M. avium* PCR	Unknown Mycobacterium spp
M. fortuitum, M. avium	Narang *et al.*, 2009	Soil and water	India	Culture, biochemical and PRA.	*M. chelonae, M. flavescens, M. abscessus, M. thermoresistable, M. phlei*
M.chelonae, M. fortuitum	Parashar *et al.*, 2009	Soil and water	Man-made water systems and	Culture, biochemical tests,,	*M. avium, M. marinum, M. manitobense, M. kansasii, M. terrae, M. smegmatis, M. flavescens*

			garbage soil, Agra India	PCR-sequencing , PRA	
M. terrae, M. vaccae/ M. vanbaalenii	Michel *et al.*, 2007	water	South Africa	Culture, PCR-sequencing.	*M. engbackii*, Unknown *Mycobacterium* spp closely related to *M. moriokaense*, Unknown *Mycobacterium* spp closely related to *M. kansasii/ M. szulgai*,
MAC, *M. fortuitum,*	Pavlik *et al.*, 2007	Mixed soil and water, dust, spider nests, feed	Pig farm, Czech republic	Culture, biochemical tests, and PCR.	*M. nonchromogenicum, M. abscessus, M. scrofulaceum*, Unknown *Mycobacterium* spp.
M. fortuitum	Chilima *et al.*, 2006	Soil, water	Households and animal enclosures, Northern Malawi	Culture, and direct PCR-sequencing	*Mycobacterium* spp closely related to: *M. smegmatis*, *M. goodii*, *M. farcinogenes*, and *M. senegalense*
M. avium, M. intracellulare, M. gordonae, M. kansasii	De Groote *et al.*, 2006	Soil	USA	Culture, PCR and PRA	*M. szulgai, M. chelonae, M. peregrinum, M. simiae, M. terrae, M. interjectum, M. flavescens, M. asiaticum, M. fortuitum, M. porcinum, M. smegmatis*
M. vanbaalenii, M. mageritense, M. chubuense, M. fredericks-bergense	Wang *et al.*, 2006	Soil from dumping site	Dumping site in Japan	Culture, PCR-sequencing	*Mycobacterium austroafricanum Mycobacterium chlorophenolicum*

M. senegalense/M. porcinum/M. septicum/M. fortuitum, M. fallax	Niva *et al.*, 2006	soil and natural water	Boreal forest and lake, Southern Finland,	Direct PCR-DGGE- sequencing assay	*Mycobacterium cookii, M. elephantis, Mycobacterium* sp. IMVSB76676
M. fortuitum, M. marinum, M. gordonae, M. avium	Beran *et al.*, 2006	Feed, water, soil	Animal feed and drinking water, soil, Czech Republic	Culture, biochemical tests, PCR (*M. avium*)	*M. triviale, M. flavescens, M. kansasii, M. terrae, M. chelonae, M. abscessus, unknown Mycobacterium* spp.
M. fortuitum complex, *M. gordonae*	Bland *et al.*, 2005	Natural water	River, South West USA	Culture on 7H11 medium, biochemical tests, PCR-sequencing, PRA (combined)	*M. avium, M. kansasii, M. scrofulaceum, M. simiae, M. gordonae, M. intracellulare, M. intermedium, M. smegmatis, M. interjectum, M. lentiflavum, M. nonchromogenicum, M. celatum, M. chitae, M. phlei, M. malmoense, M. marinum, unknown Mycobacterium* spp
M. lentiflavum, M. tusciae, M. gordonae	Torvinen *et al.*, 2004	Man-made water, biofilms	Water distribution systems, Finland	Culture, biochemical tests and PCR-sequencing	Unknown *Mycobacterium* spp
M. gordonae, M. gilvum	September *et al.*, 2004	biofilm	Man-made water system, South Africa	Culture on 7H11,PCR-sequencing	*M. abscessus, M. fortuitum, M. septicum,* Unknown *Mycobacterium spp*

M. gordonae, M. nonchromogenicum	Le Dantec *et al.* 2002b	water	(SDW) France	Culture, PCR-sequencing.	*M. aurum, M. gadium, M. fortuitum, M. peregrinum, M. intracellulare, M. flavescens, M. chelonae,* Unknown *Mycobacterium* spp
M. gordonae, M. fortuitum, M. marinum	Leoni *et al.*, 1999	Water,	Swimming pool, surfaces, Italy	Cultured on 7H10 and biochemical tests	*M. marinum, M. chelonae, M. flavescens, M. phlei, M. terrae*
M. mucogenicum, M. gordonae, M. fortuitum	Covert *et al.*, 1999	Man-made water	Drinking water systems USA	Cultured, PCR-sequencing.	*M. intracellulare, M. gastri/M. kansasii, M. fortuitum, M. peregrinum, M. scrofulaceum, M. avium, M. chelonae,* Unknown *Mycobacterium* spp
M. gordonae, M. chelonae, M. mucogenicum	Neumann *et al.*, 1997	Man-made water	Drinking water reservoirs, Germany	Cultured, Thin layer chromatography, and PRA	*M. kansasii, M. nonchromogenicum, M. huberniae, M. peregrinum*
MAC, *M. scrofulaceum, M. fortuitum*	Kamala *et al.*, 1994	Soil, natural water dust	India, Madras	Biochemical on isolates cultured on LJ.	*M. diernhoferi, M. gadium, M. parafortuitum, M. phlei, M. smegmatis, M. thermorestible, M. vaccae, M. asiaticum, M. kansasii, M. malmoense, M. szulgai, M. xenopi,* Unknown *Mycobacterium* spp
MAC, *M. scrofulaceum, M. nonchromogenicum, M. terrae, M. fortuitum*	Portaels, 1995	Natural and man-made environments (Soil, water, mud, grass)	Zaire (Democratic republic of Congo), and USA*	Culture, and biochemical tests	*M. gordonae, M. parafortuitum, M. marinum, M. chelonae**

SDW, water distribution system; PCR, polymerase chain reaction; PRA, PCR-restriction endonuclease analysis; DGGE, denaturing gradient gel electrophoresis; spp, species; *, only isolated in USA in a study by Portaels, 1995 .

1.2.6 Bovine tuberculosis: Epidemiology and Pathology

Bovine tuberculosis causes significant losses in agriculture worldwide and is a public health hazard (Smith *et al.,* 2003; Phillips *et al*., 2003; Biet *et al.,* 2005). More than 50 million cattle are infected with *M. bovis* resulting in global economic loss of approximately 3 billion dollars a year (Schiller *et al.,* 2010). This disease has been has been reported in many species including domestic cattle, sheep, goat, pig, horse, cat, dog, deer, bison, buffalo, badger, possum, hare, ferret, wild boar and feral pig, antelope, Ilama, alpaca, and nonhuman primates (O'Reilley and Darbon, 1995, Hlokwe *et al.,* 2014).

Human infections with this micro-organism occur when infected, unpasteurized milk or infected meat is ingested, or when there is close contact with animals that have the disease. Transmission by ingestion often leads to extra-pulmonary TB where infection is usually seen in the cervix and axillary lymph nodes. The aerogenous infection of humans by aerosols from infected cattle also occurs. (Cosivi *et al.,* 1998; Kazwala, 1998; Biet *et al.,* 2005; Shitaye *et al.,* 2007). The incidence of bovine tuberculosis in humans is much lower than the disease caused by the human tubercle bacillus; however, the AIDS epidemic has increased the risk of transmission of *M. bovis* to humans. In fact, HIV infection results in increased susceptibility to all forms of mycobacterial disease (Collins, 2000, Karne *et al.,* 2012). This not only poses a risk of infection for other humans, but results in livestock and other animals being exposed to higher levels of BTB (Wedlock *et al.,* 2002). *M. bovis* pulmonary infections in humans are clinically, radiologically and pathologically indistinguishable from that caused by *M. tuberculosis* (Cosivi *et al.,* 1998).

Transmission amongst animals and from humans may occur from cases with open tuberculosis via the aerogenous route, spitting, coughing, congenital route, urinating or ingestion of contaminated material (Phillips *et al.,* 2003; Ayele *et al.,* 2004; Shitaye *et al.,* 2007). The pattern of lesions observed in

slaughtered animals is indicative of the route of transmission. Transmission by inhalation of aerosols is thought to result in the formation of lesions restricted to thoracic cavity, while animals with lesions in mesenteric lymph nodes are presumed to have been infected by ingestion (Pollock and Neill, 2002). The most common route of cattle-cattle as well as wildlife-wildlife transmission is the aerogenous route facilitated by respiratory excretion and inhalation of the bacterium (horizontal transmission). Vertical transmission is another alternative way of aerogenous transmission of the bacillus from cattle-cattle or wildlife-cattle and wildlife-wildlife. It occurs by inhalation of the contaminated environmental material like dust and grazing in infected pastures (Phillips *et al.*, 2003; Biet *et al.*, 2005). Transmission by ingestion (the oral route) is considered a secondary route of *M. bovis* transmission in cattle and occurs in scavenging animals like the cheetah and lion through ingestion of contaminated meat of their prey (De Lisle *et al.*, 2002).

Transmission studies suggest that the success of transmission is dependent on various factors such as host susceptibility, infective dose, frequency of excretion, the route of infection and the period of communicability. Animal experimental studies by Buddle *et al.*, (1994), and Neill *et al.*, (1988), where cattle were infected intratracheally and intranasally with different doses of *M. bovis* provided evidence that the infective dose has a profound influence on the severity of the diseases. Dean *et al.*, (2005), demonstrated that < 10 viable bacteria are sufficient to establish TB pathology that is similar to that seen in natural infection. Transmission studies conducted with housed cattle have also shown that bacterial shedding involves low numbers of the bacilli, suggesting that natural infection is usually initiated by a relatively low dose of *M. bovis* (Neill *et al.*, 1988; McCorry *et al.*, 2005). Transmission of *M. bovis* in many species is primarily by the aerogenous route.

During the course of infection, bacteria gain access to macrophages, once present in phagosomes they prevent their fusion with lysosomes, hence can multiply undisturbed (Aldwell *et al.*, 1997). When the pathogen reaches sufficient numbers, the cell dies and the released organisms are ingested by freshly

recruited monocytes or macrophages. The infected macrophages produce cytokines and chemokines that attract other phagocytic cells including monocytes, other alveolar macrophages and neutrophils which eventually form the tubercle lesions (Dannenberg and Rook, 1994; van Crevel *et al.*, 2002; Ernst, 2012). The organism is subsequently disseminated to the lymph nodes and blood stream, and is deposited in the liver, spleen, kidney, bone, brain, meninges and the lung. Infected animals can remain for years without any clinical signs of disease even in advanced stages, until they are sent for slaughter (Collins *et al.*, 2000).

1.2.7 Control of bovine tuberculosis in cattle and wildlife animals

1.2.7.1 Test and slaughter

M. bovis eradication or reduction to very low levels in cattle has been achieved by implementation of control programs based on the test and slaughter principles, due to lack of effective vaccines. In some countries abattoir surveillance is used as a control strategy (Schiller *et al.*, 2010). Accurate diagnosis of BTB is crucial for these control strategies to be effective. With the advent of tuberculin, control programs based on test and slaughter were initiated as early as 1917 (Cousins and Roberts, 2001). These strategies included diagnosis via the tuberculin based tests, isolation of affected herds, sub-isolation of infected animals within affected herds, slaughter of the infected animals, and border testing policies. In countries with low BTB prevalence, these strategies were successful (Buddle *et al.*, 2006; Hope and Villareal-Ramos, 2008). In Australia BTB was officially eradicated in 1997 (Cousins and Roberts, 2001), and BTB prevalence was reduced from 5% to 0.0002% in USA (Palmer *et al.*, 2011). However, wildlife species such as the badger in the United Kingdom and Ireland (Gormley and Collins, 2000; Griffin *et al.*, 2005; Corner *et al.*,2011), the brush tail possum in New Zealand (Wedlock *et al.*, 2002), Cape buffalo and kudu

in South Africa (Bengis *et al.,* 2001), white-tailed deer and the bison in the United States of America and Canada, wild boar in Spain (Aranaz *et al.,* 2004), and several others elsewhere, are considered maintenance hosts for *M. bovis*. These species act as reservoirs of (re-) infection of both domestic animals and other wildlife species and thus are a severe hindrance to control and eradication programs in these countries. Once bovine tuberculosis establishes itself in a wildlife maintenance host, eradication of the disease becomes highly unlikely (Michel *et al.,* 2006).

Host immune responses are important for diagnosis and control of bovine tuberculosis

A spectrum of immune responses develops in course of time after infection with *Mycobacterium bovis* and (Schluger and Rom 1998). These are generally categorized as cellular and humoral (antibody) immune responses. The cellular immune responses consist of both innate and the adaptive components and is driven by the intracellular nature of the bacillus. T cells respond to infection through production of several cytokines such as among others interferon gamma (IFNγ) which is considered to be involved in activation of macrophages, neutrophils, monocytes and dendritic cells (Ernst, 2012). Cell mediated immune responses to the tubercle bacilli have been shown by *in vitro* assays and skin testing *in vivo* in experimental animal models including mice, guinea pigs and cattle. These models have yielded considerable information on the mechanism of immunity (Ernst, 2012). The prominent role of IFNγ, thought to be protective, is well established and therefore widely used as a marker of infection/protection**.** Other cytokines produced in *M. bovis* specific immune responses may have a role in diagnosis of BTB. These are among others, tumor necrosis factor, (TNF-α), interleukin 1 beta (IL-1ß) (Jones *et al.* 2010), IL-6, IL-12, IL-18, IL15, IL 17 (Armanda-Cortes *et al.,* 2012). It is generally accepted that cell mediated responses predominate early in the disease and Interferon gamma, measured in the IFNγ assay also

known as the Interferon gamma release assay (IGRA) almost co-incides with a positive tuberculin skin test and later on humoral responses develop, reflected by the presence of antibodies in serum as illustrated in Figure 1.5. Finally cell mediated immune responses may gradually wane. While it is generally accepted that very recently infected cattle fail to respond to the tuberculin skin test, there is an uncertainty on the actual time course of the infection/immune responsiveness. (Pollock and Neill 2002, Welsh *et al.*, 2005). The shift from cellular immune response to antibody production is associated with progression to clinical signs of BTB. (Yaersley *et al.*, 1998). The most widely used immunological diagnostic test internationally for control of bovine tuberculosis through test and slaughter strategies is the tuberculin skin test. So far the IFN gamma assay is applied either as a confirmatory test of skin test reactors or alongside the TST to increase the sensitivity (Schiller *et al.*, 2010). Both assays measure cell mediated immunity (CMI).

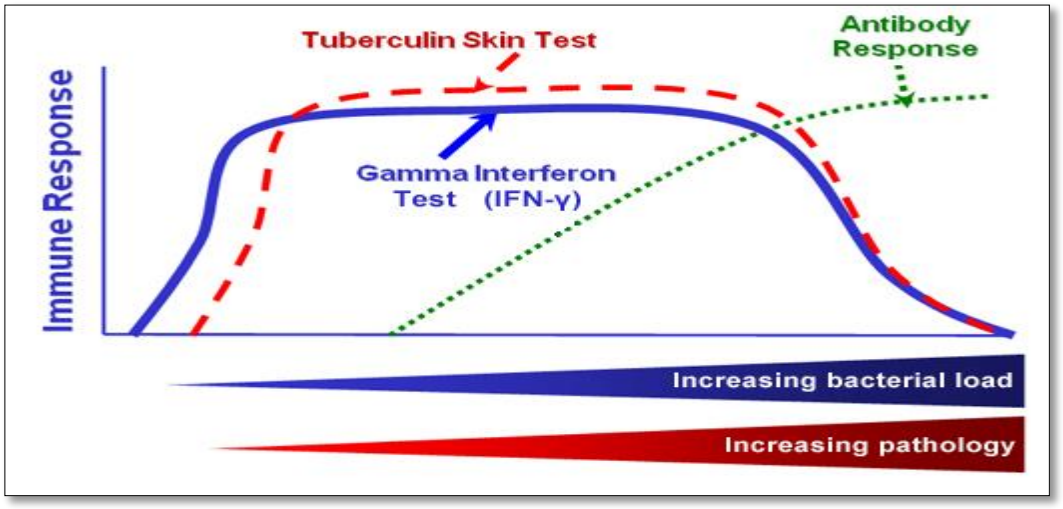

Fig 1.5: Immunology of tuberculosis (extract from Pollock and Neill, 2002)

1.2. 7.1.1 Cell mediated immunity based assays for BTB.

Tuberculin skin test (TST)

TST has been a convenient and cost effective method for assessing cell mediated immune responses to a variety of antigens (Schiller *et al.,* 2010). TST measures dermal swelling which peaks three days after intradermal injection of purified protein derivative (PPD). BTB eradication programs around the world have successfully relied on the TST to diagnose *M. bovis* infection in cattle and some wildlife species. Two types of TST are in use *i.e.* the single intradermal test (SIT) and the single intradermal comparative cervical tuberculin test (SICCT). Both rely upon the delayed type hypersensitivity response to tuberculins or PPD. The SIT uses PPD made from *M. bovis* (PPD-B) alone and can be carried out as the caudal fold test (CFT) (mainly used in countries like USA, Australia and New Zealand) or as an injection into the skin on the side of the neck. The SICCT (mainly applied in countries like South Africa and other parts of Africa) uses PPD-B and PPD prepared from *Mycobacterium avium* (PPD-A) (Vordermeier *et al.,* 2006). PPD-A is used as a control antigen representative of the background responses induced by environmental mycobacterial antigens (Schiller *et al.,* 2010). Responses to bovine and avian tuberculins are then compared according to OIE guidelines (standard interpretations) or those developed by national eradication programs (Schiller *et al.,* 2010). For standard interpretations, if the reaction to bovine tuberculin is more than 4mm greater than the reaction to avian, the animal is considered to be infected with BTB and is called a reactor (OIE-terrestrial manual, 2009). Sometimes severe interpretations involving lowering the cut-off for animals to be classed as reactors are used so as to enhance the sensitivity of the test (de la Rua-Domenech, 2006).

Several lines of evidence support very high estimated specificity of the CFT. Estimates of as high as 98% have been reported in Australia (Whipple *et al.,* 1995) and 98% in USA (Francis *et al.,* 1976). The test's

relative sensitivity remains moderate, with estimates ranging from 55.1%-90.9% when the standard interpretations are used (Neill *et al.,* 1994; Costello *et al.,* 1997). Sensitivity estimation of TST is challenging as it is always based on animal population it is applied to but not necessarily the population of animals the test will be used on. Therefore sensitivity may differ from country to country. In addition, sensitivity of the SICCT is reported to increase when severe interpretations are used instead of standard interpretation, but at the expense of reduced test specificity. SIT is found to have a relatively higher sensitivity than SICCT, but the test specificity is compromised by lack of the control of immune responsiveness to environmental mycobacteria (de la Rua-Domenech, 2006).

The main disadvantages of the TST test in Africa, especially in the wildlife context, are the need for trained technicians for correct reading and interpretation of the skin swelling and the fact that reading can only be done 72 hours after intradermal injection of tuberculin, which requires two immobilizations and potentially a period of captivity (Michel *et al.,* 2011). Test performance may vary because of differences in tuberculin doses, commercial PPD preparations used and differences in interpretation schemes (Schiller *et al.,* 2010).

Interferon-gamma (IFNγ) assay

The IFNγ assay measures the production of this Interferon gamma after antigenic stimulation of whole blood with PPD (Schiller *et al.,* 2010). As such it broadly measures the same response as the intradermal skin test. Blood samples are collected and transported to the laboratory within 8 hours of sampling where they are stimulated with PPD-B and PPD-A and incubated at 37°C. The supernatant is harvested after 24 hours and the IFNγ produced is quantified by an ELISA using the commercial BOVIGAM™ kit (Prionics, Netherlands/ Switzerland). The BOVIGAM™ assay is applied only in bovine species. In its basic form, the BOVIGAM™ test positivity is determined by comparing responses to PPD-B and PPD-

A. An advantage of the IFNγ assay is that both sensitivity and specificity can be adjusted by altering the criteria (cut-off values) for defining a positive reactor (Whipple *et al.,* 2001; Palmer *et al.,* 2006). Other practical advantages of the IFNγ assay as an *in vitro* assay include more repeat testing when necessary without having to wait for several days between the TST to avoid desensitization of reactivity of the animals towards the tuberculins. Furthermore the IFNγ assay gives an opportunity to employ a range of *M. bovis* specific (recombinant) antigens or PPDs prepared from other mycobacteria when maximizing specificity or relative sensitivity. Different studies have reported estimates of the assay sensitivity ranging from 73% - 100% and specificity from 85%-99.6% (de la Rua-Domenech, 2006). Just like with the skin test, these discrepancies could be due to among other factors, differences in the commercial PPD preparation and test protocols. Even though the relative sensitivity of IFN gamma assay is reported by several studies to be higher than that of the TST, lower test specificity raises a lot of concerns (Schiller *et al.,* 2010). Efforts to improve the specificity involved replacement of the PPD with defined antigens (Vordermeier *et al.,* 1999).With the advancement in molecular biology (and in particular the elucidation of the genomes of *M. bovis*, *M. bovis* BCG and *M. tuberculosis* and the use of comparative genomics), identification of regions in the genomes of *M. tuberculosis* and *M. bovis* which are absent in the *M. bovis* BCG strain and most NTM species provides an opportunity for the development of new diagnostic tools which can be useful in improving the specificity of the IFNγ assay (Pollock *et al.,* 2003). These *M. tuberculosis* complex 'specific' regions encode a number of proteins that are immunogenic. One example is the use of the Region of Difference 1 (RD1) in the *M. tuberculosis* complex, containing operons encoding dominant T-cell antigens. The predominant antigens in this region are the Early Secreted Antigenic Target-6 (ESAT-6), and Culture Filtrate Protein 10 (CFP 10). Genes encoding the ESAT-6 and CFP 10 antigens have been shown to be absent in *M. bovis* BCG strains and most NTM strains that have been investigated so far, but present in the members of the *M. tuberculosis* complex (Harboe *et al.,* 1996). Due to their absence in many of the species, especially *M. bovis* BCG, tthese antigens are

nowadays employed in whole blood IFNγ assays for the detection of *M. tuberculosis* complex infection (Pollock *et al*., 2003). Although the use of a single antigen or a pool of defined antigens might be more specific than the use of PPD, sensitivity might be compromised. Several studies using ESAT-6 and CFP 10 have overall demonstrated a reduced sensitivity of approximately 10% compared to PPD (Pollock *et al.,* 2000; Buddle *et al.,* 2003; Vordermeier *et al*., 2006). Therefore, a wider range of antigens in addition to antigens like ESAT-6 and CFP 10 is needed to improve sensitivity. Several other defined antigens encoded in the *M. bovis* genome and not in BCG have been evaluated as diagnostic markers for BTB. When a cocktail of ESAT-6, CFP 10 combined with Rv3615c was used an improved specificity of 97% and relative sensitivity of 89% was demonstrated (Vordermeier *et al.,* 2009).

1.2.7.1.2. Antibody based assays for BTB diagnosis:

Application of antibody-based assays for TB diagnosis and surveillance offer the possibility of convenient, flexible and cost-effective test platforms. The establishment of these assays has proven to be stagnant most probably due the slow and progressive nature of the disease; the assays cannot diagnose early infections. In the early development of these assays, the use of crude mycobacterial preparations generated satisfactory sensitivity but specificity was very poor, probably due to cross reactivity with NTM. Identification of defined immuno-dominant antigens for use in the different antibody assays offered potential to improve specificity (Schiller *et al.*, 2010). These antigen discovery efforts have led to the development of a number of antibody based assays employing single antigens, antigen cocktails or multiple epitope fusion peptides. The two most sero-dominant antigens of *M. bovis* are MPB70 and MPB83 have been applied in several of these assays. MPB83 has been the cornerstone in development of the assays employing multiple-antigens (Schiller, 2010). Antigen cocktails and multi epitope fusion proteins have been used to demonstrate improved sensitivity (Waters *et al.,* 2006; Lyashchenko *et al.,*

2008). The multiple antigen print immunoassay or MAPIA has been useful for screening responses by various host species to determine antigen recognition pattern and kinetics of responses to various antigens during the course of infection or even in response to antibiotic therapy. The optimal antigens (improved specificity and sensitivity) as defined by MAPIA, were incorporated in a lateral flow immunoassay format, called the 'Rapid test' (Greenwald *et al.*, 2003, Michel and Simões, 2008). The sensitivity and specificity estimates of the 'Rapid test' in badgers were reported to be 53% and 98% respectively, an improvement from the ELISA test employing a single MPB83 antigen (sensitivity estimates of 47% and specificity of 89%) (Greenwald *et al.*, 2003). Other promising developments include a fluorescence polarization assay (FPA) (Jolley *et al.*, 2007), a 96- well plate multiplex system (Whelan *et al.*, 2008), a dual platform assay (Green *et al.*, 2009), a chemilumiscent platform (Green *et al.*, 2009), and an improved ELISA (Schiller *et al.*, 2010). Field studies with these assays have shown to be encouraging, with high estimated specificities of >95% (Meyer and Orloski, 2007; Whelan *et al.*, 2008). However, exact estimates of test accuracy under varying herd prevalence rates are still pending and more work still needs to be done to improve the sensitivities of these assays (Schiller *et al.*, 2010). The FPA detects antibody responses to a small peptide of MPB70. Though the assay is highly specific (>99%), it has also been shown to lack sensitivity (26%), probably due to the nature of bovine immune responses to this antigen (Meyer and Orloski 2007; Ngandolo *et al.*, 2009; Schiller *et al.*, 2010). A systematic review and meta-analysis of 67 studies published between May 2006 and June 2010, based on serological TB assays for pulmonary and 25 studies of extra- pulmonary human TB revealed that, these assays produce inconsistent estimates of sensitivity (0%-100% for both pulmonary and extra pulmonary) and specificity (31%-100% for pulmonary and 59%-100% for extra pulmonary TB). The authors concluded that quality evidence still remain low for serological diagnosis of human TB (Steingart *et al.*, 2011).

1.2.7.2 Vaccination as a strategy for BTB control

Vaccination of livestock and wildlife represents an alternative to test and slaughter for control of BTB that should prevent the disease from persisting in the maintenance host on the longer term. Vaccination of livestock against BTB could be an import control strategy for BTB especially in countries with a wildlife reservoir or that cannot afford implementation of the 'test and slaughter' control strategy (Buddle *et al.*, 2013). Currently the only registered vaccine against BTB is the human vaccine, the *M. bovis* BCG. The development, successes and failures of the attenuated *M. bovis* BCG vaccine strains for human immunization against TB have been documented (Anderson and Doherty, 2005). Clinical trials evaluating the efficacy of BCG in humans have demonstrated variability in efficacy, ranging from 0% in South India and Africa to 80% in the UK (Brandt *et al.*, 2002; Black *et al.*, 2002). High levels of exposure to NTM of human populations where BCG vaccination is less effective have triggered investigation of the effect of NTM in both humans and animals. For instance, protection against pulmonary TB after BCG vaccination could not be observed in Africa and South India where NTM-PPD responders are more abundant than in UK (Black *et al.*, 2002). Likewise variable efficacy of BCG vaccination has been observed in cattle (Buddle *et al.*, 2011). There are several proposed explanations for this variability, but the most compelling hypothesis is the interference or inhibition of protection by environmental mycobacteria (Fine *et al.*, 1995; Buddle *et al.*, 2002; Thom *et al.*, 2008).

1.2.7.2.1 BCG vaccination against tuberculosis in cattle

BCG was first evaluated in cattle ten years before its delivery to a human infant in 1921 (Locht, 2010). Since the 1900s numerous experimental and field studies have been performed in cattle to investigate the use of BCG as a vaccine against BTB and efficacies reported in cattle ranged from complete success (Calmette and Guérin, 1920; 1924; Sibgatullin, 1982), minimal benefit (Haring *et al.,* 1930; Doyle and Stuart, 1958) to no benefit (Watson, 1928; Berggren, 1977; 1981). Several other studies conducted have failed to demonstrate a suitable level of efficacy of BCG in cattle (Waters *et al.,* 2012). Potential reasons for BCG failure in these field trials in general included the use of high doses of BCG (10^8-10^{10} CFU) now known to be less effective than lower doses; BCG trials were often performed in regions with high *M. bovis* prevalence and cows may have been exposed to *M. bovis* early in their lives (Waters *et al.,* 2012). Other tested parameters included the suitable routes of delivery which ranged from oral, intravenous, intradermal and subcutaneous; and also the use of killed or live bacilli, and single or booster doses (Haring *et al.,* 1930, Buddle *et al.,* 2011).

Even though the study designs, bacterial strains used as well as the doses and routes of infections in most of initial studies and the more refined recent studies varied, additional observations are still applicable, such as: single dose provides significant protection against subsequent experimental challenge with virulent *M. bovis*, field vaccination results in variable efficacy (0%-80%), live bacilli are required for protection , and re-vaccination does not improve efficacy and vaccine can be delivered orally but needs higher doses to achieve equivalent protection with parenteral administration (Buddle *et al.,* 2003; Wedlock *et al.,* 2011; Buddle *et al.,* 2011; Buddle *et al.,* 2013). In addition, in recent BCG field trials it was shown that vaccination at birth induces high levels of protection; sensitization of very young animals

to environmental mycobacteria by 6 weeks of age do not affect the effectiveness of BCG (Howard *et al.,* 2002; Buddle *et al.,* 2003; Hope *et al.,* 2005; de Lisle *et al.,* 2005; Thom *et al.,* 2008); revaccination of neonatal calves (after 6 weeks) with BCG reduces the protective effect of BCG (Buddle *et al.,* 2003); Pasteur and Danish strains provide similar efficacy (Wedlock *et al.,* 2007); 10^4 - 10^6 CFU of BCG induce similar levels of protection (Buddle *et al.,* 1995). Immunization with *Mycobacterium avium* has been shown to induce some protection against *M. bovis* (Buddle *et al.* 2002; Hope *et al.,* 2005; Bastida and Juste 2011). Highlights on the history of BTB vaccine research as reviewed by Waters *et al.,* 2012 are summarized in Table 1.2.

A recent study for the evaluation of BCG by field trials in cattle performed in Mexico indicated a reduction in the number of cattle being infected **(**Buddle *et al.,* 2011). Another study in Ethiopia demonstrated an encouraging protective effect of BCG, showing at least 56% protection of cattle against bovine tuberculosis in a natural transmission setting (Ameni *et al.,* 2010).

Another major problem with the use of BCG vaccination in cattle is that vaccination causes sensitization thus compromising diagnosis by tuberculin based tests like TST or interferon gamma assay. This is due to the fact that most antigens present in the *M. bovis* purified protein derivative (PPD) are also present in *M. bovis* BCG. Differentiation of infected from vaccinated animal (DIVA) strategies using defined antigens that are absent in BCG but present in MTBC, have been applied for the assurance of infection status prior to movement of vaccinated cattle.(Vordermeier *et al.,* 1999;Millington *et al.,* 2011; Marongui *et al.,* 2013).

1.2.7.2.2 Other candidate vaccines against BTB in cattle

In spite of efforts to test other mycobacterial strains as vaccine candidates, like *Mycobacterium microti* and *Mycobacterium vaccae*, *M. bovis* BCG is still the most promising vaccine candidate for BTB. *M. vaccae* was not shown to be effective in cattle against experimental challenge with *M. bovis* (Waters *et al.*, 2012). Practical applications of *M. microti* as a vaccine were infeasible despite the demonstrated protection superior to BCG and the fact that revaccination after 4-6 months provided boosting of the immunity, probably due to variations of its virulence in cattle (Waters *et al.*, 2012). There have been several other approaches for TB vaccine development in cattle including the use of: attenuated *M. tuberculosis* complex strains, DNA vaccines, protein sub-unit vaccines and live-virus vectored vaccines. The review by Buddle *et al.*, 2011, of vaccination trials conducted in cattle using different vaccines and showing variable effects is summarized in table 1.3.

1.2.7.2.3 BCG vaccination trials in wildlife for control of BTB

Vaccination of wildlife species is being considered by several countries as a strategy to control BTB (Buddle *et al.*, 2013). It is believed that problems associated with TB vaccination in cattle may not necessarily apply in vaccination of wildlife and protection does not have to be complete (Buddle *et al.*, 2013). In most countries, diagnostic tests are rarely available/ used for wildlife. In South Africa however, it is a regulation that buffaloes be tested for BTB and other diseases before movement (http://www.daff.gov.za). Vaccination in wildlife can, however, present several challenges. The choice of vaccine is limited as the most practical method of vaccine delivery is by oral baits and revaccination of animals may not be a practical option (Buddle *et al.*, 2011). Challenges associated with control of variables relevant to oral delivery include: dose of vaccine consumed, the number of animals receiving

the vaccine, age of vaccination, uptake by non-target species, prior exposure to *Mycobacterium* species, and vaccine viability in the field (Waters *et al.,* 2012; Buddle *et al.,* 2013). An alternative strategy for some wildlife species would be to capture, TB test, and mark, vaccinate (parenteral) and release the animal. This strategy can be applied in a limited area as it is too labor intensive and costly for large scale applications (Buddle *et al.,* 2013).

Studies have been conducted to evaluate the critical variables that influence the efficacy, safety, and application of BCG in several wildlife species including the white tailed deer, (Nol *et al.*, 2008; Palmer *et al.*, 2009), brush tail possums and ferrets (Aldwell *et al.*, 2003; Buddle *et al.,* 2006; Tompkins *et al.,* 2009; 2013), Eurasian badgers (Corner *et al.*, 2008a; b; c; Lesellier *et al*., 2009; Chambers *et al.*, 2011); wild boar (Garrido *et al.*, (2011) and African buffalo (de Klerk *et al.,* 2010).

Results on the efficacy ranged from no protection in African buffaloes (de Klerk *et al.,* 2010), poor in wild boar (Garrido *et al.,* 2011) and to a very high level of efficacy (95%) in possums (Tompkins *et al.,* 2009). Some of the observations from these trials are that there was no difference in performance between the Danish and the Pasteur strains and that there was no difference observed when a 10^7 or 10^8 CFU were used in possums (Buddle *et al.,* 2006). It was also shown that protection of badgers increased when intramuscular vaccination was increased by tenfold to 10^6 CFU (Lesellier, *et al.,* 2011). Oral or subcutaneous vaccination of white tailed deer was shown to significantly reduce the severity of the disease (Palmer *et al.,* 2007; Nol *et al.,* 2008). Another observation was that BCG could persist for nine months in deer vaccinated subcutaneously (Palmer *et al.,* 2009).

Other vaccine trials included evaluation of attenuated *M. bovis* vaccine which gave more protection in brush tail possums against experimental tuberculosis infection than BCG (Collins *et al.,* 2007). On the other hand, killed *M. bovis* as a vaccine evaluated against BTB in wild boar gave some protection, as did

the BCG (Garrido *et al.*, 2011). Re-vaccination of wild boar with BCG showed strong protective response (Gortazar *et al.*, 2014). An increased protection against BTB was observed in brush tail possum when a heat killed *M. vaccae* was used in combination with BCG (Skinner *et al.*, 2002). A summary of recent trials for wildlife vaccination is shown in table 1.4, adopted from Buddle *et al.*, 2013.

Table 1.2: Highlights in tuberculosis vaccine research from 1901-2010 (reviewed by Waters *et al.*, 2012)

Year	Findings and relevance	References
1901	Successful immunization of cattle with human-derived tubercle bacilli attenuated by lengthy propagation stating: that combating bovine tuberculosis only means a step on the road that will finally lead to the effective prevention of human tuberculosis.	http://www.nobel_prizes/medicine/laureates/1901/behring-lecture.html
1911	Vaccination with *M. bovis* (Nocard strain, 200 mg) attenuated by serial passage on glycerol soaked potato slices in ox bile (*i.e.* BCG) protects cattle (n = 9) from intravenous challenge with virulent *M. bovis*	Calmette and Guérin, 1911
1920	BCG (20 mg) vaccinated cattle have reduced numbers of lung lesions as compared to non-vaccinates in a field efficacy trial (in-contact exposure)	Calmette and Guérin, 1920; Calmette and Guérin, 1924
1930	Live bacilli (BCG) are required to develop a protective response as dead bacilli provide minimal protection	Haring *et al.*, 1930

1939	Low dose oral (5 mg) fails to protect; however, high dose (50 mg) provides moderate protection (finding confirmed by Buddle *et al.,* 2011)	Buxton and Glover, 1939
1955	BCG vaccination combined with segregation of vaccinates successfully clears infection in 28 TB-affected cattle herds	Scheller and Gaggermeier, 1959
1972	BCG provides greater protection in purebred Zebu cattle as compared to cross-bred Zebu cattle; thus, host genetic factors impact vaccine efficacy	Ellwood and Waddington 1972
2002	Exposure to environmental non-tuberculous *Mycobacterium* spp. affect BCG efficacy	Buddle *et al.,* 2002; Hope *et al.,* 2005; Horward *et al.,* 2002; de Lisle *et al.,* 2005*;* Thom *et al* 2008
2002	Modern pathology scoring system described now widely accepted	Vordermeier *et al.,* 2002
2002	First use of RD1-based DIVA approach with BCG vaccination	Vordermeier *et al.,* 2002
2003	Neonatal vaccination of cattle with BCG. Early study by Fritzsche in 1956 is confirmed by several studies in 2003-2009; neonatal vaccination with attenuated *M. bovis* is protective circumventing potential undesirable impacts of exposure to non-tuberculous Mycobacterium spp.	Buddle *et al.* 2008; Hope *et al.,* 2005; Endsley *et al.,* 2009; Fritzsche, 1956; Skinner *et al.,* 2005

2003	BCG booster immunization does not improve efficacy	Buddle *et al.*, 2003
2005 - 2010	Improved efficacy of BCG plus subunit vaccination as compared to BCG alone, provides potential for booster vaccination	Wedlock *et al.* 2005a; Vordermeier *et al.*, 2009; Skinner *et al.*, 2005; Skinner *et al.* 2003; Cai *et al.*, 2006; Wedlock *et al.*, 2005b; Maue *et al.*, 2004?
2007	Commercially prepared lyophilized BCG Danish provides equivalent protection as does frozen BCG Pasteur; thereby a licensed product is available for use in cattle	Wedlock *et al.*, 2007
2009	Vaccine-elicited T cell central memory (TcM) immune responses correlate to protection with BCG or *M. bovis* RD1 vaccines	Waters *et al.*, 2009; Vordermeier *et al.*, 2009; Whelan *et al.*, 2008
2010	Development of DIVA skin test approach for cattle	Whelan *et al.*, 2010
2011	Production of cytokines such as IL/L2 that predict vaccine efficacy when measured after vaccination but before M. bovis challenge	Bhuju *et al.*, 2012

The table was extracted from Waters *et al.*, 2012 and modified.

Table 1.3: Recent vaccination trials in cattle to assess protection against bovine tuberculosis (2005-2009) (reviewed by Buddle *et al.*, 2011)

Type of vaccine	Vaccination strategy	Protection	Protection compared to BCG [a]	References
Adjuvanted subunit vaccines	Protein	Yes	<	Wedlock *et al.* (2005a); McNair *et al.* (2007)
	Protein + BCG	Yes	>	Wedlock *et al.*, 2005b ;Wedlock *et al.*, 2008
Virus-vector vaccines	Recombinant viruses expressing mycobacterial protein + BCG	Yes	>	Vordermeier *et al.* (2009)
Mycobacterium tuberculosis complex attenuated vaccines	*M. tuberculosis* double deletion mutant	No	No protection	Waters *et al.* (2007)
	Mycobacterium bovis auxotroph	Yes	Not tested	Khare *et al.* (2007)
	M. bovis deletion mutant	Yes	=	Waters *et al.* (2009)
DNA vaccines	Mycobacterial DNA	Yes	=	Maue *et al.* (2004), Cai *et al.* (2005)
	Mycobacterial DNA + BCG	Yes	>	Skinner *et al.* 2003 and Skinner *et al.*, 2005; Maue *et al.* (2007); Cai *et al.* (2006)

This table was extracted from Buddle *et al.*, 2011.

[a] Greater than (>), less than (<) or similar (=) protection compared to BCG; these differences were not statistically assessed

Table 1.4: Vaccination / challenge trials of tuberculosis vaccines administered to wildlife by injection or oral routes

Wildlife species	Vaccine	Delivery route	Challenge	Protection (a)	References
Possum	BCG	Oral	Aerosol	Yes	Aldwell *et al.* (2003)
	BCG	Oral	Aerosol	Yes	Buddle *et al.* (2006)
	BCG	Oral	Natural exposure	Yes	Tompkins *et al.* (2009)
	Attenuated *Mycobacterium bovis*	Oral or Subcutaneous	Aerosol	Yes	Collins *et al.* (2007)
	Killed *M. vaccae*+ BCG	Intranasal Intra-conjunctival		Yes	Skinner *et al.*, 2002
Badgers	BCG	Oral	Intra-bronchial	Yes	Corner *et al.* (2010)
	BCG	Intramuscular	Intra-bronchial	Yes	Lesellier *et al.*, (2009)
	BCG	Intramuscular	Natural exposure	Reduced sero-positivity	Chambers *et al.* (2011)
White tailed deer	BCG	Subcutaneous	Intra-tonsillar	Yes	Palmer *et al.*, (2009)
	BCG	Oral or Subcutaneous	Intra-tonsillar	Yes	Nol *et al.*, (2008)
Wild boar	BCG	Oral	Oral	Not significant	Garrido *et al.*, (2011)
	Killed *M. bovis*	Oral/ Intramuscular	Oral	Not significant	Garrido *et al.*, (2011)
	BCG re-vaccination	Oral	Oropharyngeal	Reduction in lesion and culture scores	Gortazar *et al.*, 2014
African buffalo	BCG	Intramuscular	Intra-tonsillar	No protection	de Klerk *et al.*, (2010)

The table was extracted from Buddle *et al.*, 2013 and modified. BCG, bacilli Calmette Guérin; [a,] Protection is defined as a significant reduction in bacterial counts or lesion severity compared with non-vaccinated animals

1.2.8 Consequences of prior exposure of animals to NTM on *M. bovis* BCG vaccination and diagnosis of BTB

It has been hypothesized that exposure to some NTM species results in inappropriate priming of host immune responses. Exceptionally, a consistently high protective efficacy has been demonstrated when BCG is used to vaccinate neonates (less likely to be exposed to NTM) but wanes over years, and was not able to protect adult cattle (Buddle *et al.*, 2002; Thom *et al.*, 2008). Two mechanisms for the variable efficacy of BCG have been hypothesized *viz* 'blocking', which implies that the previous immunity induced by the exposure to environmental mycobacteria restricts the growth of BCG; or 'masking', which implies that BCG is unable to confer any additional immunity to that already induced by the natural NTM exposure that may not necessarily be protective (Brandt *et al.*, 2002; Weir *et al*., 2006). The possibility that cross-reactive immune responses may have a direct antagonistic effect due to antigens that are present in NTM and absent in BCG have also been investigated (Checkley *et al.*, 2011). These responses may result in reduction in antigen- specific IFNγ, an essential component of BCG vaccine induced resistance as well the diagnosis of tuberculosis. Several lines of evidence also suggest that differences in exposure to NTM and cross-sensitization to shared mycobacterial antigens may be important determining factors to the observed BCG efficacy variations (Demangel *et al.*, 2005). The possibility that various NTM species can trigger immune responses that can cross-protect against subsequent infection with pathogenic mycobacteria, was evaluated by Youmas and others in 1961 in guinea pigs, in particular against *M. tuberculosis* infection. Further studies to test the hypothesis were conducted by Palmer and Long in 1966 where they found that infection with *M. fortuitum* and *M. kansasii* could actually provide as much as 50% and 85% protective efficacy respectively against *M. tuberculosis* as that produced by BCG alone. Further evidence has subsequently shown that exposure of laboratory animals to different *Mycobacterium* species can provide some protection against infection with *M. tuberculosis* or *M. bovis*

which might interfere with immune response to subsequent BCG vaccination. *M. avium* complex (Brandt *et al.,* 2002; Poyntz *et al.,* 2014); *M. vaccae*; *M. scrofulaceum* (Black *et al.,* 2001; Demangel *et al.,* 2005) and *M. marinum* (Weir *et al.,* 2006) have been reported to induce immune responses that later interfere with BCG vaccination in mice experiments. It is suggested that since immune responsiveness to environmental mycobacteria correlates with genetic relatedness to *M. bovis* BCG, the inhibitory effect of environmental mycobacteria critically depends on the extent of cross- recognition of antigens shared with *M. bovis* BCG (Demangel *et al.,* 2005).

This is one major hindrance to control of BTB. A concern especially in Africa is that BCG is still the only available licensed vaccine against tuberculosis and there is a high TB prevalence in both humans and animals.

Similarly, the cross- reactive immune responses elicited by NTM are also thought to have a negative impact on the diagnosis of BTB using tuberculin based immunological assays (Michel, 2008; Schiller *et al.,* 2010). Likewise, *M. bovis* BCG may influence the outcome of immunological assays because of cross-reactive immune responses to antigens common to MTBC, NTM and *M. bovis* BCG, leading to false positive results (Vordermeier *et al.*, 2007; Vordermeier *et al.*, 2009). These specificity constraints are thought to be mainly associated with the use of PPD a crude mixture of proteins that may contain common epitopes present in both NTM and MTBC (including *M. bovis* BCG). Even though PPDs are widely used in immunological assays for BTB, little is known about the active components of these derivatives. Therefore, although the measure of the response to PPD is an important aid in TB diagnosis, it is possible that in some cases it only gives an indication of exposure to mycobacteria, and not necessarily the true BTB status of an animal. Even though the DIVA strategies have been introduced to differentiate between infected and vaccinated animals, these may not necessarily apply to differentiate between NTM infected and *M. bovis* infected animals. This is because the targeted genes like those

encoding for ESAT-6 and CFP 10 antigens are also present in some non-tuberculous mycobacteria, most notably *M. kansasii, M. marinum, M. leprae, M. szulgai* and *M. smegmatis* (Gey van Pittius *et al.,* 2002, Geluk *et al.,* 2004). In some of these NTM, like the *M. kansasii*, the antigen homologues have been reported to be expressed and even recognized by bovine T-cell (Vordermeier *et al.,* 2007).

Since animals are frequently exposed to NTM, tuberculin based tests as well as BCG vaccination strategies may suffer from problems of cross- reactive immune responses. (Mazurek *et al.,* 2001; Schiller *et al.,* 2010). This is a hindrance in control of BTB.

Therefore; future diagnostic assays including CMI and antibody based tests will likely employ the use of PPD for improved test sensitivity and defined antigens for improved test specificity. Santema *et al.,* (2009) have used proteomic analysis of PPD from *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *Mycobacterium avium* subsp. *avium* and *Mycobacterium bovis* in order to identify MAP specific proteins and to investigate their immunogenicity. The proteome analysis showed that the PPDs possess shared proteins which may most likely be responsible for cross- reactive immune responses. Therefore, knowledge of the composition of the PPDs may enable the rational design of tuberculins with selected immunogenic non-cross-reactive proteins or peptides. Comparative genomic and proteomic studies of the prevalent NTM species (animals most likely exposed to) in a particular region and *M. bovis* may help in selection of *M. bovis* specific immuno-dominant antigens as candidates for diagnosis of mycobacterial infections.

In South Africa, cattle and buffalo are maintenance hosts for *M. bovis*. In this study we first determined NTM prevalence and defined the abundant species in South African cattle and buffaloes and their environments in order to mitigate and understand the potential role of NTM in cross reactive -immune responsiveness of these hosts species against BTB. We identified novel NTM species which were among the abundant and characterized them by a series of phenotypic assays as well as determination of the

genetic sequences of the four of the mycobacterial housekeeping genes. Whole genomes of the abundant NTM species were deciphered and searched for genes encoding proteins that are considered immunogenic in pathogenic mycobacteria *i.e.* mainly *M. bovis.* We produced PPDs from these NTM species and determined their protein composition and compared to the proteome of commercial PPDs derived from *M. bovis* (PPD-B) and *M. avium* (PPD-A) used in TST and IFNγ assays.

1.3 References

ABUBAKAR I, PIMPIN L, ARITI C, BEYNON R, MANGTANI P, STERNE JA, FINE PE, SMITH PG, LIPMAN M, ELLIMAN D, WATSON JM, DRUMRIGHT LN, WHITING PF, VYNNYCKY E, RODRIGUES LC (2013). Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guérin vaccination against tuberculosis. *Health Technology Assessment* **17**(37):1-372.

ADÉKAMBI T, DRANCOURT M (2004). Dissection of the phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sod*A, *rec*A and *rpo*B gene sequencing. *International Journal of Systematic and Evolutionary Microbiology* 54: 2095-2105.

ADÉKAMBI T, BERGER P, RAOULT D, DRANCOURT M (2006). *RpoB* gene sequence-based characterization of emerging non-tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 56: 133-143.

ALDWELL FE, WEDLOCK DN, BUDDLE BM (1997). Sequential activation of alveolar macrophages by IFN [gamma] and LPS is required for enhanced growth inhibition of virulent *Mycobacterium bovis* but not *M. bovis* BCG. *Immunology and Cell Biology* **75:** 161-166.

ALDWELL FE, KEEN D, PARLANE N, SKINNER MA, DE LISLE GW, BUDDLE BM (2003). Oral vaccination with *Mycobacterium bovis* BCG in a lipid formulation induces resistance to pulmonary tuberculosis in possums. *Vaccine* **22:** 70-76.

ALEXANDER KA, LAVER PN, MICHEL AL, WILLIAMS M, VAN HELDEN PD, WARREN RM, GEY VAN PITTIUS NC (2010). Novel *Mycobacterium tuberculosis* complex pathogen *M. mungi*. *Emerging Infectious Diseases* **16:** 1296-1299.

AFSERDEN S, SAXEGAARD F (1992). An outbreak of tuberculosis in pigs and cattle caused by *Mycobacterium africanum*. *Veterinary Records* **131**: 51-53.

AMENI GG, VORDERMEIER HM, ASEFFA AA, YOUNG DB, HEWINSON RG (2010). Field evaluation of the efficacy of *Mycobacterium bovis* bacillus Calmette-Guérin against bovine tuberculosis in neonatal calves. *Clinical and Vaccine Immunology* **17:** 1533-1538.

AMENI G, TADESSE K, HAILU E, DERESSE Y, MEDHIN G, ASEFFA A, HEWINSON G, VORDERMEIER M, BERG S (2013). Transmission of *Mycobacterium tuberculosis* between Farmers and Cattle in Central Ethiopia. *Public Library of Science: One* **8**: e76891. doi:10.1371/journal.pone.0076891

ANDERSON P, DOHERTY TM (2005). The success and failure of BCG: implications for a novel tuberculosis vaccine. *Nature Reviews: Microbiology* **3:** 656-662.

ARANAZ A, DE JUAN L, MONTERO N, SÁNCHEZ C, GALKA M, DELSO C, ALVAREZ J, ROMERO B, BEZOS J, VELA AI, BRIONES V, MATEOS A, DOMÍNGUEZ (2004). Bovine Tuberculosis (*Mycobacterium bovis*) in wildlife in Spain. *Journal of Clinical Microbiology* **42:** 2602-2608.

ARANDAY-CORTES E, HOGARTH PJ, KAVEH DA, WHELAN AO, VILLARREAL-RAMOS B, LALVANI A, VORDERMEIER HM (2012). Transcriptional profiling of disease-induced host responses in bovine tuberculosis and the identification of potential diagnostic biomarkers. *Public Library of Science: One* **7**: e30626.

AYELE WY, NEILL SD, ZINSSTAG J, WEISS MG, PAVLIK I (2004). Bovine tuberculosis: an old disease but a new threat to Africa. *International Journal of Tuberculosis and Lung Diseases* **8:** 924-937.

BAHRAM EN, ENSIEH S, SHRAREH M, JAMSHID F, HOSSEIN F, GHASEMIAN SH, SADAT HN, TAHMINEH N, FARZANEH S, NIMA B, FATEMEH Z (2012). Isolation and phenotypic identification of non- tuberculous mycobacteria existing in Isfahan different water samples. *Advanced Biomedical Research* **1**:18

BARNES PF, CAVE DM (2003). Molecular epidemiology of tuberculosis. *Northern England Journal of Medicine* **349:** 1149-1156.

BASTIDA F; JUSTE RA (2011). Paratuberculosis control: a review with a focus on vaccination. *Journal of Immune Based Therapies and Vaccines* **9***:8* doi: 10.1186/1476-8518-9-8.

BEHR MA, WILSON MA, GILL WP, SALAMON H, ACHOONIK GK, RANE S, SMALL PM (1999). Comparative genomics of BCG vaccines by whole genome DNA microarray. *Science* **284:** 1520-1523.

BENGIS RG, KEET DF, MICHEL AL, KRIEK NP (2001). Tuberculosis caused by *Mycobacterium bovis*, in kudu (*Tragelaphus strepsiceros*) from a commercial game farm in the Malelane area of the Mpumalanga Province, South Africa. *Onderstepoort Journal of Veterinary Research* **68:** 239-241.

BERAN V, MATLOVA L, HORVATHOVA A, BARTOS M, MARAVKOVA M, PAVLIK I (2006). Mycobacteria in the animal's environment in the Czech Republic. *Veterinary Archives* **76:** S33-S39.

BERCOVIER H, VINCENT V (2001). Mycobacterium infections in domestic and wild animals due to *Mycobacterium marinum, M. fortuitum, M. chelonae, M, porcicum, M. farcinogenes, M. smegmatis, M. scrofulaceum, M. xenopi, M. kansasii, M. simiae* and *M. genavense. Revue Scientifique et technique de l'office International des Epizooties* **20:** 265-290.

BERGGREN SA (1977). Incidence of tuberculosis in BCG vaccinated and control cattle in relation to age distribution in Malawi. *British Veterinary Journal* **133:** 490-494.

BERGGREN SA (1981). Field experiments with BCG vaccine in Malawi. *British Veterinary Journal* **137:** 88-96.

BHUJU S, ARANDAY-CORTES E, VILLARREAL-RAMOS B, XING Z, SINGH M, VORDERMEIER HM (2012). Global gene transcriptome analysis in vaccinated cattle revealed a dominant role of IL-22 for protection against bovine tuberculosis. *Plos Pathogens* **8:** e1003077.

BIET F, BOSCHIROLI ML, THOREL MF, GUILLOTEAU LA (2005)**.** Zoonotic aspects of *Mycobacterium bovis* and *Mycobacterium avium-intracellulare* complex (MAC). *Veterinary Research* **36:** 411-436.

BLACK GF, WEIR RE, FLOYD S, BLISS L, WARNDORFF DK, CRAMPIN AC, NGWIRA B, SICHALI L, NAZARETH B, BLACKWELL JM, BRANSON K, CHAGULUKA SD, DONOVAN L, JARMAN E, KING E, FINE PE, DOCKRELL HM (2002). BCG –induced increase in interferon-gamma response to mycobacterial antigens and efficacy of BCG vaccination in Malawi and the UK: Two randomized controlled studies. *The Lancet* **359:** 1393-1401

BLACKWOOD KS, HE C, GUNTON J, TURENNE CY, WOLFE J, KABANI AM (2000). Evaluation of the recA sequences for identification of *Mycobacterium* species. *Journal of Clinical Microbiology* **38**:2846-2852.

BLAND CS, IRELAND JM, LOZANO E, ALVAREZ MA, PRIMM TP (2005). Mycobacterial ecology of the Rio Grande. *Applied and Environmental Microbiology* **71:** 5719-5727.

BOJALIL LF, CERBON J, TRUJILLO A (1962). Adansonian classification of mycobacteria. *Journal of General Microbiology* **28**: 333-346.

BONSU OA, LAING E, AKANMORI BD (2000). Prevalence of tuberculosis in cattle in the Dangme-West district of Ghana, public health implications. *Acta Tropica* **76:** 9-14.

BOTHA L, GEY VAN PITTIUS NC, VAN HELDEN PD (2013). Mycobacteria and disease in Southern Africa. *Transboundary and Emerging Diseases* **60**: 147-156.

BOUKARY AR, THYS E, ABATIH E, GAMATIE D, ANGO I, YENIKOYE A, SAEGERMAN C (2011). Bovine Tuberculosis prevalence survey on cattle in the rural livestock system of Torodi (Niger). *Public Library of Science: One* **6:** e24629.

BRANDT L, CUNHA JF, OLSEN AW, CHILIMA B, HIRSCH P, APPELBERG R, ANDERSON P (2002). Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and Immunity* **70:** 672-678.

BROOKS RW, PARKER BC, GRUFT H, FALKINHAM JO (1984). Epidemiology of infection by non-tuberculous mycobacteria. Numbers in Eastern States soils and correlation with soil characteristics. *The American Review of Respiratory Disease* **130:** 630-633.

BROSCH R, GORDON SV, MARMIESSE M, BRODIN P, BUCHRIESER C, EIGLMEIR K, GARNIER T, GUTIERREZ C, HEWISTON G, KREMMER K, PARSONS LM, PYM AS, SAMPER S, VAN SOOLIGEN D, COLE ST. (2002). A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *Proceedings of the National Academy of Science*: *USA* **99:** 3684-3689.

BROWN BA, SPRINGER B, STEIGRUBE VA, WILSON RW, PFYFFER GE, GARCIA MJ, MENENDEZ MC, RODRIGUEZ-SALGADO B, JOST KC Jr, CHIU SH, ONYI GO, BÖTTGER EC,

WALLACE RJ Jr (1999). *Mycobacterium wolinskyi* sp.nov. and *Mycobacterium goodii* sp.nov., two new rapidly growing *species* related to *Mycobacterium smegmatis* and associated with human wound infections: a co-operative study from the International working group on Mycobacterial Taxonomy. *International Journal of Systematic Bacteriology* **49:** 1493-1511.

BROWN-ELLIOT BA, WALLACE, RJ Jr (2002). Clinical and taxonomic status of pathogenic non-pigmented or late pigmenting rapidly growing mycobacteria. *Clinical Microbiology Reviews* **15**: 716-746.

BUDDLE BM, ALDWELL FE, PFEFFER A, DE LISLE GW, CORNER LA (1994). Experimental *Mycobacterium bovis* infection of cattle: effect of dose of *M. bovis* and pregnancy on immune responses and distribution of lesions. *New Zealand. Veterinary Journal* **42:** 167-172.

BUDDLE BM, DE LISLE GW, PFEIFFER A, ALDWELL FE (1995). Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with low dose of BCG. *Vaccine* **13:** 1123-1130.

BUDDLE BM, WARDS BJ, ALDWELL FE., COLLINS DM, DE LISLE GW (2002). Influence of sensitization to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20:** 1126-1133.

BUDDLE BM, WEDLOCK DN, PARLAVE MA, CORNER LAL, DE LISLE GW, SKINNER MA (2003). Revaccination of neonatal calves with *Mycobacterium bovis* BCG reduced the level of protection against tuberculosis induced by single vaccination. *Infection and Immunity* **71:** 6411-6419.

BUDDLE BM, ALDWELL FE, KEEN DL, PARLANE NA, HAMEL KL, DE LISLE GW (2006). Oral vaccination of brush tail possums with BCG: investigation into the factors that influence vaccine efficacy and determination of duration of immunity. *New .Zealand Veterinary Journal* **54:** 224-230.

BUDDLE BM, DENIS M, ALDWELL FE, VORDERMEIER HM, HEWINSON RG, WEDLOCK DN (2008). Vaccination of cattle with *Mycobacterium bovis* BCG by combination of systematic and oral routes. *Tuberculosis (Edinb)* **88:** 595-600.

BUDDLE BM, WEDLOCK DN, DENIS M, VORDERMEIER HM, HEWENSON RG (2011). Update on vaccination of cattle and wildlife populations against tuberculosis. *Veterinary Microbiology* **151:** 14-22.

BUDDLE BM, PARLANE NA, WEDLOCK DN, HEISER A (2013). Overview of vaccination trials for control of tuberculosis in cattle, wildlife and humans. *Transboundary and Emerging Diseases* **60:** 136-146.

BUHLER VA, POLLOCK A (1953). Human infection with atypical acid fast organisms, report of two cases with pathological findings. *American Journal of Clinical Pathology* **23:** 101-109.

BUXTON JB and GLOVER RE (1939). *Tuberculin tests in cattle.* A.R.C report series 4 London, UK: Privy Council, Agricultural Research Council.

CAI H, TIAN X, HU XD, LI SX, YU DH, ZHU YX (2005). Combined DNA vaccines formulated either in DNA or in saline protect cattle from *Mycobacterium bovis* infection. *Vaccine* **23:** 3887-3895.

CAI H, YU DH, HU XD, LI SX, ZHU YX (2006). A combined DNA vaccine-prime, BCG-boost strategy results in better protection against Mycobacterium bovis challenge. *DNA and Cellular Biology* **25**: 438–447

CALMETTE A and GUÉRIN C (1911). Experimental research on the body's defense against tuberculosis infection. *Annales de l'Institut Pasteur* **25:** 625-641.

CALMETTE A and GUÉRIN C (1920). New research experiments on vaccination of cattle against tuberculosis. *Annales de l'Institut Pasteur* **34:** 553-561.

CALMETTE A and GUÉRIN C (1924). Vaccination of cattle against tuberculosis and new method of prophylaxis of bovine tuberculosis. *Annales de l'Institut Pasteur* **38:** 371-398.

CAYROU C, TURENNE C, BEHR, MA, DRANCOURT M (2010). Genotyping of *Mycobacterium avium* complex organisms using multispacer sequence typing. *Microbiology* **156:** 687–694.

CHAMBERS MA, ROGERS F, DELAHAY RJ, LESELLIER S, ASHFORD R, DALLEY S, GOWTAGE S, DAVE D, PALMER S, BREWER J, CRAWSHAW T, CLIFTON- HADLEY R,

CARTER S, CHEESEMAN C, HANKS C, MURRAY A, PALPHRAMAND K, *et al.*(2011). Bacillus Calmette- Guérin vaccination reduces the severity and progression of tuberculosis in badgers. *Proceedings of the Royal Society B: Biological Science* **278:** 1913-1920.

CHECKELY AM, WYLLIE DH, SCRIBA TJ, GOLUBCHIK T, HILL AVS, HANEKON WA, MCSHANE H (2010). Identification of Antigens specific to non- tuberculous mycobacteria: The Mce family of proteins as a target of T cell immune responses. *Public Library of Science*: One **6:** e26434.doi**:**10.1371/journal.pone.0026434

CHEN YC, JOU R, HUANG W, LIU K, LAY C, CHANG S, TSENG C, LAI C, SU Y (2008). Bacteremia caused by *Mycobacterium wolinskyi*. *Emerging Infectious Diseases* **14:** 1818-1819.

CHILIMA BZ, CLARK M, FLOYD S, FINE PEM, HIRSCH PR (2006). Distribution of environmental mycobacteria in Karonga district, Northern Malawi. *Applied and Environmental Microbiology* **72:** 2343-2350.

CHINABUT S (1999). *Mycobacteriosis and norcadiosis*. Vol 3, Edited by W.B. Woo and P.T.K. Fish diseases and disorders: viral bacterial and fungal infections. Vol 3 CAB International, New York, NY. p 319-340.

CLEAVELAND S, MLENGEYA T, KAZWALA RR, MICHEL A, KAARE MT, JONES SL, EBLATE E, SHIRIMA GM, PACKER C (2005). Tuberculosis in Tanzanian wildlife. *Journal of Wildlife Diseases.* **41:** 446-450.

CLEAVELAND S, SHAW DJ, MFINANGA SG, SHIRIMA G, KAZWALA RR, EBLATE E, SHARP M (2007). Mycobacterium bovis in rural Tanzania: risk factors for infection in human and cattle populations. *Tuberculosis (Edinb)* **87**:30-43.

CLOUD JL, NEAL H, ROSENBERRY R, TURENNE Y, JAMA M, HILLYARD DR, CARROLL KC (2002). Identification of *Mycobacterium* spp by using commercial 16S ribosomal DNA sequencing kit and additional sequencing libraries. *Journal of Clinical Microbiology* **40:** 400-406.

COCKLE PJ, GORDON SV, LALVANI A, BUDDLE BM, HEWINSON GG, VORDERMEIER HM (2002). Identification of novel *Mycobacterium tuberculosis* antigens with potential as diagnostic reagents or subunit vaccine candidates by comparative genomics. *Infection and Immunity* **70:** 6996-7003.

COLE ST, BROSCH R, PARKHILL J, GARNIER T, CHURCHER C, HARRIS D, GORDON SV, EIGLMEIER K, GAS S, BARRY CE 3RD, *et al.*, (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537-544.

COLLINS CH, GRANGE JM, YATES MD (1984)**.** A review: mycobacteria in water. *Journal of Applied Bacteriology*. **57:** 193-211.

COLLINS CM (2000). The bovine tubercle bacillus**.** *British Journal of Biomedical Science* **57:**234-240.

COLLINS DM, DE LISLE GW, ALDWELL FE, BUDDLE BM (2007). A new attenuated *Mycobacterium bovis* vaccine protects bush tail possums (*Trichosurus vulpecula)* against experimental tuberculosis infection. *Vaccine* **25:** 4659-4664.

CORNER LAL, CLEGG TA, MORE SJ, WILLIAMS DH, O'BOYLE I, *et al.* (2008a). The effect of varying levels of population control on the prevalence of tuberculosis in badgers in Ireland. *Research in Veterinary Science* **85:** 238-249.

CORNER LAL, COSTELLO E, LESELLIER S, O'MEARA D, GORMLEY E (2008b). Vaccination of European badgers (*Meles meles*) with BCG subcutaneous and mucosal routes induces protective immunity against endobroncheal challenge with *Mycobacterium bovis. Tuberculosis* **88:** 601-609.

CORNER LAL, COSTELLO E, LESELLIER S, O'MEARA D, GORMLEY E (2008c). Experimental tuberculosis in the European badger (*Meles meles*) after endobroncheal inoculation with *Mycobacterium bovis:* II. Progression of infection. *Research in Veterinary Science* **85:** 481-490.

CORNER LA, COSTELLO E, O'MEARA D, LESELLIER S, ALDWELL FE, SINGH M, HEWINSON RG, CHAMBERS MA, GORMLEY E (2010). Oral vaccination of badgers (*Meles meles*) with BCG and protective immunity against endobronchial challenge with *Mycobacterium bovis. Vaccine* **28:** 6265-6272.

CORNER, LAL, MURPHY D, GORMLEY E (2011). *Mycobacterium bovis* infection in Eurasian badger (*Meles meles*) the disease, pathogenesis, epidemiology and control. *Journal of Computational Pathology* **144:** 1-24.

COSIVI O, GRANGE JM, DABORN CJ, RAVIGLIONE MC, FUJIKURA T, COUSINS D, ROBINSON RA, HUCHZERMEYER HFAK, DE KANTOR I, MESLIN FX (1998). Zoonotic

tuberculosis due to *Mycobacterium bovis* in developing countries. *Emerging Infectious Diseases* **4:** 59-70.

COSTELLO E, EGAN JWA, QUIGLEY FC, O'REILLY PF (1997). Performance of the single intradermal comparative tuberculin test in identifying cattle with tuberculous lesions in Irish herds. *Veterinary Records* **141**: 222-224.

COUSINS DV, ROBERTS JL (2001). Australia's campaign to eradicate bovine tuberculosis: the battle for freedom and beyond. *Tuberculosis* **81:** 5-15.

COUSINS DV, BASTIDA , CATALDI A, QUSE V, REDROBE S, DOW S, DUIGNAN P, MURRAY A, DUPONT C, AHMED N, COLLINS DM, BUTTLER WR, DAWSON D, RODRIGUEZ D, LOUREIRO J, ROMANO MI, ALITO A, ZUMARRAGA M, BERNARDELLI A (2003). Tuberculosis in seals caused by a novel member of *Mycobacterium tuberculosis* complex: *Mycobacterium pinnipedii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **53:** 1305-1314.

COVERT TC, RODGERS MR, REYES AL, STELMA GN Jr (1999)**.** Occurrence of non- tuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology* **65:** 2492-2496.

DAI J, CHEN Y, DEAN S, MORRIS JG, SALFINGER M, JOHNSON JA (2011). Multiple genome comparison reveals new loci for *Mycobacterium* species identification. *Journal of Clinical Microbiology* **49:** 144-153.

DANNENBURG AM. Jr and ROOK GAW. (1994). Pathogenesis, protection and control. *In Tuberculosis,* ASM Press, Washington DC.459-483.

DE GARINE-WICHATITSKY M, CARON A, KOCK R, TSCHOPP R, MUNYEME M, HOFMEYR M, MICHEL A (2013). A review of bovine tuberculosis at the wildlife-livestock-human interface in sub-Saharan Africa. *Epidemiology and Infection* **7:**1342-56.

DE GROOTE MA and HUITT G (2006). Infections due to rapidly growing mycobacteria. *Clinical Infectious Diseases* **42:** 1756-1963.

DE GROOTE MA, PACE NR, FULTON K, FALKINHAM JO III (2006). Relationship between Mycobacterium isolates from patients with pulmonary mycobacterial infection and potting soils. *Applied and Environmental Microbiology* **72:** 7602-7606.

DE JONG BC, ANTONIO M, GAGNEUX S (2010) *Mycobacterium africanum*—Review of an Important Cause of Human Tuberculosis in West Africa. *Public Library of Science*: *Neglected Tropical Diseases* **4**: e744. doi:10.1371/journal.pntd.0000744

DE KLERK LM, MICHEL AL, BENGIS RG, KRIEK NP, GODFROID J (2010). BCG vaccination failed to protect yearling African buffaloes (*Syncerus caffer*) against experimental intratonsilar challenge with *Mycobacterium bovis. Veterinary Immunology and Immunopathology* **137:** 84-92.

DE LA RUA-DOMENECH R., GOODCHILD AT, VORDERMEIER HM, HEWINSON RG, CHRISTIANSON KH, CLIFTON-HADLEY RS (2006). Ante mortem diagnosis of tuberculosis in

cattle: A review of the tuberculin tests, gamma interferon assay and other ancillary diagnostic techniques. *Research in Veterinary Science* **81**: 190-210.

DEAN GS, RHODES SG, COAD M, WHELAN AO, COCKLE PJ, CLIFFORD DJ, HEWINSON RG, VORDERMEIER HM (2005). Minimum infective dose of *Mycobacterium bovis* in cattle. *Infection and Immunity* **73:** 6467-6471.

DE LISLE GW, BENGIS RG, SCHITT SM, O'BRIEN DJ (2002). Tuberculosis in free-ranging wildlife; detection, diagnosis and management. *Revue scientifique et technique de l'office international des epizooties* **21:** 317-334.

DE LISLE GW, WARDS BJ, BUDDLE BM, COLLINS DM (2005).The efficacy of live tuberculosis vaccines after sensitization with *Mycobacterium avium*. *Tuberculosis (Edinb)* **85:** 73-79.

DEMANGEL C, GARNIER t, ROSENKRANDS I, COLE ST (2005). Differential effects of prior exposure to environmental mycobacteria on vaccination with *Mycobacterium bovis* BCG or a recombinant BCG strain expressing RD1. *Infection and Immunity* **73:** 2190-2196.

DEVULDER G, PEROUSE DE MONTCLOS M, FLANDROIS JP (2005). *International Journal of Systematic and Evolutionary Microbiology* **55:** 293-302.

DOYLE TM and STUART P (1958). Vaccination of cattle with BCG. *British Veterinary Journal* **114:** 3-10.

DURNEZ L, EDDYANI M, MGODE GF, KATAKWEBA A, KATHOLI CR, MACHANG'U RR, KAZWALA RR, PORTAELS F, LEIRS H (2008). First detection of mycobacteria in African rodents and insectivores using stratified pool screening. *Applied Environmental Microbiology* **74:** 768-773.

DURNEZ L, SADIKI H, KATAKWEBA A, MACHANG'U RR, KAZWALA RR, LEIRS H, PORTAELS F (2009). The prevalence of *Mycobacterium bovis*-infection and atypical mycobacterioses in cattle in and around Morogoro, Tanzania. *Tropical Animal Health Production* **41:** 1653-1659.

DURNEZ L, SUYKERBUYK P, NICOLAS V, BARRIÈRE P, VERHEYEN E, JOHNSON RC, LEIRS H, PORTAELS F (2010) Terrestrial small mammals as reservoirs of Mycobacterium ulcerans in Benin. Applied Environmental Microbiology **76**: 4574-4577.

DURNEZ L, KATAKWEBA A, SADIKI H, KATHOLI CR, KAZWALA RR, MACHANG'U RR, PORTAELS F, LEIRS H. Mycobacteria in terrestrial small mammals on cattle farms in Tanzania. *Veterinary Medicine International* doi:10.4061/2011/495074.

DVORSKA L, BULL TJ, BARTOS M, MATLOVA L, SVASTOVA P, WESTON RT, KINTR J, PARMOVA I, VAN SOOLINGEN D, PAVLIK I (2003). A standardized restriction fragment length polymorphism (RFLP) method for typing *Mycobacterium avium* isolates links IS*901* with virulence for birds. *Journal of Microbiological Methods* **55:** 11-27.

DYE C and FLOYD K (2006). *Tuberculosis*. Edited by: Jamison DT, Breman JG, Measham AR, *et al*., Disease Control Priorities in Developing Countries. 2nd edition. Washington (DC): World Bank.

ELLWOOD DC and WADDINGTON FG (1972). A second experiment to challenge the resistance to tuberculosis in BCG vaccinated cattle in Malawi. *British Veterinary Journal* **128:**619-626.

ENDSLEY JJ, WATER WR, PALMER MV, NONNECKE BJ, THACKER TC, JACOBS WR **Jr, *et al*** (2009). The calf model of immunity for development of a vaccine against tuberculosis. *Veterinary Immunology and Immunopathology* **128:** 199-204.

ERNST JD, TREVEJO-NUNEZ G, BANAIEE N (2007). Genomics and evolution, pathogenesis and diagnosis of tuberculosis. *The Journal of Clinical Investigations* **117:** 1738-1745.

ERNST J (2012). The immunological life cycle of tuberculosis. *Nature Reviews: Immunology* **12:** 581-591.

FALKINHAM JO III, NORTON CD, LECHEVALLIER MW (2001). Factors influencing numbers of *Mycobacterium avium, Mycobacterium intracellulare* and other mycobacteria in the water distribution systems. *Applied and Environmental Microbiology* **67:** 1225-1231.

FARHAT M, GREENWAY C, PAI M, MENZIES D (2006). False- positive tuberculin tests: what is the effect of BCG and non-tuberculous mycobacteria? *International Journal of Tuberculosis and Lung Disease.* **10:** 1192-1204.

FINE PE (1995). Variation in protection by BCG: implications of and for heterologous immunity. *Lancet* **346:** 1339-1345.

FISHER O, MATLOVA L, DVOSRKA L, SVASTOVA P, BARTL J, MELICHAREK I, WESTON RT, PAVLIK I (2001). Diptera as vectors of mycobacterial infections in cattle and pigs. *Medical and Veterinary Entomology* **15:** 208-211.

FISHER OA, MATLOVA L, BARTL J, DVOSRKA L, SVASTOVA P, DU MAINE R, MELICHAREK I, BARSTOS M, PAVLIK I(2003a). Earthworms (Oligochaeta, Lumbricidae) and mycobacteria. *Veterinary Microbiology* **91:** 325-338.

FISHER OA, MATLOVA L, DVOSRKA L, SVASTOVA P, PAVLIK I (2003b). Nymphs of the Oriental cockroach, *Blatta orientalis* as passive vectors of causal agents of avian tuberculosis and paratuberculosis. *Medical and Veterinary Entomology* **17:** 145-150.

FISHER OA, MATLOVA L, DVOSRKA L, SVASTOVA P, BARTL J, WESTON RT, PAVLIK I (2004a). Blowflies *Calliphora vicina* and *Lucilia sericata* as passive vectors of *Mycobacterium avium* subsp *avium*, *M. a. paratuberculosis* and *M. hominissuis*. *Medical and Veterinary Entomology* **18:** 116-122.

FISHER OA, MATLOVA L, DVOSRKA L, SVASTOVA P, PERAL DL, WESTON RT, BARTOS M, PAVLIK I (2004b).Beetles as possible vectors of infections *Mycobacterium avium* species. *Veterinary Microbiology* **102:** 247-255.

FRANCIS J, SEILER RJ, WILKEI WI, O'BOYLE DO, LUMSDEN MJ, FROST AJ (1978). The sensitivity and specificity of various tuberculin tests using bovine PPD and other tuberculins. *Veterinary Records* **103:** 420-435.

FRITZSCHE K (1956). Further results with modified BCG vaccination of calves. Zentralbl Veterinärmed **3:** 63-74

GARRIDO JM, SEVILLA IA, BELTRÁN-BECK B, MINGUIJON E, BALLESTEROS C, GALINDO RC, BOADELLA M, LYASHCHENKO KP, ROMERO B, GEIJO MV, RUIZ-FONS F, ARANAZ A, JUSTE RA, VICENTE J, DE LA FUENTE J, GORDÁZAR C (2011). Protection against tuberculosis in European badger vaccinated with heat-inactivated *Mycobacterium bovis. Public Library of Science: One* **6:** e24905.

GORTAZAR C, BELTRÁN-BECK B, GARRIDO JM, ARANAZ A, SEVILLA IA, BOADELLA M, LYASHCHENKO KP, GALINDO RC, MONTORO V, DOMÍNGUEZ L, RAMÓN JUSTE R , DE LA FUENTE J (2014). Oral re-vaccination of Eurasian wild boar with Mycobacterium bovis BCG yields a strong protective response against challenge with a field strain. *BioMed Central: Veterinary Research* **10:**96 doi: 10.1186/1746-6148-10-96

GELUK A, VAN MEIJGAARDEN E, FRANKEN KL, WIELES B, AREND SM, FABER WR, NAAFS B, OTTENFOFF TH (2004). Immunological cross-reactivity of the *Mycobacterium leprae* CFP-10 with its homologue in *Mycobacterium tuberculosis. Scandinavian Journal of Immunology* **59:** 66-70**.**

GEY VAN PITTIUS NC, WARREN RM, VAN HELDEN PD (2002**).** Esat-6 and CFP 10: What is diagnosis? *Infection and Immunity* **70:** 6509-6511.

GEY VAN PITTIUS NC, PERRETT KD, MICHEL AL, KEET DF, HLOKWE T, STREICHER EM, WARREN RM, VAN HELDEN PD (2012). Infection of African buffalo (*Syncerus caffer*) by Oryx bacillus, a rare member of the antelope clade of *Mycobacterium tuberculosis* complex. *Journal of Wildlife Diseases* **48:** 849-857.

GORMLEY E and COLLINS JD (2000). The development of wildlife control strategies for eradication of tuberculosis in cattle in Ireland. *Tuberculosis and Lung Diseases* **80:** 229-236.

GREEN LR, JONES CC, SHERWOOD AL, GARKAVI I, CANGELOSI GA, THACKER TC, PALMER MV, WATERS WR, RATHE CR (2009). Single antigen serological testing for bovine tuberculosis. *Clinical and Vaccine Immunology*. **16:** 1309-1313.

GREENWALD R, ESFANDIARI J, LESELLIER S, HOUGHTON R, POLLOCK J, AAGAARD C, ANDERSON P, HEWINSON RG, CHAMBERS M, LYASHCHENKO K (2003). Improved sero-detection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multi-antigen test formats. *Diagnosis and Microbiology of Infectious Diseases* **46:** 197-203.

GRIFFIN JM, WILLIAMS DH, KELLY GE, CLEGG TA, O'BYLE I, COLLINS JD, MORE SJ (2005). The impact of badger removal on the control of tuberculosis in cattle herds in Ireland. *Preventive Veterinary Medicine* **67**:237-266.

GUTIERREZ MC, SUPPLY O, BROSCH R (2009). Pathogenomics of mycobacteria. *Genome Dynamics*. **6:** 198-210.

HALL-STOODLEY L and LAPPIN-SCOTT H (1998). Biofilm formation by rapidly growing mycobacterial species *Mycobacterium fortuitum. FEMS Microbiology Letters* **168:** 77-84.

HARBOE M, OETTINGER T, WIKER HG, ROSENKRANDS I, ANDERSEN P (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infection and Immunity* **64:** 16-22.

HARING CM, TRAUM J, HAYES FM, HENRY BS (1930). Vaccination of calves against tuberculosis with Calmette-Guérin culture, BCG. *Hilgardia* **4:** 307-394.

HARMSEN D, DOSTAL, S, ROTH A, NIEMANN S, ROTHGANGER J, SAMMETH M, ALBERT J, FROSCH M, RICHTER E (2003). RIDOM: Comprehensive and public sequence database for identification of Mycobacterium species. *Biomed Central: Infectious Diseases*. **3:** 26.

HARVELAAR AH, BERWALD LG, GROOTHUIS DG, BASS JG (1985). Mycobacteria in semi-public swimming pool and whirl pools. *Zentralblatt fur Bacteriology and Microbiology Hygiene* 180**:** 505-514.

HLOKWE TN, VAN HELDEN P, MICHEL AL (2014). Evidence of increasing intra and inter- specifies transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? *Preventive Veterinary Medicine* **115:** 10-17.

HOEFSLOOT W, VAN INGEN J, ANDREJAK C, ANGEBY K, BAURIAUD R, BEMER P, BEYLIS N, BOEREE MJ, CACHO J, CHIHOTA V, CHIMARA E, CHURCHYARD G, CIAS R, DAZA R, DALEY CL, DEKHUIJZEN PN, DOMINGO D, DROBNIEWSKI F, ESTEBAN J, FAUVILLE-DUFAUX M, FOLKVARDSEN DB, GIBBONS N, GÓMEZ-MAMPASO E, GONZALEZ R, HOFFMANN H, HSUEH PR, INDRA A, JAGIELSKI T, JAMIESON F, JANKOVIC M, JONG E, KEANE J, KOH WJ, LANGE B, LEAO S, MACEDO R, MANNSÅKER T, MARRAS TK, MAUGEIN J, MILBURN HJ, MLINKÓ T, MORCILLO N, MORIMOTO K, PAPAVENTSIS D, PALENQUE E, PAEZ-PEÑA M, PIERSIMONI C, POLANOVÁ M, RASTOGI N, RICHTER E, RUIZ-SERRANO MJ, SILVA A, DA SILVA MP, SIMSEK H, VAN SOOLINGEN D, SZABÓ N, THOMSON R, TÓRTOLA FERNANDEZ T, TORTOLI E, TOTTEN SE, TYRRELL G, VASANKARI T, VILLAR M, WALKIEWICZ R, WINTHROP KL, WAGNER D (2013). The geographic diversity of non-tuberculous mycobacteria isolated from pulmonary samples: an NTM-NET collaborative study. *European Respiratory Journal.* **42:** 1604-1613.

HOPE JC, THOM ML, VILLAREAL-RAMOS B, MEAD E, VORDERMEIER HM, HEWINSON RG, HOWARD CJ (2005). Vaccination of neonatal calves with *Mycobacterium bovis* BCG induces protection against intra nasal challenge with virulent *M. bovis. Clinical and Experimental Immunology* **139:** 48-56.

HOPE JC and VILLAREAL-RAMOS B (2008). Bovine TB and development of new vaccines. *Comparative. Immunology. Microbiology and Infectious Diseases*. **31:** 77-100.

HORSBURGH CR (1991)**.** Current Concepts- *Mycobacterium avium* complex infections in acquired immunodeficiency syndrome. *The New England Journal of Medicine.* **324:** 1332-1338**.**

HOWARD CJ, KWONG LS, VILLARREAL- RAMOS B, SOPP P, HOPE JC (2002). Exposure to *Mycobacterium avium* primes immune system of calves for vaccination with *Mycobacterium bovis* BCG. *Clinical and Experimental Immunology* **120**: 48-56.

HUARD RC, FABRE M, DE HAAS P, LAZZARINI LCO, VAN SOOLINGEN D, COUSINS D, HO JL (2006). Novel genetic polymorphisms that further delineate the phylogeny of the *Mycobacterium tuberculosis* complex. *Journal of Bacteriology* **188:** 4271-4287.

JOLLEY ME, NASIR MS, SURUJBALLI OP, ROMANOWSKA A, RENTERIA TB, DE LA MA, LIM A, BOLIN SR, MICHEL AL, KOSTOVIC M, CORRIGAN EC (2007). Fluorescence polarization assay for detection of antibodies to *Mycobacterium bovis* in sera. *Veterinary Microbiology* **120:** 113-121.

JONES GJ, PIRSON C, HEWINSONRG, VORDERMEIER HM (2010). Simultaneous measurement of antigen-stimulated interleukin-1 beta and gamma interferon production enhances test sensitivity for the detection of *Mycobacterium bovis* infection in cattle. *Clinical and Vaccine Immunology* **17**:1946-1951.

KAMALA T, PARAMASIVAN CN, HERBET D, VENKATESAN P, PRABHAKA R. (1994). Isolation and identification of environmental mycobacteria in the *Mycobacterium bovis* BCG area of South India. *Applied and Environmental Microbiology* **60**: 2180-2183.

KAMERBEEK J, SCHOULS L, KOLK A, VAN AGTERVELD M, VAN SOOLINGEN D, KUIJPER S, BUNSCHOTEN A, MOLHUIZEN H, SHAW R, GOYAL M, VAN EMBDEN J (1997).Simultaneous detection and strain differentiation of *Mycobacterium bovis* for diagnosis and epidemiology. *Journal of Clinical Microbiology* **36:** 937-943.

KANKYA C, MUWONGE A, DJØNNE B, MUNYEME M, OPUDA-ASIBO J, SKJERVE E, OLOYA J, EDVARDSEN V, JOHANSEN T (2011). Isolation of non- tuberculous mycobacteria from pastoral ecosystems of Uganda: Public health significance. *Biomed Central: Public Health* **11**: 320

KASAI H, EZAKI T, HARAYYAMA S (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *Journal of Clinical Microbiology* **38**: 301-308.

KAZWALA RR, DABORN CJ, KUSILUKA LJM, JIWA SFH, SHARP JM, KAMBARAGE DM (1998). Isolation of Mycobacterium species from raw milk of pastoral cattle of southern highlands of Tanzania. *Tropical Animal Health and Production* **30:** 233–239.

KEMPSELL KE, JI YE, ESTRADA IC, COLSTON MJ, COX RA (1992). The nucleotide sequence of the promoter 16S rRNA and spacer region of the ribosomal RNA operon of *Mycobacterium tuberculosis* and comparison with *Mycobacterium leprae* precursor rRNA. *Journal of General Microbiology* **138:** 1717-1727.

KARNE SS, SANGLE SA, KIYAWAT DS, DHARMASHALE SN, KADAM B, BHARDWAJ RS (2012). *Mycobacterium avium-intracellulare* brain abscess in HIV positive patient. *Annals of Indian Academy of Neurology* **15**: 54-55.

KHARE S, HONDALUS MK, NUNES J, BLOOM BR, ADAMS LG (2007). *Mycobacterium bovis* Delta leuD auxotroph- induced protective immunity against tissue colonization, burden and distribution in cattle intranasally challenged with *Mycobacterium bovis. Vaccine* **25:** 1743-1755.

KIM H, KIM SH, SHIM TS, KIM MN, BAI GH, PARK YG, LEE SH, CHAE GT, CHA CY, KOOK YH, KIM BJ (2005). Differentiation of Mycobacterium species by analysis of the heat-shock protein 65 gene (hsp65). *International Journal of Systematic and Evolutionary Microbiology* **55:** 1649-1656.

KIRSCHNER RA Jnr, PARKER BC, FALKINHAM JO (1992). Epidemiology of infection by non-tuberculous mycobacteria. *Mycobacterium avium, Mycobacterium intracellulare,* and *Mycobacterium scrofulaceum* in acid brown- water swamps of the southern United States and their association with environmental variables. *American Reviews of Respiratory Diseases* **145:** 271-275

LE DANTEC C, DUGUET J-P, MONTIEL A, DUMOUTIER N, DUBROU S, VINCENT V (2002a). Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. *Applied and Environmental Microbiology* **68:** 1025-1032.

LE DANTEC C, DUGUET J-P, MONTIEL A, DUMOUTIER N, DUBROU S, VINCENT V (2002b). Occurrence of mycobacteria in water treatment lines and in water distribution systems. *Applied and Environmental Micribiology* **68:** 5318-5325.

LEONI E, LEGNANI P, MUCCI MT, PIRANI R (1999). Prevalence of mycobacteria in a swimming pool environment. *Journal of Applied Microbiology.* **87:** 683-688.

LESELLIER S, CORNER L, COSTELLO E, LYASHCHENKO K, GREENWALD R *et al*. (2009). Immunological responses and protective immunity in BCG vaccinated badgers following endobronchial infection with *Mycobacterium bovis. Vaccine* **27:** 402-409.

LESELLIER, PALMER S, GOWGATE-SEQUIERA S, ASHFORD R, DALLEY D, DAVÉ, D, WEYER U, SALGUERO FJ, NUNEZ A, CRAWSHAW T, CORNER LA, HEWINSON RG, CHAMBERS MA (2011). Protection of Eurasian badgers (*Meles meles*) from tuberculosis after intra-muscular vaccination with different doses of BCG. *Vaccine* **29:** 3782-3790.

LIESACK W, PITULLE C, SELA S, STACKEBRANDT E (1990) Nucleotide sequence of the 16S rRNA from *Mycobacterium leprae*. *Nucleic Acids Research* **18**:5558.

IIVANAINEN EK, MARTIKAINEN PJ, VÄÄNÄNEN PK, KATILA ML (1993). Environmental factors affecting the occurrence of mycobacteria in brooks waters. *Applied and Environmental Microbiology* **59:** 398-404.

IIVANAINEN EK, MAERTIKAINEN PJ, RÄISÄNEN ML, KATILA ML (1997). Mycobacteria in boreal coniferous forest soils. *FEMS Microbiology and Ecology* **23:**

LIVANAINEN E, NORTHRUP J, ARBEIT RD, RISTOLA M, KATILA ML, VON REYN CF (1999). Isolation of mycobacteria from indoor swimming pools in Finland. *Acta Pathologica Microbiologica et Immunologica Scandinavica* **107:** 193-200.

LOCHT C (2010). *The history of BCG*: Editors Nor NM, Acosta A, Sarmiento ME. The art and Science of tuberculosis vaccine development. Shah Alam. Malaysia: Oxford University Press. p 149-167.

LYASHCHENKO KP, GREENWALD R, ESFANDIARI J, CHAMBERS MA, VINCENTE J, GORTAZAR C, SANTOS N, CORREIA-NEVES M, BUDDLE BM, JACKSON R, O'BRIEN DJ, SCHMITT S, PALMER MV, DELAHAY RJ, WATERS WR (2008)**.** Animal-side serologic assay for rapid detection of *Mycobacterium bovis* infection in multiple species of free ranging wildlife. *Veterinary Microbiology* **132:** 283-292.

MACHACKOVA M, MATLOVA L, LAMKA J, SMOLIK J, MELICHAREK I, HANZLIKOVA M, DOCEKAL J, CVETNIĆ Ž, NAGY G, LIPIEC M, OCEPEK M, PAVLIK I (2003): Wild boar (*Sus scrofa*) as a possible vector of mycobacterial infections: review of literature and critical analysis of data from Central Europe between 1983 and 2001.*Veterinary*. *Medicine*. **48:** 51-65.

MACHACKOVA M, SVASTOVA P, LAMKA J, PARMOVA I, LISKA V, SMOLIK J, FISCHER OA, PAVLIK I. (2004): Paratuberculosis in farmed and free-living wild ruminants in the Czech Republic in the years 1999–2001. *Veterinary Microbiology* **101:** 225–234.

MALAMA S, MUNYEME M, MWANZA S, MUMA JB (2014). Isolation and characterization of non-tuberculous mycobacteria from humans and animals in Namwala district of Zambia. *BioMed Central Research Notes*. **7:** 622.

MARCO I, DOMINGO M, LAVI S (2000). Mycobacterium infection in a captive- reared Capercaillie (*Tetrao urogallus*). *Avian Disease* **44:** 227-230.

MARTÍN-CASABONA N, BAHRMAND AR , BENNEDSEN J , OSTERGAARD THOMSEN V , CURCIO M, FAUVILLE-DUFAUX M , FELDMAN K, HAVELKOVA M, KATILA ML , KÖKSALAN K, PEREIRA MF, RODRIGUES F , PFYFFER GE, PORTAELS F, ROSSELLÓ URGELL J, RÜSCH-GERDES S , TORTOLI E , VINCENT V, WATT B (2004). Non-tuberculous mycobacteria: Patterns of isolation. A multi-country retrospective survey. *International Journal of Tuberculosis and Lung Diseases* **8***:*1186-1193.

MARONGUI L, DONINI M, TOFFALI L, ZENARO E, DUSI S (2013). ESAT-6 and HspX improve effectiveness of BCG to induce human dendric cells-dependant Th1 and NK cells activation. *Public Library of Science:* O*ne* **8:** e75684.doi:10.1371/journal.pone.0075684.

MARRAZI MG, CHAPGIER A, DEFILIPPI A, PISTOIA V, MANGINI S, SAVIOLI C, ACQUA AD, FEINBERG J, TORTOLI E, CASANOVA J (2009). Disseminated *Mycobacterium scrofulaceum* infection in a child with interferon-γ- receptor 1 deficiency. *International Journal of Infectious Diseases* **14:** e167-e170.

MAUE AC, WATER WR, PALMER MV, WHIPPLE DL, MINION FC, BROWN WC, ESTES DM. (2004). CD80 and CD86, but not CD154, augment DNA vaccine-induced protection in experimental bovine tuberculosis. *Vaccine* **23:** 769-779.

MAUE AC, WATERS WR, PALMER MV, NONNECKE BJ, MINION FC, BROWN WC, NORIMINE J, FOOTE MR, SCHERERM CFC, ESTES DM (2007). An ESAT-6 CFP10 DNA vaccine administered in conjunction with *Mycobacterium bovis* BCG confers protection to cattle challenged with virulent *M. bovis. Vaccine* **25:** 4735-4746.

MAZUREK GH, LOBUE PA, DALEY CL, BERNARDO J, LARDIZABAL AA, BISHAI WR, IADEMARCO MF, ROTHEL JS (2001). Comparison of a whole blood γ interferon assay with tuberculin skin testing for detecting latent *Mycobacterium tuberculosis* infection. *Journal of the American Medical Association* **286:** 1740-1747.

MCCORRY T, WHELAN AO, WELSH MD, MCNAIR J, WALTON E, BRYSON DG, HEWINSON RG, VORDERMEIER HM, POLLOCK JM (2005). Shedding of *Mycobacterium bovis* in the nasal mucus of cattle infected experimentally with tuberculosis by the intranasal and intratracheal routes. *Veterinary Records* **157**: 613–618.

MCNAIR J, WELSH MD, POLLOCK JM (2007). The immunology of bovine tuberculosis and progression towards improved disease control strategies. *Vaccine* **25:** 5504-5511.

MENENDEZ MC, GARCIA MJ, NAVARRO MC, GONZALEZ-Y-MERCHAND JA, RIVERA-GUTIERREZ S, GARCIA- SANCHEZ L, COX RA (2002). Characterization of an rRNA operon (*rrnB*) of *Mycobacterium fortuitum* and other mycobacterial species: implications for the classification of mycobacteria. *Journal of Bacteriology* **184:** 1078-1088.

MEYER RM, ORLOSKI KA (2007). An evaluation of new technologies for diagnosis of *Mycobacterium bovis* infection in cattle. *Proceedings of the United States Annual Health Association Annual Meeting* **111:** 743-744.

MICHALAK K, AUSTIN C, DIESEL S, BACON MJ, ZIMMERMAN P, MASLOW JN (1998). *Mycobacterium tuberculosis* infection as a zoonotic disease: transmission between humans and elephants. *Emerging Infectious Diseases* **4:** 283-287.

MICHEL AL, BENGIS RG, KEET DF, HOFMEYER M, DE KLERK LM, CROSS PC, JOLLES AE, COOPER D, WHYTE IJ, BUSS P, GODFROID J (2006)**.** Wildlife tuberculosis in South African conservation areas. *Veterinary Microbiology* **112:** 91-100.

MICHEL AL, DE KLERK L, VAN PITTIUS NCG, WARREN RM, VAN HELDEN PD (2007). Bovine tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. *BMC Veterinary Research* **3:** doi: 10.1186/1746-6148-3-23.

MICHEL AL, SIMÕES M (2009). Comparative field evaluation of two rapid immuno-chromatographic tests for the diagnosis of bovine tuberculosis in African buffaloes (*Syncerus caffer*). *Veterinary Immunology and Immunopathology* **127:** 186-189.

MICHEL AL, COOPER D, JOOSTE J, DE KLERK LM, JOLLES A (2011). Approaches towards optimizing the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine* **98**: 142-151.

MIGNARD S, FLANDROIS JP (2007). Identification of *Mycobacterium* using EF-Tu encoding (tuf) gene and the tmRNA encoding (ssrA) gene. *Journal of Medical Microbiology* **56:** 1033-1041.

MILLINGTON KA, FORTUNE SM, LOW J, GARDES A, HINGLEY-WILSON SM, WICKREMASINGHE M, KON, OM, LALVANI A (2011). Rv3615 is a highly immunodominant RD1 (Region of difference)-dependent secreted antigen specific for *Mycobacterium tuberculosis* infection. *Proceedings of the National Academy of Science* **108:** 5730-5735.

MIRSAEIDI M, FARSHIDPOUR M, ALLEN MB, EBRAHIMI G, FALKINHAM JO (2014). Highlights on advances in non-tuberculous mycobacterial disease in North America. *Biomed Research International*

MOSTOWY S, COUSINS D, BRINKMAN J, ARANAZ A, BEHR MA (2002). Genomic Deletions suggest a phylogeny for *Mycobacterium tuberculosis* complex. *Journal of Infectious Diseases* **186:** 74-80.

MÜLLER B, STEINER B, BONFOH B, FANÉ A, SMITH NH, ZINSSTAG J (2008).. Molecular characterisation of Mycobacterium bovis isolated from cattle slaughtered at the Bamako abattoir in Mali. *Biomed Central: Veterinary Research* **4:** 26.

NARANG R, NARANG P, MENDIRATTA DK (2009). Isolation and identification of non-tuberculous mycobacteria from water and soil in central India. *Indian Journal of Microbiology* **27:** 247-250.

NEILL, SD, HANNA J, O' BRIEN JJ, MCCRACKEN RM (1988). Excretion of *Mycobacterium bovis* by experimentally infected cattle. *Veterinary Records* **123:** 340-343.

NEILL, SD, CASSIDY J, HANNA J, MACKIE DP, POLLOCK JM, CLEMENT A, WALTON E, BRYNSON DG (1994). Detection of *Mycobacterium bovis* infection in skin test- negative cattle with bovine interferon gamma assay. *Veterinary Records* **135**: 134-135.

NEUMANN M, SCHULZE-ROBBECKE R, HAGENAU C, BEHRINGER K (1997). Comparison of methods for isolation of mycobacteria from water. *Applied Environmental Microbiology* **63:** 547-552.

NGANDOLO BN, MÜLLER B, DIGUIMBAYE-DJAÏBE C, SCHILLER I, MARG-HAUFE B, CAGIOLA M, JOLLEY M, SURUJBALLI O, AKAKPO AJ, OESCH, ZINSSTAG (2009). Comparative assessment of fluorescence polarization and tuberculin skin testing for the diagnosis of bovine tuberculosis in Chadian cattle. *Preventive Veterinary Medicine* **89:** 81-89.

NIEMANN S, HARMSEN D, RUSCH-GERDES S, RICHTER E (2010). Differentiation of Clinical *Mycobacterium tuberculosis* complex isolates by *gyrB* DNA sequence polymorphism analysis. *Journal of Clinical Microbiology*. **38:**3231-3234

NIVA M, HERNESMAA A, HAAHTELA K, SALKINOJA-SALONEN M, SIVONEN K, HAUKKA K (2006). Actinobacterial communities of boreal forest soil and lake water are rich in mycobacteria. *Boreal Forest Research* **11**: 45-53.

NOL P, PALMER MV, WATERS WR, ALDWELL FE, BUDDLE BM, TRIANTIS JM, LINKE LM, PHILLIPS GE, THACKER TC, RHYAN JC, DUNBAR MR, SALMAN MD (2008).Efficacy of oral and parenteral routes of *Mycobacterium bovis* bacilli Calmette Guérin vaccination against experimental bovine tuberculosis in white tailed deer (*Odocoileus virginianus*): a feasibility study. *Journal of Wildlife Diseases* **44:** 247-259.

O'REILLY LM, DABORN CJ (1995). The epidemiology of *Mycobacterium bovis* infections in animals and man: a review. *Tuberculosis and Lung Diseases.* **76:** 1-46.

OCEPEK M, PATE M, ŽOLNIR-DOVČ M, POLJAK M (2005). Transmission of *Mycobacterium tuberculosis* from human to cattle. *Journal of Clinical Microbiology* **43:** 3555-3557.

OIE Terrestrial Manual 2014. Bovine Tuberculosis. Chapter 2.4.7. Online at: http://www.oie.int.

OLOYA J, MUMA JB, OPUDA-ASIBO J, DJØNNE B, KAZWALA R, SKJERVE E (2007). Risk factors for herd-level bovine-tuberculosis seropositivity in transhumant cattle in Uganda. *Preventive Veterinary Medicine* **80**: 318-329.

PALMER CE, LONG MW (1966). Effects of infection with atypical mycobacteria on BCG vaccination and tuberculosis. *American Review of Respiratory Disease* **94:**553-568.

PALMER MV, WATERS WR, THACKER TC, GREENWALD R, ESFANDIARI J, LYASHCHENKO KP (2006). Effects of different tuberculin skin testing regimes on gamma interferon and antibody

responses in cattle experimentally infected with *Mycobacterium bovis. Clinical and Vaccine Immunology* **13:** 387-394.

PALMER MV, THACKER TC, WATERS RW (2007). Vaccination of white tailed deer (*Odocoileus* virginianus) with *Mycobacterium bovis* bacillus Calmette Guérin. *Vaccine* **25:** 6589-6597.

PALMER MV, THACKER TC, WATERS WR (2009). Vaccination with *Mycobacterium bovis* BCG strains Danish and Pasteur in white tailed deer (*Odocoileus virginianus*) experimentally challenged with *M. bovis. Zoonoses Public Health* **56:** 243-251.

PALMER MV, WATERS WR (2011). Bovine tuberculosis and establishment of an eradication program in the United States: role of veterinarians. *Veterinary and Medical International* **2011:** 816345

PANTEIX G, GUTIERREZ MC, BOSCHIROLI ML, ROUVIERE M, PLAIDY A, PRESSAC D, PORCHERET H, CHYDERIOTIS G, PONSADA M, VAN OORTEGAM K, SALLOUM S, CABUZEL S, BAÑULS AL, VAN DE PERRE P, GODREUIL S (2010). Pulmonary tuberculosis due to *Mycobacterium microti:* a study of six recent cases in France. *Journal of Medical Microbiology* **59:** 984-989.

PARASHAR D, CHAUHAN DS, SHARMA VD, CHAUHAN A, CHAUHAN AVS, KATOCH VM (2004). Optimization of procedures for isolation of mycobacteria from soil and water samples obtained in northern India. *Indian Journal of Medical Research* **70:** 3751-3753.

PARASHAR D, DAS R, CHAUHAN DS, SHARMA VD, LAVANIA M, YADAV VS, CHAUCAN SVS, KATOCH VM (2009). Identification of environmental mycobacteria isolated in Agra, north India by convectional and molecular approaches. Indian Journal of Medical Research **129:** 424-431.

PARSONS S, SMITH SGD, MARTINS Q, HORSNELLD WGC, GOUSE TA, STREICHERA EM, WARREN RM, VAN HELDEN PD, GEY VAN PITTIUS NC (2008). Pulmonary infection due to the dassie bacillus (Mycobacterium tuberculosis complex sp.) in a free-living dassie (rock hyrax—Procavia capensis) from South Africa. *Tuberculosis* **88:** 80-83.

PARSONS SDC, DREWE JA, GEY VAN PITTIUS NC, WARREN RM, VAN HELDEN PD (2013). Novel cause of tuberculosis in meerkats in South Africa. *Emerging Infectious Diseases* doi:http://dx.doi.org/10.3201/ied1912.130268.

PARK HY, KOH WJ, KWON OJ, LEE NY, SHIM YM, PARK YK, BAI GH, MUN HS, KIM BJ (2007). Pulmonary Disease caused by *Mycobacterium xenopi:* The first case in Korea. *Yonsei Medical Journal.* **48:** 871-875.

PARTE AC (2014). List of Prokaryotic names with Standing in Nomenclature (LPSN). Online at: http://www.bacterio.net/

PAVLIK I, MATOVA L, DVORSKA L, BARTL J, OKTABCOVA L, DOCEKAL J, PARMOVA I (2003). Tuberculosis lesions in pigs in the Czech Republic during 1990-1999: occurrence, causal factors and economic loses. *Veterinarni Medicina* **14:** 113-125.

PAVLIK, I., MATLOVA, L., DVORSKA, L., SHITAYE, J.E., PARMOVA, I. (2005). Mycobacterial infections in cattle and pigs caused by *Mycobacterium avium* complex members and atypical mycobacteria in Czech Republic during 2000-2004. *Veterinary Medicine* **50:** 281-290.

PAVLIK I, MATLOVA L, GILAR M, BARTL J, PARMOVA I, LYSAK F, ALEXA A, DVORKA-BARTOSOVA L, SVEC V, VRBAS V, HORVATHOVA A (2007). Isolation of conditionally pathogenic mycobacteria from the environment of one pig farm and the effectiveness of preventive measures between 1997 and 2003. *Veterinarinarni Medicina* **52**: 392-404.

PHILLIPS CJ, FOSTER CR, MORRIS PA, TEVERSON R (2003). The transmission of *Mycobacterium bovis* infection to cattle. *Research in Veterinary Science* **74:** 107-122.

PHILLIPS MS, VON REYN FC (2001). Nosocomial Infections due to nontuberculous Mycobacteria. *Clinical Infectious Diseases* **33:** 1363-1374

POLLOCK JM, GIRVIN RM, LIGHTBODY KA, CLEMENTS RA, NEILL SD, BUDDLE BM, ANDERSON P (2000)**.** Assessment of defined antigens for diagnosis of bovine tuberculosis in skin test-reactor cattle. *Veterinary Records* **146:** 659-665.

POLLOCK JM and NEILL SD (2002). *Mycobacterium bovis* infection and tuberculosis in cattle. *The Veterinary Journal* **163:** 115-127.

PONTIROLI A, KHERA TT, OAKLEY BB, MASON S, DOWD SE, TRAVIS ER, ERENSO G, ASEFFA A, COURTENAY O, WELLINGTON EMH (2013). Prospecting environmental mycobacteria: Combined molecular approaches reveal unprecedented diversity. *Public Library of Science*: *One* **8**: e68648.doi:10.1371/journal.pone.0068648.

PORTAELS F (1995). Epidemiology of mycobacterial diseases. In: Mycobacterial diseases of the skin. *Clinics in Dermatology*. Elsevier Sciences Inc, New York, Ed. M. Schuster. **13**: 207-222.

POYNTZ HC, STYLIANOU E, GRIFFITHS KL, MARSAY L, CHECKLEY AM, MCSHANE H (2014). Non-tuberculous mycobacteria have diverse effects on BCG efficacy against *Mycobacterium tuberculosis*. *Tuberculosis (Edinburgh, Scotland)* **94**: 226-237.

PULCINI C, VANDENBUSSCHE E, PODGLAJEN I, SOUGAKOFF W, TRUFFOT-PERNOT C, BUU-HOÏ A, VARON E, MAINARDI J (2006). Hip Prosthesis infection due to *Mycobacterium wolinskyi*. *Journal of Clinical Microbiology* **44:** 3463-3464.

PRIMM TP, LUCERO CA, FALKINHAM JO III (2004)**.** Health impacts of environmental Mycobacteria. *Clinical Microbiology Reviews* **17:** 98-106.

PYM AS, BRODIN P, BROSCH R, HUERRE M, COLE ST (2002). Loss of RD1contributed to the attenuation of the live tuberculosis vaccines, *Mycobacterium bovis* BCG and *Mycobacterium microti*. *Molecular Microbiology* **46:** 707-717.

RAHBAR M, LAMEI A, BABAZADEH H, YAVARI SA (2010). Isolation of rapid growing mycobacteria from soil and water in Iran. *African Journal of Biotechnology* **9:** 3618-3621.

RASTOGI N, LEGRAND E, SOLA C (2001). The mycobacteria: an introduction to nomenclature and pathogenesis. *Revue scientifique et technique* **20:** 21-54.

RODRÍGUEZ S, BEZOS J, ROMERO B, DE JUAN L, ÁLVAREZ J, CASTELLANOS E, MOYA N, LOZANO F, JAVED MT, SÁEZ-LLORENTE JL, LIÉBANA E, MATEOS A, DOMÍNGUEZ L, ARANAZ A(2011). *Mycobacterium caprae* Infection in Livestock and Wildlife, Spain. *EMERGING Infectious Diseases* **17** doi: 10.3201/eid1703.100618

ROTH A, FISCHER M, HAMID ME, MICHALKE S, LUDWIG W.MAUCH H (1998). Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *Journal of Clinical Microbiology* 36: 139–147.

RUNYON EH (1959). Anonymous mycobacteria in pulmonary disease. *Medical Clinics of North America*. **43**: 273-290.

SANTEMA W, OVERDIJK M, BARENDS J, KRIJGSVELD J, RUTTEN V, KOETS A (2009). Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative proteomic analysis of Mycobacterium tuberculins. *Veterinary Microbiology* **138:** 191-196.

SCHELLER H, GAGGERMEIER G (1959). Vaccination of cattle in herds infected with TB with the 'strain P' turbercle' bacillus described by Gräub. *Veterinary Bulletin* **26:** 183.

SCHERER, L., SPERHACKE, R.O., ROSSETTI, M.L.R., RUFFINO-NETTO, A., KRITSKI, L.A**.** **(**2011). Usefulness of the polymerase chain reaction dot blot assay, used with Ziehl Neelsen staining for the rapid and convenient diagnosis of pulmonary tuberculosis in human immune deficiency virus seropositive and sero-negative individuals. *Infectious Disease Reports.* **3:** doi:10.4081/idr.2011.e3

SCHILLER I, OESCH B, VORDERMEIER HM, PALMER MV, HARRIS BN, ORLOSKI KA, BUDDLE BM, THACKER TC, LYASHCHENKO KP, WATERS WR (2010). Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and Emerging Diseases* **57**: 205-220.

SCHLUGER NW, ROM WN (1998). The host immune response to tuberculosis. *American Journal of Respiratory Critical Medicine* **157:** 697-691.

SEPTEMBER SM, BRÖZEL VS, VENTER SN (2004). Diversity of non-tuberculoid *Mycobacterium* species in biofilms of urban and semi urban drinking water distribution systems. *Applied and Environmental Microbiology* **70**: 7571-7573.

SEXTON P and HARRISON AC (2008). Susceptibility to nontuberculous mycobacterial lung disease. *European Respiratory Journal* **31:** 1322-1333.

SHINNICK TM and GOOD RC (1994)**.** Mycobacterial taxonomy. *European Journal of Clinical Microbiology and Infectious Diseases.* **13:** 884-901.

SHITAYE JE, TSEGAYE W, PAVLIK I (2007). Bovine tuberculosis infection in animal and human populations in Ethiopia: a review. *Veterinary Medicine* **8**: 317-322.

SHITAYE JE, HORVÁTHOVÁ A, BARTOSÓVÁ- MORÁVKOVÁ M, KAEVSKA M, DONNELLY N, PAVLIK I (2009). Distribution of non-tuberculous mycobacteria in environmental samples from a slaughterhouse and in raw and processed meats. *Czech Journal of Food Science* **27:** 194-202.

SCHULZE-RÖBBECKE R, JANNING B, FISCHEDER R. (1992). Occurrence of mycobacteria in biofilm samples. *Tubercle and Lung Disease* **73:**141-144.

SNIADACK DH, OSTROFF-SM, KARLIX MA, SMITHWICK RW, SCHWARTZ B, SPRAUER MA, SILCOX-VA, GOOD RC (1993). A nosocomial pseudo-outbreak of *Mycobacterium xenopi* due to a contaminated potable water supply: Lessons in prevention. *Infection Control and Hospital Epidemiology.* **14:** 636-641.

SIBGATULLIN RS (1982). Eradicating tuberculosis from large cattle breeding complexes by using BCG vaccine Development of efficient prophylactic and treatment methods for animal infectious diseases. *Proceeding of Kazan Veterinary Institute*. Union of Soviet Socialist Republics, vol 60: 62.

SKINNER MA, KEEN DL, PARLANE NA, YATES GF, BUDDLE BM (2002). Increased protection against bovine tuberculosis in the brush tail possum (*Trichosurus vulpecula*) when BCG is administered with killed *Mycobacterium vaccae. Tuberculosis (Edinb)* **82:** 15-22.

SKINNER MA, BUDDLE BM, WEDLOCK DN, KEEN DL, DE LISLE, TASCON RE, FERRAZ JC, LOWRIE DB, COCKLE PJ, VORDERMEIER HM, HEWINSON RG (2003) A DNA prime-*Mycobacterium bovis* BCG boosts vaccination strategy in cattle induces protection against bovine tuberculosis. *Infection and Immunity* **71:** 4901-4907.

SKINNER MA, BUDDLE BM, WEDLOCK DN, DE LISLE GW, COOKE MM, TASCON RE, FERRAZ JC, LOWRIE DB, VORDERMEIER HM, HEWINSON RG, BUDDLE BM (2005). The order of a prime-boost vaccination of neo-natal calves with *Mycobacterium bovis* BCG and a DNA vaccine encoding mycobacterial proteins Hsp65, Hsp70 and Apa is not critical for enhancing protection against bovine tuberculosis. *Infection and Immunity* **73:** 4441-4444.

SMITH NH, DALE J, INWALD J, PALMER S, GORDON S, HEWINSON RG, SMITH JM (2003). The population structure of *Mycobacterium bovis* in Great Britain: Clonal expansion. *Public Library of Science: Pathogens* **9:** 15271-15275**.**

SMITH NH, KREMER K, INWALD J, DALE J, DRISCOL JR, GORDON SV, VAN SOOLINGEN D, HEWINSON RG, SMITH JM (2006a). Ecotypes of the *Mycobacterium tuberculosis* complex. *Journal of Theoretical Biology* **239:** 220-225.

SMITH NH, GORDON SV, DE LA RUA-DOMENECH R, CLIFTON-HADLEY RS, HEWINSON G (2006b). Bottlenecks and broomsticks: the molecular evolution of *Mycobacterium bovis. Nature Reviews of Microbiology* **4:** 670-681.

STAHL DA and URBANCE JW (1990). The division between fast-growing and slow-growing species corresponds to natural relationships among the mycobacteria. *Journal of Bacteriology* **172**: 116-124.

STEED K, FALKINHAM JO (2006). Effect of growth in biofilm on chlorine susceptibility of *Mycobacterium avium* and *Mycobacterium intracellulare. Applied and Environmental Microbiology* **72**:4007-4011.

STEELE JH (1995). Regional and country status report. . Editors: Thoen CO and JH Steele JH. *Mycobacterium bovis* infection in animals and humans. Iowa State University Press. Ames, IA. p 169-172.

STEINGART KR, FLORES LL, DENDUKURI N, SCHILLER I, LAAL S, RAMSAY A, HOPEWELL PC, PAI M. (2011). Commercial serological tests for the diagnosis of active pulmonary and extra pulmonary tuberculosis: an updated systematic review and meta-analysis. *Public Library of Science: Medicine*. **8(**8):e1001062).

TAKEWAKI S, OKUZUMI K, ISHIKO H, NAKAHARA K, OHKUBO A, NAGAI R (1993). Genus-specific polymerase chain reaction for the mycobacterial dnaJ gene and species-specific oligonucleotide probes. *Journal of Clinical Microbiology.* **31**: 446-450.

TARARA R, SULEMAN MA, SAPOLSKY R, WABOMBA MJ, ELSE JG (1985). Tuberculosis in wild olive baboons, *Papio cynocephalus anubis* (Lesson), in Kenya. *Journal of Wildlife Diseases* **21:** 137-140.

TSCHOPP R, BERG S, ARGAW K, GADISA E, HABTAMU M, SCHELLING E, YOUNG D, ASEFFA A, ZINSSTAG J (2010). Bovine Tuberculosis in Ethiopian wildlife. *Journal of Wildlife Diseases* **46:** 753-762.

TELENTI A, MARCHESI F, BALZ M, BALLY F, BOTTGER EC, BODMER T. (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *Journal of Clinical Microbiology* **31**: 175-178.

THEGERSTROM J, MARKLUND BI, HOFFNER S, AXELSSON-OLSSON D, KAUPPINEN J, OLSEN B (2005). *Mycobacterium avium* with the bird type IS*1245* RFLP profile is commonly found in wild and domestic animals, but rarely in humans. *Scandinavian Journal of Infectious Diseases* **37:** 15-20.

THOM M, HOWARD C, VILLAREAL-RAMOS B, MEAD E, VORDERMEIER HM, HOPE J (2008). Consequences of prior exposure to environmental mycobacteria on BCG vaccination and diagnosis of tuberculosis infection. *Tuberculosis (Edinb)* **88:** 324-334.

THOMSON RM, CARTER R, TOLSON C, COULTER C, HUYGENS F, HARGREAVES M (2013). Factors associated with isolation of non- tuberculous mycobacteria (NTM) from a large municipal water system in Brisbane, Australia. *Biomed Central: Microbiology* **13:** doi: 10.1186/1471-2180-13-89.

TOMPKINS DM, RAMSEY DSL, CROSS ML, ALDWELL FE, DE LISLE GW, BUDDLE BM (2009). Oral vaccination reduces the incidence of bovine tuberculosis in a free-living wildlife species. *Proceedings of the Royal Society: Biological Science* **276:** 2987-2995.

TOMPKINS DM, BUDDLE BM, WHITFORD J, CROSS ML, YATES GF, LAMBETH G (2013). Sustained protection against tuberculosis conferred to a wildlife host by single dose oral vaccination**.** *Vaccine* **31:** 893-899.

TORTOLI E, BESOZZI G, LACCHINI C, PENATI V, SIMONETTI MT, ELMER S (1998). Pulmonary infection due to *Mycobacterium szulgai:* case report and review of literature. *European Respiratory Journal* **11:** 975-977.

TORTOLI E (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clinical Microbiology Reviews* **16**: 319-354.

TORTOLI E (2006). The new mycobacteria: an update. *FEMS Microbiology.* **48:** 159-178.

TORVINEN E, SOUMALAINEN S, LEHTOLA MJ, MIETTINEN IT, ZACHEUS O, PAULIN L, KATILA M-L, MARTIKAINEN PJ (2004). Mycobacteria in water and loose deposits of drinking water distribution systems in Finland. *Applied and Environmental Microbiology* **70**: 1973-1981.

TROESCH A, NGUYEN H, MIYADA CG, DESVARENNE S, GINGERAS TR, KAPLAN PM, CROS P, MABILAT C (1999)**.** *Mycobacterium* species identification and rifampin resistance testing with High-Density DNA Probe Arrays. *Journal of Clinical Microbiology* **37:** 49-55.

TRUMAN RW, SINGH P, SHARMA R., BUSSO P, ROUGEMONT J, PANIZ-MONDOLFI A., KAPOPOULOU A, BRISSE S, SCOLLARD DM, GILLIS TP, COLE ST. (2011). Probable zoonotic leprosy in the southern United States. – *New English Journal of Medicine*, **364:** 1626-1633

TSUKAMURA **(**1966). Adonsonian classification of mycobacteria. *Journal of General Microbiology* **45:** 253-273.

VAN CREVEL R, OTTENHOFF TMN, VAN DER MEER, JWM (2002). Innate Immunity to *Mycobacterium tuberculosis. Clinical Microbiology Reviews* **15:** 294-309.

VAN HELDEN PD, PARSONS SDC, GEY VAN PITTIUS NC (2009). 'Emerging' mycobacteria in South Africa. *Journal of South African Veterinary Association.* **80**: 210-214.

VAN INGEN J, BOEREE MJ, DE LANGE WCM, DE HAAS PEW, DEKHUIJZEN PNR, VAN SOOLINGEN D (2008). Clinical relevance of *Mycobacterium szulgai* in the Netherlands. *Clinical Infectious Diseases* **46:** 1200-1205.

VAN INGEN J, BOEREE JM, DEKHUIJZEN PNR, VAN SOOLINGEN D (2009). Environmental sources of rapid growing non-tuberculous mycobacteria causing disease in humans. *Clinical Microbiology and Infections* **15:** 888-893**.**

VAN SOOLINGEN D, HOOGENBOEZEM T, DE HAAS PE, HERMANS PW, KOEDAM MA, TEPPEMA KS, BRENNAN PJ, BESRA GS, PORTAELS F, TOP J, SCHOULS LM, VAN EMBDEN JD (1997). A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, Canetti: characterization of an exceptional isolate from Africa. *International Journal of Systematic Bacteriology* **47:** 1236-1245.

VAN SOOLINGEN D, VAN DER ZANDEN AG, DE HAAS PE, NOORDHOEK GT, KIERS A, FOUDRAINE NA, PORTAELS F, KOLK AH, KREMER K, VAN EMBDEN JC (1998). Diagnosis of *Mycobacterium microti* infections among humans by using novel genetic markers. *Journal of Clinical Microbiology* **36:** 1840-1845

VORDERMEIER HM, COCKLE PC, WHELAN A, RHODES S, PALMER N, BAKKER D, HEWINSON RG (1999). Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. *Clinical and Diagnostic Laboratory Immunology* **6:** 675-682.

VOEDERMEIER MM, CHAMBERS MA, COCKLE PJ, WHELAN AO, SIMMONS J, HEWINSON RG (2002). Correlation of ESAT-6 –specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection and Immunity* **70:** 3026-3032.

VORDERMEIER HM, HUYGEN K, SINGH M, HEWINSON RG, XING Z (2006). Immune response induced in cattle by vaccination with a recombinant adenovirus expressing mycobacterial antigen 85A and *Mycobacterium bovis* BCG. *Infection and Immunity* **74**: 1416-1418.

VORDERMEIER HM, VILLARAEAL-RAMOS B, COCKLE PJ, MCAULAY M, RHODES SG, THACKER T, GILBERT SC, MCSHANE H, HILL AVS, XING A, HEWINSON RG (2009). Viral Booster vaccines improve *Mycobacterium bovis* BCG- induced protection against bovine tuberculosis. *Infection and Immunity* **77:** 3364-3373.

WANG Y, OGAWA M, FUKUDA K, MIYAMOTO H, TANIGUCHI H (2006). Isolation and identification of mycobacteria from soils at an illegal dumping site and landfills in Japan. *Microbiology and Immunology* **50:** 513-524.

WARREN RM, VAN PITTIUS NCG, BARNARD M, HESSELING A, ENGELKE E, DE KORK M, GUTIERREZ MC, CHEGE GK, VICTOR TC, HOAL EG, VAN HELDEN PD (2006). Differentiation of *Mycobacterium tuberculosis* complex by PCR amplification of genomic regions of difference. *International Journal of the Tuberculosis and Lung Diseases.* **10:** 818-822.

WATERS WR, PALMER MV, THACKER TC, PAYEUR JB, HARRIS NB, MINION FC, GREENWALD R, EFSANDIARI J, ANDERSON P, MCNAIR J, POLLOCK JM, LYASHCHENKO KP (2006). Immune responses to defined antigens of *Mycobacterium bovis* in cattle experimentally infected with *Mycobacterium kansasii. Clinical and Vaccine Immunology* **13:** 611-619.

WATERS WR, PALMER MV, NONNECKE BJ, THACKER TC, SCHERER CFC, ESTES DM, JACOBS Jr WJ, GLATMAN-FREEDMAN A, LARSEN MH (2007). Failure Δ*Mycobacterium tuberculosis ΔRD1ΔpanCD* double deletion mutant in a neonatal calf aerosol *M. bovis* challenge model: comparisons to responses elicited by *M. bovis* bacillus Calmette Guérin. *Vaccine* **25** 7832-7840

WATERS WR, PALMER MV, NONNECKE BJ, THACKER TC, CAPINOS SCHERER CF, ESTES DM, HEWINSON RG, VORDERMEIER HM, BARNES SW, FEDERE GC, WALKER JR, GLYNNE RJ, HSU T, WEINRICK B, BIERMANN K, LARSEN MH, JACOBS WR Jr (2009). Efficacy and immunogenicity of *Mycobacterium bovis* ΔRD1 against aerosol *M. bovis* infection in neonatal calves. *Vaccine* **27:** 1201-1209.

WATERS RW, PALMER MV, BUDDLE BM, VORDERMEIER MH (2012). Bovine tuberculosis vaccine research: Historical perspectives and recent advances. *Vaccine* **30**: 2611-2622.

WATSON EA (1928). Research on Bacillus Calmette –Guérin and experimental vaccination against bovine tuberculosis. *Journal of American Veterinary and Medical Association* **73:** 799-816.

WEDLOCK DN, SKINNER MA, DE LISLE GW, BUDDLE BM (2002a). Control of *Mycobacterium bovis* infections and the risk of human populations. *Microbes and Infection* **4:** 471-480.

WEDLOCK DN, DENIS M, SKINNER MA, KOACH J, DE LISLE GW, VORDERMEIER HM, HEWINSON RG, VAN DRUNEN LITTLE VAN DER HURK S, BABIUK LA, HECKER R, BUDDLE BM (2005). Vaccination of cattle with CpG oligodeoxynucleotide- formulated mycobacterial protein

vaccine and *Mycobacterium bovis* BCG induces levels of protection against bovine tuberculosis superior to vaccination with BCG alone. *Infection and Immunity* **73:** 3540-3546.

WEDLOCK DN, SKINNER MA, VORDERMEIER HM, HEWINSON RG, HECKER R, LITTEL-VAN DEN HURK SD, BUDDLE BM. (2005). Vaccination of cattle with *Mycobacterium bovis* culture filtrate proteins and CpG oligodeoxynucleotides induces protection against bovine tuberculosis. *Veterinary Immunology and Immunopathology*. **106:** 53–63

WEDLOCK DN, DEIS M, VORDERMEIER HM, HEWINSON RG, BUDDLE BM (2007). Vaccination of cattle with Danish and Pasteur strains of *Mycobacterium bovis* BCG induce different levels of IFN gamma post vaccination, but induce similar levels of protection against bovine tuberculosis. *Veterinary Immunology and Immunopathology* **118:** 50-58.

WEDLOCK DN, ALDWELL FE, D LISLE GW, VORDERMEIER HM, HEWINSIN RG, BUDDLE BM (2011). Protection against bovine tuberculosis induced by oral vaccination of cattle with *Mycobacterium bovis* BCG is not enhanced by co-administration of mycobacterial protein vaccines. *Veterinary Immunology and Immunopathology* **144:** 220-227.

WEIR RE., BLACK GF, NAZARETH B, FLOYD S, STENSON S, STANLEY C, BRANSON K, SICHALI L, CHAGULUKA SD, DONOVAN L, CRAMPIN AC, FINE PEM, DOCKRELL, HM. (2006). The influence of previous exposure to environmental mycobacteria on the interferon-gamma response to bacille Calmette–Guérin vaccination in southern England and northern Malawi. *Clinical and Experimental Immunology*. **146:** 390-399.

WELSH MD, CUNNINGHAM RT, CORBETT DM, GIRVIN MR, MCNAIR J, SKUCE RA, BRYSON DG, POLLOCK JM (2005). Influence of pathological progression on the balance between cellular and humoral immune responses in bovine tuberculosis. *Immunology* **114:** 101-111.

WHELAN OA, WRIGHT DC, CHAMBERS MA, SINGH M, HEWINSON RG, VORDERMEIER HM (2008). Evidence for enhanced central memory priming by live *Mycobacterium bovis* BCG vaccine in comparison to with killed BCG formulations. *Vaccine* **26:** 166-173

WHELAN AO, CLIFFORD D, UPADHYAY B, BRENDAN EL, MCNAIR J, HEWINSON GR, *et al* (2010). Development of a skin test for bovine tuberculosis for differentiating infected from vaccinated animals. *Journal of Clinical Microbiology* **48:** 3176-3181.

WHIPPLE DL, BOLIN CA, DAVIS AJ, JARNAGIN JL, JOHNSON DC, NABORS RS, PAYEUR JB, SAARI DA, WILSON AJ, WOLF MM (1995). Comparison of the sensitivity of the caudal fold and commercial γ –interferon assay for diagnosis of bovine tuberculosis. *American Journal of Veterinary Research* **56:** 415-419.

WHIPPLE DL, PALMER MV, SLAUGHTER RE, JONES SL (2001). Comparison of purified protein derivatives and effect of skin testing on results of a commercial gamma interferon assay for diagnosis of tuberculosis in cattle. *American Journal of Veterinary Research* **13:** 117-122.

WOLINSKY E (1995). Mycobacterial lymphadenitis in children: a prospective study of 105 non-tuberculous cases with long term follow-up. *Clinical Infectious Diseases* **20:** 954-963.

YAERSLEY D, EGAN J, COSTELLO E., O'REILLY PF, HEWINSON RG (1998). An evaluation of an anamnestic ELISA for the detection of tuberculous cattle. *Irish Veterinary Journal* **51:** 303-306.

YOUMANS, GP, PARLETT RC, YOUMANS AS. (1961). The significance of the response of mice to immunization with viable unclassified mycobacteria. *American Review of Respiratory Diseases* **83:** 903-905.

YAMADA-NODA M, OHKUSU K, HATA H, SHAH MM, NHUNG PH, SUN XS, HAYASHI M, EZAKI T (2007). *Mycobacterium* species identification: – a new approach via *dnaJ* gene sequencing. *Systematic and Applied Microbiology* **30:** 453-462.

ZELAZYN AM, CALHOUN LB, LI L, SHEAR YR, FISCHER SH, (2005). Identification of Mycobacterium species by SecA sequences. *Journal of Clinical Microbiol* **43:** 1051-1058.

1.4 Problem statement

South African livestock and wildlife are exposed to environmental mycobacteria, whether concurrently with *M. bovis* or alone. These NTM may potentially elicit broadly cross-reactive immune responses which may interfere with current standard diagnostic assays for BTB, namely TST, the IFNγ and serological tests.

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1.5 Hypothesis

There are highly prevalent NTM species in South Africa which possess immunogenic proteins with a potential to induce significant cross-reactive immune responses in cattle and African buffaloes. These immune responses interfere with the current immuno-diagnostic assays for BTB, *viz* TST, IFNγ assay, as well as serological assays.

1.6 Objectives of the study

1. To investigate the diversity, distribution and prevalence of NTM in South African cattle, African buffaloes and their environments by conducting a survey from samples (bovine and buffalo tissues, nasal and pharyngeal swabs, soil, water and milk) collected from different regions.

2. To characterize the selected abundant NTM isolates by molecular and phenotypic methods.

3. To decipher the genomes of the selected prevalent NTM species and compare them to those of pathogenic mycobacteria, *viz M. bovis* and *M. tuberculosis* with a particular focus on genes encoding immunogenic proteins.

4. To conduct comparative proteomic analysis of PPD derived from the selected prevalent NTM species to the commercially available PPDs (Bovine and Avian PPDs) used in the tuberculin based tests.

5. To investigate the potential of the selected NTM species to elicit an immune response using combined genomic and proteomic analysis.

CHAPTER 2. PREVALENCE AND DISTRIBUTION OF NON-TUBERCULOUS MYCOBACTERIA (NTM) IN CATTLE, AFRICAN BUFFALOES (SYNCERUS CAFFER) AND THEIR ENVIRONMENTS IN SOUTH AFRICA

***GCEBE N, RUTTEN V, GEY VAN PITTIUS NC, MICHEL A (2013). Transboundary and Emerging Diseases* 60:74-84.**

2.1 Abstract

It has been hypothesized that a variety of NTM species to which livestock and wildlife species are naturally exposed, induce broadly cross reactive anti-mycobacterial immune responses which interfere with current standard diagnostic assays. NTM have also been implicated in *Mycobacterium bovis* specific immune responsiveness, hence potentially the development of tuberculosis. Cattle and African buffaloes are both maintenance hosts of bovine tuberculosis (BTB) in South Africa, yet the effective diagnosis and control in these species may be hampered by adverse effects of NTM. As part of an investigation of the role of NTM in the immune responsiveness of cattle and African buffalo to NTM, we conducted a countrywide survey to establish the prevalent NTM species and their distribution in the natural environments (water and soil) of cattle and African buffaloes. A total of 1123 samples (water, soil, nasal and pharyngeal swabs) were collected for Mycobacterium isolation. In addition, NTM isolated from tissue samples between 1991 and 2011 were included in the analysis. Mycobacteria were isolated from 56% of the samples from the countrywide survey. A total of 420 NTM isolates from soil, water, animal tissues, and animal derived swab samples were genotyped with the following results: 302 belonged to 40 known NTM species, 79 were found to be closely related to 23 known NTM species, and 38 isolates were found to be potential novel species that are not currently listed in the RIDOM and NCBI BLAST databases. The four NTM species or closely related groups most frequently isolated in this survey included *Mycobacterium terrae* (11.2% of isolates), a group of mycobacteria closely related to *Mycobacterium moriokaense* (referred to as *M. moriokaense*-like) (8.1 % of isolates), *Mycobacterium nonchromogenicum* (7.4% of isolates) and *Mycobacterium vaccae/ M. vanbaalenii* (5.2% of isolates). The phylogenetic analysis of the *M. moriokaense* like isolates, based on the 16s rRNA sequences, revealed at least 8 clusters, possibly associated with 8 different NTM species. Our findings provide account of NTM species diversity and associated prevalences in cattle and African buffaloes and their

environments in South Africa. The identification of the most prevalent NTM species in this study will allow for a targeted investigation of their effects on host immune responses.

Keywords: NTM, *Mycobacterium nonchromogenicum, Mycobacterium moriokaense, Mycobacterium terrae, Mycobacterium vaccae, M. vanbaalenii*

2.2 Introduction

It is hypothesized that different non-tuberculous Mycobacterium (NTM) species induce broadly cross reactive immune responses in livestock and wildlife species to which they are exposed. These immune responses interfere with current standard diagnostic assays, namely the skin test and the interferon γ-assay (Michel, 2008; Michel *et al.*, 2010; Schiller *et al.*, 2010), as well as with the efficacy of the *M. bovis* BCG vaccine against tuberculosis (Brandt *et al.*, 2002; Weir *et a*l., 2006). The use of purified protein derivative (PPD), a crude mixture of proteins that may contain epitopes common in NTM as well as *M. bovis*, in the tuberculin skin test (TST) and the gamma interferon assay, is thought to be the cause of these cross reactive immune responses (Schiller *et al.*, . 2010). The efficacy of *M. bovis* BCG vaccine varies from about 70% to 0% protection in cattle (Berggren, 1981; Vordermeier *et al.*, 2002; Ameni *et al.*, 2010). Variability of the efficacy of *M. bovis* BCG is also observed in humans, ranging from 80% to 0%, with consistently low efficacy in many tropical regions of the world (Fine, 1989; Hart and Sutherland; Ponnighaus *et al.*, 1992; Arbeláez *et al.*, 2000). However, a consistent high efficacy is seen when BCG is used to vaccinate newborns (Rodrigues *et al*, 1991; Colditz *et al.*, 1995). It is hypothesized that exposure to NTM may affect the efficacy of *M. bovis* BCG due to antigens that are common to both BCG and NTM that may provide cross-priming effects with either positive or negative consequences (Kamala *et al.*, 1996; Brandt *et al.*, 2002; Young *et al.*, 2007). Similarly, NTM may influence the

development of tuberculosis in animal hosts after natural infection (Buddle *et al.,* 2002; Brandt *et al.,* 2002). NTM distribution in animal populations and the epidemiology of these bacterial species is poorly understood, compared to that of *M. bovis*, but it is assumed that NTM species isolated from animals and their environment in a particular region represent the distribution of the NTM species in that region. This may give the opportunity to study immune responsiveness due to NTM as well as the consequences of these immune responses for experimental vaccination and disease progression after infection with *M. bovis.* Worldwide, cattle are the main hosts of *M. bovis* amongst domestic animals (Cousins, 2001). In South Africa, African buffaloes (*Syncerus caffer*) are considered the most important wildlife maintenance host for *M. bovis* (de Vos *et al.,* 2001; Rodwell *et al.,* 2001), and *M. bovis* has been isolated from buffalo on numerous occasions in South African national parks and private game reserves (Bengis *et al.,* 1996; Michel *et al.,* 2006; 2009, Hlokwe *et al.,* 2011). Sequencing and phylogenetic analysis of the Mycobacterium house- keeping genes, like the 16S rRNA has been used routinely to identify NTM and investigate their diversity (Harmsen *et al.,*2003) . This study aimed to identify the NTM species diversity and associated prevalences in cattle and African buffalo, and their environments in South Africa, by sequencing of the 16S rRNA of the isolated NTM as well as phylogenetic analysis of the very closely related sequences.

2.3 Materials and Methods

2.3.1 Sample collection

A countrywide survey was conducted for sample collection for NTM isolation between April 2010 and December 2012. In addition NTM isolates collected from tissue samples between the years 1991-2011 and stored at -20°C were used. Samples from both the country wide survey and the stored isolates were collected at 119 sites distributed across geographic and climatic regions in South Africa. Sampling sites were selected by accordingly identified State Veterinary Offices and largely represented convenience sampling.

A total of 1123 samples (570 nasal and pharyngeal swabs, 143 water samples and 410 soil samples) were collected during the country wide survey (during no particular season) from Kruger National Park, private game reserves, and livestock farms (communal and commercial) across South Africa. The criteria for sample collection during this survey were: 1) Samples included water, soil, and nasal/pharyngeal swabs from cattle at each sampling site; 2) country wide distribution of sampling sites including all geographical and climatic areas; 3) In pertinent cases, isolation of two or more NTM species from the same sample would be considered as separate isolates.

Nasal and pharyngeal swab samples were collected from live buffalo and cattle, as well as soil and water from the habitats of these animals. Soil samples were collected from the cattle grazing camps, crush pens, kraals, commonages, and buffalo capture bomas and around parks where buffalo are seen. In certain cases only environmental samples were collected due to logistical constraints in obtaining samples from animals. Water samples were collected from dams where these animals drink, animal drinking pans,

troughs and stagnant river sites. From each site soil samples were collected at 5-25cm depth, and 150ml of water samples were collected in sterile sampling bottles, about 25cm deep. Nasal and pharyngeal swabs were collected using sterile cotton swabs, and then immediately immersed in distilled water or phosphate buffer saline (PBS) (PH= 7) in the field. These were collected from a minimum of 5 randomly selected animals per site. The samples were kept at 4°C until they were processed for NTM isolation.

Tissue samples (lymph nodes, liver, spleen and lungs, with or without visible lesions) from cattle were collected during routine meat inspection by state veterinarians or meat inspectors according to standard operating procedures at abattoirs. Tissue samples from buffalo were collected as part of bovine tuberculosis research projects, using approved sampling protocols, and sent to ARC-OVI Tuberculosis laboratory for diagnosis of mycobacterial diseases by culture. These were: lymph nodes, liver and lungs and spleen.

2.3.2 Isolation of NTM from different sample types

For nasal or pharyngeal swabs, the samples were soaked in 7 ml of sterile distilled water overnight. The swab was then discarded and the solution centrifuged at 2360 g for 10 min at room temperature. Hydrochloric acid (HCl) at a final concentration of 2% was added to the pellet and the sample was incubated at room temperature for 10 min. The solution was then centrifuged for another 10 min at 2360 g at room temperature, where after the supernatant was discarded. The pellet was washed with 7 ml distilled water and centrifuged again for 10 min at 2360 g at room temperature.

For soil and water samples, a modified protocol by Livanainen, (1995), was used for processing of soil and water samples for mycobacterial isolation. For soil, 20 ml of distilled water was added to 5 g of soil in a 50 ml Falcon tube, and shaken by hand for at least 1 min. The solution was allowed to stand at room

temperature for 30 min to allow the sediment to settle. The sample was centrifuged at 800 x g for 5 min. The turbid supernatant was transferred to a clean tube and centrifuged again at 3900 g for 30 min at room temperature. The supernatant was discarded; 20 ml of 2% NaOH was added to the pellet and incubated at room temperature for 10 min. The suspension was centrifuged at 3900 g for 10 min at room temperature. The supernatant was discarded and the pellet was suspended in 20 ml of 5% oxalic acid. The suspension was allowed to stand at room temperature for 15 min and again centrifuged at 3900 g for 10 min. The supernatant was discarded, 20 ml of water was added and the suspended pellet was centrifuged at 3900 g for 10 min at room temperature. Thereafter, the supernatant was discarded, leaving approximately 1ml fluid with the pellet. For water samples, an aliquot of 100 ml from each water sample was allowed to settle at room temperature for 30 min, after which approximately 50 ml of the upper part was transferred to a clean 50 ml Falcon tube and centrifuged at 3900 g for 30 min. The supernatant was discarded and the tube refilled with approximately the remaining 50 ml from the 100 ml aliquot, and centrifuged again at 3900 g for 30 min at room temperature. The supernatant was discarded and the procedure for processing of soil samples as described above was followed.

For all the sample types the sediment was inoculated onto Löwenstein Jensen (LJ) slopes supplemented with glycerol and an antibiotic cocktail of PolymyxinB, AmphotericinB, Carbenicillin and Trimethoprim (PACT) (National Health Laboratories, South Africa, and Becton Dickinson, Germany). Two LJ slopes of each sample were incubated at 37˚C and monitored for colony growth of mycobacteria at 2 days, 7 days, and thereafter weekly for 10 weeks. When growth of bacteria was observed, based on morphology of mycobacterial colonies (Csillag, 1961), individual colonies were selected for Ziehl Neelsen staining, and suspensions of the acid fast bacteria were prepared for further identification using polymerase chain reaction (PCR) and the remaining cultures were stored at -20°C for further investigations. Tissue samples were processed for mycobacterial isolation according to Bengis *et al*. 1996. In short, ±5g of sample was suspended in 10ml of distilled water and homogenized. Two aliquots of 7ml were decontaminated with

1% HCl and 2% NaCl respectively for 10 min. These were centrifuged, the supernatant was discarded and the pellet was then washed with distilled water followed by centrifugation. The resulting pellets were inoculated on LJ slopes and incubated at 37˚C.

The sources of the isolates were classified into the source categories, i) "environmental" samples from soil, water and, since isolation of NTM from swabs could be an indication of contamination from the environment, also swabs and ii) "animal" the tissue samples.

2.3.3 *In vitro* amplification and sequencing of the 16S rRNA for identification of mycobacteria

Individual colonies from cultures were picked and boiled culture suspensions were prepared as DNA templates for PCR amplification of the 16S rRNA of mycobacteria. A 25 µl PCR reaction mixture (Qiagen HotStar Taq, Germany) was prepared, containing 12.9 µl deionised water, 2.5 µl of 10x PCR buffer {Tris.Cl, KCl, $(NH_4)_2$, SO_4}, 2 µl $MgCl_2$, 1 µl dNTP's (10 mM), 0.1 µl Taq Polymerase, 5 µl of 5x Q-solution, 1 µl of each forward and reverse primers (50 pmol) and 1-2 µl of DNA template. Primers 16S-F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 16S-R (5' -GCG ACA AAC CAC CTA AGA G -3') were used for the 16S ribosomal RNA amplification (Harmsen *et al.*, 2003). The PCR cycling parameters were as follows: initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 60 °C for 30 sec and elongation at 72°C for 30 sec, and a final extension at 72°C for 10 min. The PCR products of 577bp were sent to the DNA sequencing unit at the Central Analytical Facility of Stellenbosch University, South Africa for sequencing of the forward strands using an ABI sequencer (Applied Biosystem Inc.). Sequences were then analysed using the RIDOM 16S ribosomal RNA database (RIDOM-www.ridom-rdna.de) and NCBI BLAST (www.blast.ncbi.nlm.nih.gov/Blast.cgi) for mycobacterial speciation.

2.3.4 Phylogenetic analyses of the NTM isolates

Phylogenetic analyses of isolates based on the partial 16S rRNA (577bp) of a variety of *M. moriokaense*-like isolates were performed using Molecular Evolutionary Genetics Analysis (MEGA) platform (www.megasoftware.net) (version 5.05). Two *M. moriokaense* isolates were also included in the analysis, and a *Nocardia* spp sequence retrieved from Genbank (www.ncbi.nlm.nih.gov) was used as an out-group species. Sequence alignments were performed using Clustalw (Thompson *et al.*, 1994). Phylogenetic trees were constructed using the neighbor-joining method (Saitou and Nei, 1987). The p-distance parameters were used as a substitution model. A thousand bootstrap replicates were run. The neighbour-joining trees were also compared to trees constructed using the maximum likelihood method and Kimura-2 was used as a substitution model.

2.4 Results

2.4.1 Sample collection and NTM isolation

Samples were collected from 114 cattle farms, 2 wildlife conservation areas (Kruger National Park, and Hluhluwe Imfolozi Park) and 3 private game farms, during the country wide survey (fig 2.1). At eight of the 114 cattle farms, only soil and water samples were collected due to difficulty in accessing animals.

Six hundred and twenty nine isolates of acid fast bacteria were detected from 1123 samples (including all sampling sites) collected during the countrywide survey (56% detection rate). These were 285 isolates from 570 samples from nasal and pharyngeal swabs (50 % detection rate) , 262 from 410 soil samples (66% detection rate) and 82 from 143 water samples (57% detection rate). Hundred and five (9.3%) cultured samples were contaminated before growth of mycobacteria. Stored NTM isolates from tissue (n=91) and soil (n=2), were derived from samples in the Hluhluwe Imfolozi Park, Kruger National Park, 1 game farm and 32 cattle farms during routine diagnostic isolation of mycobacteria. The NTM isolates from the tissue samples were isolated in pure culture.

2.4.2 Characterization of NTM isolates

A total of 420 out of the 629 mycobacterium isolates were sequenced for species identification. Of these 420 isolates 160 were recovered from soil (n=160) from water (n=66) from swabs (n=103) and from

tissue (n=91) (Table 2.1). We could not analyse all the 629 isolates mainly for budget purposes. The remaining 209 isolates were stored away.

Table 2. 1 a: NTM species identified per sample type from environmental and animal sources

NTM species	Environmental source			Animals	Total : n
	soil:n (%) [a]	water: n (%) [a]	swabs : n (%) [a]	n (%) [a]	
M. acapulcensis	2(1.25)	0(0)	9 (8.65)	8 (8.8)	19
M. asiaticum	1(0.625)	0 (0)	2 (1.9)	3 (3.3)	6
M. avium complex	6 (3.8)	3(4.5)	0 (0)	16 (17.6)	25
M. chitae	0(0)	0 (0)	1 (0.96)	3 (3.3)	4
M. confluentis	2(1.25)	0 (0)	0 (0)	1 (1.1)	3
M. duvalii	2(1.25)	0 (0)	3(2.9)	3 (3.3)	8
M. flavescens/ M. novocastrense	0(0)	0(0)	1(0.96)	3 (3.3)	4
M. fortuitum complex	8(5)	0 (0)	0	8 (8.8)	16
M. goodii	1(0.625)	0(0)	0(0)	2 (2.2)	3
M. gordonae	2(1.25)	0 (0)	0 (0)	1 (1.1)	3
M. interjectum	1(0.625)	0(0)	0(0)	1(1.1)	2
M. intermedium	4(2.5)	2(3)	0(0)	1 (1.1)	7
M. moriokaense	4(2.5)	3(4.5)	9 (8.65)	2 (2.2)	18
M. moriokaense-like	6(3.8)	6 (9)	13 (12.5)	9 (9.9)	34
M. nonchromogenicum	16(10)	11(16.7)	2(1.9)	2 (2.2)	31
M. palustre	1(0.625)	0 (0)	0(0)	2(2.2)	3
M. pulveris/ M. elephantis	0(0)	0(0)	1(0.96)	3 (3.3)	4
M. septicum/ M. peregrinum	7 (4.4)	0 (0)	1 (0.96)	1(1.1)	9
M. simiae	1(0.625)	4(6)	0(0)	5 (5.5)	10
M. szulgai	0(0)	2 (3)	0 (0)	1 (1.1)	3
M. terrae	33(20.6)	7(10.6)	5(4.8)	2 (2.2)	47
M. triviale	1(0.625)	1(1.5)	0(0)	1 (1.1)	3
M. vaccae/ M. vanbaalenii	3(1.9)	0(0)	17(16.3)	2 (2.2)	22
Unidentified NTM	28(17.5)	18 (27.3)	29 (27.9)	8 (8.8)	83
Total	129	57	93	88	367

n, number of isolates belonging to the respective NTM species; NTM, non-tuberculous mycobacteria; a, percentage of isolates per sample type. *Mycobacterium avium* complex include *M. avium* subp avium/paratuberculosis, *M. vulneris*, *M. intracellulare* and *M. colombiense*. *Mycobacterium fortuitum* complex include *M. fortuitum fortuitum* and *M. fortuitum acetamidolyticum*. **This table was modified into 2.1a , 2.1b and 2.1c after publication for the purpose of the thesis**

Table 2. 1 b: NTM species isolated from environmental sources only

NTM species	Soil	Water	Swab	Total n
M. arupense	1(0.625)	0 (0)	0(0)	1
M. austroafricanum	1(0.625)	1 (1.5)	0 (0)	2
M. engbackii	3(1.9)	2 (3)	0(0)	5
M. flouroanthenivorans	1 (0.625)	0(0)	0 (0)	1
M. holsaticum	0(0)	0 (0)	2 (1.9)	2
M. kumamotonense	3(1.9)	0 (0)	0 (0)	3
M. madagascariense	0(0)	1(1.5)	0 (0)	1
M. monasence	0(0)	0 (0)	1(0.96)	1
M. nebraskense/ M. gastri/ M. bohemicum/ M. kansasii/ M. malmoense	0(0)	1 (1.5)	0 (0)	1
M. neoaurum	3(1.9)	0 (0)	3(2.9)	6
M. paraffinicum	14(8.75)	3(4.5)	0 (0)	17
M. parafortuitum	1(0.625)	0 (0)	4 (3.8)	5
M. senuense	1(0.625)	0 (0)	0 (0)	1
M. thermoresistible	1(0.6)	0 (0)	0(0)	1
M. triplex/ M. monteferionse	2(1.25)	1(1.5)	0 (0)	3
Total	31	9	10	50

n, number of isolates belonging to the respective NTM species; NTM, non-tuberculous mycobacteria; a, percentage of isolates per sample type.

Table 2. 1 c: NTM species isolated from animal sources only

NTM species	n(%)[a]
M. chelonae/ abscessus	1 (1.1)
M. lacticola	1 (1.1)
M. wolinsky	1 (1.1)
Total	3

n, number of isolates belonging to the respective NTM species; NTM, non-tuberculous mycobacteria; a, percentage of isolates per sample type. .

2.4.3 NTM species diversity and associated prevalence

Identification of NTM to species level was performed by sequencing of a partial region of their 5' 16S rDNA sequence. Forty species were identified from 303 isolates based on their availability on the NCBI and RIDOM databases. In addition, a total of 117 NTM isolates did not belong to any species represented in these databases. Of these, 79 were closely related to 23 known species of which 34 were identified as closely related to *Mycobacterium moriokaense* (here referred to a *M. moriokanese*-like isolates). Finally 38 isolates were identified as potentially novel species as they were not related to any species represented in the databases. Of the isolates not belonging to species represented on the databases, 34 were derived from soil, 24 from water, 42 from swabs and 17 from tissue. Of the 40 characterised NTM species, 37 were from environmental sources (water, soil and swabs), and 25 from animal tissue samples. Most of the NTM species recovered from the environmental sources were also found in animal tissue, except for *M. parafortuitum, M. kumanotonense, M. arupense, M. holsaticum, M. paraffinicum, M. austroafricanum, M. neoaurum, M. senuense, M. floroanthenivorans, M. monasence, M. engbackii, M. triplex/ M. montefirionse, M. thermoresistible, M. madagascariense* and *M. nebraskense/M. gastri/ M. bohemicum/ M. kansasii/ M. malmoense* which were not isolated from animal tissues. These species except *M. paraffinicum, parafortuitum, M. neoaurum* and *M. engbackii* were, however, detected at very low frequencies of <1% of total isolates. Isolates identified as *M. wolinsky, M. lacticola and M. chelonae or M. abscessus* were isolated from animal sources but not from the environment. These isolates were also detected at low frequency of <1.1% of total isolates. Twenty eight of the known NTM species were recovered from soil, 14 from water, and 16 from animal swabs. Ten NTM species were isolated from both soil and water samples and these are *M. nonchromogenicum, M. terrae, M. triviale, M. intermedium, M. paraffinicum, M. moriokaense, M.*

austroafricanum, M. engbackii, M. triplex or *M. monteferionse* and *Mycobacterium avium* complex (MAC)), including *M. avium* subsp *avium, M. colombiense, M. vulneris* and *M. intracellulare*. Except for *M. fortuitum* complex (*M. fortuitum* subsp *fortuitum* and *M. fortuitum* subsp *acetamidolyticum*) and *M. septicum or M. peregrinum* whose detection rate were 5% and 4.4%, respectively (percentage per sample type), the NTM isolates detected from either soil or water occurred at very low frequencies of <1.9% (percentage per sample type). Ten NTM species and *M. moriokaense* –like isolates were detected from both swab samples and either soil and water. These are *M. nonchromogenicum, M. terrae, M. vaccae* or *M. vanbaalenii, M. duvalii, M. acapulcensis, M. morioakense, M. septicum, M. asiaticum, M. parafortuitum* and *M. neoaurum.* Some isolates belonging to the closest related species could not be differentiated from each other by partial sequencing of the 16S rRNA gene, including *M. triplex* and *M. montefiorense; M. septicum* and *M. peregrinum; M. vaccae* and *M. vanbaalenii; M. flavesence and M. novocastrense; M. pulveris* and *M. elephantis, M. chelonae* and *M. abscessus* as well as *M. nebraskense M. kansassii, M. gastri, M. bohemicum* and *M. malmoense.* The species identified by the RIDOM and NCBI databases and their prevalence are shown in figure 2.2; their distribution across sample types (water, soil, nasal and pharyngeal swabs and animal tissue) from different sample source (environmental and animal) are shown in Tables 2.1 a and 2.1 b and 2.1c. In cases where samples were collected from both the animals and their environments the same NTM species were occasionally isolated from both sources. For example, three *M. moriokaense* and three *M. moriokaense* –like isolates were isolated from 16 buffalo in Hluhluwe iMfolozi Park (HiP), and 1 isolate of each NTM species was isolated from eight soil samples collected from the same Park. Isolates from the four most commonly isolated species are in decreasing order: *M. terrae* (n=47)*;* a group of isolates belonging to species closely related to *M. moriokaense:* referred to as *M. moriokaense*-like isolates (n=34); *M. nonchromogenicum* (n=31); and *M. vaccae / M. vanbaalenii* (n=22). The distribution of these isolates across sample sources is summarised in Table 2.2. All four of these NTM

species/groupings were isolated from both environmental as well as animal sources. Members of *Mycobacterium avium* complex (MAC) represented 6.2% of the isolates and were isolated from both sample sources, while *M. fortuitum* complex isolates represented 3.8% of the isolates and were also isolated from both sample sources.

Table 2.2: Sample source distribution of the four most frequently isolated NTM species

NTM species	**Sample sources**		
	Environmental source: n (%)a	Animal source: n(%)a	total: n (%)b
M. moriokaense-like isolates	25 (7.6)	9(10)	34 (8.1)
M. nonchromogenicum	29(8.7)	2(2.2)	31 (7.4)
M. terrae	45 (13.6)	2(2.2)	47 (11.2)
M. vaccae/ M. vanbaalenii	20(6.1)	2(2.2)	22 (5.2)

n, number of isolates belonging to the respective NTM species; NTM, non-tuberculous mycobacteria; a, percentage of isolates per sample source; b, Percentage of isolates per total number of isolates (420).

2.4.4 Phylogenetic analysis of multiple *M. moriokaense*-like isolates

Phylogenetic classification of *M. moriokaense*- like isolates is shown in Figure 2.3. The neighbour joining tree shows genotypes belonging to 8 or more different clusters. One of the *M. moriokaense* isolates (*M. moriokaense* C-22) grouped with the one cluster consisting of 13 (38%) *M. moriokaense*–like isolates. These groupings were also supported by analysis using a maximum likelihood tree (results not shown).

2.5 Discussion and conclusion

NTM have been isolated ubiquitously in nature, i.e. in the environment: in soil (Covert *et al.,* 1999; Martin-Casabona *et al.*, 2004), and water (Collins *et al.*, 1984; Shitaye *et al.*, 2009), as well as in animals and humans (Kazwala *et al*., 1998; Berg *et al.*, 2009). Some NTM species have occasionally been isolated from animals in the Kruger National Park and the Hluhluwe iMfolozi Park in South Africa (van Helden *et al.,* 2005; Michel, 2007; Michel *et al.,* 2008) but no countrywide NTM survey was conducted. The species diversity and prevalence of NTM detected in this study in the environments of cattle and free-ranging buffaloes in South Africa were defined. NTM species were recovered from samples collected from geographically and climatically different sites in the natural environments of cattle and African buffaloes in South Africa, indicating their distribution in the environment. Twenty seven per cent of known NTM species isolated from environmental samples were found to be shared between soil and water on one hand and animal swabs on the other hand, suggesting that NTM are readily exchanged between these two environments (water and soil) and the animals' mucous membranes. When comparing isolates from animal tissues, and from environmental sources (water and soil and animal swabs), we found that 55% of the known NTM species occurred in both animal and environmental samples. This number is likely to be higher due to additional isolates from both sources which were classified as unknown species. This confirms the well accepted fact that the main niche of NTM is the environment, and animals are thought to be infected from environmental sources through inhalation or ingestion (Primm *et al*., 1994). Furthermore most isolates detected only from either the environmental or animal samples were detected at very low frequencies (<1.1%). The isolation of NTM from animal tissues and the correlation between the isolates from tissue of animals and those from environmental samples, also suggests that most species in this study have the ability to be transmitted from the environment to animals, where they either cause colonisation without causing pathological changes or they may, in some cases,

lead to disease. The four most frequently isolated NTM species in this study were *M. terrae, M. nonchromogenicum, M. moriokaense* –like isolates and *M. vaccae/ M. vanbaalenii,* whose effects on the immune responsiveness of animals and humans are unknown. *M. vaccae* has been suggested to enhance immune function, hence making it helpful in treatment of TB patients (Yang *et al.,* 2011). NTM closely related to *M. moriokaense* have been isolated previously in a buffalo and environmental samples in South Africa (Michel *et al.,* 2007). The occurrence of these *M. moriokaense*-like species, as well as the discovery of several other unknown NTM in South Africa suggest the occurrence of a number of uncharacterised Mycobacterium species in these environments and warrants further characterisation of these species. The abundance of *M. moriokaense*-like isolates prompted us to investigate their phylogenetic relatedness especially in view of their potential immune modulating role in cattle and buffaloes. The phylogenetic analysis of these isolates based on their 16S rRNA revealed at least 8 groups, suggesting that we may have isolated at least 8 different NTM species that are closely related to *M. moriokaense*. Studies on isolation of NTM from bovine tissue sources in Africa have mainly focused on either cattle from slaughter houses or on NTM that were coincidentally isolated from animal lesions while looking for *M. bovis* (Kazwala *et al.,* 1998; Tschopp *et al.,* 2010). In one of these studies in Ethiopian cattle, more than 40% of culture positive isolates were NTM and *M. nonchromogenicum* was isolated as a predominant species (Berg *et al.,* 2009). A study from Chad published in 2006, found *M. nonchromogenicum* together with MAC and *M. fortuitum* to be common in humans and cattle (Diguimbaye-Djaibé *et al.,* 2006). *M. terrae* was isolated as a frequently occurring species in a study on Ethiopian wildlife (Tschopp *et al.,* 2010), and was isolated from cattle in Tanzania (Kazwala, *et al.,* 1998; Cleaveland *et al,* 2005), and in the wildlife environment in South Africa (Michel *et al.,* 2007). In Uganda Kankya *et al.,* 2011 isolated non-tuberculous mycobacteria in 15.5% of environmental samples, they identified 15 NTM species, from 48 samples, and *M. nonchromogenicum, M. fortuitum* complex, *M. avium* complex and *M. gordonae* were identified as most frequently detected species. A study in Zaire

also identified *M. nonchromogenicum* and *M. terrae* to be among the most prevalent NTM species in the environment (Portaels, 1995). *M. nonchromogenicum* has also been detected in small mammals and cattle in Tanzania (Durnez *et al.,* 2011). This suggests that *M. nonchromogenicum* and *M. terrae* are ubiquitous NTM species in Africa although percentages differ in different countries Identification of 40 known NTM species, 79 unknown isolates related to other NTM species in the RIDOM and NCBI databases, as well as 38 unknown, potential novel species and a 56% overall Mycobacterium detection rate in environmental samples served to demonstrate that the diversity of NTM species in South Africa is high. Other studies performed in central, south and north India, even though their NTM detection rates (43% , 54%, 40% respectively) were comparable to that observed in this study, only 7 , 20 and 10 different characterised NTM species were recovered from 60 ,717 and 291 environmental samples respectively. (Kamala *et al.,* 1994; Narang *et al.,* 2009; Parashar *et al.,* 2009). All three of these studies employed culture based methods followed by PCR restriction analysis of the Mycobacterium housekeeping genes. Similarly in another study conducted in Ohio (1999), 33% NTM detection rate led to identification of 18 NTM species from 139 environmental samples using culture and 16S rDNA PCR-sequencing assay (Covert *et al.,* 1999). In another study in Iran (2004) 10 NTM species were identified from 72 NTM isolates recovered in 490 environmental samples (Rahbar *et al.* 2010). In this study, NTM detection was performed using phenotypic assays. In a study conducted in Czech Republic between 2003 and 2004, 12 characterised NTM species were detected from 400 isolates that were recovered in 1389 environmental samples and detection was done by a combination of biochemical test on cultured isolates as well as PCR that detected mycobacteria to genus level (Beran *et al.,* 2006). Different reported diversities in the different countries could have been influenced by the differences in the detection techniques, as it is a fact that molecular based detection methods like PCR- sequencing assays employed in this study offer more accurate results than the classical phenotypic assays (Turenne *et al.,* 2001)

M. avium PPD is used mostly for application in both the comparative TST and the gamma interferon test for TB in most countries based on the knowledge that *M. avium* is among the most frequently isolated NTM in both animals and humans and has been shown to prime immune responsiveness (Prichard, 1988; Wood and Jones, 2001; Palmer *et al.,* 2006). *M. fortuitum* PPD is used in the modified gamma interferon assay based on the isolation of *M. fortuitum* from IFNγ test positive buffaloes in the Kruger National Park (Michel *et al.,* 2008; 2011). Although potentially cross reactive among NTM, members of the *Mycobacterium avium* complex (MAC) (6.2%) as well as *M. fortuitum* (3,8%) complex, were not isolated as frequent as the abundant species defined here, therefore, their exclusive use as environmental controls in TST and gamma interferon in South Africa may be reconsidered to include further investigation of the prevalent NTM. The identification of these abundant and common NTM species now allows a targeted investigation of their effects on host immune response and BCG vaccination.

In conclusion, we have for the first time shown that NTM are highly abundant and diverse in the environment of South African cattle and buffalo and potential novel NTM species exist in South Africa for which no information is yet available on the international databases. We have shown that NTM are readily exchanged between water or soil and animals via their mucous membranes. Even though we have classified the swabs as environmental samples, they may have colonised and stimulated the host's immune response in some cases. We have also shown that certain NTM species occur more frequently in the environment than others *viz M. terrae, M. nonchromogenicum, M. vaccae/ M. vanbaalenii* and *M. moriokaense* –like isolates. This suggests that cattle and buffalo are not only exposed to a variety of NTM species, but they are exposed more to some species than to others, with potential implications for TB diagnosis, vaccination and *M.bovis* infection due to cross reactive immune responsiveness

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2.6 References

AMENI G, VORDERMEIER M, ASEFFA A, YOUNG DB, HEWINSON RG (2010). Field evaluation of the efficacy of *Mycobacterium bovis* Bacillus Calmette-Gue´rin against Bovine Tuberculosis in neonatal calves in Ethiopia. *Clinical and Vaccine Immunology* **17:** 1533-1538.

ARBELÁEZ MP, NELSON KE, MUÑOZ A (2000). BCG vaccine effectiveness in preventing tuberculosis and its interaction with human immunodeficiency virus infection. *International Epidemiology Association* **29:** 1085-1091.

BRANDT L, CUNHA JF, OLSEN AW, CHILIMA B, HIRSCH P, APPELBERG R, ANDERSON P (2002). Failure of *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and Immunity* **70:** 672-678.

BENGIS RG, KRIEK NP, KEET DF, RAATH JP, DE VOS V, HUCHZERMEYER HF (1996). An outbreak of bovine tuberculosis in a free ranging African buffalo (Syncerus caffer-sparrman) population in the Kruger National Park. A preliminary report. *Onderstepoort Journal of Veterinary Research* **63:** 15-18.

BERAN V, MATLOVA L, HORVATHOVA A, BARTOS M, MORAVKOVA M, PAVLIK I (2006). Mycobacteria in the animal's environment in the Czech Republic. *Veterinary Archives (Suppl)* **76:** S33-S39.

BERG S, FIRDESSA R, HABTAMU M, GADISA E, MENGISTU A, *et al.* (2009). The burden of mycobacterial disease in Ethiopian cattle: Implications for public health. *Plos One* **4(4)**: edoi: 10.1371/ journal.pone.0005068.

BERGGREN SA (1981). Field experiment with BCG vaccine in Malawi. *British Veterinary Journal* **137:** 88-94.

BRANDT L, CUNHA JF, OLSEN AW, CHILIMA B, HIRSCH P, APPELBERG R, ANDERSON P (2002). Failure of the *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and Immunity* **70:** 672-678.

BUDDLE B M, WARDS BJ, ALDWELL FE, *et al,* (2002). Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20:** 1126-1133.

CLEAVELAND S, MLENGEYA T, KAZWALA RR, MICHEL A, KAARE MT, JONES SL, EBLATE E, SHIRIMA GM, PACKER C (2005). Tuberculosis in Tanzanian wildlife. *Journal of Wildlife Diseases* **41:** 446-453

COLDITZ GA, BERKEY CS, MOSTELLER F, BREWER TF, WILSON ME, BURDICK E, FINEBERG HV (1995). The efficacy of bacillus Calmette-Guérin vaccination of newborns and infants in prevention of tuberculosis: meta-analysis of the published literature. *Pediatrics* **96:** 29-35.

CSILLAG (1961) Morphological and Biochemical features of mycobacteria. *Journal of General Microbiology* **24:** 261-272.

COLLINS CH, GRANGE JM, YATES MD (1984). A review: mycobacteria in water. *Journal of Applied Bacteriology* **57:** 193-211.

COUSINS DV (2001). *Mycobacterium bovis* infection and control in domestic livestock. *Revue Scientifique et Technique –Office International Des Epizooties* **20:** 71-85

COVERT TC, RODGERS MR, REYES AL, STELMA GN JR (1999). Occurrence of non- tuberculous mycobacteria in environmental samples. *Applied and Environmental Microbiology* **65:** 2492-2496.

DE VOS V, BENGIS RG, KRIEK NP, MICHEL A, KEET DF, RAATH JP, HUCHZERMEYER HF (2001). The epidemiology of tuberculosis in free ranging African buffalo (*Syncerus caffer*) in the Kruger National Park, South Africa. *Onderstepoort Journal of Veterinary Research* **68:** 119-130.

DIGUIMBAYE-DJAIBÉ C, VINVENT V, SHELLING E, HILTY M, NGANDOLO R, *et al.*, (2006). Species identification of non-tuberculous mycobacteria from humans and cattle of Chad. *Schweiz Arch Tierheilkd* **148:** 251-256.

DURNEZ L, KATAKWEBA A, SADIKI H, KATHOLI CR, KAZWALA RR, MACHANG'U RR, PORTAELS F, LEIRS H. (2011). Mycobacteria in terrestrial small mammals on cattle farms in Tanzania. Veterinary Medicine International. doi:10.4061/2011/495074

FINE PE (1989). The BCG story: lessons from the past and implications for the future. *Reviews of Infectious Diseases* **11:** S353-S359.

HARMSEN D, DOSTAL, S, ROTH A, NIEMANN S, ROTHGANGER J, SAMMETH M, ALBERT J, FROSCH M, RICHTER E (2003)**.** RIDOM: Comprehensive and public sequence database for identification of Mycobacterium species. *Biomed Central*: *Infectious Diseases* **3:** 26.

HART PD, SUTHERLAND I (1977). BCG and vole bacillus vaccines in the prevention of tuberculosis in adolescence and early adult life. *British Medical Journal* **2:** 293-295.

HLOKWE TM, JENKINS AO, STREICHER EM, VENTER EH, COOPER D, GODFROID J, MICHEL A (2011)**.** Molecular characterisation of *Mycobacterium bovis* isolated from African buffaloes (*Syncerus caffer*) in Hluhluwe- iMfolozi Park in KwaZulu-Natal South Africa. *Onderstepoort Journal of Veterinary Research* **78:** doi: 10,4102/ojvr.v78i1.232.

KAMALA T, PARAMASIVAN CN, HERBERT D, VENKATESAN P, PRABHAKAR R (1996). Immune response and modulation of immune response induced in the guinea-pigs by *Mycobacterium avium* complex (MAC) and *M. fortuitum* complex isolates from different sources in the south Indian BCG trial area. *Indian Journal of Medical Research* **103:** 201-211.

KANKYA C, MUWONGE A, DJØNNE B, MUNYEME M, OPUDA-ASIBO J, SKJERVE E, OLOYA J, EDVARDSEN V, JOHANSEN TB (2011). Isolation of non-tuberculous mycobacteria from pastoral ecosystems of Uganda: Public Health significance. *Biomed Cenral: Public Health* **11:** 320

KAZWALA RR, DABORN CJ, KUSILUKA LJM, JIWA SFH, SHARP JM, KAMBARAGE DM (1998): Isolation of Mycobacterium species from raw milk of pastoral cattle of southern highlands of Tanzania. *Tropical Animal Health and Production* **30:** 233–239.

IIVANAINEN E, MARTTIKAINEN PJ, PAVLIK I (1995). Effect of freezing of water samples on viable counts of environmental mycobacteria. *Letters of Applied Microbiology* **21:** 257-260.

IIVANAINEN E (1995). Isolation of mycobacteria from acidic forest soil samples. Comparison of culture methods. *Journal of Applied Bacteriology* **78:** 663-668.

MARTÍN-CASABONA N, BAHRMAND AR , BENNEDSEN J , OSTERGAARD THOMSEN V , CURCIO M, FAUVILLE-DUFAUX M , FELDMAN K, HAVELKOVA M, KATILA ML , KÖKSALAN K, PEREIRA MF, RODRIGUES F , PFYFFER GE, PORTAELS F, ROSSELLÓ URGELL J, RÜSCH-GERDES S , TORTOLI E , VINCENT V, WATT B (2004). Non-tuberculous mycobacteria: Patterns of isolation. A multi-country retrospective survey. *International Journal of Tuberculosis and Lung Diseases* **8***:*1186-1193.

MICHEL AL, BENGIS RG, KEET DF, HOFMEYER M, DE KLERK LM, CROSS PC, JOLLES AE, COOPER D, WHYTE IJ, BUSS P, GODFROID J (2006). Wildlife tuberculosis in South African conservation areas: Implications and challenges. *Veterinary Microbiology* **112:** 91-100.

MICHEL AL, COETZEE ML, KEET DF, MARE L, WARREN R, COOPER D, BENGIS RG, KREMER K, VAN HELDEN P (2009). Molecular epidemiology of *Mycobacterium bovis* isolates from free- ranging wildlife in South African game reserves. *Veterinary Microbiology* **133:** 335-343.

MICHEL AL, DE KLERK L, GEY VAN PITTIUS NC, WARREN RM, VAN HELDEN PD (2007). Bovine tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into

water and exposure to environmental mycobacteria. *BioMed Central*: *Veterinary Research.* **3:** doi: 10.1186/1746-6148-3-23.

MICHEL AL (2008)**.** *Mycobacterium fortuitum* infection interference with *Mycobacterium bovis* diagnostics: natural infection cases and a pilot experimental infection. *Journal of Veterinary and Diagnostic Investigations* **20:** 501-503.

MICHEL AL, COOPER D, JOOSTE J, DE KLERK LM, JOLLES A (2011). Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine* **98:** 142-151.

NARANG R, NARANG P, MENDIRATTA DK (2009). Isolation and identification of non-tuberculous mycobacteria from water and soil in central India. *Indian Journal of Medical Microbiology* doi:10.4103/0255-0857.53208.

ORME IM, ROBERTS AR, COLLINS FM (1986). Lack of evidence for reduction in the efficacy of subcutaneous BCG vaccination in mice infected with non-tuberculous mycobacteria. *Tubercle* **67:** 41-46.

PALMER MV, WATERS WR, THACKER TC, GREENWALD R, ESFANDIARI J, LYASHCHENKO KP (2006). Effects of different tuberculin skin testing regimes on gamma interferon and antibody

responses in cattle experimentally infected with *Mycobacterium bovis. Clinical and Vaccine Immunology* **13:** 387-394.

PARASHAR D, DAS R, CHAUHAN DS, SHARMA VD, LAVANIA M, YADAV VS, CHAUCAN SVS, KATOCH VM (2009). Identification of environmental mycobacteria isolated in Agra, north India by convectional and molecular approaches. *Indian Journal of Medical Research* **129:** 424-431.

PONNIGHAUS JM, FINE PE, STERNE JA, WILSON JR, MSOSA E, *et al.* (1992). Efficacy of BCG vaccine against leprosy and tuberculosis in northern Malawi. *Lancet* **339:** 636-639.

PORTAELS F (1995). Epidemiology of mycobacterial diseases. In: Mycobacterial diseases of the skin. Clinics in Dermatology. Elsevier Sciences Inc, New York, Ed. M. Schuster. **13**: 207-222

PRICHARD DG (1988). A century of bovine tuberculosis 1888-1988: conquest and controversy. *Journal of Computational Patholology* **99:** 357-399.

PRIMM TP, LUCERO CA, FALKINHAM JO III (2004). Health impacts of environmental Mycobacteria. *Clinical Microbiology Reviews* **17:** 98-106.

RAHBAR M, LAMEI A, BABAZADEH H, YAVARI SA (2010). Isolation of rapid growing mycobacteria from soil and water in Iran. *African Journal of Biotechnology* **9:** 3618-3621**.**

RODRIGUES LC, GILL N, SMITH PG (1991). BCG vaccination in the first year of life protects children of Indian subcontinent ethic origin against tuberculosis in England. *Journal of Epidemiology and Community Health* **45:** 78-80.

RODWELL TC, KRIEK NP, BENGIS RG, WHYTE IJ, VILJOEN PC, DE VOS V, BOYCE WM (2001). Prevalence of bovine tuberculosis in African buffalo at Kruger National Park. *Journal of Wildlife Diseases* **37:** 258-264.

SATOU N, NEI M (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology Evolution* **4:** 406-425

SCHILLER I, OESCH B, VORDERMEIER HM, PALMER MV, HARRIS BN, ORLOSKI KA, BUDDLE BM, THACKER TC, LYASHCHENKO KP, WATERS WR (2010). Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and Emerging Diseases* **57**: 205-220.

SHITAYE JE, HORVÁTHOVÁ A, BARTOSÓVÁ, MORÁVKOVÁ M., KAEVSKA M, DONNELLY N, PAVLIC I (2009). Distribution of non-tuberculous mycobacteria in environmental samples from a slaughterhouse and in raw and processed meats. *Czech Journal of Food Science* **27:** 194-202.

THOMPSOM JD, HIGGINS DG, GIBSON TJ (1994). CLUSTAW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position- specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22:** 4673-4680.

TSCHOPP R, BERG S, ARGAW K, GADISA E, HABTAMU M, SCHELLING E, YOUNG D, ASEFFA A, ZINSSTAG J (2010). Bovine Tuberculosis in Ethiopian wildlife. *Journal of Wildlife Diseases* **46:** 753-762.

TURENNE CY, TSCHETTER L, WOLFE J, KABANI A (2001). Necessity of quality - controlled 16S rRNA gene sequence databases: Identifying non – tuberculous *Mycobacterium* species. *The American Society of Microbiology* **39**: 3637- 3648.

VAN HELDEN PD, GEY VAN PITTIUS N C, WARREN RM, MICHEL A, HLOKWE T, MORAR D, GODFROID J, DU PLESSIS EC, BENGIS R (2008). Pulmonary infection due to *Mycobacterium goodii* in a spotted hyaena (*Crocuta crocuta*) from South Africa. *Journal of Wildlife Diseases* **44**:151-154.

VOEDERMEIER MM, CHAMBERS MA, COCKLE PJ, WHELAN AO, SIMMONS J, HEWINSON RG (2002). Correlation of ESAT-6 –specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Infection Immunology* **70:** 3026-3032.

WEIR RE, BLACK GF, NAZARETH B, FLOYD S, STENSON S, STANLEY C, BRANSON K, SICHALI L, CHAGULUKA SD, DONOVAN L, CRAMPIN AC, FINE PEM, DOCKRELL HM (2006). The influence of previous exposure to environmental mycobacteria on the interferon-gamma response to bacille Calmette–Guérin vaccination in southern England and northern Malawi. *Clinical and Experimental Immunology* **146:** 390-399.

WOOD PR**,** JONES (2001). Bovigam: an invitro cellular diagnostic assay for bovine tuberculosis. *Veterinary Microbiology* **40:** 125-135.

YANG X-Y, CHEN Q-F, LI Y-P, WU S-M (2011). *Mycobacterium vaccae* as an adjuvant therapy to anti-tuberculosis chemotherapy in never-treated tuberculosis patients: A Meta analysis. *Plos One* **6(9):** e23826.doi:10.1371/journal.pone.0023826.

YOUNG SL, SLOBBE L, WILSON R, BUDDLE BM, DE LISLE GW, BUCHAN GS (2007). Environmental strains of *Mycobacterium avium* interfere with immune responses associated with *Mycobacterium bovis* BCG vaccination. *Infection and Immunity* **75:** 2833-2840.

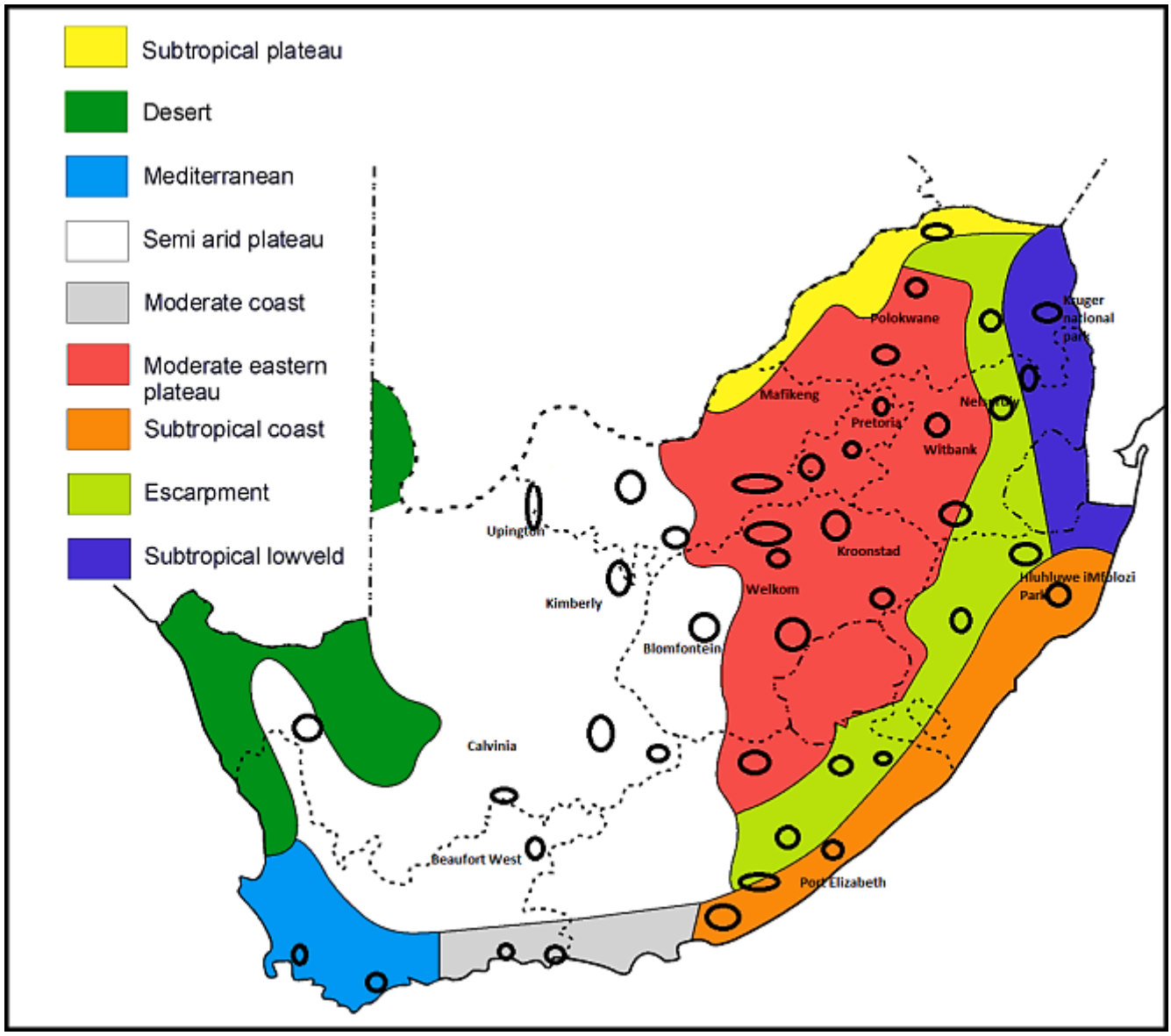

Fig 2.1: Geographical and climatical distribution of sampling sites for country wide survey in South Africa. Sampling sites are indicated by (O).

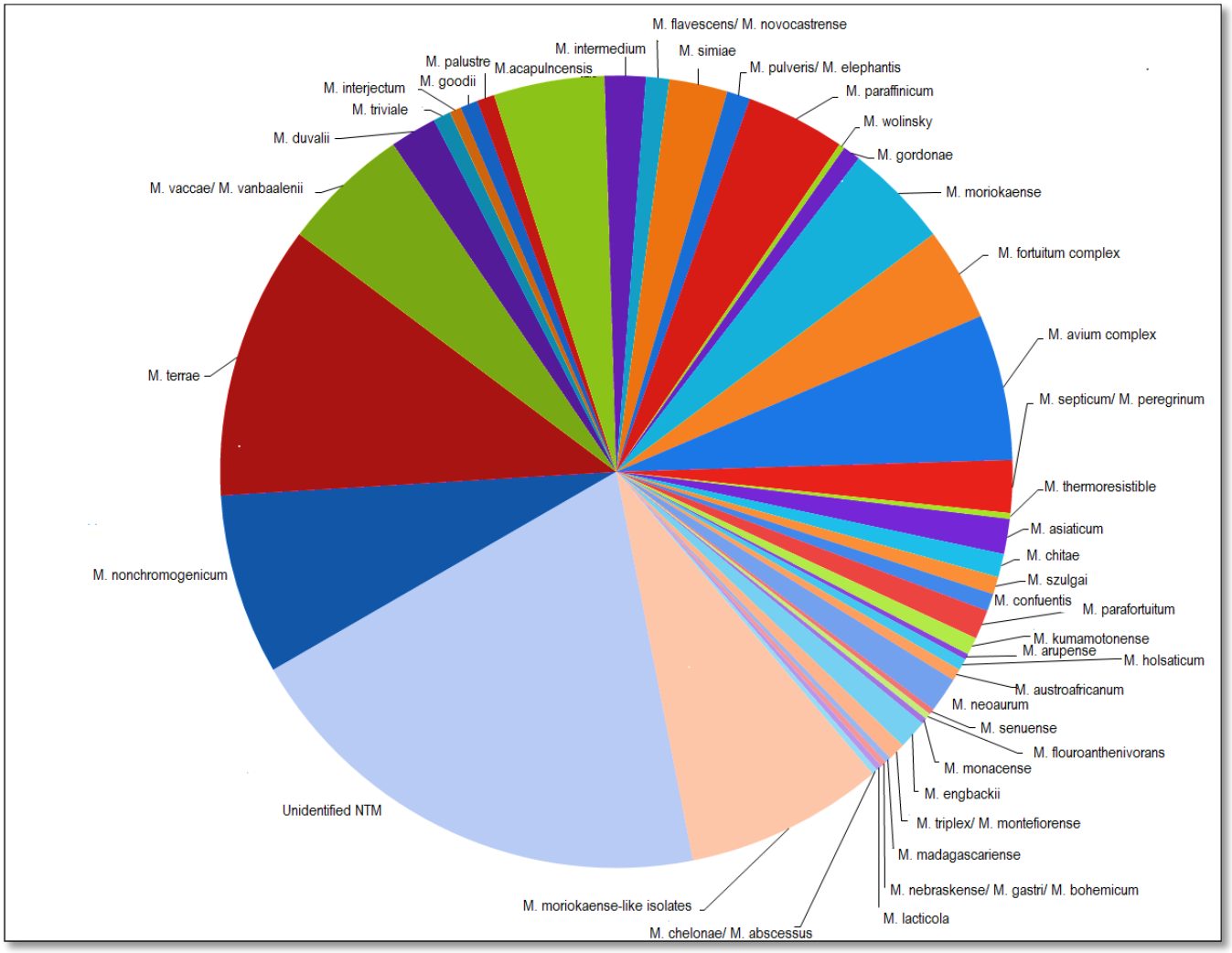

Fig 2.2: NTM pie chart diagram indicating NTM prevalence

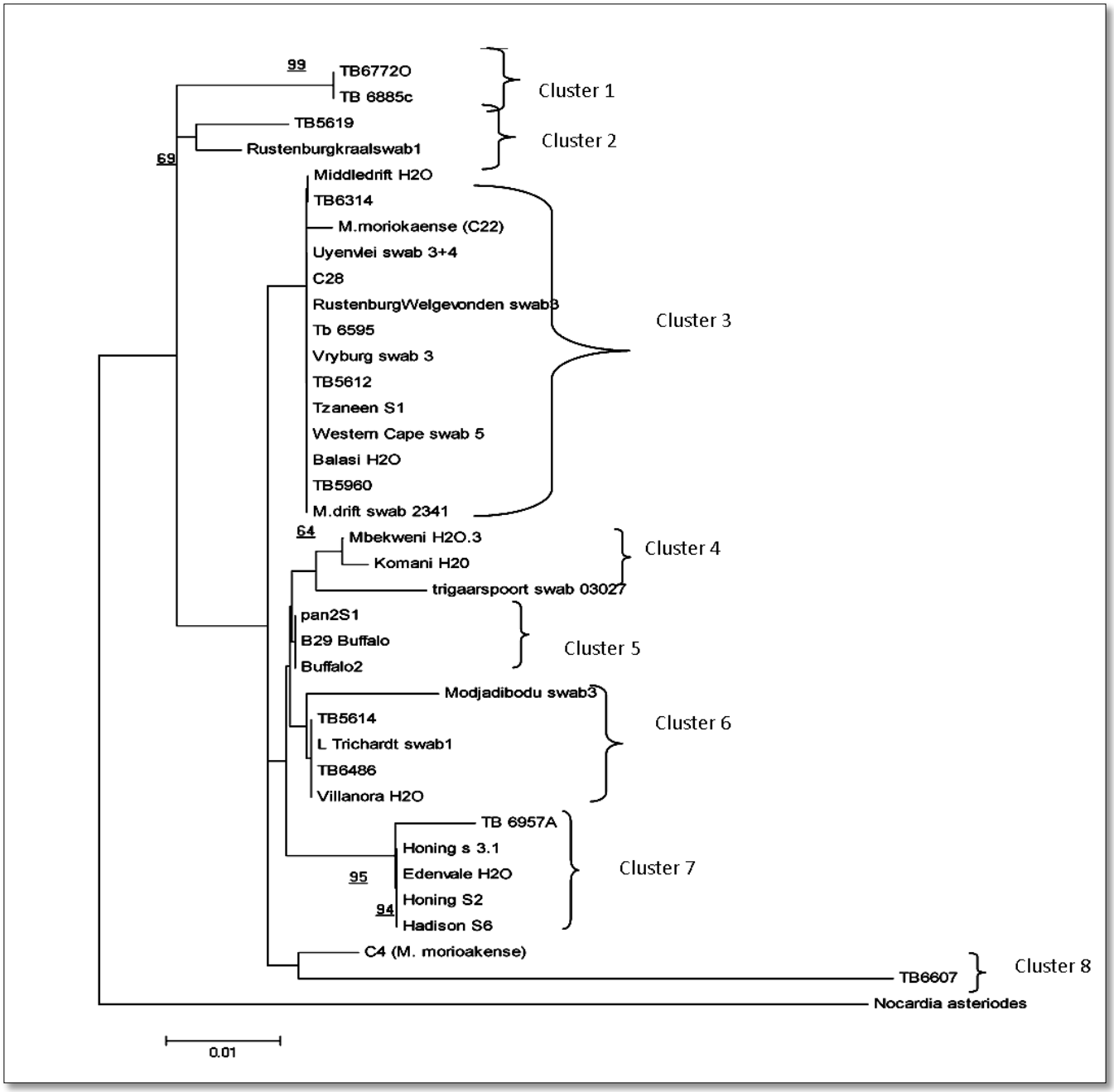

Fig 2.3: Phylogenetic tree of Mycobacterium moriokaense-like isolates based on the partial 16S rRNA gene sequences. One thousand bootstrap replicates were run. The tree was constructed using MEGA v5.05, and the criterion was neighbour joining. Branch support values on the nodes are shown as percentages, and values <60% are not shown. Nocardia spp. was used as an outgroup sequence.

CHAPTER 3. TWO NOVEL SPECIES OF NON-TUBERCULOUS MYCOBACTERIA REVEALED BY MULTIPLE GENE SEQUENCE CHARACTERIZATION

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3.1 Abstract

Non-tuberculous mycobacteria (NTM) are ubiquitous in the environment and an increasing number of NTM species have been isolated and characterised from both humans and animals, highlighting the zoonotic potential of these bacteria. Host exposure to NTM may impact on cross-reactive immune responsiveness which may affect diagnosis of bovine tuberculosis and may also play a role in the variability of the efficacy of *Mycobacterium bovis* BCG vaccination against tuberculosis. In this study we characterized 18 NTM isolates originating from water, soil, nasal swabs of cattle and African buffalo as well as bovine tissue samples. These isolates were previously identified during an NTM survey and were found to be among the four most abundant NTM. They had not been described to species level and were all found to be closely-related to *Mycobacterium moriokaense*. A polyphasic approach that includes phenotypic characterisation, antibiotic susceptibility profiling, sequencing, and phylogenetic analysis of three gene loci, *viz hsp6*5, *sodA*, and *rpoB* was employed to characterize these isolates. Sequence data analysis of the three gene loci revealed that these isolates belong to unique *Mycobacterium* species. This evidence was further supported by several differences in phenotypic characteristics between the isolates and the closely related species. Further evidence that the isolates belonged to novel species was provided by their phylogenetic position in the neighbour joining trees of the individual gene sequences which revealed the existence of two distinct clusters, representing two novel species. We proposed the names, *Mycobacterium malmesburii* sp.nov. and *Mycobacterium komanii* sp.nov. for these two new species. Isolation of these novel NTM species from different regions in the environment, as well as animal tissue samples confirms the existence of as yet unidentified NTM species that animals are exposed to. Their relevance needs to be investigated, especially in light of cross- reactive immune responses that they may elicit.

Key words: **Non-tuberculous mycobacteria, *Mycobacterium malmesburii* sp. nov., *Mycobacterium komanii* sp. nov., multiple gene sequences**

3.2 Introduction

Currently more than 100 non-tuberculous mycobacterial species are listed in public databases (http//:www.bacterio.net). An increasing number of NTM species has recently been isolated and characterized from both humans and animals, highlighting the zoonotic potential of these species (reviewed by Tortoli *et al.*, 2006, van Helden *et al.*, 2009 and Botha *et al.*, 2013). An increased interest in these organisms has developed in view of the potential impact of host exposure to NTM on cross-reactive immune responsiveness which may affect diagnosis of bovine tuberculosis (BTB) (Michel, 2008; Michel *et al.*, 2011; Schiller *et al.*, 2010) and may also play a role in the variability of the efficacy of *Mycobacterium bovis* BCG vaccination against tuberculosis (Buddle *et al.*, 2002; Brandt *et al.*, 2002; Weir *et al*., 2006). NTM are ubiquitous in the environment and there is no evidence of animal-to-animal or human-to-human transmission (Primm *et al*., 1994; Falkinham, 2002). Characterization of NTM isolates from environmental as well as clinical samples is important as these may lead to identification of emerging pathogens. For instance, *Mycobacterium avium* was essentially a rare human pathogen before the acquired immune deficiency syndrome (AIDS) pandemic, and since then this NTM species has become a very important pathogen of AIDS patients (Karne *et al.*, 2012). Isolation of a large number of NTM from human and animal samples does not contradict the hypothesis that the environment is the main niche of NTM (Falkinham, 2002). The reference molecular method for the identification of mycobacteria is the sequencing of the 16S rDNA, which has contributed to the discovery of novel NTM isolates beyond possibilities offered by conventional methods such as phenotypic characterisation (Turenne *et al.*, 2001). However, this approach has its limitations as there are instances where the sequence of this gene has been found to be similar, if not identical between species of the genus. For example, analysis of the 16S rDNA sequence could not delineate *Mycobacterium triplex* and *Mycobacterium montefiorense; Mycobacterium septicum* and *Mycobacterium peregrinum;*

Mycobacterium vaccae and *Mycobacterium vanbaalenii; Mycobacterium flavesence and Mycobacterium novocastrense; Mycobacterium pulveris* and *Mycobacterium elephantis; Mycobacterium chelonae* and *Mycobacterium abscessus* as well as *Mycobacterium nebraskense Mycobacterium kansassii, Mycobacterium gastri, Mycobacterium bohemicum* and *Mycobacterium malmoense* (Gcebe *et al.*, 2013). In addition ambiguous results can be obtained with sequencing of the 16S rDNA due to the possible presence of two copies of ribosomal RNA with different sequences in the same organism, making it necessary to find alternative specific sequences (Adékambi and Drancourt, 2004; Adékambi *et al.*, 2006). Other DNA sequences, loci or genes present in all mycobacteria have been described for the differentiation of species, among others, the *hsp65* (Telenti *et al.*, 1993; Senna *et al.*, 2008); *gyrB* (Kasai *et al.*, 2000), *recA*, (Blackwood *et al.*, 2000; Adekambi *et al.*, 2004); *rpoB* (Adékambi *et al.*, 2003; 2006), *dnaK* (Dai *et al.*, 2011) and *sodA (*Adékambi and Drancourt, 2004). It has been shown that multiple locus analysis provides a more detailed and accurate identification of *Mycobacterium* species than the use of a single locus (Devulder *et al.*, 2005). We previously described the prevalence of NTM isolates in cattle, African buffaloes and their environments in South Africa and found a group of isolates (closest related by analysis of 16S rDNA to *Mycobacterium moriokaense*) that were not well described in literature at the species level. These isolates were found to be among the four most abundant NTM species (8.1%) in the study comprising of 6% isolated from environmental and 2.1% from animal samples. The other abundant NTM species were *Mycobacterium terrae* (11.2%), *Mycobacterium nonchromogenicum* (7.4%) and *M. vaccae / M. vanbaalenii* (5.2%) (Gcebe *et al.*, 2013). From these results it can be hypothesized that cattle and African buffaloes are likely to be more frequently exposed to these species than to other NTM. This has potential implications for BTB diagnosis due to cross reactive immune responsiveness of host animals to these species, as shown by Michel *et al.*, 2011. The aim of the present study was to conduct an in depth characterisation of this group of isolates closely related to *M.*

moriokaense. The investigation included phenotypic characterization, antimicrobial susceptibility profiling, and sequence based analysis of three mycobacterial housekeeping genes.

3.3 Materials and Methods

3.3.1 Mycobacterium cultures

Eighteen isolates were recovered from soil, water, nasal and pharyngeal swabs of cattle and buffalo, as well as tissue samples of cattle during a survey conducted between April 2010 and December 2012 to determine the prevalence and distribution of NTM, and analysis of isolates from routine diagnostic samples collected in different geographic and climatic regions of South Africa (Table 3.1). All the cultures were maintained on either Middlebrook 7H11 agar plates (Becton Dickinson, USA) supplemented with 0.1% OADC (Merck Chemicals, South Africa), and glycerol as well as on Löwenstein Jensen (LJ) slopes supplemented with glycerol and an antibiotic cocktail of PolymyxinB, AmphotericinB, Carbenicillin and Trimethoprim (PACT) (Becton Dickinson, USA) for subsequent biochemical testing.

Table 3. 1: Origin of NTM isolates used in the study

NTM Isolate ID	Sample type	Climatic region in South Africa	Location and GPS co-ordinates
Pan2S1	Soil	Subtropical coast	Hluhluwe Imfolozi park: 28°13'11" S, 31°57'07" E
Honing S2	Soil	Semi-arid plateau	Kimberly 24° 44'3" S, 24°46'19" E
Balasi H_2O	Water	Escarpment	Bisho 32°50'58"S, 27°26'17"E
Villanora H_2O	Water	Moderate Eastern plateau	Lephalale 23°40'S, 27°45'E
Komani H_2O	Water	Escarpment	Queenstown 31°51'S, 26°53'E
Mbekweni H_2O.3	Water	Escarpment	Queenstown 31°51'S, 26°53'E
Swab 242	Bovine nasal swab	Moderate Eastern Plateau	Frankfort 27°30'14"S, 27°35'58"E
Middledrift swab 2341	Bovine nasal swab	Escarpment	Middledrift 32°49'7"S, 26°59'15"E
W. Cape swab 5	Bovine nasal swab	Mediterranean climate	Malmesbury 33°27'4.7"S, 18°43'19.06"E
Trigaarspoort swab 03027	Bovine nasal swab	Moderate Eastern Plateau	Pretoria 25°39'15.4"S, 28°56'51.8"E
Vryburg swab3	Bovine nasal swab	Semi-arid	26°58'S, 24°54'E
Uyenvlei swab3+4	Bovine nasal swab	Moderate eastern plateau	Lephalale 23°40′S 27°45′E
C28	Buffalo pharyngeal swab	Subtropical coast	Hluhluwe Imfolozi park: 28°13'11" S, 31°57'07" E
C4	Buffalo pharyngeal swab	Subtropical coast	Hluhluwe Imfolozi park: 28°13'11" S, 31°57'07" E
TB 6607	Buffalo nasal swab	Subtropical cost	Hluhluwe Imfolozi park: 28° 13'11 S, 31°57'07 E
TB 5612	Bovine lymph node	Sub-tropical coast	Bergville

			28°43'48"S, 29°21'0.2"E
TB 5960A	Bovine lymph node	Escarpment	East London 32°59'S, 27°52'E
TB 5614	Bovine lymph node	Subtropical coast	Bergville 28°43'48"S, 29°21'0.2"E

3.3.2 Phenotypic characterisation of the isolates

Slopes and plates were incubated at 37°C, 45°C and 25°C in order to evaluate the ability of each isolate to grow at different temperatures. We also observed colony morphology (rough or smooth), pigmentation, acid fastness and different growth rates of the isolates. The cultures for all the biochemical as well as antibiotic susceptibility/ resistance tests were grown at 37°C.

Sodium chloride (NaCl) tolerance (5%) of each isolate was evaluated by supplementing the Middlebrook 7H11 agar with 5% NaCl and observing the growth of each isolate against media that was not supplemented with 5% NaCl, after 2-10 days. The isolates were tested for the ability to reduce nitrate, for urease activity and niacin accumulation, using the commercial test strips (Becton Dickinson, USA) according to the manufacturer's instructions. They were also tested for the 3 day and 14 day arylsulphatase activity (Wayne, 1961). We tested all the isolates for pyrazinamidase activity as described by Singh *et al.,* 2007, except that Middlebrook 7H11 agar was used and the culture incubation period was 4 days. In addition, the isolates were also tested for the ability to hydrolyze Tween 80 using the method described by Kilburn *et al.,* 1973. Semi quantitative catalase activity of the isolates was tested using 3% hydrogen peroxide and observing formation of gas bubbles (Kent and Kubica, 1985). Finally the isolates were tested for their ability to hydrolyze aesculin, for citrate utilization (National Health Laboratory Services, South Africa) as well as their ability to utilize the following sugars as sole carbon

sources: D-mannitol, inositol, L-rhamnose and L-arabinose (Selecta media, South Africa). Since no standard antibiotics are prescribed for taxonomic classification of NTM, only eight of those antibiotics that have been used in literature for classification of NTM were applied in this study (Brown -Elliot, 2002). Antibiotic susceptibility to amikacin (30 µg), cefoxitin (10 µg), ciprofloxacin (10 µg), clarithromycin (15 µg) , doxycycline (30 µg) , imipenem (10 µg), amoxylin (30 µg) and tobramycin (10 µg) (Oxoid LTD, UK) was determined for all the isolates, using a modified Kirby Bauer disk diffusion method on Middlebrook 7H11 agar plates supplemented with 0.1% OADC and incubated for up to 3 days (for the rapid growing mycobacteria) and 10 days (for the slow growing mycobacteria) at 37°C, after which the zones of inhibition were measured (Brown-Eliot and Wallace, 2002). Since other antibiotics used in this study are not routinely applied to rapidly growing mycobacteria (RGM) in a clinical context; we reported the profiles as size of the inhibition zones. If a minimum of 3 mm zone of inhibition is obtained, that was interpreted as inhibition.

3.3.3 Genetic and phylogenetic analysis of the isolates

Genetic markers targeted:

In addition to the partial sequencing of the 16S rRNA gene, used previously to identify the isolates (Gcebe *et al.,* 2013), three molecular targets including a 439 bp region of the gene encoding the 65 kDA heat shock protein (*hsp65*) (Telenti *et al.,* 1993), part (764 bp) of the RNA polymerase beta subunit (*rpoB*) gene (Adékambi *et al.,* 2003; 2006) and part (464 bp) of the superoxide dismutase (*sodA)* gene (Adékambi and Drancourt, 2004) were amplified and sequenced.

Polymerase Chain Reaction (PCR)

Boiled culture suspensions from individual isolates were used as DNA template in the various PCR protocols.

***Hsp65* PCR**: Tb11 (5'ACCAACGATGGTGTGTCCAT 3') and Tb12 (5' CTTGTCGAACCGCATACCCT 3') primers were used for the amplification of the *hsp65* gene fragment. A 50 µl PCR mixture was prepared, containing 28.5 µl de-ionized water, 3 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 4.75 µl of 10x PCR buffer (160 mM) (Tris -HCl, $MgCl_2$, Tween 20, $(NH_4)_2$, SO_4), 0.75 µl Taq DNA Polymerase (5 U/ µl) (Supertherm ^{TM}), 1 µl of each forward and reverse primers (50 pmol) and 10 µl of DNA template. The PCR cycling parameters were as follows: Fourty five cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min and final extension at 72°C for 10 min.

***RpoB* PCR**: MycoF (5' GGCAAGGTCACCCCGAAGGG 3') and MycoR (5' AGCGGCTGCTGGGTGATCATC 3') primers were used for amplification of *rpoB* gene fragment. A 50 µl PCR mixture was prepared as described for the *hsp65* PCR. The cycling parameters were as follows: Initial denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 30 s, elongation at 72°C for 90 s, and final extension at 72°C for 5 min.

***SodA* PCR**: SodF (5'ACATCTCGGGTCAGATCAACGACG 3') and SodR (5'GACGTTCTTGTACTGCAGGTA 3') primers were used for the amplification of the *sod*A gene fragment. A 50 µl PCR mixture was prepared as described for the *hsp65* PCR. The cycling parameters

were as follows: Initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, elongation at 72°C for 2 min, and final extension at 72°C for 5 min.

***Sequencing and phylogenetic analysis*:**

The amplicons were sequenced at the Central Analytical Facility of Stellenbosch University, South Africa, using the same primer sequences that were used for amplification of the respective gene fragments. Sequences from both strands were edited manually and pairwise alignments undertaken using the BioEdit Sequence alignment editor (version 7.1.9) and Molecular Evolutionary Genetics Analysis (MEGA) platform (www.megasoftware.net) (version 6) (Tamura *et al.,* 2013). The resulting consensus sequences were analysed on the NCBI BLAST platform for species identification (www.blast.ncbi.nlm.nih.gov/Blast.cgi) by megablast. For phylogenetic analysis, all the sequences were first trimmed at both the 5' and the 3' ends to encompass the most corresponding gene fragment sequences of mycobacteria deposited in Genbank. The resulting fragments used for phylogenetic analysis was 424 bp for *hsp-65* gene, 711 bp for *rpoB* and 411 bp for *sodA*. The resulting fragment used for the concatenated nucleotide sequences in the following order (*hsp65*, *rpoB* and *sodA*) was 1546 bp. Multiple sequence alignments of the individual genes as well as the concatenated sequences were performed using Clustalw (Thompson *et al.,* 1994) from MEGA (version 6), to determine percentage similarity between each of the gene sequences. Evolutionary divergences between the sequences were estimated using maximum the composite likelihood method. Phylogenetic trees resulting from individual gene fragment sequences of the isolates and those of other mycobacteria, (both slow and rapidly growing) retrieved from Genbank (www.ncbi.nlm.gov/genbank), were constructed using the neighbour-joining method (Saitou and Nei, 1987) and validated using the maximum composite likelihood method. One thousand

bootstrap replicates were run and *Nocardia farcinica* species DSM43665 and *Nocardia brasiliensis* ATCC 700358 were used as the outgroup.

3.3.4 Genbank nucleotide accession numbers

The partial gene sequences (16S rRNA, *hsp-65*, *rpo*B and *sodA*) for the two proposed novel species, *viz* *Mycobacterium malmesburii* sp. nov. and *Mycobacterium komanii* sp. nov. were deposited in Genbank (www.ncbi.nlm.gov/genbank) and the following accession numbers provided**:** 16S rRNA, KJ 873241*; hsp-65*, KJ 873243*; rpoB*, KJ 873245*; sodA*, KJ 873247 for *M. malmesburii* sp.nov and **16S** rRNA, KJ 873240*; hsp-65*, KJ 873242*; rpoB*, KJ 873244*; sodA*, KJ 873246 for *M. komanii* sp.nov.

3.4 Results

3.4.1 Phenotypic characteristics and antibiotic susceptibility profiles

Phenotypic characteristics of the isolates in comparison with those of *M. moriokaense, M. elephantis, M. novocastrense, M. flavescens,* and *Mycobacterium arupense* strains are summarised in Table 3.2. The antibiotic susceptibility profiles are summarized in Table 3.3. Colonies of all the other isolates except for isolate TB 6607 appeared on LJ slants and Middlebrook 7H11 agar at 25°C, 37°C and 45°C in 2-5 days. Colonies for isolate TB 6607 appeared at 10 days and showed growth at 25°C, 37°C and 45°C. All the isolates showed a yellow pigment, except for isolate Uyenvlei swab 3, which showed an orange pigment. Except for isolate TB 6607 which appeared as smooth colonies, all the other isolates appeared rough on both LJ slants and Middlebrook 7H11 agar. All isolates were positive for the catalase activity, Tween

80 hydrolysis and the 14 day aryl sulphatase activity tests except for isolate Vryburg swab 3 which was negative for the 14 day aryl sulphatase activity. Isolates Honing S2, Uyenvlei swab 3 and TB 6607 were positive for the 3 day aryl sulphatase activity test, whereas the others were negative. All the isolates did not grow in the presence of 5% NaCl and neither did they utilize D-mannitol, inositol, L-rhamnose and L-arabinose as sole carbon sources. The isolates were negative for the aesculin hydrolysis and citrate activity tests except for isolate Honing S2 which was positive for the aesculin hydrolysis test. All the isolates were positive for pyrazinamidase activity tests, urease and nitrate reductase activity except for isolate swab 242 which was negative for nitrate reductase. For the niacin production test all the isolates with the exception of isolate swab 242 were negative.

The identified differences in phenotypic characteristics of the isolates with those of the closely related species *i.e. M. moriokaense, M. novocastrense, M. flavescens, M. elephantis* and *M. arupense,* illustrated in Table 3.2, include pigmentation, growth ability as well as other biochemical traits. The most apparent feature that distinguished the isolates from both *M. moriokaense,* and *M. arupense* was pigmentation, as all the isolates appeared to be scoto- chromogens whereas both these NTM were previously reported to be non-chromogenic (Cloud *et al.*, 2006; Turenne *et al.*, 2002; Tortoli, 2003). Other characteristics that further segregated the isolates from *M. arupense* was their ability to degrade urea as well as to reduce nitrate to nitrite (except for isolate Swab 242). *M. arupense* was previously reported to lack these traits (Cloud *et al.*, 2006). Contrary to what is reported for *M. novocastrense, M. flavescens* as well as *M. elephantis,* all the isolates were unable to grow in the presence of 5% NaCl (Tortoli, 2003; Shojaei et al., 1997; Bojalil *et al.*, 1962; Turenne *et al.*, 2002; Tortoli *et al.*, 2004). In addition, the ability of the isolates to grow at 45°C further separated them from *M. flavescens* as well as *M. arupense,* which were previously reported not to grow at this temperature (Tortoli *et al.*, 2004; Cloud *et al.*, 2006).

The antibiotic susceptibility profiles determined for the isolates in this study have only been used as a tool for taxonomic classification. All isolates with the exception of Uyenvlei swab 3 which was not inhibited by ciprofloxacin and amikacin, showed zones of inhibition of >20 mm, to the two antibiotics as well as doxycycline. Most of the isolates were not inhibited by imipenem (except TB 5614 and TB 5612, both showing inhibition zones of 10 mm), tobramycin (except Honing S2 and Western Cape swab 5 showing inhibition zones of 20 mm and 12 mm, respectively), amoxylin (except TB 5960A, C4 and Trigaarspoorts swab 03027 for which 10 mm inhibition zones were observed) and cefoxitin (except for Pan2S1, where a 20 mm zone of inhibition was observed). Isolates TB 6607 ; TB 5614 ; Uyenvlei swab 3 ; C4 ; Vryburg swab3; Middledrift swab 2341 ; and Villanora H_2O were not inhibited by clarithromycin whereas the growth of the other isolates, i.e. TB 5960A, C28, TB 5612, W. Cape swab 5, Pan2S1, Trigaarspoort swab 03027, Swab242, Balasi H_2O, Honing S2, Komani H_2O, and Mbekweni H_2O.3 was inhibited by the antibiotic.

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Table 3.2: Comparison of phenotypic characteristics of the NTM isolates with those of the closely related species

NTM ID/ species	Source	Growth rate (days)	Pigment	Growth at:	Biochemical features										Utilization of sugars					
				25°C 37°C 45°C	Semi quantitative Catalase	5% NaCl tolerance	Tween 80 hydrolysis	Arylsulphatase (14ays)	Arylsulphatase (3days)	Urease	Niacin	Nitrate reduction	Pyrazinamidase activity	Citrate	Aesculin	D-mannitol	Inositol	L-rhamnose	L-arabinose
M. moriokaense (tested in the lab)	Ref	< 7	-	+ + + + nd	+	-	+	-	+	+	nd	+	+	-	+	-	-	nd	nd
M. moriokaense (e, f)	Ref	< 7	-	+ + nd	-, +	+	nd	nd	-, +	+	nd	+	+	-	-	+, -	+	+	+
M. novocastrense (a, c)	Ref	< 7	+	+ + +	+	+	+	nd	v	+	-	+	nd	nd	nd	nd	nd	nd	nd
M. flavescens (b, g, h)	Ref	< 7	+	+ + -	+	+	+	+	-	+	-	+	+	+	nd	+, v	-	-	nd
M. arupense (d)	Ref	< 7	-	+ + -	nd	-	+	+	-	-	-	-	-	nd	nd	nd	nd	nd	nd

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M. elephantis a,b	Ref	< 7	+	+	+ ++	+		+	+	-	-	+		+	+	nd	nd	-	-	nd	nd
TB 5960A	Bo tissue	< 7	+	+	+ ++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
TB 6607	Bu swab	> 7	+	+	+ ++	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-
TB 5614	Bo tissue	< 7	+	+	+ ++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
C28	Bu swab	< 7	+	+	+ ++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
C4	Bu swab	< 7	+	+	+++	+	+	-	+	+	-	+	--	+	+	-	-	-	-	-	-
Swab 242	Bu swab	< 7	+	+	+++	+	+	-	+	+	-	+	+	-	+	-	-	-	-	-	-
TB 5612	Bo tissue	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
W. cape swab 5	Bo swab	< 7		+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Pan2soil1	soil	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Trigaarspoort swab03027	Bo swab	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-

Vryburg swab3	Bo swab	< 7	+	+	+++	+	-	-	+	-	-	+	-	+	+	-	--	-	-	-	-
Balasi H_2O	water	< 7	+	+	+++	+	-	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Honing S2	soil	< 7	+	+	+++	+	+	-	+	+	+	+	-	+	+	-	+	-	-	-	+
Villanora H_2O	water	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Komani H_2O	water	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Mbekweni $H_2O.3$	water	< 7	+	+	+++	+	+	-	+	+	-	+	-	+	+	-	-	-	-	-	-
Middledrift swab 2341	Bo swab	< 7	+	+	+++	+	+	-	+	+	-	+	+	+	+	-	-	-	-	-	-
Uyenvlei swab 3+4	Bo swab	< 7	+	+	+++	+	+	-	+	+	+	+	-	+	+	-	+	-	-	-	-

a, data retrieved from Tortoli , 2003; b, Data retrieved from Turenne *et al.*, 2002; c, Shojaei et al., 1997; d, data taken from Cloud *et al.*, 2006; e, data retrieved from Tsukamura *et al.*, 1986; f, data taken from Adékambi *et al.*, 2006; g, data taken from Tortoli *et al.*, 2004; h, data taken from Bojalil *et al.*, 1962; v, variable results based on a and c; +, positive; -, negative; +++, optimum growth; Bo, bovine; Bu, buffalo; Ref, reference strain; swab, nasal swab.

Table 3.3: Antibiotic susceptibility profiles of the NTM isolates

Isolate ID	Ciprofloxacin (10 µg)	Clarithromycin (15µg)	Imipenem (10 µg)	Tobramycin (10 µg)	Doxycycline (30 µg)	Amoxylin (30 µg)	Amikacin (30 µg)	Cefoxitin (10 µg)
	Inhibition zone diameters in millimeter							
TB 5960A	20	20	0	1	20	10	20	0
TB 6607	20	3	0	0	20	3	20	0
TB 5614	20	0	10	3	20	0	20	0
Uyenvlei swab3	0	0	0	3	20	0	20	0
C28	20	10	0	3	20	3	20	3
C4	20	0	0	3	20	10	20	0
TB 5612	20	20	10	0	20	20	20	0
W. cape swab 5	20	10	0	12	20	0	20	0
Pan2soil1	20	20	0	3	20	0	20	20
Trigaarspoort swab 03027	20	20	0	3	20	10	20	3
Vryburg swab3	20	0	0	3	20	0	20	0
Balasi H_2O	20	10	0	3	20	0	20	0
Swab 242	20	20	0	3	20	3	20	0

Honing S2	20	20	0	20	20	0	20	0
Komani H_2O	20	20	0	3	20	0	20	0
Mbekweni $H_20.3$	20	20	0	3	20	0	20	0
Middledrift swab 2341	20	0	0	3	20	0	20	0
Villanora H_2O	20	0	0	0	20	0	20	0

Inhibition zone: The diameter of the clear zone around the antimicrobial disc, indicating bacterial growth inhibition by the respective antibiotic**.**

3.4.2 Genetic characteristics and phylogenetic analysis

The 18 NTM isolates were shown to have a 95% - 98% sequence similarity with *M. moriokaense* based on partial 16S rDNA sequence (Gcebe *et al.*, 2013). Of the 18 isolates, 16 had at least 97% 16S rDNA sequence similarity to that of *M. moriokaense,* except isolates TB 6607 and Honing S2, with 95.6% and 96.5% sequence similarity to that of *M. moriokaense,* respectively. The sequence analysis data of the isolates for different gene fragments is summarised in Table 3.4 and (highest sequence similarities to known *Mycobacterium* species were reported). The distance matrices (Table 3.5-3.7) illustrate the estimated divergence between the respective gene sequences (*hsp65*, *rpo*B and *sod*A).

When we characterised the isolates in this study by sequencing of their *hsp65* gene (439 bp fragment), 10/18 isolates (namely, Middledrift swab 2341, Balasi H_2O, Vryburg swab3, W. Cape swab 5, TB 5612,

Swab 242, TB 5960A, C28, Uyenvlei swab 3 and C4) showed 95% sequence similarity to *Mycobacterium novocastrense*. Alignment of the *hsp65* gene fragment showed only a 0.3% sequence divergence that occurred between isolate Uyenvlei swab 3 and the other nine isolates and no variation was observed among the nine isolates (Table 3.5). Honing S2 showed 93% similarity to *Mycobacterium novocastrense* and *Mycobacterium flavescens*; Villanora H_2O showed 96% sequences similarity to *M. novocastrense*; whereas isolate TB 6607 showed 99% sequence similarity to *Mycobacterium* isolates, *Mycobacterium* sp G1368 or *Mycobacterium* sp variant Ms430 (sequences deposited in the Genbank database). Isolates Mbekweni H_2O.3, TB 5614, Trigaarspoort swab 03027, and Pan2S1 showed 98% similarity to *M. novocastrense;* while Komani H_2O exhibited 97% sequence similarity to *M. novocastrense*. A 0.5% sequence *M. novocastrense* divergence was the maximum observed between Pan2S1 and Komani H_2O (Table 3.5).

Partial sequence analysis of the *rpoB* gene identified these same 10 isolates as well as isolates Honing S2, Komani H_2O and Villanora H_2O to share 95% sequence similarity with *M. novocastrense*, *M. flavescens* and *M. arupense.* Despite these isolates sharing same percentage sequence similarities with *M. novocastrense*, there were higher sequence variations between each of the three isolates (Honing S2, Komani H_2O and Villanora H_2O) and the other ten isolates, than among the ten isolates. Komani H_2O showed at least 5% sequence divergence from the other ten isolates while Villanora H_2O and Honing S2 exhibited at least 3% and 7.1% divergence from the other ten isolates, respectively. The maximum sequence variation that occurred among the ten isolates was 2.2%, and was observed between isolates Uyenvlei swab 3 and TB 5960A (Table 3.6). Isolates TB 5614 showed 92% sequence similarity of the partial region of the *rpoB* gene to that of *M. arupense*, *M. moriokaense* and *M. barrasiae* whereas Mbekweni H_2O.3, Trigaarspoort swab 03027 and Pan2S1 showed 96% similarity to *M. novocastrense,*

M. flavescens and *M. arupense.* A 0.9% sequence divergence was observed between isolates Mbekweni H_2O.3 and Trigaarspoort swab 03027 as well as between isolates Mbekweni H_2O.3 and Pan2S1. On the other hand, isolate TB 6607 showed 99% sequence similarity of the *rpo*B gene fragment to *M. flavescens.*

Characterization of the isolates by sequencing of the partial fragment of the *sodA* gene showed eight isolates from the group of ten together with isolates Pan2S1, Honing S2, Trigaarspoort swab 03027, Villanora H_2O, Honing S2, Mbekweni H_2O.3 and Middledrift swab 03027 to share between 91% and 93% sequence similarity with *M. flavescens* and *M. novocastrense.* Two isolates *viz* Balasi H_2O and TB 5612 could not be amplified with the *sodA* primers used. The other isolates TB 6607 had *sodA* gene sequence that was 99% similar to *M. elephantis* whereas isolate TB 5614, shared 97% sequence similarity of the *sod*A gene fragment with *M. elephantis.* Table 3.7 illustrates *sodA* sequence divergence between the isolates.

Phylogenetic relatedness of the isolates and other *Mycobacterium* species, based on individual gene fragments, *viz hsp65*, *rpoB*, and *sodA* as well as concatenated gene sequences are illustrated by the phylogenetic trees in Fig 3.1 A-D. Phylogenetic analysis of the isolates based on the *hsp65* gene fragment revealed the 10/18 isolates, namely Middledrift swab 2341, Balasi H_2O, Vryburg swab3, W. Cape swab 5, TB 5612, Swab 242, TB 5960A, C28, Uyenvlei swab 3 and C4 to form a single cluster and were designated, *M. malmesburii* with a single branch of isolate Uyenvlei swab 3, indicating the observed sequence variation between this isolate and the others in the cluster (Fig 3.1A). The highest level of bootstrap support value (100%) identified these isolates as belonging to the same *Mycobacterium* species. Despite that these isolates showed 95% sequence similarity to *M. novocastrense,* they did not cluster phylogenetically with this species. Isolates Komani H_2O, Pan2S1, Trigaarspoort swab 03027 and Mbekweni H_2O.3 clustered together with a minimum bootstrap support value of 86% and were

designated *M. komanii*. This cluster did not group with any other *Mycobacterium* species. Both *M. novocastrense* and *M. flavescens* as well as *M. elephantis* and *M. pulveris* were positioned in between the two clusters i.e. *M. malmesburii* and *M. komanii*. The branch supporting isolates TB 5614 and Honing S2 in the tree showed a rather low bootstrap value of 39% between them but they did not group with any of the other isolates or known NTM species. Isolates Villanora H_2O and TB 6607 did not group with each other or with any of the other isolates or known NTM species.

Figure 3.1B illustrates the phylogenetic analysis of the isolates and other *Mycobacterium* species based on the *rpoB* gene fragment. In this analysis, both the *M. malmesburii* and *M. komanii* clusters, showed 96% and 99% intra-cluster bootstrap support values respectively, separated by two of their closest related species, namely *M. novocastrense* and *M. flavescens.* Isolate TB 6607 clustered together with *M. flavescens*. Isolates Villanora H_2O, TB 5614, and Honing S2 did not group with any of the other isolates.

Figure 3.1C illustrates the phylogenetic tree, based on the *sodA* gene fragment. This analysis revealed 8 isolates of the *M. malmesburii* cluster branch with bootstrap support value of 94%, with the exception of Balasi H_2O and TB 5612, which could not amplify. Clustering of the *M. komanii* isolates was supported by a minimum bootstrap value of 100%. *M. novocastrense* and *M. flavescens* separated these two clusters. Isolates TB 6607 and TB 5614 clustered together with *M. elephantis* whereas Villanora H_2O and Honing S2 did not cluster with any of the isolates included in this analysis.

The phylogenetic tree derived from the concatenated sequences also displayed the *M. malmesburii* cluster to be distinct from the *M. komanii* cluster with bootstrap values of 100% observed for each branch

supporting the clusters (Table 3.1D). Isolates TB 6607, Honing S2, Villanora H_2O and TB 5614 were positioned separate from the other isolates.

As these two clusters clearly represented two novel species not previously described in literature, we proposed the names *Mycobacterium malmesburii sp.nov.* (for the *M. malmesburii* cluster isolates) and *Mycobacterium komanii sp.nov.* (for the *M. komanii* cluster isolates) respectively**.**

Table 3.4: Genetic characterisation of the NTM isolates by sequencing of different Mycobacterium house -keeping genes, showing highest homology results

Isolate ID	**16S rRNA**	***hsp* 65**	***rpo*B**	***sod*A**
TB 5960A	98% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, / M. arupense* and. *novocastrense*	93% *M. flavescens/ M. novocastrense*
TB 6607	95.6% *M. moriokaense*	99% *Mycobacterium* sp G1368, *Mycobacterium* sp variant Ms430	99% *M. flavescens*	99% *M. elephantis*
TB 5614	97% *M. moriokaense, M. brasiliense*	98% *M. novocastrense*	92% *M. arupense, M. moriokaense* and. *barrasiae*	98% *M. elephantis*
C28	97.7% *M. morioakense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens* and *M. novocastrense*
C4	98% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	93% *M. flavescens*
Swab242	97.4% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens*
TB5612	98% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	No amplification
W. cape swab 5	97.9% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens* and *M. novocastrense*
Pan2soil1	97.7% *M. moriokaense*	98% *M. novocastrense*	96% *M. flavescens, M. novocastrense* and *M. arupense*	92% *M. flavescens*
Trigaarspoort swab 03027	98% *M. moriokaense*	98% *M. novocastrense*	96% *M. flavescens, M. arupense* and. *M. novocastrense*	92% *M. flavescens*
Vryburg swab3	98.% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens* and *M. novocastrense*

Balasi H_2O	97.9% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens/M arupense* and *M. novocastrense*	No amplification
Honing s2	96.5% *M. moriokaense*	93% *M. flavescens/ M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	91% *M. flavescens*
Komani H_2O	97.2% *M. moriokaense* and *M. brasiliensis*	98% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	93% *M. flavescens* and *M. novocastrense*
Uyenvlei swab 3+4	97.7% *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens* and *M. novocastrense*
Mbekweni H_2O. 3	98.% *M. moriokaense*	98% *M. novocastrense*	96% *M. flavescens, M. arupense* and *M. novocastrense*	92% *M. flavescens* and *M. novocastrense*
Middledrift swab2341	98 % *M. moriokaense*	95% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	93% *M. flavescens*
Villanora H_2O	*98% M. moriokaense,* and *M. barrassiae*	96% *M. novocastrense*	95% *M. flavescens, M. arupense* and *M. novocastrense*	93% *M. flavescens* and *M. novocastrense*

Chapter Three

Table 3.5: Estimates of evolutionary divergence between *hsp65* gene sequences

	Estimates of Evolutionary Divergence between hsp65 gene sequences																																												
Isolate ID/ species																																														
1.Western_Cape_swab_5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41				
2.C28	0.000																																													
3.M.drift_swab_2341	0.000	0.000																																												
4. c4	0.000	0.000	0.000																																											
5.swab_242	0.000	0.000	0.000	0.000																																									
6.TB5612	0.000	0.000	0.000	0.000	0.000																																								
7.Balasi_H20	0.000	0.000	0.000	0.000	0.000	0.000																																							
8.TB5960A	0.000	0.000	0.000	0.000	0.000	0.000	0.000																																						
9. Vryburg_swab3	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000																																					
10.Uyenvlei_swab_3	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003	0.003																																				
11.Trigaarspoort_swab_03027	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.057																																			
12.Komani_H2O	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.055	0.003																																		
13.Mbekweni_H2O.3	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.055	0.057	0.000	0.003																																	
14.Pan2s1	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.055	0.003	0.005	0.003																																
15.M._novocastrense_CIP_105546_65_(_AF547862)	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.052	0.055	0.023	0.025	0.023	0.020																															
16. M._holsaticum_(AY438084)	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.083	0.085	0.055	0.052	0.055	0.055	0.044																															
17.M._gadium_strain_CIP_105388_(AF547835)	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.105	0.108	0.060	0.063	0.060	0.060	0.052	0.063																															
18.M._elephantis_(_AF547828)	0.082	0.082	0.082	0.082	0.082	0.082	0.082	0.082	0.082	0.085	0.052	0.049	0.052	0.049	0.041	0.057	0.077																															
19.TB5614	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.069	0.071	0.018	0.020	0.018	0.020	0.025	0.060	0.060	0.060																															
20.TB6607	0.113	0.113	0.113	0.113	0.113	0.113	0.113	0.113	0.113	0.116	0.072	0.069	0.072	0.072	0.075	0.089	0.089	0.100	0.069																															
21.Mycobacterium_mageritense_strain_CIP_104973_(AY458070)	0.130	0.130	0.130	0.130	0.130	0.130	0.130	0.130	0.130	0.133	0.095	0.097	0.095	0.095	0.092	0.103	0.063	0.112	0.097	0.110																															
22.M._moriokaense_CIP105393_(AF547857)	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.091	0.093	0.055	0.057	0.055	0.055	0.052	0.055	0.044	0.082	0.063	0.086	0.069																															
23.Villanora_H2O	0.036	0.036	0.036	0.036	0.036	0.036	0.036	0.036	0.036	0.039	0.028	0.026	0.028	0.026	0.036	0.066	0.074	0.060	0.039	0.083	0.100	0.060																															
24.Honing_S2	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.103	0.106	0.060	0.058	0.060	0.063	0.066	0.094	0.095	0.103	0.058	0.113	0.131	0.094	0.074																															
25.Mycobacterium_flavescens_strain_CIP_104533_(_AF547831)	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.058	0.060	0.033	0.036	0.033	0.031	0.013	0.055	0.066	0.055	0.039	0.089	0.094	0.055	0.047	0.077																															
26.Mycobacterium_arupense_strain:CST7052_(GTC2730)	0.173	0.173	0.173	0.173	0.173	0.173	0.173	0.173	0.173	0.176	0.146	0.143	0.146	0.143	0.128	0.150	0.121	0.143	0.137	0.143	0.115	0.158	0.144	0.164	0.141																															
27.Mycobacterium_barrassiae_CIP_108545_(AY859679)	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.090	0.093	0.060	0.063	0.060	0.060	0.052	0.060	0.066	0.087	0.060	0.086	0.091	0.020	0.065	0.094	0.055	0.168																															
28.Mycobacterium_phlei_strain_CIP_105389(AF547866)	0.079	0.079	0.079	0.079	0.079	0.079	0.079	0.079	0.079	0.082	0.074	0.071	0.074	0.074	0.071	0.074	0.068	0.102	0.071	0.115	0.093	0.065	0.057	0.098	0.074	0.137	0.071																															
29.Mycobacterium_confluentis_strain_CIP_105510_(AF547822)	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.149	0.115	0.118	0.115	0.115	0.095	0.112	0.088	0.115	0.106	0.130	0.085	0.117	0.124	0.146	0.104	0.085	0.121	0.120																															
30.Mycobacterium_simiae_strain_ATCC_25275_(JF491306)	0.108	0.108	0.108	0.108	0.108	0.108	0.108	0.108	0.108	0.111	0.099	0.102	0.099	0.097	0.082	0.103	0.109	0.097	0.103	0.133	0.115	0.103	0.099	0.130	0.097	0.112	0.108	0.129	0.127																															
31.Mycobacterium_smegmatis_strain_ATCC_19420_(AY458065)	0.135	0.135	0.135	0.135	0.135	0.135	0.135	0.135	0.135	0.138	0.100	0.103	0.100	0.100	0.091	0.097	0.068	0.100	0.106	0.109	0.036	0.080	0.111	0.143	0.106	0.120	0.102	0.099	0.079	0.103																															
32.Mycobacterium_pulveris_strain_CIP_106804_(AF547869)	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.054	0.057	0.068	0.065	0.068	0.065	0.063	0.071	0.097	0.041	0.074	0.118	0.124	0.094	0.071	0.120	0.076	0.175	0.105	0.108	0.142	0.108	0.120																															
33.Mycobacterium_goodii_strain_ATCC_700504_(AY458071)	0.138	0.138	0.138	0.138	0.138	0.138	0.138	0.138	0.138	0.141	0.100	0.103	0.100	0.100	0.094	0.109	0.071	0.109	0.100	0.103	0.041	0.088	0.105	0.136	0.109	0.118	0.105	0.096	0.082	0.106	0.015	0.129																															
34.Mycobacterium_gastri_strain_CIP_104530_(_AF547836)	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.115	0.097	0.095	0.097	0.097	0.086	0.092	0.100	0.095	0.103	0.110	0.089	0.106	0.097	0.137	0.097	0.116	0.111	0.127	0.115	0.066	0.092	0.112	0.095																															
35.Mycobacterium_kansasii_strain_ATCC_12478_(AF434739)	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.112	0.115	0.106	0.103	0.106	0.106	0.092	0.103	0.109	0.106	0.106	0.122	0.098	0.115	0.100	0.141	0.103	0.122	0.115	0.124	0.116	0.071	0.097	0.118	0.103	0.020																															
36.Mycobacterium_szulgai_strain_CIP_104532_(AF547878)	0.117	0.117	0.117	0.117	0.117	0.117	0.117	0.117	0.117	0.120	0.113	0.111	0.113	0.113	0.099	0.105	0.119	0.108	0.117	0.141	0.132	0.128	0.117	0.136	0.111	0.115	0.128	0.126	0.117	0.065	0.119	0.107	0.123	0.060	0.068																															
Mycobacterium_scrofulaceum_strain_CIP_105416_(AF547871)	0.088	0.088	0.088	0.088	0.088	0.088	0.088	0.088	0.088	0.091	0.102	0.100	0.102	0.100	0.083	0.091	0.123	0.085	0.105	0.130	0.127	0.114	0.094	0.139	0.094	0.122	0.108	0.120	0.124	0.052	0.114	0.091	0.117	0.060	0.069	0.047																															
Mycobacterium_gordonae_strain_CIP_104529_(AF547840)	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.142	0.145	0.134	0.136	0.134	0.134	0.118	0.124	0.119	0.118	0.140	0.133	0.124	0.133	0.137	0.172	0.131	0.118	0.139	0.155	0.118	0.086	0.112	0.130	0.112	0.072	0.086	0.060	0.074																															
Mycobacterium_triviale_strain_DSM_44153_(AF547883)	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.115	0.118	0.102	0.100	0.102	0.102	0.094	0.083	0.110	0.097	0.114	0.137	0.110	0.112	0.103	0.143	0.106	0.151	0.120	0.112	0.127	0.125	0.109	0.124	0.121	0.092	0.104	0.121	0.113	0.138																															
Mycobacterium_terrae_strain_CIP_104321_(AF547879)	0.133	0.133	0.133	0.133	0.133	0.133	0.133	0.133	0.133	0.136	0.130	0.127	0.130	0.130	0.109	0.115	0.100	0.115	0.121	0.149	0.085	0.123	0.118	0.153	0.124	0.083	0.141	0.103	0.100	0.077	0.079	0.133	0.085	0.069	0.075	0.085	0.083	0.089	0.096																															
Mycobacterium_nonchromogenicum_strain_DSM_44164_(AF547861)	0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.168	0.171	0.131	0.133	0.131	0.131	0.122	0.134	0.103	0.134	0.131	0.133	0.099	0.132	0.133	0.160	0.137	0.066	0.151	0.118	0.088	0.103	0.091	0.168	0.080	0.106	0.121	0.111	0.118	0.094	0.147	0.074																															
Nocardia_farcinica_strain_DSM_43665_(_AY756523)	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.159	0.134	0.131	0.134	0.134	0.122	0.118	0.124	0.133	0.134	0.124	0.124	0.127	0.125	0.160	0.122	0.140	0.127	0.133	0.140	0.146	0.145	0.174	0.136	0.128	0.131	0.146	0.140	0.144	0.137	0.134	0.124																															

Table 3.6: Estimates of evolutionary divergence between the *rpoB* gene sequences

|	Estimates of Evolutionary Divergence between the rpoB gene Sequences																																									
Isolate ID/ species																																										
1.western_cape_swab5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42
2.swab242	0.000																																									
3.Balasi_h2O	0.000	0.000																																								
4.C28	0.004	0.004	0.004																																							
5.Middledrift_swab_2341	0.004	0.004	0.004	0.009																																						
6.TB5960A	0.013	0.013	0.013	0.009	0.018																																					
7.C4	0.006	0.006	0.006	0.010	0.001	0.019																																				
8.Uyenvlei_swab3	0.009	0.009	0.009	0.013	0.007	0.022	0.006																																			
9.Vryburg_swab3	0.006	0.006	0.006	0.001	0.010	0.010	0.012	0.015																																		
10.TB5612	0.004	0.004	0.004	0.000	0.009	0.009	0.010	0.013	0.001																																	
11.Komani_H2O	0.052	0.052	0.052	0.057	0.050	0.065	0.052	0.052	0.058	0.057																																
12.Villanora_H2O	0.036	0.036	0.036	0.041	0.032	0.050	0.033	0.036	0.042	0.041	0.047																															
13.Mycobacterium_moriokaense_CIP_105393_(AY859699)	0.122	0.122	0.122	0.127	0.117	0.136	0.119	0.122	0.129	0.127	0.092	0.123																														
14.TB5614	0.109	0.109	0.109	0.114	0.104	0.120	0.102	0.106	0.116	0.114	0.096	0.099	0.088																													
15.TB6607	0.054	0.054	0.054	0.058	0.049	0.065	0.050	0.052	0.060	0.058	0.047	0.060	0.101	0.102																												
16.Honing_S2	0.073	0.073	0.073	0.078	0.070	0.086	0.071	0.074	0.079	0.078	0.046	0.067	0.104	0.108	0.057																											
17.Pan2s1	0.052	0.052	0.052	0.057	0.050	0.065	0.052	0.052	0.058	0.057	0.000	0.047	0.092	0.096	0.047	0.046																										
18.Trigaarspoort_swab_03027	0.052	0.052	0.052	0.057	0.050	0.065	0.052	0.052	0.058	0.057	0.000	0.047	0.092	0.096	0.047	0.046	0.000																									
19.Mbekweni_H2O.3	0.062	0.062	0.062	0.062	0.060	0.070	0.062	0.062	0.063	0.062	0.009	0.054	0.103	0.106	0.057	0.055	0.009	0.009																								
20.M._flavescens_CIP_104533_(AY859698)	0.052	0.052	0.052	0.057	0.047	0.063	0.049	0.052	0.058	0.057	0.046	0.059	0.103	0.101	0.007	0.055	0.046	0.046	0.056																							
21.M._arupense_strain_FI-0127_(JN571225)	0.166	0.166	0.166	0.167	0.170	0.169	0.171	0.174	0.169	0.167	0.178	0.177	0.169	0.172	0.174	0.179	0.178	0.178	0.182	0.174																						
22.M.novocastrense_CIP_105546_(AY859704)	0.057	0.057	0.057	0.062	0.052	0.070	0.054	0.057	0.063	0.062	0.051	0.055	0.115	0.122	0.039	0.057	0.051	0.051	0.060	0.038	0.172																					
23.M.elephantis_CIP_106831_(AY859702)	0.121	0.121	0.121	0.124	0.121	0.133	0.123	0.126	0.126	0.124	0.127	0.111	0.105	0.130	0.115	0.124	0.127	0.127	0.138	0.113	0.160	0.112																				
24.Mycobacterium_gadium_CIP_105388_(AY859703)	0.116	0.116	0.116	0.122	0.111	0.131	0.113	0.115	0.123	0.122	0.105	0.119	0.074	0.117	0.099	0.115	0.105	0.105	0.116	0.101	0.169	0.113	0.125																			
25.Mycobacterium_holsaticum_CIP_107786_(AY859705)	0.133	0.133	0.133	0.136	0.133	0.146	0.135	0.139	0.138	0.136	0.132	0.126	0.112	0.124	0.122	0.129	0.132	0.132	0.143	0.117	0.184	0.129	0.067	0.145																		
26.Mycobacterium_mageritense_strain_ATCC_700351_(JF706630.1)	0.129	0.129	0.129	0.134	0.126	0.138	0.127	0.129	0.136	0.134	0.132	0.130	0.131	0.142	0.139	0.137	0.132	0.132	0.143	0.137	0.162	0.134	0.142	0.134	0.161																	
27.Mycobacterium_simiae_strain_ATCC_25275_(GQ153313)	0.144	0.144	0.144	0.150	0.141	0.157	0.143	0.146	0.152	0.150	0.156	0.148	0.144	0.149	0.154	0.157	0.156	0.156	0.166	0.152	0.160	0.161	0.143	0.136	0.151	0.151																
28.Mycobacterium_barrassiae_CIP_108545_(AY859696)	0.107	0.107	0.107	0.112	0.102	0.121	0.104	0.108	0.114	0.112	0.087	0.104	0.043	0.083	0.102	0.099	0.087	0.087	0.097	0.104	0.177	0.113	0.121	0.079	0.123	0.144	0.148															
29.Mycobacterium_phlei_CIP_105389_(AY859700)	0.110	0.110	0.110	0.111	0.110	0.120	0.112	0.115	0.113	0.111	0.114	0.112	0.097	0.111	0.114	0.113	0.114	0.114	0.121	0.112	0.158	0.119	0.105	0.120	0.116	0.125	0.139	0.126														
30.Mycobacterium_confluentis_strain_CIP_105510_(EU109298)	0.137	0.137	0.137	0.143	0.137	0.150	0.139	0.137	0.145	0.143	0.149	0.134	0.157	0.159	0.153	0.166	0.149	0.149	0.161	0.153	0.138	0.155	0.145	0.169	0.159	0.117	0.148	0.171	0.127													
31.Mycobacterium_smegmatis_strain_ATCC_19420_(AY262735)	0.138	0.138	0.138	0.143	0.137	0.147	0.139	0.140	0.145	0.143	0.127	0.140	0.129	0.140	0.149	0.151	0.127	0.127	0.138	0.147	0.136	0.147	0.152	0.138	0.167	0.089	0.149	0.135	0.141	0.123												
32.Mycobacterium_pulveris_CIP_106804_(AY859701)	0.133	0.133	0.133	0.133	0.134	0.143	0.132	0.136	0.135	0.133	0.129	0.130	0.107	0.138	0.128	0.132	0.129	0.129	0.136	0.126	0.169	0.125	0.038	0.136	0.072	0.153	0.160	0.128	0.112	0.167	0.162											
33.Mycobacterium_goodii_strain_ATCC_700504_(AY262736)	0.135	0.135	0.135	0.141	0.135	0.148	0.137	0.135	0.142	0.141	0.123	0.141	0.127	0.136	0.137	0.138	0.123	0.123	0.134	0.137	0.158	0.137	0.166	0.136	0.163	0.083	0.157	0.129	0.145	0.128	0.046	0.172										
34.Mycobacterium_nonchromogenicum_strain_ATCC_19530_(JN881351	0.143	0.143	0.143	0.145	0.147	0.150	0.149	0.151	0.146	0.145	0.151	0.158	0.144	0.155	0.155	0.158	0.151	0.151	0.159	0.153	0.052	0.157	0.143	0.155	0.161	0.136	0.149	0.154	0.130	0.123	0.123	0.151	0.137									
35.Mycobacterium_kansasii_ATCC_12478_(HQ880687)	0.180	0.180	0.180	0.182	0.180	0.190	0.182	0.188	0.184	0.182	0.186	0.187	0.165	0.173	0.188	0.178	0.186	0.186	0.192	0.186	0.168	0.188	0.152	0.183	0.178	0.195	0.120	0.167	0.162	0.175	0.205	0.165	0.212	0.161								
36.Mycobacterium_szulgai_strain_ATCC_29716_(JN881348)	0.152	0.152	0.152	0.158	0.152	0.165	0.154	0.160	0.159	0.158	0.154	0.165	0.142	0.158	0.155	0.146	0.154	0.154	0.166	0.154	0.147	0.161	0.145	0.145	0.158	0.158	0.095	0.157	0.110	0.158	0.153	0.162	0.159	0.142	0.137							
37.Mycobacterium_terrae_strain_ATCC_15755(JF346876)	0.155	0.155	0.155	0.156	0.158	0.156	0.160	0.162	0.158	0.156	0.153	0.162	0.145	0.177	0.165	0.168	0.153	0.153	0.157	0.165	0.065	0.164	0.142	0.159	0.173	0.149	0.153	0.158	0.140	0.120	0.145	0.151	0.153	0.064	0.165	0.154						
38.Mycobacterium_vaccae_strain_ATCC_15483_(JF923624)	0.139	0.139	0.139	0.141	0.139	0.146	0.141	0.139	0.143	0.141	0.142	0.136	0.144	0.140	0.149	0.150	0.142	0.142	0.150	0.145	0.167	0.151	0.155	0.138	0.157	0.092	0.136	0.147	0.118	0.115	0.098	0.167	0.098	0.157	0.202	0.141	0.158					
39.Mycobacterium_triviale_strain_ATCC_23290_(JF712873)	0.169	0.169	0.169	0.171	0.173	0.181	0.175	0.176	0.173	0.171	0.170	0.176	0.162	0.189	0.171	0.186	0.170	0.170	0.178	0.171	0.124	0.172	0.152	0.151	0.168	0.179	0.155	0.180	0.159	0.140	0.182	0.159	0.186	0.128	0.171	0.158	0.123	0.177				
40.Mycobacterium_gastri_strain_ATCC_15754_(JN986748)	0.171	0.171	0.171	0.171	0.171	0.179	0.173	0.177	0.173	0.171	0.171	0.171	0.158	0.159	0.174	0.167	0.171	0.171	0.175	0.172	0.157	0.176	0.146	0.165	0.167	0.187	0.112	0.157	0.153	0.167	0.190	0.157	0.192	0.143	0.029	0.131	0.148	0.191	0.157			
41.Mycobacterium_scrofulaceum_strain_ATCC_19981_(GQ153305)	0.139	0.139	0.139	0.145	0.139	0.152	0.141	0.145	0.146	0.145	0.138	0.149	0.116	0.138	0.145	0.142	0.138	0.138	0.149	0.145	0.137	0.145	0.133	0.136	0.153	0.144	0.103	0.136	0.106	0.138	0.145	0.142	0.145	0.114	0.133	0.092	0.121	0.142	0.140	0.128		
42.Mycobacterium_gordonae_strain_ATCC_14470_(JF346873)	0.143	0.143	0.143	0.148	0.146	0.156	0.148	0.149	0.150	0.148	0.135	0.156	0.121	0.142	0.146	0.139	0.135	0.135	0.147	0.145	0.152	0.154	0.138	0.133	0.153	0.145	0.094	0.136	0.109	0.149	0.137	0.139	0.143	0.134	0.131	0.073	0.138	0.135	0.138	0.118	0.089	
43.Nocardia_farcinica_(AB243742)	1.020	1.020	1.020	1.036	1.020	1.031	1.025	1.021	1.040	1.036	1.027	1.030	1.005	1.044	1.058	1.054	1.027	1.027	1.043	1.057	1.206	1.092	1.053	1.044	1.092	1.106	1.052	0.986	1.065	1.119	1.072	1.089	1.100	1.138	1.147	1.115	1.140	1.152	1.174	1.146	1.074	1.044

Table 3. 7: Estimates of evolutionary divergence between *sodA* sequences

	Estimates of Evolutionary Divergence between sodA Sequences																																								
Isolate ID/ species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37				
1.TB5614																																									
2.TB6607	0.013																																								
3.Villanora_H2O	0.119	0.122																																							
4.Honing_S2	0.118	0.115	0.091																																						
5. Mbekweni_H2O.3	0.097	0.106	0.076	0.097																																					
6.Pan2s1	0.109	0.118	0.079	0.106	0.027																																				
7. Komani_H2O	0.097	0.106	0.076	0.097	0.000	0.027																																			
8.Trigaarspoort_swab03027	0.097	0.106	0.076	0.097	0.000	0.027	0.000																																		
9.Vryburg_swab_3	0.122	0.122	0.044	0.094	0.076	0.094	0.076	0.076																																	
10.Swab242	0.125	0.126	0.047	0.097	0.079	0.097	0.079	0.079	0.003																																
11.Western_Cape_swab5	0.125	0.126	0.047	0.097	0.079	0.097	0.079	0.079	0.003	0.000																															
12.C28	0.125	0.126	0.047	0.097	0.079	0.097	0.079	0.079	0.003	0.000	0.000																														
13.Middledrift_swab2341	0.113	0.119	0.041	0.094	0.076	0.088	0.076	0.076	0.016	0.019	0.019	0.019																													
14.TB5960A	0.116	0.126	0.038	0.094	0.073	0.091	0.073	0.073	0.019	0.016	0.016	0.016	0.019																												
15.C4	0.113	0.122	0.038	0.094	0.076	0.088	0.076	0.076	0.030	0.033	0.033	0.033	0.013	0.016																											
16.Uyenvlei_swab3	0.129	0.129	0.050	0.100	0.082	0.100	0.082	0.082	0.005	0.003	0.003	0.003	0.022	0.019	0.036																										
17.Mycobacterium_mageritense_strain_CIP_104973_(AY4	0.138	0.148	0.131	0.147	0.134	0.157	0.134	0.134	0.125	0.128	0.128	0.128	0.131	0.125	0.128	0.131																									
18.Mycobacterium_novocastrense_strain_CIP_105546(AY	0.119	0.125	0.079	0.100	0.073	0.090	0.073	0.073	0.085	0.088	0.088	0.088	0.088	0.082	0.088	0.091	0.148																								
19.Mycobacterium_flavescens_strain_CIP_104533_(AY544	0.122	0.131	0.076	0.085	0.073	0.085	0.073	0.073	0.085	0.088	0.088	0.088	0.082	0.079	0.082	0.091	0.157	0.078																							
20.Mycobacterium_simiae_strain_CIP_104531_(AY544864	0.161	0.161	0.157	0.158	0.164	0.180	0.164	0.164	0.154	0.157	0.157	0.157	0.154	0.145	0.144	0.161	0.151	0.154	0.154																						
21.Mycobacterium_gadium_strain_CIP_105388_(DQ47331	0.154	0.155	0.147	0.154	0.141	0.167	0.141	0.141	0.125	0.128	0.128	0.128	0.125	0.125	0.125	0.131	0.115	0.137	0.141	0.151																					
22.Mycobacterium_elephantis_strain_CIP_106831_(AY544	0.016	0.003	0.125	0.118	0.109	0.121	0.109	0.109	0.119	0.122	0.122	0.122	0.116	0.122	0.119	0.126	0.145	0.128	0.134	0.158	0.151																				
23.Mycobacterium_bovis_strain_CIP_105234_(AY544809)	0.224	0.241	0.265	0.254	0.262	0.276	0.262	0.262	0.261	0.261	0.261	0.261	0.254	0.254	0.247	0.265	0.262	0.265	0.254	0.219	0.268	0.245																			
24.Mycobacterium_moriokaense_strain_CIP_105393_(AY5	0.170	0.164	0.154	0.176	0.184	0.194	0.184	0.184	0.157	0.153	0.153	0.153	0.156	0.150	0.153	0.157	0.187	0.188	0.170	0.180	0.197	0.167	0.283																		
25.Mycobacterium_arupense_strain_DSM_44942_(EU191	0.171	0.175	0.185	0.171	0.168	0.184	0.168	0.168	0.182	0.185	0.185	0.185	0.178	0.179	0.172	0.189	0.185	0.178	0.178	0.144	0.192	0.171	0.249	0.221																	
26.Mycobacterium_gastri_strain_CIP_104530_(AY544825)	0.231	0.238	0.253	0.257	0.249	0.264	0.249	0.249	0.257	0.257	0.257	0.257	0.250	0.254	0.247	0.258	0.260	0.265	0.268	0.241	0.264	0.242	0.102	0.284	0.246															
27.Mycobacterium_kansasii_type_1_(EU127837)	0.218	0.225	0.247	0.250	0.246	0.257	0.246	0.246	0.250	0.250	0.250	0.250	0.239	0.243	0.236	0.250	0.258	0.262	0.262	0.249	0.258	0.228	0.115	0.268	0.251	0.044														
28.Mycobacterium_szulgai_strain_CIP_104532_(AY544867	0.242	0.253	0.288	0.261	0.300	0.311	0.300	0.300	0.284	0.288	0.288	0.288	0.277	0.281	0.270	0.292	0.304	0.289	0.266	0.266	0.275	0.257	0.124	0.313	0.284	0.160	0.141													
29.Mycobacterium_scrofulaceum_strain_CIP_105416_(_A	0.141	0.145	0.154	0.171	0.154	0.170	0.154	0.154	0.154	0.158	0.158	0.158	0.147	0.151	0.145	0.161	0.168	0.141	0.167	0.137	0.171	0.148	0.199	0.208	0.141	0.202	0.206	0.241												
30.Mycobacterium_gordonae_strain_CIP_104529_(AY5448	0.128	0.129	0.144	0.157	0.148	0.164	0.148	0.148	0.151	0.154	0.154	0.154	0.154	0.148	0.148	0.158	0.151	0.157	0.147	0.147	0.173	0.125	0.256	0.205	0.162	0.248	0.239	0.278	0.138											
31.Mycobacterium_triviale_strain_DSM_44153_(AY544872	0.129	0.132	0.145	0.129	0.128	0.138	0.128	0.128	0.138	0.142	0.142	0.142	0.135	0.132	0.132	0.145	0.157	0.152	0.148	0.141	0.155	0.128	0.246	0.216	0.128	0.242	0.224	0.269	0.141	0.154										
32.Mycobacterium_terrae_strain_CIP_104321_(AY544868)	0.150	0.160	0.157	0.150	0.144	0.153	0.144	0.144	0.157	0.161	0.161	0.161	0.151	0.148	0.144	0.164	0.154	0.141	0.167	0.122	0.147	0.157	0.209	0.198	0.094	0.222	0.213	0.249	0.131	0.157	0.103									
33.Mycobacterium_nonchromogenicum_strain_DSM_4416	0.138	0.148	0.182	0.165	0.145	0.168	0.145	0.145	0.159	0.162	0.162	0.162	0.165	0.162	0.162	0.165	0.158	0.168	0.175	0.144	0.171	0.145	0.245	0.209	0.097	0.238	0.235	0.268	0.155	0.165	0.100	0.085								
34.Mycobacterium_pulveris_strain_CIP_106804_(AY54485	0.076	0.076	0.122	0.122	0.132	0.141	0.132	0.132	0.122	0.125	0.125	0.125	0.116	0.119	0.113	0.129	0.171	0.135	0.132	0.178	0.172	0.079	0.246	0.171	0.182	0.242	0.214	0.254	0.152	0.161	0.155	0.168	0.173							
35.Mycobacterium_goodii_strain_CIP_106349_(AY544826)	0.144	0.148	0.125	0.141	0.128	0.141	0.128	0.128	0.132	0.135	0.135	0.135	0.132	0.122	0.125	0.138	0.128	0.138	0.122	0.141	0.132	0.144	0.261	0.187	0.155	0.249	0.238	0.275	0.164	0.147	0.122	0.148	0.139	0.148						
36.Mycobacterium_smegmatis_strain_ATCC_19420_(AY4	0.135	0.138	0.128	0.144	0.132	0.141	0.132	0.132	0.128	0.131	0.131	0.131	0.128	0.122	0.132	0.134	0.128	0.132	0.135	0.147	0.135	0.135	0.280	0.184	0.168	0.276	0.265	0.291	0.164	0.160	0.132	0.158	0.155	0.142	0.050					
37.Mycobacterium_phlei_strain_CIP_105389_(AY544856)	0.144	0.141	0.163	0.164	0.154	0.170	0.154	0.154	0.147	0.151	0.151	0.151	0.138	0.154	0.141	0.154	0.173	0.163	0.154	0.163	0.181	0.138	0.245	0.204	0.187	0.241	0.238	0.307	0.173	0.171	0.161	0.163	0.167	0.151	0.177	0.174				
38.Nocardia_brasiliensis_strain_ATCC_700358_(DQ40203	0.175	0.185	0.182	0.192	0.162	0.185	0.162	0.162	0.175	0.178	0.178	0.178	0.172	0.165	0.158	0.182	0.181	0.182	0.178	0.178	0.154	0.182	0.273	0.227	0.168	0.277	0.262	0.318	0.191	0.174	0.132	0.141	0.128	0.186	0.158	0.161	0.188			

3.4.3 Description of *Mycobacterium malmesburii* sp. nov.

Mycobacterium malmesburii is named after a town (Malmesbury) in South Africa, where one of the isolates of this species originated from.

The organisms are acid fast bacilli. Colonies form a yellow-orange pigment in the dark, and grow on LJ slants supplemented with PACT, as well as on Middlebrook 7H11 at 25°C, 37°C and 45°C, within 2-5 days but optimally at 37°C. The bacteria are positive for semi quantitative catalase test (>45 mm foam), Tween 80 hydrolysis, nitrate reductase, urease and pyrazinamidase activity tests. Different isolates show variable activity for the 3 day and 14 day arylsulphatase tests, as well as niacin production, but were most were negative for the 3 day test, positive for the 14 day test and negative for niacin accumulation. The bacteria do not grow in the presence of 5% NaCl and neither do they utilize D-mannitol, inositol, L-rhamnose and L-arabinose as sole carbon sources. The organisms are negative for the aesculin hydrolysis and citrate activity tests. Growths of most strains is not inhibited by imipenem, tobramycin, cefoxitin, amikacin and amoxylin, but are inhibited by ciprofloxacin, clarithromycin and doxycycline. These bacteria are mainly found in the environment at the escarpment, semi-arid plateau, moderate eastern plateau, subtropical coastal regions, as well as areas with mediterranean climate in South Africa. Isolates from bovine lymph node samples have been encountered in multiple parts of the country. No clinical relevance has been defined for this species.

3.4.4 Description of *Mycobacterium komanii* sp. nov.

Mycobacterium komanii is named after a town in South Africa where one of the isolates originated from. Komani is the Xhosa name for Queenstown (South Africa). These bacteria grow at 25°C, 37°C and 45°C on LJ slants supplemented with PACT, as well as on Middlebrook 7H11 within 2-5 days, but optimally at 37°C. The colonies form yellow pigments in the dark. The bacteria are positive for semi quantitative catalase test (>45 mm foam), Tween 80 hydrolysis, nitrate reductase, urease, pyrazinamidase, 3 day and 14 day arylsuphatase activity tests. The organisms do not grow in the presence of 5% NaCl; neither do they utilize D-mannitol, inositol, L-rhamnose and L-arabinose as sole carbon sources, and are negative for niacin accumulation test. The organisms are negative for aesculin hydrolysis and citrate activity tests. Growth of these bacteria is inhibited by ciprofloxacin, clarithromycin, doxycycline and amikacin, but not by imipenem, tobramycin, amoxylin and cefoxitin (for most strains). The bacteria were isolated from the environment at the escarpment, moderate eastern plateau as well as subtropical coastal regions of South Africa.

3.5 Discussion

In a survey to determine the prevalence of NTM in cattle, African buffalo and their environments in South Africa, we found a group of NTM isolates that had not been previously described in literature. These were among the four most abundant species in this survey and were found to be closely related to *M. moriokaense,* by analysis of the 16S rDNA sequences (Gcebe *et al*, 2013). It was the aim of this study to conduct an in-depth characterisation of 18 of these NTM isolates. Multiple gene sequence characterisation, as well as phylogenetic analysis of three other gene loci, revealed that 14/18 of these

belong to two novel NTM species. We proposed *M. malmesburii* sp. nov. (comprised of 10/18 isolates) and *M. komanii* sp. nov. (comprised of 4/18 isolates) as names for these two new species. The clustering of the isolates of the same proposed new species on the phylogenetic tree was supported by high bootstrap values (>85% for each gene sequence as well as concatenated sequences). This together with the absence of significant phylogenetic clustering neither between the clusters of the new species nor with known *Mycobacterium* species, formed the basis of our conclusion that the two clusters represent two novel NTM species. Greater sequence divergence of the three gene fragments (*hsp65*, *rpoB*, and *sodA*) of the isolates and the closely related species also suggested that these are unique sequences of novel species.

Further supporting evidence that the isolates represent novel species was that, even though they shared some common phenotypic characteristics with their closely related species *i.e. M. moriokaense*, *M. novocastrense*, *M. flavescens* and *M. arupense* there were clear differences, like pigmentation, biochemical as well as growth characteristics suggesting that these isolates do not belong to any of the known species. Even though the isolates of the two novel NTM species could be clearly differentiated from their closest related species by phenotypic characterisation, these profiles could not differentiate them from each other, suggesting that these two species are very closely related. The other remaining isolates did not seem to belong to any of the two novel species, neither did they belong to any other known *Mycobacterium* species, but they could not be classified as novel species as they occurred as a single isolates only (as per recommendations by Christensen *et al.*, 2001 and Janda *et al.*, 2002: a minimum of 5-10 isolates from geographically unrelated areas). Antibiotic susceptibility profiles of the isolates were not able to separate them unequivocally from their closest relatives, but were useful in the description of the novel species.

The polyphasic approach employed in this study showed clear differences between the two proposed novel species and their closest related species. Determining the 16S rDNA sequence is a very important first molecular based step which has been shown to contribute towards the discovery of new species (Cloud 2006). However sequencing of multiple genes together with phenotypic characterisation has revealed advantages over use of a single gene, in the description of the novel species. Phylogenetic analysis of the different *Mycobacterium* housekeeping genes has long been used in taxonomic studies. This analysis based on the three loci, was able to separate isolates belonging *M. malmesburii* sp. nov. from those of *M. komanii* sp. nov. It has been reported that trees derived from the *rpo*B gene are not as robust as those derived from the 16S rDNA and *hsp65* sequences, but in this study they gave useful taxonomic classification of the isolates (Devulder, 2005).

The identification of novel NTM species from the environment (in different regions of South Africa) of cattle and African buffaloes as well as from tissue samples, confirms the existence of a number of as yet unidentified NTM species that animals are exposed to. Their relevance needs to be further investigated, especially in light of cross-reactive immune responses they may elicit as shown by Michel *et al.*, 2011. Our motivation to further characterize this group of isolates was that, they appear to be among the abundant in cattle, African buffaloes and their environments in South Africa. Therefore animals are more likely to be exposed to the NTM species, with potential implications in BTB diagnosis and vaccination due to cross reactive immune responsiveness of hosts to these *Mycobacterium* species (and consequently development of BTB).

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3.6 References:

ADÉKAMBI T, COLSON P, DRANCOURT M (2003). *rpoB*- based identification of nonpigmented and late- pigmented rapidly growing mycobacteria. *Journal of Clinical Microbiology* **41**: 5699-5708.

ADÉKAMBI T, DRANCOURT M (2004). Dissection of the phylogenetic relationships among 19 rapidly growing *Mycobacterium* species by 16S rRNA, *hsp65*, *sod*A, *rec*A and *rpo*B gene sequencing. *International Journal of Systematic and Evolutionary Microbiology* **54**: 2095-2105.

ADÉKAMBI T, BERGER P, RAOULT D, DRANCOURT M (2006). *rpoB* gene sequence-based characterisation of emerging non - tuberculous mycobacteria with descriptions of *Mycobacterium bolletii* sp. nov., *Mycobacterium phocaicum* sp. nov. and *Mycobacterium aubagnense* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* **56**: 133-143.

ADÉKAMBI T, RAOULT D, DRANCOURT M (2006). *Mycobacterium barrassiae* sp. nov., a *Mycobacterium morioakense* group species associated with chronic pneumonia. *Journal of Clinical Microbiology* **44**: 3493-3498.

BLACKWOOD KS, HE C, GUNTON J, TURENNE CY, WOLFE J, KABANI AM. (2000) Evaluation of the recA sequences for identification of *Mycobacterium* species. *Journal of Clinical and Microbiology* **38**: 2846-2852.

BOJALIL LF, CERBON J, TRUJILLO A (1962). Adonsonian classification of mycobacteria. *Journal of General Microbiology* **28:** 333-346.

BOTHA L, GEY VAN PITTIUS NC, VAN HELDEN PD (2013). Mycobacteria and disease in Southern Africa. *Transboundary and Emerging Diseases* **60**: 147-156.

BRANDT L, CUNHA JF, OHLSEN AW, CHILIMA B, HIRSSCH R, APPELBERG R, ANDERSON P (2002). Failure of *Mycobacterium bovis* BCG vaccine: some species of environmental mycobacteria block multiplication of BCG and induction of protective immunity to tuberculosis. *Infection and. Immunology* **70**: 672-678.

BROWN-ELLIOT BA, WALLACE RJ JR. (2002). Clinical and taxonomic status of pathogenic nonpigmented or late pigmenting rapidly growing mycobacteria. *Clinical Microbiology Reviews* **15**: 716-746.

BUDDLE BM, WARDS BJ, ADWELL FE, COLLINS DM, DE LISLE GW (2002). Influence of sensitisation to environmental mycobacteria on subsequent vaccination against bovine tuberculosis. *Vaccine* **20**: 1126-1133.

CHRISTENSEN H, BISGAARD M, FREDERIKSEN W, MUTTERS R, KUHNERT P, OLSEN JE (2001). Is characterisation of a single isolate sufficient for valid publication of a new genus or species?

Proposal to modify recommendations 30b of the Bacteriological code (1990 revision). *International Journal of Systematic and Evolutionary Microbiology* **51**: 2221-2225.

CLOUD JL, MEYER JJ, POUNDER JI, JOST KC JR, SWEENEY A, CARROLL KC, WOODS GL (2006). *Mycobacterium arupense* sp. nov., a non-chromogenic bacterium isolated from clinical specimes. *International journal of systematic and evolutionary microbiology* **56**: 1413-1418.

DAI J, CHEN Y, DEAN S, MORRIS JG, SALFINGER M, JOHNSON JA (2011). Multiple genome comparison reveals new loci for *Mycobacterium* species identification. *Journal of Clinical Microbiology* **49:** 144-153.

DEVULDER G, PÉROUSE DE MONTCLOS M, FLANDROIS JP (2005). A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. *International Journal of Systematic and Evolutionary Microbiology* **55**: 293-302.

FALKINHAM JO (2002). Non- tuberculous mycobacteria in the environment. Clin Chest Med **29**: 529-551.

FELSENSTEIN J (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* **39**: 783-791.

GCEBE N, RUTTEN V, GEY VAN PITTIUS NC, MICHEL A (2013). Prevalence and distribution of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their environments in South Africa. *Transboundary and Emerging Diseases* **60**:74-84.

JANDA JM, ABBOTT SL (2002). Bacterial identification for publication: when is enough enough? *Journal of Clinical Micribiology* **40**: 1887-1891.

KARNE SS, SANGLE SA, KIYAWAT DS, DHARMASHALE SN, KADAM B, BHARDWAJ RS (2012). *Mycobacterium avium-intracellulare* brain abscess in HIV positive patient. *Annals of Indian Academy of Neurology* **15**: 54-55.

KASAI H, EZAKI T, HARAYYAMA S. (2000). Differentiation of phylogenetically related slowly growing mycobacteria by their *gyrB* sequences. *Journal of Clinical Microbiology* **38**: 301-308.

KENT PT, KUBICA GP (1985). Public health mycobacteriology: a guide for the level III laboratory. Centres for disease Control, US department of Health and Human Services, Atlanta.

KILBURN JO, O'DONNELL KF, SILCOX VA, DAVID HL (1973). Preparation of a stable mycobacterial tween hydrolysis test substrate. *Applied Microbiology* **26**: 836.

MICHEL AL, COOPER D, JOOSTE J, DE KLERK LM, JOLLES A (2011). Approaches towards optimising the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine* **98**: 142-151.

MICHEL AL (2008). *Mycobacterium fortuitum* infection interference with *Mycobacterium bovis* diagnostics: natural infection cases and a pilot experimental infection. *Journal of Veterinary Diagnostic Investigations* **20**: 501-503.

PRIMM TP, LUCERO CA, FALKINHAM JO III (2004). Health impacts of environmental mycobacteria. *Clinical Microbiology Reviews* **17:** 98-106.

SATOU N, NEI M (1987). The neighbour-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology Evolution* **4**: 406-425.

SCHILLER I, OESCH B, VORDERMEIER HM, PALMER MV, HARRIS BN, ORLOSKI KA, BUDDLE BM, THACKER TC, LYASHCHENKO KP, WATERS WR (2010). Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and Emerging Diseases* **57**:205-220.

SENNA SG, BATTILANA J, COSTA JC, SILVA MG, DUARTE RS, FONSECA LS, SUFFYS PN, BOGO MR (2008). Sequencing of the *hsp65* gene for identification of *Mycobacterium* species isolated from environmental and clinical sources in Rio de Janeiro, Brazil. *Journal of Clinical Microbiology* **46**: 3822-3825.

SHOJAEI H, GOODFELLOW M, MAGEE JG, FREEMAN R, GOULD FK, BRIGNALL CG (1997). *Mycobacterium novocastrense* sp. nov., a rapidly growing photochromogenic Mycobacterium. *International Journal of Systematic Bacteriology* **47**: 1205-1207.

SINGH P, WESLEY C, JADAUN GPS, MALONIA KS, DAS R, UPADHYAY P, FAUJDAR J, SHARMA P, GUPTA P, MISRA AK, SINGH K, CHAUNHAN DS, SHARMA VD, GUPTA UD, VENKATESAN K, KATOSH VM (2007). Comparative evaluation of Löwenstein- Jensen proportion method, Bact/ Alert 3D system, and enzymatic pyrazinamidase assay for pyrazinamide susceptibility testing of *Mycobacterium tuberculosis. Journal of Clinical Microbiology* **45:** 76-80.

TAMURA K, NEI M, KUMAR S (2004). Prospects for inferring very large phylogenies by using the neighbour joining method. *Proceedings of the National Academy of Science* **101**: 11030-11035.

TAMURA K, STECHER G, PETERSON D, FILIPSKI A, KUMAR S (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology Evolution* **30**: 2725-2729.

TELENTI A, MARCHESI F, BALZ M, BALLY F, BOTTGER EC, BODMER T (1993). Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *Journal of Clinical Microbiology* **31**: 175-178.

THOMPSOM JD, HIGGINS DG, GIBSON TJ (1994). CLUSTAW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position- specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

TORTOLI E (2003). Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clinical Microbiology Reviews* **16**: 319-354.

TORTOLI E (2006). The new mycobacteria: an update. *FEMS* **48**:159-178.

TORTOLI E, RINDI L, BARTOLONI A, GARZELLI C, MANFRIN V, MANTELLA A, PICCOLI P, SCARPARO C (2004). Isolation of a novel sequevar of *Mycobacterium flavescens* from the synovial fluid of an AIDS patient. *Clinical Microbiology Infections* **10**: 1017-1019.

TSUKUMURA M, YANO I, IMAEDA T (1986). *Mycobacterium moriokaense* sp. nov., a rapidly growing non-pathogenic mycobacterium. *International Journal of Systematic Bacteriology* **36:** 333-338.

TURENNE C, CHEDORE P, WOLFE J, JAMIESON F, MAY K, KABANI A (2002). Phenotypic and molecular characterisation of Clinical isolates of *Mycobacterium elephantis* from human specimens. *Journal of Clinical Microbiology* **40**: 1230-1236.

TURENNE CY, TSCHETTER L, WOLFE J, KABANI A (2001). Necessity of quality - controlled 16S rRNA gene sequence databases: Identifying non – tuberculous *Mycobacterium* species. *The American Society of Microbiology* **39**: 3637- 3648.

VAN HELDEN PD, PARSONS SDC, GEY VAN PITTIUS NC (2009). 'Emerging' mycobacteria in South Africa. *Journal of International South African Veterinary Association* **80**: 210-214.

WALLACE RJ Jr., SWENSON JM, SILCOX VA, GOOD RC **(**1982). Disk diffusion testing with polymyxin and amikacin for differentiation of *Mycobacterium fortuitum* and *Mycobacterium chelonei*. *Journal of Clinical Microbiology* **16**: 1003–1006.

WAYNE LG (1961). Recognition of *Mycobacterium fortuitum* by means of the 3-day phenolphthalein sulfatase test. *The American Journal of Clinical Pathology* **36**: 185-197.

WEIR RE, BLACK GF, NAZARETH B, FLOYD S, STENSON S, STANLEY C, BRANSON K, SICHALI L, CHAGULUKA SD, *et al.*, (2006). The influence of previous exposure to environmental mycobacteria on the interferon-gamma response to bacille Calmette–Guérin vaccination in southern England and northern Malawi. *Clinical and Experimental Immunology* **146**: 390-399.

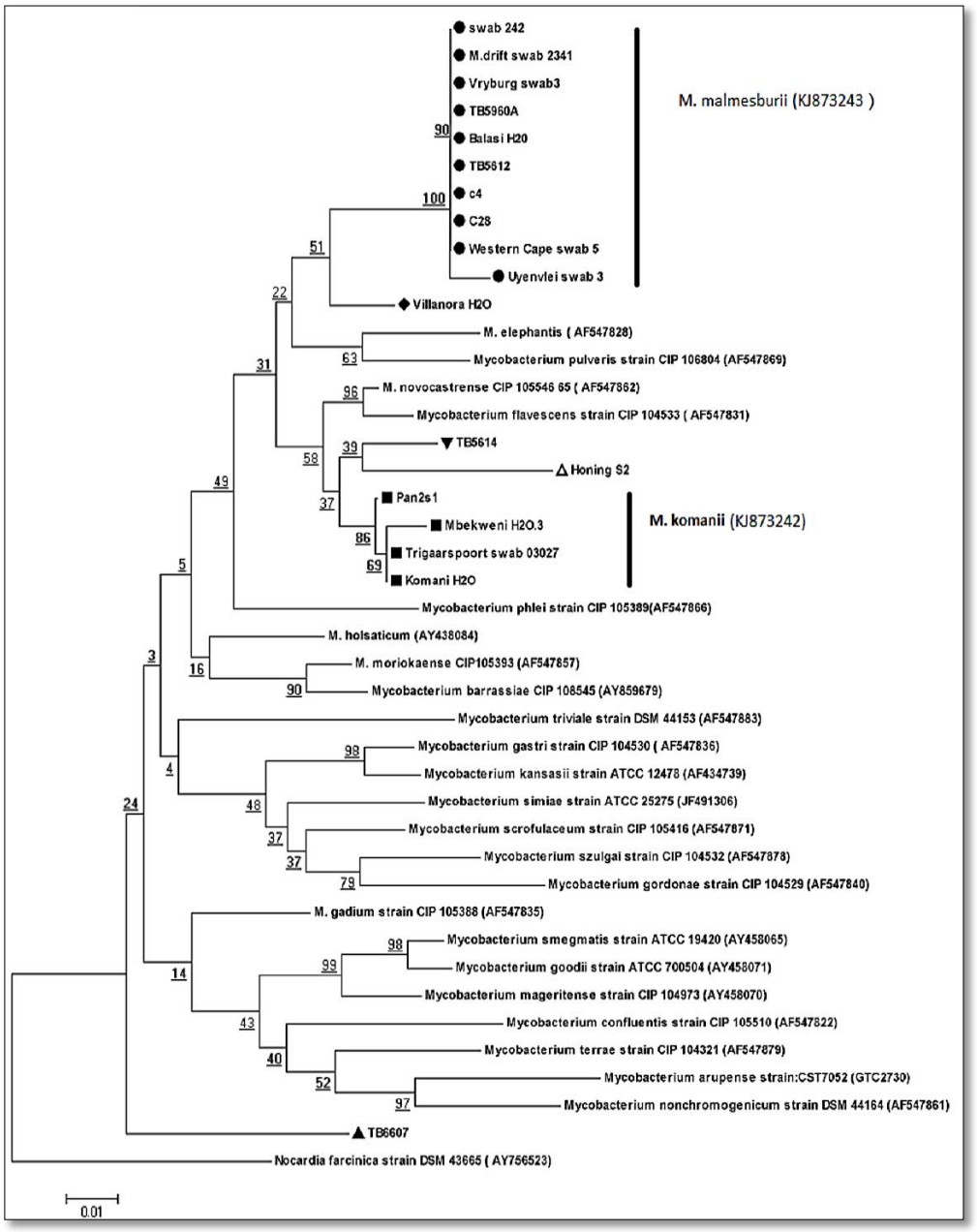

Fig 3.1A: Phylogenetic tree constructed using neighbour joining method, illustrating the position of the isolates. Genbank accession numbers for the sequences are shown in parenthesis. The tree is based on the partial *hsp65* gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Nocardia spp was used as an out group sequence.

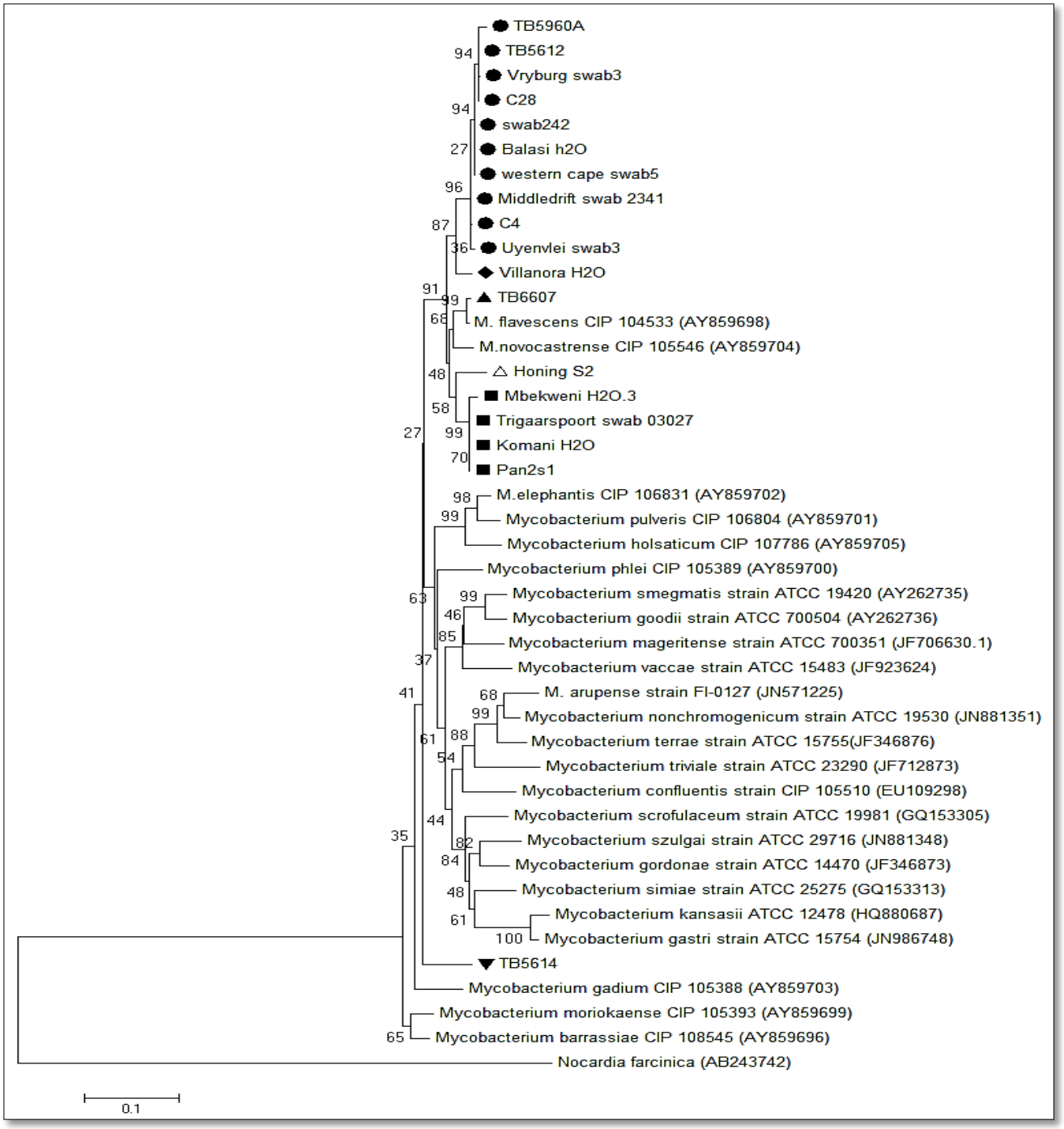

Fig 3.1B: Phylogenetic tree constructed using neighbour joining method, illustrating the position of the isolates. Genbank accession numbers for the sequences are shown in parenthesis. The tree is based on the partial *rpo*B gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. V5.05.Norcadia spp was used as an out group sequence.

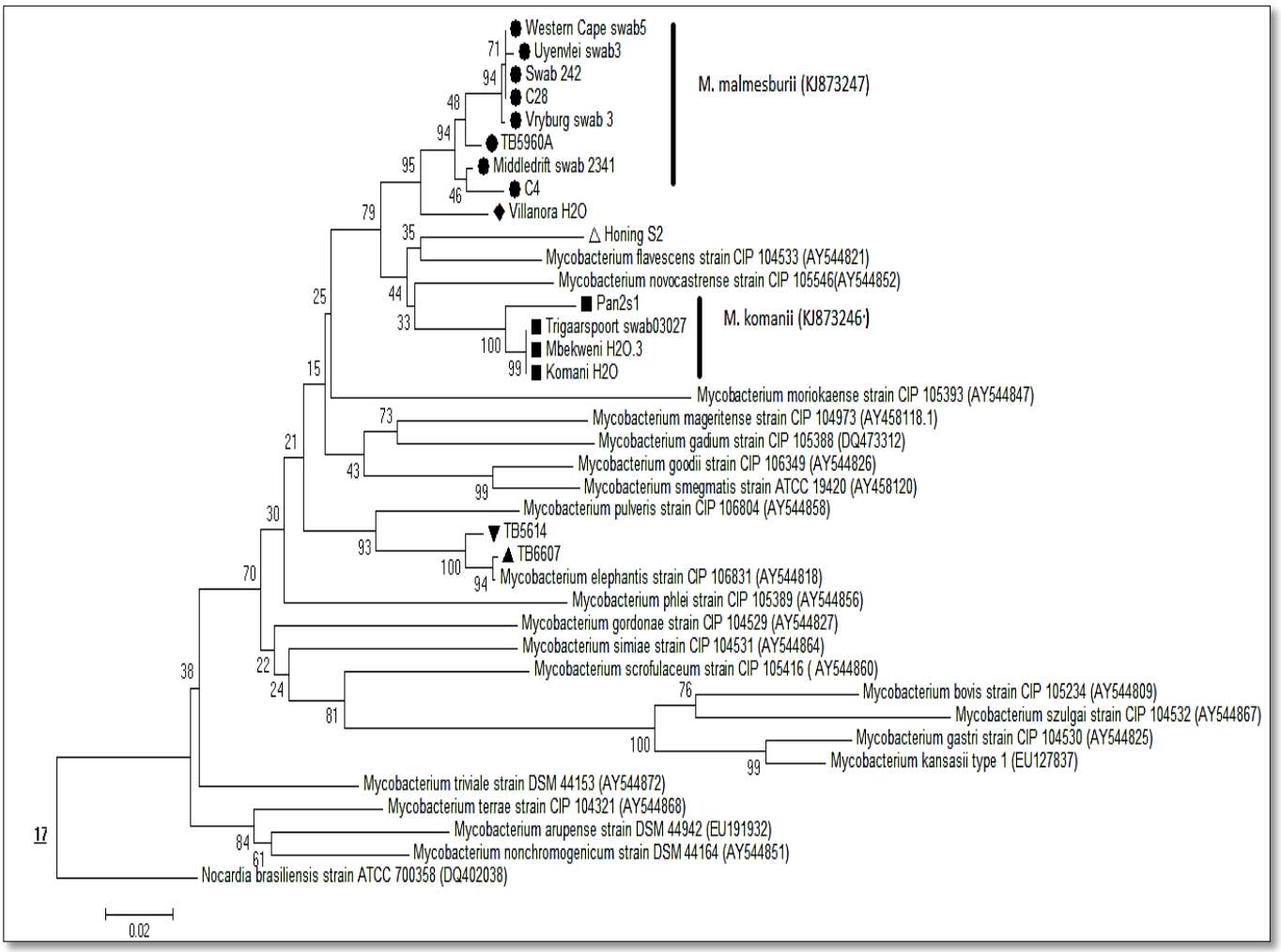

Fig 3.1C: Phylogenetic tree constructed using neighbour joining method, illustrating the position of the isolates. Genbank accession numbers for the sequences are shown in parenthesis. The tree is based on the partial *sod*A gene sequences. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. .Nocardia spp was used as an out group sequence.

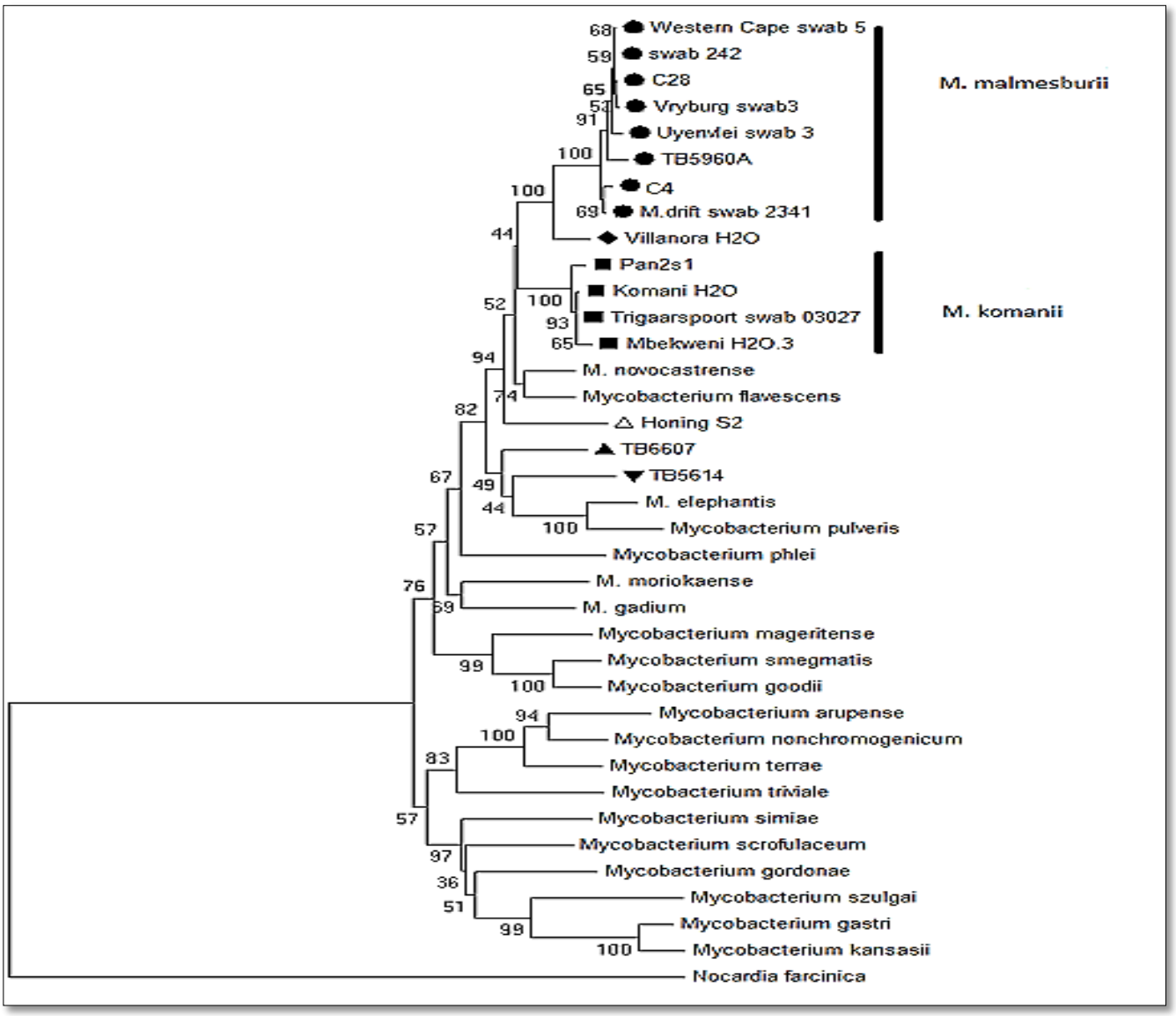

Fig 3.1D: Phylogenetic tree constructed using neighbour joining method, illustrating the position of the isolates. The tree is based on concatenated sequences of: *hsp65* (424bp), *rpoB (*711bp) and *sodA* (411bp) gene fragments. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) and are in the units of the number of base substitutions per site. Nocardia spp was used as an out group sequence.

CHAPTER 4. COMPARATIVE GENOMICS OF FOUR NTM SPECIES AND M. BOVIS / M. TUBERCULOSIS: OCCURRENCE OF SHARED GENES WITH EMPHASIS ON THOSE ENCODING IMMUNOGENIC PROTEINS

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CONTENTS OF CHAPTER 4 and CHAPTER 5 WERE SUBMITTED FOR PUBLICATION

4.1 Abstract

It is not known whether colonization of a host with NTM expressing immunogenic proteins that are to a certain degree homologous to those of pathogenic mycobacteria, without actual infection, could lead to significant cross- reactive immune responses that may interfere with diagnosis of BTB. In this study, we employed comparative genomics of four NTM species *viz Mycobacterium komanii* sp. nov., *Mycobacterium malmesburii* sp. nov., *Mycobacterium nonchromogenicum*, *Mycobacterium fortuitum* ATCC 6841 and *Mycobacterium bovis/ Mycobacterium tuberculosis* to assess their potential to elicit cross-reactive immune responses against *M. bovis* antigens by searching the NTM genomes for the presence of shared genes shown to be encoding for immunogenic proteins in *M. bovis*. The four NTM species had *esxA* and *esxB* sequences. Five bovine T-cell recognized epitopes of *M. bovis* ESAT-6 (*esxA*) were detected in *M. fortuitum* and *M. nonchromogenicum* genome while four CFP 10 (*esxB*) immunogenic epitopes were detected in *M. komanii* sp. nov. and *M. malmesburii* sp.nov. genomes. Genes of the ESX-3 locus (*esxG*, *esxH*) and *esxR* were also found in the four NTM species. Two of the five *M. bovis* immunogenic epitopes of *esxR* showed 100% similarity to *M. fortuitum, M. malmesburii* sp. nov. and *M. komanii* sp.nov. sequences. Full cross recognition of these NTM *esxR* epitopes is therefore highly likely, and may lead to misdiagnosis of BTB. The *mpb70* orthologs as well as four *M. bovis* immunogenic epitopes were identified in *M. malmesburii* sp.nov. and *M. komanii* sp.nov. *CanA, mpb64, Rv1120c* and *dnaK* sequences were also detected in all the four NTM while *mpb63, tpx* and *hspX* occurred in *M. nonchromogenicum* and *M. fortuitum*.. This study has laid a foundation into investigation of these NTM and their cross-reactivity with *M. bovis* immunogenic proteins.

Key words: NTM, Esx family, esx-1, esx-2, esx-3, esx-4 esx-5, mpb70, mpb63, mpb64, Rv1315, NTM, M. fortuitum, M. malmesburii sp.nov., M. komanii sp.nov., M. nonchromogenicum, M. bovis, M. tuberculosis, mpb83, espC, cross-reactive immune responses, BTB immunodiagnosis.

4.2 Introduction

The hypothesized interference of NTM and the TB vaccine strain, BCG, in tuberculin based assays for B(TB) immuno-diagnosis has triggered investigation of 'specific' or defined antigens which are uniquely present in pathogenic mycobacteria, mainly *M. bovis* and *M. tuberculosis* and absent in *M. bovis* BCG and NTM. Comparative genomics has led to the identification and characterisation of certain genetic regions in the genomes of mycobacteria that are absent in *M. bovis* BCG and most NTM, but present in *M. tuberculosis* and *M. bovis*. The genomes of *M. tuberculosis* and of *M. bovis* are 99.95% identical. Several studies employing different techniques of genomic biology such as bacterial artificial chromosome array, DNA micro- array, subtractive genomic hybridization and whole genome sequence analysis revealed a number of genomic regions of difference (RD) among *M. bovis, M. tuberculosis* and *M. bovis* BCG (Mahairas *et al.*, 1996; Ganguly *et al.*, 2008). Examples of these genomic regions deleted during the evolution of *M. bovis* BCG are: RD1, RD2, RD3 and RD14 encompassing 29 open reading frames (ORF) (Maharais *et al.*, 1996; Ganguly *et al.*, 2008).

Proteins encoded in these regions in pathogenic mycobacteria are investigated on the assumption that they may be involved in the virulence of pathogenic mycobacteria and in activation of cells involved in host immune responses and therefore hold potential as candidates for the diagnosis of B (TB). Since host humoral responses against tuberculosis only appear at later stages of infection most studies have mainly focused on antigens that are targets for cell mediated immune responses. Open reading frame sequences for these proteins encoded in these regions are selected and peptides or conjugate proteins derived from these sequences are synthesized and tested for their immunogenicity (Skjøt *et al*., 2002; Cockle *et .al* 2002). Since RD1 deletion seems to have been the early event in the attenuation of the vaccine strain, proteins within this region are the most studied. The RD1 which is 9.5 kb in size and comprises of nine

open reading frames, Rv3871 to Rv3879c (annotation of the *M. tuberculosis* genome) or Mb3900 to Mb3908c (annotation of the *M. bovis* genome). The two most predominant proteins, namely the 6 kDa early secreted target antigen (ESAT-6) also known as ESXA, (encoded by *Rv3875* in *M. tuberculosis* and *Mb3905* in the *M. bovis* genome); and the 10 kDa culture filtrate protein (CFP 10) also known as ESXB (encoded by *Rv3874* in the *M. tuberculosis* genome and *Mb3904* in the *M. bovis* genome) are being widely investigated as candidate vaccines and used as antigens in the diagnosis of B(TB) (Pym *et al.,* 2003; Vordermeier *et al.,* 2006; Ganguly *et al.,* 2008). PPE 68 encoded by *Rv3873* or *Mb3903* has also been identified as an immunogenic component of RD1. ESAT-6 and to a lesser extent CFP 10 and PPE 68 have been shown to induce T-cell responses in animal models (Demangel *et al.,* 2004; Mustafa *et al.,* 2014). Functional studies have shown that RD1 constitutes a specialised system for secretion in mycobacteria, involving genes lying inside the RD1 locus as well as the regions flanking this RD. The system is named the ESAT-6 secretion system (Brondin *et al.,* 2004). Secretory proteins that subvert host defences and impair the development of protective immunity have since been the focus of research aimed at development of TB vaccines and immunodiagnostic assays, because they are thought to have a potential to induce protective immunity and immune responses of diagnostic value (Ize and Palmer; 2006; Ganguly *et al.,* 2008; Marongui *et al.,* 2013). In addition, proteins of the Proline Glutamate (PE) or Proline Proline Glutamate (PPE) family which are localised to the cell surface or secreted have been investigated as TB vaccine and diagnostic candidates (Akhter *et al.,* 2012).The availability of the whole genomes of *M. bovis*, *M. tuberculosis* and *M. bovis* BCG has contributed immensely to the identification of a number of these immunogenic proteins. Among proteins involved in secretion are the 14kDa heat shock protein HSPX encoded by *Rv2031c/Mb2057c,* the probable conserved secreted protein TB22.2/ MPB64 encoded by *Rv3036c/Mb3062c,* TB 10.3 encoded by *esxR/ Rv3019c/Mb3045c,* TB 10.4 encoded by *esxH/Rv0288/Mb0296*, *esxI/ Rv1037c/ Mb1066c*, *esxJ/ Rv1038/ Mb1067*, EspC encoded by

Rv3615c/Mb3645c, *Rv2660c/Mb2678c*, Tpx encoded *Rv1932/Mb1967*, CanA encoded *by Rv1284/Mb1315*, hypothetical protein, *Rv1120c/Mb1151c* and secreted antigenic proteins, Ag85 complex (Millington *et al.*, 2011; Marongui *et al.*, 2013).

Other antigens that have been shown to play a pivotal role in host humoral immune response and have been investigated for their potential as candidates for B (TB) diagnosis are MPB70/ MPT70, MPB83/ MPT83, MPB63/ MPT63. These proteins are highly homologous (amino acid similarities of 98%-100%) within the members of the *Mycobacterium tuberculosis* complex (including *M. bovis* BCG) (Ganguly *et al.*, 2008).

The two predominant antigens, MPB70, a major secreted immunogenic protein and MPB83, cell surface lipoprotein are highly expressed in *M. bovis* and considerably less abundantly expressed in *M. tuberculosis*. Some strains of *M. bovis* BCG express only minute amounts of MPB70 and MPB83, *viz* the Pasteur, Danish 1331, Glaxo, Tice and Beijing strains. *M. bovis* BCG strains expressing high levels of MPB70 and MPB83 are the Tokyo, Moreau, Russian, Sweden, Birkhaug and Romanian strains (Wiker 2009). These proteins including MPB80 are encoded by two genes in an operon of six genes spanning from *Rv2871- Rv2876* (in the *M. tuberculosis* genome) or *Mb2896-Mb2901* (in the *M. bovis* genome). MBP83 is encoded by *Rv2873/Mb2897* in the operon while MPB70/80 is encoded by *Rv2875/Mb2900*. Several studies using recombinant MPB83 in assays such as MAPIA and lateral flow immunochromatography have confirmed MPB83 to be among the most promising antigens for sero-diagnosis of BTB in a range of animal species such as cattle, badgers, white tailed deer, wild boar and possums (Goodger *et al.*, 1994; Greenwald *et al.*, 2003; Liu *et al.*, 2007; Lerselier *et al.*, 2008; Lyashchenko *et al.*, 2008). It has also been established that the antibodies to MPB83 can be detected

relatively earlier (4 weeks) than the anti-MPB70 response which usually develops after 18-22 months post infection (Wiker *et al.,* 2009).

Cell mediated immune responses of humans and animals to these antigens have also been explored for diagnosis of tuberculosis. The T-cell response to MPB70 is much better characterised than the T-cell response to MPB83 (Wiker, 2009). It was shown that MPB70 causes strong skin test reactivity upon sensitisation of guinea pigs with BCG, but not with *M. tuberculosis* (Nagai *et al.,* 1986). Roche *et al. (*1994), also showed that vaccinated individuals demonstrated T-cell responses to MPB70 *in vitro*. Other studies have reported that individuals infected with *M. tuberculosis* show T-cell responses to MPB70. In these studies MPB70 was shown to induce IFNγ as well as tumour necrosis factor (Al Attiya *et al.,* 2003; 2006). Studies in cattle infected with *M. bovis* also demonstrated T cell immune responses to MPB70. (Wood *et al.,* 1994; Pollock *et al.,* 1994). On the other hand, MPB83 has been shown to induce stronger T-cell immune responses in *M. bovis* infected animals than MPB70 (Vordermeier *et al.,* 1999).

Genes encoding antigenic proteins like ESAT-6, CFP 10 and PPE 68 have been shown not to be specific to MTBC but also occur in *M. leprae* as well as in NTM like *M. kansasii, M. marinum, M. smegmatis, M. szulgai* (Sorensen *et al.,* 1995; Harboe *et al.,* 1996; Gey van Pittius *et al.,* 2001). Even though the amino acid sequences of the corresponding proteins vary among these species, expression of these proteins was detected most notably in *M. kansasii* (Vordermeier *et al.,* 2007). Likewise, homologues of MPB70 and MPB83 in *M. kansasii* and MPB70 in *Nocardia asteroids* were found, and these proteins were demonstrated to be expressed in these species (Woolford *et al.,* 1997). In addition, *M. kansasii* homologues have been described for genes encoding for other immunogenic antigens of *M. bovis*/ *M. tuberculosis* such as TB10.4 encoded by *Rv 0288*/ *esxH*, and TB 10.3/ *esxR* (Skjøt *et al.,* 2002). Studies have shown that some of these proteins are not only expressed in the NTM species but that cattle

experimentally infected with *M. kansasii* also demonstrate cell mediated immune responses to ESAT- 6 and CFP 10 as well as antibody responses to a cocktail of ESAT- 6, CFP 10 and MPB83 (Waters *et al.*, 2010). Contrary in a study by Demangel *et al* (2005), mice sensitised with *M. scrofulaceum*, *M. avium* or *M. vaccae* did not produce cellular immune responses cross reacting with ESAT- 6 probably because the *esat*-6 gene is not available in the genomes of these mycobacterial species (Demangel *et al.*, 2005).

Potential NTM specific immunogenic antigens and their role in priming T cell immune response were assessed by Checkley *et al.* (2011). This study used a bioinformatics comparative genomics approach (comparing whole genome sequences of the most commonly isolated NTM in humans to those of MTBC) to identify antigens specific to NTM and use them as targets of T cell immune responses to NTM infection. In this study a cluster of mammalian cell entry (Mce) proteins was shown to be specific to NTM and low level responses to these proteins were detected in NTM infected individuals (Checkley *et al.*, 2011).

The aim of the present study was to compare the genomes of the four NTM species *viz M. nonchromogenicum*, *M. fortuitum*, *M. malmesburii* sp.nov. and *M. komanii* sp.nov. to those of M. *bovis* and *M. tuberculosis*. The comparison was done in order to investigate the occurrence of shared genes, known to encode for immunogenic proteins in *M. bovis*/ *M. tuberculosis*, in the NTM genomes. The NTM genomes were searched for genes for *Mycobacterium* secretory proteins mainly from the ESAT - 6 family as well as MPB70, MPB63 and MPB83 proteins and other proteins. The NTM selected for this analyses, except *M. fortuitum*, were found to be among the most frequently occurring NTM species in cattle, African buffalo and their environments in South Africa (Gcebe *et al.*, 2013) and a PPD derived from *M. fortuitum* is used in the modified BOVIGAM^{TM} assay in South Africa as an additional NTM tuberculin to the one derived from *M. avium* derived (Michel *et al.*, 2011).

4.3 Materials and Methods

4.3.1 Origin of bacterial cultures

M. malmesburii sp.nov. strain WCM 7299, *M. komanii* sp.nov. strain GPK 1020 and *M. nonchromogenicum* strain NCK 8460 were isolated during a NTM survey in South Africa (Gcebe *et al*, 2013). The two former strains were identified and characterised as novel NTM species in chapter three of this thesis. The *M. nonchromogenicum* strain was isolated from soil and species identification was done by PCR and sequencing of 16S rDNA, *hsp65*, *rpoB* and *sodA*. *M. fortuitum* ATCC 6841 was supplied by the Tuberculosis laboratory of the ARC-Onderstepoort Veterinary Institute and its species identity was confirmed by PCR- sequencing assays targeting 16S rDNA, *hsp65*, *rpoB*, and *sod*A.

4.3.2 DNA extraction for whole genome sequencing

DNA was extracted from bacterial cultures in solid media using the Qiagen nucleic acid extraction kit (Whitehead Scientific, South Africa).

4.3.3 Library preparation and sequencing

Genomic DNA paired -end libraries were generated using the Nextera DNA sample preparation kit (Illumina) and indexed using the Nextera index kit (Illumina). Sequencing was performed as paired end reads (2x250bp) employing the Illumina Miseq system using the Miseq reagent kit v2 at the Agricultural Research Council.

4.3.4 Sequence quality control and trimming

Adapter contents were removed using Cutadapt[2]. All reads were trimmed for quality with Trimmomatic (sliding window=4, trimming when average quality<15), clipped at 20-150 bases for *M. fortuitum* and *M. malmesburii* sp.nov. or 20-240 bases for *M. nonchromogenicum* and *M. komanii* sp.nov. using the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/); and reads with less than 80% bases with a quality score of less than 20 were removed (FASTX Toolkit). FASTQC was used to analyse the sequence quality of the processed reads at the Centre for Proteomics and Genomics Research (CPGR, South Africa).

4.3.5 Whole genome sequence analysis:

4.3.5.1 Alignment

To gauge the similarity of the NTM species to *M. bovis* AF2122/97 (NC 002945.3) and *M. tuberculosis* H37Rv (NC 000962.2), alignment of each of the processed sets of reads to these reference genomes

was performed using BWA (Li and Durbin, 2009).

4.3.5.2 De Novo assembly

Assembly of the sequencing reads was performed using SPAdes, which mainly involves construction of DNA sequences of contigs and the mapping of reads to contigs (Bankevich *et al.,* 2012). Each assembly was evaluated using QUAST (Gurevich *et al.,* 2013).

4.3.5.3 Annotation

The assembled genome sequences were annotated using Prokka annotation pipeline (Seemann, 2014). This involved predicting tRNA, rRNA and mRNA genes in the sequences and assigning putative gene products to the protein-coding genes (CDSs) based on their similarity to sequences in a database of curated Mycobacterium genes.

4.3.5.4 Comparison of NTM predicted proteins to those of reference genomes

The list of annotated genes for each NTM was compared to those of the reference genomes (*M. bovis* AF2122/97 and *M. tuberculosis* H37Rv). Basic local alignment search tool of proteins (BLASTP) (Altschul *et al.,* 1990; 1997) was used to identify putative homologous proteins as the best Basic local alignment search tool (BLAST) hits between each pair of genomes.

4.3.5.5 Genomic similarity search among the NTM strains, *M. bovis* and *M. tuberculosis*

To examine the similarities among the four newly sequenced genomes and the two reference genomes, *viz M. bovis* AF2122/97 and *M. tuberculosis* H37Rv, pairwise BLAST was performed between the full sets of amino acid sequences for all strains. The similarity of a pair of strains was estimated as a proportion of protein- coding genes that have homologues in both strains (*i.e*. number of genes shared/ total number of genes). To estimate the similarity for only the highly conserved genes, the same metric was calculated using the subset of the best BLAST hits with amino acid sequence identity ≥90% that were aligned along ≥ 50% of the sequence length.

4.3.5.6 Investigating the closest sequence relatives of the NTM strains

BLAST search of the largest contigs from each NTM assembly was performed against the NCBI Genbank database to identify the closest known sequence relative of each NTM species. BLAST searches were also performed for the annotation CDS features for each NTM species against the reference genomes for their respective best hits and visualized using BLAST Ring Image Generator (BRIG) (Alikhan *et al.*, 2011).

4.3.5.7 Genome assembly visualisation

BLAST Ring Image Generator (Alikhan *et al.*, 2011) was used to visualize the pairwise BLAST results of CDS features in *M. bovis* and each NTM annotation against that of *M. tuberculosis* and against the respective closest NTM relative.

4.3.5.8 Identification of immunogenic proteins in NTM annotations

BLAST searches were conducted to quantify the similarity of the annotated amino acid sequences in the NTM assemblies (*M. fortuitum*, *M. malmesburii* sp.nov., *M. komanii* sp.nov. and *M. nonchromogenicum*) to immunogenic proteins including the Esx family (ESX-1 to ESX-5) in *M. bovis*, *M. tuberculosis* and *M. smegmatis*, (see table 4.1 for the list of the targeted proteins). BLASTP searches against NCBI Genbank database were also conducted for the respective NTM protein sequences to quantify similarity to other sequences in the database. Amino acid and nucleotide sequences of the *M. bovis*, *M. tuberculosis* and *M. smegmatis* proteins were retrieved from Genolist database (http://Genolist.pasteur.fr/Tuberculist). Multiple amino acid sequence alignments of the individual amino acid sequences (ESAT-6 and CFP 10 [ESX-1], TB10.4, TB10.3 [ESX-3], MPB70 and CanA) from different mycobacteria were performed using Clustalw (Thompson *et al.*, 1994) from MEGA (version 6), to assess sequence similarities of the *M. bovis* and the NTM homologues at immunogenic epitope level. NTM protein homologues that showed amino acid sequence similarities of <50% to the respective *M.bovis* antigen were not included in the alignments.

Table 4.1: List of target genes (other than the e encoding immunogenic proteins in *M. bovis* and *M. tuberculosis*

Name/Gene ID	Species	RefSeq/Genbank	Locus Tag	Protein product
espC	*M. tb*	WP_003899599.1	Rv3615c	ESX-1 secretion associated protein EspC
	M. bovis		Mb3645c	hypothetical protein
dnaK	*M. tb*	WP_003401814.1	Rv0350	chaperone protein DnaK
	M. bovis		Mb0358	molecular chaperone DnaK
mpt63/ *mpb63*	*M. tb*	WP_003409684.1	Rv1926c	immunogenic protein Mpt63
	M. bovis		Mb1961c	hypothetical protein
mpt70/ *mpb70*	*M. tb*	WP_003414644.1	Rv2875	major secreted immunogenic protein Mpt70
	M. bovis		Mb2900	major secreted immunogenic protein Mpb70
mpt83/ *mpb83*	*M. tb*	WP_003900588.1	Rv2873	cell surface lipoprotein
	M. bovis		Mb2898	cell surface lipoprotein MPB83 (p23)
canA	*M. tb*	WP_003406616.1	Rv1284	beta carbonic anhydrase
	M. bovis		Mb1315	hypothetical protein
tpx	*M. tb*	WP_003409700.1	Rv1932	thiol peroxidase
	M. bovis		Mb1967	thiol peroxidase
mpt64 *mpb64*	*M. tb*	WP_003415939.1	Rv3036	secreted protein
	M. bovis	WP_010950816.1	Mb3062c	hypothetical protein
	M. tb	WP_003405876.1	Rv1120c	hypothetical protein

	M. bovis	WP_010950495.1	Mb1151c	hypothetical protein
	M. tb	WP_003913382.1	Rv2660c	hypothetical protein
	M. bovis	WP_010950756.1	Mb2678c	hypothetical protein
	M. tb	NP-21710.1	Rv2654c	antitoxin
	M. bovis	WP_003913382.1	Mb2659c	prophage integrase
hsp20/	*M. tb*	WP_003900838	Rv2031c	Heat shock protein, hsp20
hspX	*M. bovis*		Mb2057c	Heat shock protein, hspX
esxA	*M.tb*	WP_000339993.1	Rv3875	6 kDa early secretory antigenic target (ESAT-6)
	M. bovis		Mb3905	6 kDa early secretory antigenic target (ESAT-6)
esxB	*M. tb*	WP_003399940.1	Rv3874	10 kDa culture filtrate antigen LHP (CFP10)
	M. bovis		Mb3904	10 kDa culture filtrate antigen LHP (CFP10)
esxC	*M.tb*	WP_003899750	Rv3890c	ESAT-6 like protein EsxC (ESAT-6 like protein 11)
	M. bovis		Mb3919c	Putative esat-6 like protein 11
esxD	*M.tb*	WP_003400035.1	Rv3891C	Possible esat-6 like protein EsxD
	M. bovis		Mb3920C	Conserved hypothetical protein
esxG	*M.tb*	NP_214801.1	Rv0287	ESAT-6 like protein EsxG
	M. bovis	NP_853959.1	Mb0295	Hypothetical protein
esxH	*M. tb*	WP_023369886.1	Rv0288	ESAT-6 like protein EsxH

	M. bovis		Mb0296	low molecular weight protein antigen 7 CFP7 (TB10.4)
esxQ	*M. tb*	WP_003912054.1	Rv3017	ESAT-6 like protein EsxQ
	M. bovis		Mb3042	ESAT-6 like protein 8
esxR	*M. tb*	WP_003415340.1	Rv3019	ESAT-6 like protein EsxR
	M. bovis		Mb3045	ESAT-6 like protein 9 (TB10.3)
esxS	*M. tb*	WP_003415342.1	Rv3020c	ESAT-6 like protein EsxS (PE28)
	M. bovis		Mb3046c	PE family protein
esxT	*M. tb*	WP_003900059.1	Rv3444C	ESAT-6 like protein EsxT
	M. bovis		Mb3474C	Hypothetical protein
esxU	*M. tb*	WP_003900060.1	Rv3445C	ESAT-6 like protein EsxU
	M. bovis		Mb3475C	Hypothetical protein
esxV	*M. tb*	NP_218136.1	Rv3619c	ESAT-6 like protein EsxV
	M. bovis		-	-
esxM	*M. tb*	WP_003408810.1	Rv1792	ESAT-6 like protein EsxM
	M. bovis		Mb1820	Hypothetical protein
esxO	*M. tb*	NP_216862.1	Rv2346	ESAT-6 like protein EsxO
	M. bovis	NP_856024.1	Mb2375	ESAT-6 like protein 6
esxL	*M. tb*	WP_003898766.1	Rv1198	ESAT-6 like protein EsxL
	M. bovis		Mb1230	ESAT-6 like protein 4
esxI	*M. tb*	WP_023363993.1	Rv1037c	ESAT-6 like protein EsxI
	M. bovis		Mb1066c	ESAT-6 like protein 1

esxN	*M. tb*	WP_003408840	Rv1793	ESAT-6 like protein EsxN
	M. bovis		Mb1821	ESAT-6 like protein 5
esxK	*M. tb*	WP_003421604.1	Rv1197	ESAT-6 like protein EsxK
	M. bovis		Mb1229	ESAT-6 like protein 3
esxP	*M. tb*	WP_003900502	Rv2347c	ESAT-6 like protein EsxP
	M. bovis			
esxW	*M. tb*	WP_003899603	Rv3620c	ESAT-6 like protein EsxW
	M. bovis			
esxJ	*M. tb*	NP_215554.1	Rv1038c	ESAT-6 like protein EsxJ
	M. bovis	NP_854723.1	Mb1067c	ESAT-6 like protein 2
esxE	*M. tb*	WP_003400094.1	Rv3904c	ESAT-6 like protein EsxE
	M. bovis		Mb3934c	Hypothetical protein
esxF	*M. tb*	WP_003400100	Rv3905	ESAT-6 like protein EsxF

4.4. Results

De Novo assembly, sequence annotation, genome similarity check, alignment as well as comparison to *M. bovis* and *M. tuberculosis* reference genomes of the four NTM reads (*M. fortuitum, M. nonchromogenicum, M. malmesburii* sp.nov, and *M. komanii* sp.nov.) (referred to as the 'four NTM' in the different result sections) is reported. The occurrence of the predicted NTM Esx-family proteins and the targeted immunogenic proteins in the four NTM genomes as well as other NTM is also reported. Multiple amino acid sequence alignment and comparison at epitope level of the four NTM homologues

of ESAT-6, CFP 10, CanA, MPB70, ESXG and ESXR/ESXH, to those of *M. smegmatis*, *M. bovis* and *M. tuberculosis* is also reported.

4.4.1 Alignment of the four NTM reads with *M. bovis* and *M. tuberculosis* genomes

The results of the alignment of the NTM reads (DNA sequences) and *M. bovis* or *M. tuberculosis* are summarised in Table 4. 2. From these results it is clear that there is very little similarity (ranging from 8.83% between *M. malmesburii* sp.nov. and *M. tuberculosis* to 18.69% between *M. komanii* sp.nov. and *M. tuberculosis* at DNA level) between any of the sets of the NTM sample reads and the reference genomes *i.e. M. bovis* (NC 002945.3) and *M. tuberculosis* (NC 000962.3), hence De Novo assembly was performed. High GC contents of 66.19% -67.33% which are typical of mycobacterial genomes were observed in the NTM genomes.

Table 4.2: Alignment matrices of the four NTM genomes to those of *M. bovis* and *M. tuberculosis*

NTM species	Strain ID	Reads mapped to *M. bovis* (%)	Reads mapped to *M. tuberculosis* (%)	GC content (%)
M. fortuitum	ATCC 6841	10.58	10.63	66.19
M. nonchromogenicum	NCK 8460	15.71	15.8	66.25
M. malmesburii sp.nov.	WCM 7299	8.83	8.89	67.4
M. komanii sp.nov.	GPK 1020	18.61	18.69	67.33

4.4.2 De Novo assembly results

The summary of the assembly results is shown in Table 4.3. The assembly for *M. komanii* sp.nov. produced 63 contigs, its largest contig was 292 570 bases in size. While the assembly for *M. nonchromogenicum* produced the largest contig of all the samples (425 774 bases) it also produced the largest number of contigs. The overwhelming majority of the sequenced bases fall within the few largest contigs, but each assembly also produced a large number of very short contigs. The assembly of *M. fortuitum* and *M. malmesburii* sp.nov. resulted in high total number of contigs (179 and 255 respectively). This is probably due to the small reads sizes present in these datasets (125 bases) compared to those of *M. nonchromogenicum* and *M. komanii* sp.nov (210 bases). Both *M. komanii* sp.nov. and *M. nonchromogenicum* sp.nov. assemblies displayed improved N50 (the length for which the set of contigs that length or longer contains at least half the assembly bases) and L50 (the number of contigs of length ≥ N50) metrics over that of *M. fortuitum* and *M. malmesburii* sp. nov. assemblies. These results suggest that the order of assembly quality from highest to lowest is: *M. komanii* sp. nov., *M. nonchromogenicum*, *M. fortuitum* and *M. malmesburii* sp. nov.

Table 4.3: Summary of the De Novo assembly results

NTM Species	Strain ID	number of contigs (n)	Largest contig (bp)	Total Length (bp)	L50	N50
M. fortuitum	ATCC 6841	179	195 004	6 275 573	27	82 193
M. nonchromogenicum	NCK 8460	642	425 774	6 623 407	17	131 682
M. malmesburii sp.nov.	WCM 7299	255	135 683	5 426 606	42	42 651
M. komanii sp.nov.	GPK 1020	63	298 570	5 370 343	12	162 386

4.4.3 Assembled genome sequence annotation

The summary of the annotation is provided in Table 4.4. The numbers of protein coding genes, sequences encoding tRNA and rRNA and signal peptides identified in each of the four NTM genome are indicated. The whole genome sequences were deposited into the European Nucleotide Archive (ENA) the accession numbers are also indicated in Table 4.4.

Table 4.4: Assembled genome annotation, showing number of CDS, tRNA, rRNA and signal peptides

NTM species	Strain ID	**Accession numbers**	CDS	tRNA	rRNA	Signal peptides
M. fortuitum	ATCC 6841	CVRX01000001-CVRX01000338	6097	66	3	499
M. nonchromogenicum	NCK 8460	CVTC01000001-CVTC01002268	6903	70	3	544
M. malmesburii sp.nov.	WCM 7299	CVTB01000001-CVTB01000324	5298	54	2	401
M. komanii sp.nov.	GPK 1020	CVTA01000001-CVTA01000065	5141	53	2	408

NTM, non-tuberculous mycobacterium, CDS, protein coding genes

4.4.4 Comparison of predicted proteins of the four NTM to those of reference genomes.

Table 4.5 summarizes the results for comparison of the four NTM genomes to the reference genomes (*M. bovis* AF2122/97 and *M. tuberculosis* H37Rv). The number of putative homologues between each NTM and the two reference genomes as well as the number of genes for which no homologues were found (mainly hypothetical proteins) are indicated in the table. A higher number of gene homologues seem to be shared between *M. bovis*/ *M. tuberculosis* and each NTM than there were unique genes identified for each *Mycobacterium* species.

Table 4.5: Gene list comparison of the four NTM and *M. bovis/ M. tuberculosis* reference genomes

NTM Species	Strain ID	Genes shared with		Genes in NTM but not in *M. bovis* and *M.tb*		Genes not in the NTM but found in *M. bovis* and *M.tb*	
		M. tb n	*M. bovis* n	*M. tb* n	*M. bovis* n	*M. tb* n	*M. bovis* n
M. fortuitum	ATCC 6841	4245	4238	813	811	1910	1916
M. nonchromogenicum	NCK 8460	4732	4722	785	787	2355	2364
M. malmesburii sp.nov.	WCM 7299	3872	3865	866	860	1468	1476
M. komanii sp.nov.	GPK 1020	3862	3846	896	891	1359	1371

M.tb, M. tuberculosis

4.4.5 Esx family orthologs in NTM genomes (including the four newly sequenced NTM genomes and other NTM sequences available in NCBI Genbank database)

The four NTM annotations displayed the presence of the *esx* genes situated within the ESX-1 locus (*esxA* and *esxB*) as well as *esx* gene orthologs of the ESX-4 locus (Table 4.6). Orthologs of *esxA* and *esxB* were detected in *M. fortuitum* (71.58% and 64% similar to *M. bovis* respectively, at amino acid level), *M. malmesburii* sp.nov. (displaying 50% and 57.73% similarity to *M. bovis* respectively at amino acid level),

M. nonchromogenicum (displaying 71.58 and 64% similarity to the *M. bovis* orthologs respectively) and *M. komanii* sp.nov. (48.75% and 57.73% similar to the *M. bovis* at amino acid level). Interestingly, the BLAST searches using these sequences revealed the existence of their orthologs in additional NTM species, most notably *M. vulneris* (98% similarity to *M. fortuitum* at amino acid level for both genes), *M. mageritense* (82% and 74% similarity to *M. fortuitum* respectively, at amino acid level) and *M. farcinogenes* (96% and 92% similarity to *M. fortuitum* respectively, at amino acid level). The *esx* genes of the ESX-2 locus were not detected in any of the four NTM annotations. The *esx* genes of the ESX-3 locus (*esxG, esxH*) as well as *esxR* were found in the different NTM annotations while *esxS* and *esxQ* were both absent from all four NTM genomes as illustrated in table 4.6. The only NTM species that was found to harbor *esx* gene orthologs of the ESX-5 locus (*EsxN)* as well as *esxK* was *M. nonchromogenicum* (Table 4.6).

Table 4.6: Orthologs of the *esx genes* in the four NTM genomes

Locus	***esx* gene tag**	**Percentage homology of amino acid sequences deducted from the different NTM *esx* genes to *M. bovis***			
		M. komanii sp. nov.	*M. malmesburii* sp.nov	*M. fortuitum*	*M. nonchromogenicum*
ESX-1	*esxA/Rv3875/Mb3905*	48.75	50	75.79	71.58
	esxB/ Rv3874/Mb3905	57.73	57.73	64	64
ESX-2	*esxC/Rv3890c/Mb3919c*	no	no	no	no
	esxD/Rv3891c/Mb3920c	no	no	no	no
ESX-3	*esxG/ Rv0287/Mb0295*	78.35	78.35	81.5	81.05

	esxH/ Rv0288/Mb0296	no	no	75.79	77.94
ESX-4	*esxT/Rv3444c/Mb3474c*	73.4	73.96	73.96	73.96
	esxU/Rv3445c/ Mb3475c	60.75	56.01	60.42	60.42
ESX-5	*esxM/Rv1792/Mb1820*	no	no	no	no
	EsxN/ Rv1793/Mb1821	no	no	no	85.11
Elsewhere in the genome	*esxR/Rv3019c/Mb3045c*	75	64.79	no	no
	esxQ/Rv3017/Mb3042	no	no	no	no
	esxS/Rv3020c/Mb3046c	no	no	no	no
	esxV/Rv3619c/	no	no	no	no
	esxO/Rv 2346/Mb2375	no	no	no	no
	esxL/ Rv 1198/Mb1230	no	no	no	no
	esxI/ Rv1037c/Mb1066c	no	no	no	no
	esxK/Rv1197/Mb1229	no	no	no	85.54
	esxP/Rv2347c	no	no	no	no
	esxW/ Rv 3620c	no	no	no	no
	esxJ/Rv1038c/Mb1067c	no	no	no	no
	esxE/Rv3904c/Mb3934c	no	35.5	no	no
	esxF/Rv3905/Mb3935c	no	41.07	no	No

no, no ortholog found

4.4.6 Genes encoding homologues of immunogenic proteins (Outside the *esx* cluster) shared between the NTM and *M. bovis* genomes

Orthologs of genes encoding immunogenic proteins in *M. bovis* and *M. tuberculosis* were identified from the four sequenced NTM genomes. The respective amino acid sequence homologies of these target proteins are shown in table 4.7. Only amino acid sequences of NTM showing homology of ≥50% to those of *M. bovis/ M. tuberculosis* are described. Orthologs of *tpx* were identified in *M. fortuitum* and *M. nonchromogenicum* while *dnaK* and *canA (Mb1315/ Rv0284)* were identified in all four NTM species. Other *canA* gene sequence orthologs (sequence positions: *Mb3301, Mb3619c, Mb3555c* in the *M. bovis* genomes) were also identified in NTM. Most of these were found to be shared among the four NTM species but the *Mb3301* ortholog was not identified in *M. malmesburii* sp.nov. and *M. komanii* sp.nov.

The *mpb70* orthologs were identified in both *M. malmesburii* sp.nov. and *M. komanii* sp.nov. and the corresponding amino acid sequences of this gene were found to be 97.4% homologous between the two NTM species and 63% between the two NTM and *M. bovis*.

Two copies of *mpb63* were identified only in *M. fortuitum* and *M. nonchromogenicum*. Orthologs of *mpb64* were identified in all the four NTM genomes. While orthologs of *hspX* were identified in only *M. fortuitum* and *M. nonchromogenicum.*

Table 4.7: Orthologs of genes encoding *M. bovis* / *M. tuberculosis* immunogenic proteins and their copy numbers in each of the four NTM genomes. Only genes of NTM showing >50% amino acid (AA) sequence similarity to those of *M. bovis* are were described

Protein name/gene code	**M. komanii sp.nov** Percentage aa homology to *M. bovis* (copy numbers)	***M. nonchromogenicum*** Percentage aa homology to *M. bovis* and (copy number)	***M. malmesburii* sp.nov** Percentage aa homology to *M. bovis* and (copy number)	***M. fortuitum*** Percentage aa homology to *M. bovis* and (copy number).
espC	no	no	no	no
dnaK	92 (1)	92.42 (1)	91.3 (1)	92 (1)
mpt63	no	52/33.72* (2)	no	52/33.72* (2)
mpt70/ mpb70	63 (1)	no	63 (1)	no
mpt83/ mpb83	no	no	no	no
canA/ Mb1315c	77.78 (1)	79(1)	76.07(1)	79 (1)
tpx/ Rv1932	no	84	no	84
TB22.2/*mpt64/mpb64*	55.56 (1)	59.8	53.62 (1)	59.81 (1)
Rv1120c/ Mb1151c/	65 (1)	57.75	64 (1)	57.75 (1)
Rv2660c/ Mb2678c	no	no	no	no
Rv2659c	no	no	no	no
hspX/hsp20/Mb2057c	no	57/41.67 (2)	no	57/41 (2)
Rv2654c	no	no	no	no

no, no or <50% amino acid homology identified, * shows a second copy of the sequence ortholog which in all cases was <50% similar to the *M. bovis* homologue at amino acid level.

4.4.7 Comparison of amino acid sequence of the *M. bovis* ESAT-6, CFP 10, TB9.8, MPB70, TB10.4, TB10.3, and CanA to the NTM homologues, as well as immunogenic epitopes (ESAT, CFP 10, TB10.3, TB10.4, and MPB70)

ESAT-6 (*esxA*)

The amino acid sequence alignment of *M. fortuitum, M. nonchromogenicum, M. smegmatis, M. bovis/ M. tuberculosis esxA/* ESAT-6 homologues is demonstrated in Fig 4. 1a. Immunogenic epitopes of *M. bovis* ESAT-6 as demonstrated by Vordermeier *et al.*, (2000, 2003 and 2007) were found in *M. fortuitum, M. nonchromogenicum* as well as in *M. smegmatis* orthologs. Comparison of *M. bovis, M. smegmatis, M. nonchromogenicum* and *M. fortuitum* immunodominant epitopes revealed the following results: amino acid sequence similarities of 13/16 (81.28%) for the epitope at position 1-16 ; 9/17 (52.9%) and 10/17 (58.8%) amino acid residues. were identical between *fortuitum* and *M. bovis* and between *M. nonchromogenicum* and *M. bovis* respectively for the epitope at position 16-32, while 12/17 (70%) for the epitope at position 47-64; 12/19 (63%) for the epitope at position 56-74; 15/17 (88%) for the epitope at position 65-81 and finally 11/15 identity between *M. fortuitum* and *M. bovis,* and 12/15 between *M. nonchromogenicum* and *M. bovis* for epitope at position 81-95. Amino acid sequence similarities of 90% and 95% for the ESAT-6 were observed between *M. smegmatis* and *M. fortuitum* as well as *M. smegmatis* and *M. nonchromogenicum* respectively.

A 90% amino acid sequence similarity of ESAT-6 was observed between *M. smegmatis* and *M. fortuitum*. When we compared the *M. smegmatis, M. nonchromogenicum* and *M. fortuitum* ESAT-6 orthologs at immunogenic epitope level, the following percentages of similarities were observed: 100% identity was

observed for epitope at position 1-16 (88.2%), 29/31 (93.5) for epitope at position 16-32, 23/26 (88.4%) for epitope position 65-95 and finally 15/17 (88%) for epitope at position 57-72.

CFP 10 (*esxB*)

Comparison of amino acid sequences of the CFP 10 / *esxB* homologues of *M. fortuitum, M. smegmatis, M. malmesburii* sp.nov, *M. nonchromogenicum, M. komanii* sp.nov, *M. bovis* and *M. tuberculosis* is illustrated by the amino acid alignment in Fig 4.1b and immunogenic epitopes as demonstrated by Vordermeier *et al., (*2003; 2007) are underlined.

Comparing the *esxB* amino acid sequences of *M. bovis*, and the NTM orthologs revealed the following: for epitope at position 1-19, 14/19 (73.7%) amino acid residues were identical between *M. bovis, M. smegmatis, M. nonchromogenicum* and *M. fortuitum*, while only 12/19 (63%) amino acid residues were identical between *M. bovis, M. komanii* sp.nov and *M. malmesburii* sp.nov. For epitope at position 13-28, 11/ 16 (68.75%) amino acids were found to be identical between *M. smegmatis, M. fortuitum, M. nonchromogenicum* and *M. bovis* while 10/16 (62.5%) were identical between *M. komanii* sp. nov., *M. malmesburii* p. nov. and *M. bovis*. The epitope at position 28-44, had 9/16 (56.25%) amino acid residues identical between *M. bovis, M. fortuitum, M. nonchromogenicum* and *M. smegmatis*, while 8/16 (50%) were identical between *M. bovis, M. komanii* sp.nov. and *M. malmesburii* sp.nov. The epitope at positon 55-72, showed sequences identity of 12/16 (75%) of amino acid residues between *M. bovis, M. fortuitum, M. smegmatis, M. nonchromogenicum, M. komanii* sp. nov. and *M. malmesburii* sp. nov. For the epitope at position 56-76, 12/20 (60%) amino acids were identical between all five NTM and *M. bovis.* Finally, for epitope at position 76-93, 9/18 (50%) of the amino acid residues were identical between *M. bovis, M.*

fortuitum, M. nonchromogenicum and *M. smegmatis,* while 6/18 (33.3%) were identical between *M. bovis, M. komanii* sp. nov. and *M. malmesburii* sp. nov.

TB10.3 (*esxR*) and TB 10.4 (*esxH*)

Alignment of the NTM, *M. bovis* and *M. tuberculosis* EsxR and EsxH amino acid sequences is illustrated in Fig 4.1c. The epitope at position 1 -12, was found to be 100% identical for all the species except a deletion at position 2-in *M. nonchromogenicum*. The epitope at position 5-13 was also 100% identical between all the species except for *M. nonchromogenicum* where a deletion of this region was observed, while the epitope at position 6-16, 09/11 (84.6%) amino acid residues were similar between *M. smegmatis, M. fortuitum* and *M. bovis/ M. tuberculosis* and . The epitope at position 51-59 had 7/9 (77.7%) amino acid residues that were similar between all the NTM species. For the epitope at position 54-62, 8/9 (88.8) amino acid residues were similar between all species for epitope.

MPB70 (*Rv2875*)

Alignment of the corresponding amino acid sequences of the *mpb70* gene orthologs of *M. komanii* sp. nov., *M. malmesburii* sp.nov, *M. bovis* and *M. tuberculosis* is illustrated in Fig 4.1d. Immunogenic epitopes of *M. bovis* MPB70 described by Pollock *et al.,* 2004 are underlined .A high degree of sequence variability (85%) was observed for the region at position 1-60 between the two NTM and *M. bovis* MPB70. This region was 90% similar between the two NTM species. Amino acid sequence similarities (71% for *M. komanii* sp. nov. and 68.5% for *M. malmesburii* sp.nov) were observed between each of the

two NTM species and *M. bovis* for region at position 61-222. For the immunogenic epitopes 'KTNSSLLTSILTYHVVAG' and 'GVSTANATVYMIDSVLMPPA described by Pollock *et al.*'1994, there was sequence identity of 15/18 (83.3%) and 16/20 (80%) respectively between *M. bovis* and *M. komanii* sp.nov and 83.3 % and 85% amino acid similarity respectively between *M. bovis* and *M. malmesburii* sp.nov.

CanA (*Rv1284/Mb1315*)

Alignment of the corresponding amino acid sequences of *canA* (*Mb1315/ Rv1284*) orthologs of the NTM species, *M. bovis* and *M. tuberculosis* is illustrated in Fig4.1e. The amino acid sequence similarities between the different NTM orthologs and *M. bovis canA* range between 76.07%-79%. The alignments show higher sequence similarities for sequences stretching from position 55-102 (95%) and also at position 131-162 (93.75%) than other regions of the protein.

TB9.8 (*EsxG*)

Alignment of the amino acid sequences of the *esxG* orthologs of the NTM species and *M. bovis* is illustrated in Fig 4.1f. *M. nonchromogenicum* and *M. fortuitum* EsxG orthologs showed the highest amino acid sequence similarity to those *M. bovis* and *M. tuberculosis* with 81.05% and 81.5% of their amino acid residues identical to those of *M. bovis* and *M. tuberculosis*, respectively, followed by orthologs of *M. komanii* sp.nov.and *M. malmesburii* each displaying 78.35% similarity to those of *M. bovis* and *m. tuberculosis*.

4.4.8 Closest sequence relatives of the novel NTM genomes *M. malmesburii* sp.nov. and *M. komanii* sp. nov.

Table 4.8 shows the BLAST results for each of these NTM. , *Mycobacterium fortuitum* and to a lesser extent, *M. nonchromogenicum* have the closest resemblance to *M. smegmatis* MC^2 155 (accession number CP001663.1) with *M. malmesburii* sp.nov. displaying similarity to *M. rhodesiae* (CP003169.1). *M. komanii* sp. nov. seems to be the most novel organism scoring its highest BLAST score with *Mycobacterium.* sp. JLS (CP000580.1).

Table 4.8: BLAST results for the largest NTM contig against Genbank nucleotide database (E-value <1x10^6) for closest sequence relative search.

NTM species	Species hit	BLAST score	Query coverage	Identity
M. fortuitum	*M. smegmatis* MC^2 155	11121	65%	81%
M. nonchromogenicum	*M. smegmatis* MC^2 155	8822	69%	83%
M. malmesburii sp.nov.	*M. rhodesiae* NBB3	8503	52%	81%
M. komanii sp.nov.	*Mycobacterium* sp.JLS	5565	45%	81%

4.4.9 Genome similarity among the NTM strains, M. bovis and M. tuberculosis

Genome similarity among the NTM strains as well as between NTM, *M. bovis* and *M. tuberculosis* is demonstrated by the metrix in Fig 4.2a and b. Fig 4.2a shows the proportion of all protein coding genes

shared among the strains, and Fig 4.2b demonstrates the proportion of protein coding genes with 90% sequence identity aligned along ≥ 50% of their length. Among the NTM, *M. malmesburii* sp.nov. shared more CDS with M. *komanii* sp.nov than with the other two NTM species. Likewise, *M. fortuitum* and *M. nonchromogenicum* exhibited genome similarity to each other. However, the shared CDS of *M. komanii* sp.nov. and *M. malmesburii* are not as highly homologous as those shared between *M. fortuitum* and *M. nonchromogenicum.*

4.4.10 Genome assembly visualisation by alignment to reference genomes (*i.e. M. rhodesiae, M. smegmatis* MC^{2}155, *M. tuberculosis* H37Rv and *Mycobacterium.* sp. JLS)

Visualization of the alignment of the *M. bovis* and the predicted CDS regions of the four NTM species, *i.e. M. nonchromogenicum, M. malmesburii* sp.nov, *M. komanii* sp.nov. and *M. fortuitum* to those of *M. tuberculosis* using BLAST Ring Image Generator is given in Fig 4.3, while alignment to each NTM predicted CDS to their closest relatives is shown by the images in Fig 4.4 a, 4.4 b, 4.5 and 4.6 for *M. fortuitum* predicted CDS aligned to those of *M. smegmatis* MC^{2}155, *M. nonchromogenicum* CDS aligned to those of *M. smegmatis* MC^{2}155, *M. malmesburii* sp.nov. CDS aligned to those of *M. rhodesiae*, and *M. komanii* sp.nov. CDS aligned to those of *Mycobacterium* sp. JLS respectively. The position of the immunogenic proteins of interest in the reference genomes are also highlighted. While it is common knowledge that most of the predominantly studied immunogenic genes in *M. bovis* and *M. tuberculosis* also occur in *M. smegmatis*, our BLAST searches also revealed that single copies of genes encoding for ESAT-6, CFP 10, HSPX and TB22.2/MPB64 as well as three of each of MPB70 and CanA do occur in *Mycobacterium* sp. JLS. In *M. rhodesiae*, a single copy of MPB70 and five copies of CanA were detected.

4.5 Discussion

In this study we compared whole genome sequences of four NTM species to those of *M. tuberculosis* and *M. bovis,* mainly focusing on the presence of shared genes in NTM encoding immunogenic proteins in *M. bovis/ M. tuberculosis*. Three of these species were previously described as among the abundant NTM in South Africa, and *M. fortuitum* ATCC 6841 derived tuberculin PPD is used in the modified BOVIGAM^{TM} assay (Michel *et al.,* 2011; Gcebe *et al.,* 2013). All of these NTM have been isolated from cattle and African buffaloes as well as their environments in South Africa in a study by Gcebe *et al.,* 2013. While two of these NTM are novel RGM (*M. malmesburii* sp. nov. and *M. komanii* sp.nov) species, *M. nonchromogenicum* (slow growing Mycobacterium (SGM) and *M. fortuitum* (RGM) have also been isolated from cattle tissue in Great Britain, France and Northern Ireland as well as from buffalo in South Africa (Pollock and Anderson 1997; Hughes *et al.,* 2005; Vordermeier *et al.,* 2007; Michel *et al.,* 2007; Biet *et al.,* 2014) where they may interfere with diagnosis of bovine tuberculosis. These two species were also reported to be among the most frequently isolated NTM in cattle in other parts of Africa (Diguimbaye-Djaibe *et al.,* 2006*;* Berg *et al.,* 2009). Though colonisation in cattle in the absence of disease is known to occur, it is not known whether it can lead to T-cell activation (Pollock and Anderson, 1997; Vordermeier *et al.,* 2007). Given the latter, exposure of cattle to these NTM species may constitute a possible source of cross-reactive immune response to tuberculin and may lead to false positive diagnosis of BTB. This highlighted the need to investigate these NTM in much more detail for their genetic make-up and subsequently assess their potential to induce immune responses potentially cross-reactive with MTBC antigens. The comparative genomics approach employed in this study was aimed mainly at searching for shared genes encoding immunogenic proteins of *M. bovis* and *M. tuberculosis* in

the NTM. We elucidated and described the draft genomes of *M. nonchromogenicum, M. komanii* sp. nov., *M. malmesburii* sp. nov., and *M. fortuitum* ATCC 6841. As was expected there was very little similarity observed between the NTM genomes and those of *M. bovis* and *M. tuberculosis*. The NTM's closest relatives were found to be other NTM species; *viz M. smegmatis, M. rhodesiae* and *Mycobacterium* sp. JLS. However, though there were huge differences between the genomes of NTM and those of *M. bovis* as well as *M. tuberculosis*, orthologs of CDS of some of the targeted immunogenic proteins of *M. bovis* that have been studied for their role in BTB specific diagnosis existed in NTM. Proteins of the Esx family, in particular those encoded by *esxA* and *esxB* have been extensively investigated for their role in immune response owing to their presence in MTBC and absence in *M. bovis* BCG vaccine and hence their application for differentiation of *M. bovis* infected from vaccinated animals (Vordermeier *et al.*, 2007; 2009). In NTM, it is mainly in pathogenic slow growing NTM that are phylogenetically closely related to MTBC; like *M. kansasii* and *M. marinum* as well as in *M. leprae* that ESAT-6 and CFP 10 have been investigated for their role in cross-reactive immune responses (Geluk *et al.*, 2002; Vordermeier *et al*., 2007). The occurrence of the *esx* genes of the ESX-1 locus (*esxA* and *esxB*) in all four of the annotated non- pathogenic NTM that were sequenced in this study and its gene orthologs in other NTM genomes available in the NCBI Genbank database, like *M. vulneris* (slow growing NTM); *M. farcinogenes* (slow growing NTM), and *Mycobacterium sp.* JLS (RGM), suggests that perhaps, despite sequence differences, the *esx* genes of this locus is not only a characteristic of pathogenic, slow growing mycobacteria, but does occur in several non-pathogenic RGM. This view supports several studies that detected orthologs of these genes in other non-pathogenic RGM like *M. smegmatis, M. ridhayense, M. flavescens* and *M. mageritense* (Harboe *et al.*, 1996, Colangeli *et al*., 2000; Gey van Pittius *et al*., 2001; van Ingen *et al*., 2009). Expression of the *esxA* and *esxB* orthologs in non-pathogenic RGM have not been investigated, despite their occurrence in several of these species.

However, if expressed in these NTM they could induce cross reactive immune responses that interfere with BTB diagnosis. It was not surprising that the NTM orthologs of these proteins were more similar to each other than to MTBC proteins as they are phylogenetically more closely related to each other than to members of MTBC (Devulder *et al.,* 2005). For instance, *M. fortuitum, M. nonchromogenicum, M. malmesburii* sp.nov. and *M. komanii* sp.nov. were found to harbor protein coding sequences of EsxA and EsxB that are highly similar to those of *M. smegmatis.* A 90% amino acid sequence similarity was observed between the *M. fortuitum* and the *M. smegmatis* orthologs of EsxA, while a 71.58% and an 80% were seen between these respective NTM orthologs and that of *M. bovis*. A similar finding was observed with EsxB orthologs. The protein products of the non-pathogenic RGM *esxA* and *esxB* orthologs also need to be investigated for their role in cross- reactive immune responses.

The *esx* genes situated within the ESX-2 locus were not found in any of the four NTM annotations while orthologs of the *esx* genes of the ESX-4 locus were found in all four NTM. The functions of the proteins of both ESX-2- and ESX-4 are unknown. The existence of the *esx* genes situated in the ESX-4 locus in all the four NTM also supports the comparative genomics studies which suggested that the ESX loci in mycobacteria evolved from a series of duplication events, where ESX-4 was the progenitor. Absence of the ESX-2 locus seems to be a characteristic of the non -pathogenic RGM as this locus is also absent in the genome of *M. smegmatis* (Gey van Pittius, 2001).

Esx family proteins encoded in the ESX-3 *i.e.* EsxH, EsxG as well as theis homologue, EsxR have also been investigated as vaccine candidates and markers for TB diagnosis due to their immunogenic characteristic and have been shown to be highly homologous (>75% at amino acid level) (Skjøt *et al.,* 2000; Hoang *et al.,* 2013). Protein coding sequences of EsxR, EsxG and EsxH were found in the four NTM genomes in this study with homologies at amino acid level of as high as 81.05% to *M. bovis* (*M.*

fortuitum and *M. nonchromogenicum* EsxG) and lowest homology of 64.79 % was observed between the *M. malmesburii* sp.nov. and *M. bovis* EsxR. Comparative genomics study by Gey van Pittius *et al* 2001 have noted the occurrence of ESX-3 locus genes in *M. smegmatis*, *M. leprae*, and *M. avium* (Gey van Pittius *et al.*, 2001).

ESX-5 proteins are known to be necessary for the secretion of PE/ PPE proteins, in *Mycobacterium marinum* (Abdalla *et al.*, 2008). Among the four NTM, only *M. nonchromogenicum* was found to harbor the predicted proteins sequences, EsxN of the ESX-5 locus as well as EsxK. It was reported that some genes of the ESX-5 locus are also absent in *M. bovis* BCG and were shown to be absent in avirulent *M. smegmatis* but present in *M. avium* and in pathogenic *M. leprae*. Therefore the use of proteins of this locus as candidates for TB diagnosis could be limited to areas were *M. leprae* and *M. avium* infections are not predominant.

The occurrence of other CDS of the predominant *M. bovis* immunogenic proteins, like CanA (Rv1315) (identified in all four NTM), HspX, TpX and Mpb63 (all identified in *M. fortuitum* and *M. nonchromogenicum*), MPB70 (identified in *M. malmesburii* sp.nov. and *M. komanii* sp.nov), and MPB64 (identified in all four NTM in the annotated NTM, including their closely related species also suggest the potential of these proteins to be expressed and therefore causing cross- reactive immune responses with *M. bovis*. CDS of MPB70, MPB64, HspX and CanA were also detected in *M. rhodesiae* and it was also discovered that *Mycobacterium* sp JLS harbors three copies of *mpb70 as* well as three copies of *CanA*, while *M. rhodesiae* genome had five copies of *canA*. These proteins could potentially be expressed in these species. This could also have implications for their use as markers for BTB diagnosis where exposure to these NTM is endemic.

Based on the overall protein sequence homologies of the antigens we could not judge whether the NTM homologues will give rise to cross-reactive immune responses. We consequently checked the existence

in NTM of epitopes shown to be immunogenic in *M. bovis* and *M. tuberculosis.* Amino acid sequence alignment of the EsxA and EsxB of the newly sequenced NTM in this study, as well as *M. smegmatis, M. bovis* and *M. tuberculosis* respectively was done to investigate their similarities at "immunogenic" epitope level. In this analysis, we found that the six bovine T-cell recognized epitopes of *M. bovis* ESAT-6 (EsxA), described by Vordermeier *et al.,* 2003 and 2007 had similarities to those of *M. fortuitum* and *M. nonchromogenicum* (showing sequence similarity of as high as 81.28% and as low as 52.9%). Likewise a certain degree of sequence similarity between the six *M. bovis* CFP 10 (EsxB) immunogenic epitopes and those of the NTM species (highest similarity of 75% observed between all NTM and *M. bovis* and lowest similarity of 50% between *M. komanii* sp.nov., *M. malmesburii* sp.nov. and *M. bovis.*) was observed. Still, with sequence homologies of less than 100% between the *M. bovis* immunogenic epitopes and those of the NTM, it was difficult to unambiguously predict T-cell cross-recognition. Vordermeier *et al.,* 2004, had demonstrated that changing a single amino acid residue within a 20-mer amino acid peptide could result in peptide derivative not being recognized by T cells specific for the wild type peptide (Vordermeier *et al.,* 2004). Conversely, Vordermeier *et al.,*2007 also demonstrate that sequence identity between epitopic regions from unrelated mycobacterial antigens of >50% in the 16-20 mer regions indicated cross-reactivity at epitopic level in cattle, however, on the other hand Hewinson *et al.,* 2006 showed that other peptides that displayed similar degrees of sequence identity were not cross-reactive. Antigen cross-recognition has also been observed with *M. leprae* ESAT -6 and CFP 10 on *M. tuberculosis* patients despite very low sequence identity (36% and 40% respectively at amino acid level) to *M. tuberculosis* homologue of ESAT -6 (Geluk *et al.,* 2002; Geluk *et al.,* 2004). Therefore antigen cross- recognition as well as species specificity of these NTM proteins need to be investigated at individual peptide level recognition by T- cells from infected animals.

Comparison of EsxR and EsxH amino acid sequences at immunogenic epitope level, revealed higher sequence similarities in the epitopes of NTM and those of *M. bovis* than the predicted Esx family protein sequences of the ESX-1 locus. A sequence similarity of 100% was observed between two of the five *M. bovis* immunogenic epitopes of EsxR and those of *M. fortuitum, M. malmesburii* sp. nov. and *M. komanii* sp.nov. Full cross- recognition of these NTM EsxR epitopes is therefore highly likely, and may lead to misdiagnosis of BTB. The other three EsxR/EsxH epitopes shown to be immunogenic in *M. bovis* also exist in the three NTM showing similarity of as low as 77.7%.

Two highly immunogenic epitopes of the *M. bovis* MPB70 described by Pollock *et al*., 2004 were also observed in *M. malmesburii* sp.nov and *M. komanii* sp.nov. predicted MPB70 homologues with high sequence similarities of 83.3% and 80% at amino acid level respectively. The occurrence of these immunogenic epitopes in these NTM predicted antigen sequences, despite sequence differences may lead to cross-reactive immune responses and therefore need to be tested on animal models for their immunogenicity.

The occurrence of immunogenic antigens in RGM and epitope similarities to *M. bovis* may also explain the interference of *M. fortuitum* in BTB diagnosis in cattle and African buffalo in South Africa which was first noted by isolation of this NTM species from BTB free buffalo which showed positive results when tested using PPD based BOVIGAM (Michel *et al.,* 2008). These cross-reactive immune responses were possibly due to these shared antigens between *M. bovis* and *M. fortuitum* harboring immunogenic epitope homologues as shown in this study. Subsequent inclusion of a PPD derived from *M. fortuitum* in the modified BOVIGAM assay has led to successful identification of cattle and buffalo exposed to this species and possibly other NTM (Michel *et al.,* 2011, Michel *et al., unpublished*). Therefore use of these antigens as markers for BTB diagnosis may be hampered by exposure of cattle and buffalo to these NTM. Their use as markers for BTB diagnosis warrants further investigation taking into consideration pre-

exposure of animals to these NTM especially in regions where they are endemic, especially considering the fact that *M. fortuitum* is widely distributed across the globe and has been found to infect cattle and buffalo from different countries.

In this study no orthologs of MPB83 and EspC were identified in the four NTM genome annotations. The *mpb70*, *mpb83* and *espC* genes have not been described in other non- pathogenic RGM before. In NTM, MPB70, EspC and MPB83 orthologs of *M. kansasii* and *M. marinum* have been detected and MPB70 and MP83 have been found to be expressed in these species (Woolford *et al.*, 1997). Perhaps MPB83 and EspC proteins are specific to pathogenic mycobacteria and might play a role in virulence. Therefore the use of these antigens for diagnosis of BTB may possibly only be hampered by exposure of animals to other pathogenic mycobacteria and not complicated by non-pathogenic mycobacteria.

In conclusion we have identified NTM orthologs of genes encoding predominant immunogenic proteins in *M. bovis viz* the Esx family, like ESAT-6, CFP 10, EsxH, EsxR and EsxG as well as MPB70, in the four non-pathogenic NTM sequenced in this study and in several other non-pathogenic NTM available in the NCBI database. The identification of the *esx* genes situated within ESX-1, ESX-3 and ESX4 and outside these loci in the four sequenced NTM in this study confirms and extends observations from other studies and further suggests that the presence of the *esx* genes in these three loci is a characteristic of both pathogenic and non-pathogenic mycobacteria. The absence of *esx* genes of the ESX-2 locus in all the newly sequenced NTM as well as the *esx* genes of the ESX-5 locus in the RGM species in this study also confirms the absence of the *esx* genes of these loci in other NTM species such as *M. smegmatis.* NTM homologues of the *M. bovis* immunogenic epitopes were also detected even though there were sequence differences, except for two epitopes in the EsxR sequence, and hence we could not unambiguously predict T-cell recognition. *M. fortuitum* and *M. nonchromogenicum* ESAT- 6 and CFP

10 amino acid sequences revealed higher homology to those of the *M. bovis* proteins than the *M. malmesburii* sp. nov., and *M. komanii* sp.nov. orthologs. These two genes were also found in *Mycobacterium* sp. JLS and other NTM in the Genbank database like *M. vulneris*, *M. mageritense*, and *M. flavescens* Orthologs of *esxG*, *esxH* and *esxR* were also found in the four NTM species. Therefore investigations of antigens of the Esx family for BTB diagnosis should also seriously consider their occurrence in NTM. The MPB70 orthologs of *M. malmesburii* sp.nov. and *M. komanii* sp.nov. as well as their closely related NTM *viz M. rhodesiae* and *Mycobacterium* sp. JLS were identified, and the gene encoding this protein has not been described in non-pathogenic RGM before and therefore this study has laid a foundation for further investigation of this protein as a diagnostic marker for BTB considering the occurrence of its gene and possibility of its expression in these RGM species and T-cell recognition.

Genes encoding CanA, DnaK, Mpb64 were also detected in all the four NTM as well *Mycobacterium* sp. JLS, while *canA* was also detected in *M. rhodesiae.*

The impact of these proteins on the T-cell responses needs to be investigated, especially *M. fortuitum* homologues, considering that its isolation from animals from different countries. This study has laid a foundation into investigation of these NTM and their cross-reactivity with *M. bovis* immunogenic proteins. It will also contribute to investigations and development of BTB as well as NTM specific diagnostic markers for use in immunological assays.

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4.6 References

AKHTER Y, EHEBAUER MT, MUKHOPADHYAY S, HASNAIN S (2012). The PE/ PPE multigene family codes for virulence factors and is possible source of mycobacterial antigenic variation: Perhaps more. *Biochimie* **94:** 110-116.

AL-ATTIYA R, MUSTAFA AS, ABAL AT, MADI NM, ANDERSON P (2003). Restoration of mycobacterial antigen induced proliferation and interferon gamma responses in peripheral blood mono nuclear cells of tuberculosis patients upon effective chemotherapy. *FEMS Immunology and Medical Microbiology* **38:** 249-256

AL-ATTIYAH R, MADI N, EL-SHAMY AS, WIKER H, ANDERSEN P, MUSTAFA A (2006). Cytokine profiles in tuberculosis patients and healthy subjects in response to complex and single antigens of *Mycobacterium tuberculosis. FEMS Immunology and Medical Microbiology* **47:** 254-261.

ALEXEY G, SAVELIEV V, VYAHHI N, TESLER G (213). "QUAST: Quality Assessment Tool for Genome Assemblies." *Bioinformatics (*Oxford, England*)* **29**: 1072–1075.doi:10.1093/bioinformatics/btt086.

ALIKHAN NF, PETTY NK, ZAKOUR NLB, BEATSON SA (2011). "BLAST Ring Image Generator (BRIG): Simple Prokaryote Genome Comparisons." *Biomed Central: Genomics* **12**: 402–12.doi:10.1186/1471-2164-12-402.

ALTSCHUL F, GISH W, MILLER W, MYERS EW, LIPMAN DJ (1990). "Basic Local Alignment Search Tool." *Journal of Molecular Biology* **215**: 403–10. doi: 10.1016/S0022-2836(05)80360-2.

ALTSCHUL SF, MADDEN TL, SCHÄFFER AA, ZHANG J, ZHANG Z, MILLER W, LIPMAN DJ (1997). "Gapped BLAST and PSI-BLAST: A New Generation of Protein Database Search Programs." *Nucleic Acids Research* **25** (17): 3389–3402. doi:10.1093/nar/25.17.3389.

BANKEVICH A, NURK S, ANTIPOV D, GUREVICH AA, DVORKIN M, KULIKOV AS, LESIN VM, NIKOLENKO SI, PHAM S, PRJIBELSKI AD, PYSHKIN AV, SIROTKIN AV, VYAHHI N, TESLER G,[5] MAX A. ALEKSEYEV MA, PEVZNER PA (2012). "SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing." *Journal of Computational Biology* **19**: 455–477. doi:10.1089/cmb.2012.0021.

BERG S, FIRDESSA R, HABTAMU M, GADISA E, MENGISTU A, YAMUAH L, AMENI G, VORDERMEIER M, ROBERTSON BD, SMITH NH, ENGERS H, YOUNG D, HEWINSON RG, ASEFFA A, GORDON SV. (2009). The burden of mycobacterial disease in Ethiopian cattle: Implications for public health. *Public Library of Science*: *One* **4(4)**: edoi: 10.1371/ journal.pone.0005068

BRONDIN P, ROSENKRANDS I, ANDERSON P, COLE ST, BROSCH R (2004). ESAT-6 proteins: protective antigens and virulence factors? *Trends Micribiology* **12:** 500-508.

CHECKELY AM, WYLLIE DH, SCRIBA TJ, GOLUBCHIK T, HILL AVS, HANEKON WA, MCSHANE H (2010). Identification of Antigens specific to non- tuberculous mycobacteria: The Mce

family of proteins as a target of T cell immune responses. *Public Library of Science*: One **6** (10): e26434.doi**:**10.1371/journal.pone.0026434

COLANGELI R, SPENCER JS, BIFANI P, WILLIAMS A, LYASHCHENKO K, KEEN MA, HILL PJ, BELISLEJ, GENNARO ML (2000). MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis,* elicits tuberculosis specific delayed type hypersensitivity in guinea pigs. *Infection and Immunity* **68:** 990-993.

DEMANGEL C, BRONDIN P, COCKLE PJ, BROSCH R, MAJLESSI L, LECLERC C, COLE ST (2004). Cell envelope protein PPE68 contributes to *Mycobacterium tuberculosis* RD1 immunogenicity independently of a 10 kilo Dalton culture filtrate protein and ESAT-6. *Infection and Immunity* **72:** 2170-2176.

DIGUIMBAYE-DJAIBÉ C, VINVENT V, SHELLING E, HILTY M, NGANDOLO R, MAHAMAT HH, PFYFFER G, BAGGI F, TANNER M, ZINSSTAG J. (2006). Species identification of non-tuberculous mycobacteria from humans and cattle of Chad. *Schweiz Arch Tierheilkd* **148:** 251-256

GANGULY N, SIDDIQUI I, SHARMA P (2008). Role of *M. tuberculosis* RD-1 region encoded secretory proteins in protective response and virulence. *Tuberculosis* **88:** 510-517.

GCEBE N, GEY VAN PITTIUS NC, RUTTEN V, MICHEL A (2013). Prevalence and distribution of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their environments in South Africa. *Transboundary and Emerging Diseases* **60:** 74-84.

GELUK A, VAN MEIJGAARDEN KE, FRANKEN KLMC, SUBRONTO YW, WIELES B, AREND SM, SAMPIAIO EP, DE BOER T, FABER WR, NAAFS B, OTTENHOGFF THM (2002). Identification and characterisation ESAT-6 homologue of *Mycobacterium leprae* and T-cell cross reactivity with *Mycobacterium tuberculosis. Infection and. Immunity* **70:** 2544-2548.

GELUK, A., VAN MEIJGAARDEN, K.E, FRANKEN, K.L, WIELES, B., AREND, S.M, FABER, W.R, NAAFS, B., AND OTTENHOFF, T.H. (2004). Immunological cross-reactivity of the Mycobacterium leprae CFP-10 with its homologue in Mycobacterium tuberculosis. *Scandinavian Journal of Immunology* **59**: 66-70.

GEY VAN PITTIUS NC, GAMIELDIEN J, HIDE W, BROWN GD, SIEZEN RJ, BEYERS A (2001). The ESAT-6 gene cluster of *Mycobacterium* and other high GC Gram-positive bacteria. *Genome Biology* **2:** research0044.1-0044.18

GEY VAN PITTIUS NCG, WARREN R, VAN HELDEN PD (2002). ESAT-6 and CFP-10. What is diagnosis? *Infection and Immunity* **70:** 6509-6511.

GOODGER J, NOLAN A, RUSSEL WP, DALLEY DJ, THORNS CJ, STUART FA, CROSTON P, NEWELL DG (1994). Sero-diagnosis of *Mycobacterium bovis* infection in badgers: development of an indirect ELISA using a 25 kDA antigen. *Veterinary Records* **135:** 82-85.

GREENWALD R, ESFANDIARI J, LESELLIER S, HOUGHTON R, POLLOCK J, AAGAARD C, ANDERSEN P, HEWINSON RG, CHAMBERS M, LYASHCHENKO K (2003). Improved serodetection of *Mycobacterium bovis* infection in badgers (*Meles meles*) using multi antigen test formats. *Diagnostic Microbiology and Infectious Diseases* **46:** 197-203

HARBOE M, OETTINGER T, WIKER HG, ROSENKRANDS I, ANDERSEN P (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infection and Immunity* **64:** 16-22.

HEWINSON RG, VORDERMEIER HM, SMITH NH, GORDON SV (2006). Recent advances in our knowledge of *Mycobacterium bovis:* a feeling for the organism. *Veterinary Microbiology* **112:** 127-139.

HOANG T, AAGAARD C, DIETRICH J, CASSIDY JP, DOLGANOV G, SCHOOLNIK GK, LUNDBERG CV, AGGER EM, ANDERSON P (2013). Esat-6 (esxA) and TB10.4 (esxH) based vaccines for pre and post- exposure tuberculosis vaccination. *Public Library of Science*: One **8 (12):** e80579.doi:10.137/journal.pone.0080579.

HUGHES MS, BALL NW, MCCARROLL J, ERSKINE M, TAYLOR MJ, POLLOCK JM, SKUCE RA, NEILL SD (2005), Molecula analysis of mycobacteria other than the *M. tuberculosis* complex isolated from Northern Ireland cattle. *Veterinary Microbiology* **108:** 101-112.

IZE B, PALMER T (2006). Microbiology. Mycobacteria's export strategy. *Science* **313:** 1583-1584.

LERSELIER S, CORNER L, COSTELLO E *et al*., (2008). Antigen specific immunological responses of badgers (Meles meles) experimentally infected with *Mycobacterium bovis. Veterinary Immunology and Immunopathology* **122:** 35-45.

LI H, DURBIN R (2009). "Fast and Accurate Short Read Alignment with Burrows-Wheeler Transform." *Bioinformatics* **25**: 1754–60. doi:10.1093/bioinformatics/btp324.

LIU S, GUO S, WANG C, SHAO M, ZHANG X, GUO Y, GONG Q (2007). A novel fusion protein-based indirect enzyme- linked immunosorbent assay for the detection of bovine tuberculosis. *Tuberculosis* (Edinb) **87:** 212-217.

LYASHCHENKO KP, GREENWALD R, ESFANDIARI J, CHAMBERS MA, VICENTE J, GORTAZAR C, SANTOS N, CORREIA-NEVES M, BUDDLE BM, JACKSON R, O'BRIEN DJ, SCHMITT S, PALMER MV, DELAHAY RJ, WATERS WR (2008). Animal side serological assay for rapid detection of *Mycobacterium bovis. Veterinary Immunology and Immunopathology* **122:** 35-45.

MAHAIRAS GG, SABO PJ, HICKEY MJ, SING DC, STIVER CK (1996). Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis. Journal of Bacteriology* **178:** 1274-1282.

MARONGUI L, DONINI M, TOFFALI L, ZENARO E, DUSI S (2013). ESAT-6 and HspX improve effectiveness of BCG to induce human dendric cells- dependant Th1 and NK cells activation *Public Library of Science* :*One* **8:** e75684.doi:10.1371/journal.pone.0075684.

MATSUMOTO S, MATSUO T, OHARA N, HOTOKEZAKA H, NAITO M, MINAMI J, YAMADA T (1995). Cloning and sequencing of a unique antigen MPT70 from *Mycobacterium tuberculosis* H37Rv and expression in BCG using *E. coli*-mycobacteria shuttle vector. *Scandinavian Journal of Immunology* **41:**281-287.

MICHEL AL, DE KLERK L, VAN PITTIUS NCG, WARREN RM, VAN HELDEN PD (2008). Bovine tuberculosis in African buffaloes: observations regarding *Mycobacterium bovis* shedding into water and exposure to environmental mycobacteria. *Biomed Central: Veterinary Research* **3:** doi: 10.1186/1746-6148-3-23.

MICHEL AL, COOPER D, JOOSTE J, DE KLERK LM, JOLLES A (2011). Approaches towards optimizing the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine* **98**: 142-151.

MILLINGTON KA, FORTUNE SM, LOW J, GARDES A, HINGLEY-WILSON SM, WICKREMASINGHE M, KON, OM, LALVANI A (2011). Rv3615 is a highly immunodominant RD1 (Region of difference)-dependent secreted antigen specific for *Mycobacterium tuberculosis* infection. *Proceedings of National Academy of Science* **108:** 5730-5735.

MUSTAFA AS (2014). Characterisation of a cross reactive, immunodominant and HLA-promiscuous epitope of *Mycobacterium tuberculosis* –specific major antigenic protein PPE68. *Public Library of Science: One* **9(8):** e103679.doi:10.1371/journal.pone.0103679

NAGAI S, MIURA K, TOKUNAGA T, HARBOE M (1986). MPB70, a unique antigen isolated from culture filtrate of BCG sub strain Tokyo. Dev Biol Stand **58:** 511-516.

POLLOCK JM, ANDERSON P (1997). The potential of ESAT-6 antigen secreted by virulent mycobacteria for specific diagnosis of tuberculosis. J. Infect. Dis. **175,** 1251-1254.

POLLOCK JM, DOUGLAS AJ, MACKIE DP, NEILL SD (2004). Identification of bovine T-cell epitopes for three *Mycobacterium bovis antigens:* MPB70, 19, 000 MW, and MPB57. *Immunology* **82:** 9-15.

PYM AS, BRODIN P, MAJLESII L, BROSCH R, DEMANGEL C, WILLIAMS A, GRIFFITHS KE, MARCHAL G, LECLERC C, COLE ST (2003). Recombinant BCG exporting ESAT-6 confers enhanced protection against tuberculosis. *National Medicine* **9:** 533-539.

ROCHE PW, TRICCAS JA, AVERY DT, FIFIS T, BILLMAN-JACOBE H, BRITTON WJ (1994). Differential T cell responses to mycobacteria-secreted proteins distinguish vaccination with bacilli Calmette-Guérin from infection with *Mycobacterium tuberculosis. Journal of Infectious Diseases* **170:** 1326-1330.

SEEMANN T (2014). "Prokka: Rapid Prokaryotic Genome Annotation." *Bioinformatics* (Oxford University Press) **30**: 2068–69. doi:10.1093/bioinformatics/btu153.

SKJØT RL, OETTINGER T, ROSENKRANDS I, RAVN P, BROCK I, JACOBSEN S, ANDERSON P (2000). Comparative evaluation of low molecular mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infection and Immunity* **68:** 214-220.

SKJØT RLV, BROCK I, AREND SM, MUNK ME, THEISEN M, OTTENHOFF THM, ANDERSON P (2002). Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. *Infection and Immunity* **70:** 5446-5453.

SORENSEN AL, NAGAI S, HOUEN G, ANDERSEN P, ANDERSEN AB (1995). Purification and characterization of a low-molecular-mass T-cell antigen secreted by Mycobacterium tuberculosis. *Infection and Immunity* **63:**1710-1717.

VAN INGEN J, DE ZWAAN R, DEKHUIJZEN R, BOEREE M, VAN SOOLINGEN D (2009). Region of difference 1 in non- tuberculous *Mycobacterium* species adds a phylogenetic and taxonomic character. *Journal of Bacteriology***191**: 5865-5867.

VORDERMEIER HM, BROWN J, COCKLE PJ, FRANKEN WPJ, AREND SM, OTTENHOFF THM, JAHANS K, HEWINSON RG (2007). Assessment of cross - reactivity between *Mycobacterium bovis*

and *M. kansasii* ESAT-6 and CFP 10 at the T-cell epitope level. *Clinical and Vaccine Immunology* **14:** 12.3-1209.

VORDERMEIER HM, CHAMBERS MA, COCKLE PJ, WHELAN AO, SIMMONS J, HEWINSON RG (2002). Correlation of ESAT-6 specific gamma interferon production with pathology in cattle following *Mycobacterium bovis* BCG vaccination against experimental bovine tuberculosis. *Immunology* **70:** 3026-3032.

VORDERMEIER HM, COCKLE PC, WHELAN A, RHODES S, HEWINSON RG (2000). Towards the development of diagnostic assays to discriminate between *Mycobacterium bovis* infection and BCG vaccination in cattle. *Clinical and Infectious Diseases* **30:** S291-S298.

VORDERMEIER HM, COCKLE PC, WHELAN A, RHODES S, PALMER N, BAKKER D, HEWINSON RG (1999). Development of diagnostic reagents to differentiate between *Mycobacterium bovis* BCG vaccination and *M. bovis* infection in cattle. *Clinical Diagnosis and Laboratory Immunology* **6:** 675-682.

VORDERMEIER M, WHELAN AO, HEWINSON RG (2003). Recognition of mycobacterial epitopes by T cells across mammalian species and use of a program that predicts Human HLA-DR binding peptides to predict bovine epitopes. *Infection and Immunity* **71:** 1980-1987.

VORDERMEIER HM, HUYGEN K, SINGH M, HEWINSON RG, XING Z (2006).Immune response induced in cattle by vaccination with recombinant adenovirus expressing mycobacterial antigen 85A and *Mycobacterium bovis* BCG. *Infection and Immunity* **74:** 1416-1418.

WATERS WR, WHELAN AO, LYASHCHENKO KP, GREENWALD R, PALMER MV, HARRIS BN, HEWINSTON RG, VORDERMEIER HM (2010). Immune responses in cattle inoculated with *Mycobacterium bovis, Mycobacterium tuberculosis,* or *Mycobacterium kansasii. Clinical and Vaccine Immunology* **17:** 247-252.

WIKER (2009). MPB70 and MPB83- major antigens of *Mycobacterium bovis. Scandinavian Journal of Immunology* **69:** 492-499

WOOLFORD AJ, HEWINSON RG, WOODWARD M, DALE JW (1997). Sequence heterogeneity of an mpb70 gene analogue in *Mycobacterium kansasii. FEMS Microbiology Letters* **148:** 43-48.

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M. fortuit   MTEQVWNFAG IEGGASEIQG AVGTTAGLLD EGKGSLASLA SAWGGSGSEA
M. bovis     MTEQQWNFAG IEAAASAIQG NVTSIHSLLD EGKQSLTKLA AAWGGSGSEA
M. tubercu   MTEQQWNFAG IEAAASAIQG NVTSIHSLLD EGKQSLTKLA AAWGGSGSEA
M. smegmat   MTEQVWNFAG IEGGASEIHG AVSTTAGLLD EGKASLTTLA SAWGGTGSEA
M. nonchro   MTEQVWNFAG IEGGASEIQG AVGTTAGLLD EGKGSLASLA SAWGGSGSEA
             **** ***** **  ** *>*  *     *** *** **> **  ****>****

                                  ....|....| ....|....| ....|....|
....|....| ....|
                       60         70         80         90
M. fortuit   YQAVQTRWDN TSNELNQALQ NLAQTISEAG QTMSQTEAGV TGMFA
M. bovis     YQGVQQKWDA TATELNNALQ NLARTISEAG QAMASTEGNV TGMFA

M. tubercu   YQGVQQKWDA TATELNNALQ NLARTISEAG QAMASTEGNV TGMFA
M. smegmat   YQAVQARWDS TSNELNLALQ NLAQTISEAG QTMAQTEAGV TGMFA
M. nonchro   YQAVQTRWDN TSNELNQALQ NLAQTISEAG QTMSQTEAGV TGMFA
             ** **  **  *  *** *** *** ****** * *>***  * *****
```

Fig 4.1a: Alignment of EsxA AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), MC^2 155 (M. smegmat), *M. nonchromogenicum* (M. nonchro), *M. bovis* and *M. tuberculosis* (M. tubercu). * represents identical sequences observed in all species, and > represents same AA residue in at least one of the NTM species. Highly immunogenic epitopes of bovine as described by Vordermeier et al., 2000, 2003, 2007 are underlined.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                           10         20         30         40         50
M. fortuit       MAQMNTDAAV LAKEAANFER ISGELKSVIA QVESTGGTLA AQMQGQAGTA
M. tubercu       MAEMKTDAAT LAQEAGNFER ISGDLKTQID QVESTAGSLQ GQWRGAAGTA
M. bovis         MAEMKTDAAT LAQEAGNFER ISGDLKTQID QVESTAGSLQ GQWRGAAGTA

M. smegmat       MAAMNTDAAV LAKEAANFER ISGELKGVIA QVESTGSALA AQMVGQAGTA
M. malmesb       M-AMNTDVAV LAKEAANFER IGGELRAVIG HVESTAGALS AQLVGEAGSA
M. komanii       M-AMNTDVAV LAKEAANFER IGGELRAVIG HVESTAGALS AQLVGEAGSA
M. nonchro       MAQMNTDAAV LAKEAANFER ISGELKSVIA QVESTGGTLA AQMQGQAGTA
                 *> * **>*  ** ** **** *>* *>  *  >**** > *   *  * **>*

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                           60         70         80         90        100
M. fortuit       AQAALARFHE AADKQIQELN EISTNIHTSG TQYSSTDEDQ AGNLASSMNI
M. tubercu       AQAAVVRFQE AANKQKQELD EISTNIRQAG VQYSRADEEQ QQALSSQMGF
M. bovis         AQAAVVRFQE AANKQKQELD EISTNIRQAG VQYSRADEEQ QQALSSQMGF

M. smegmat       AQAALARFHE AAAKQVQELN EISANIHTSG TQYTSTDEDQ AGTLASSMNI
M. malmesb       AQAALMRFHE AAVRQVQALD DISANIHSAG AQYAAMDSDG SAALSSAMQF
M. komanii       AQAALMRFHE AAVRQVQALD DISANIHSAG SQYAAMDSDG SAALSSAMQF
M. nonchro       AQAALARFHE AADKQIQELN EISTNIHTSG TQYSSTDEDQ AGNLASSMNI
                 ****  ** * ** >* *>*> >**>**  >*  **>  *> >    *>*>* *
```

Fig 4.1b: Alignment of EsxB AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), *M. nonchromogenicum* (M. nonchro), *M. smegmatis* MC^2 155 (M. smegmat), *M. malmesburii* sp.nov. (M. malmesb), *M. komanii* sp. nov., *M. bovis* and *M. tuberculosis* (M. tubercu). * represents same aa residue identified across all species, and > represents same aa residues in at least one NTM species, *M. bovis* and *M. tuberculosis*, + represents aa sequence identical in *M. fortuitum, M. smegmatis, M. bovis* and *M. tuberculosis*, - represents same aa residues in *M. malmesburii* sp.nov.and *M. komanii* sp.nov., *M. bovis* and *M. tuberculosis*. Highly immunogenic epitopes of bovine as described by Vordermeier et al., 2003; 2007 are underlined.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|

                          10         20         30         40         50
M. nonchro-H    V--------- ---------- --------GA DIASEQAALS AAWQGDTGLT
M. bovis-R      MSQIMYNYPA MMAHAGDMAG YAGTLQSLGA DIASEQAVLS SAWQGDTGIT

M. smegmat-H    MSQIMYNYPA MLAHAAEMNT YSGALHAVGA DIAAEQHALA SAWQGDTGMT
M. fortuit-R    MSQIMYNYPA MLAHAGEMNT YSAALHAVGA DIASEQAALS SAWQGDTGMT
M. tubercu-R    MSQIMYNYPA MMAHAGDMAG YAGTLQSLGA DIASEQAVLS SAWQGDTGIT
M. malmesb-R    MSQIMYNYPA MLAHSGEMAG YAGALHAVGA DIASEQAALA GAWQGDTGTT
M. komanii-R    MSQIMYNYPA MLAHSGEMAG YAGALHAVGA DIASEQAALA GAWQGDTGTT
                ********** ****+>>*-- *-- *   ** ***>***+** ***+*******

                ....|....| ....|....| ....|....| ....|....| ....|..
                        60         70         80         90
M. nonchro-H    YQAWQAQWNQ AMEELVRAYH AMATTHETNT LSMLARDNAE AARWG-
M. bovis-R      YQGWQTQWNQ ALEDLVRAYQ SMSGTHESNT MAMLARDGAE AAKWGG-

M. smegmat-H    YQAWQAQWNQ AMEELVRAYR AMATTHEQNT MAMSARDQAE GAKWG--
M. fortuit-R    YQAWQAQWNQ AMEELTRAYR AMASTHEMNT MSMSARDAAE GAKWG-
M. tubercu-R    YQGWQTQWNQ ALEDLVRAYQ SMSGTHESNT MAMLARDGAE AAKWGG-
M. malmesb-R    YQAWQAQWNL ALEELVRAYR AMAATHEQNT MTMSARDQAE GAKWGG
M. komanii-R    YQAWQAQWNL ALEELVRAYR AMAATHEQNT MTMSARDQAE GAKWGG
                ** ** ***+ *-***>***   *  *** ** > * *** **  *>**
```

Fig 4.1c: Alignment of EsxR/ EsxH AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), *M. smegmatis* MC^{2}155 (M. smegmat), *M. malmesburii* sp.nov (M. malmesb), *M. komanii* sp. nov., *M. nonchromogenicum* (M. nonchro), *M. bovis* and *M. tuberculosis* (M. tubercu). The following symbols at the bottom of each alignment stand for: * indicates same AA residue identified across all species, and > indicates same sequences in at least one of the NTM and *M. bovis/ M. tuberculosis*, -, indicates same AA sequences in *M. komanii* sp.nov. , *M. malmesburii* sp. nov., and *M. bovis/ M. tuberculosis*, and +, indicates same AA residues in *M. smegmatis, M. fortuitum, M. bovis* and *M. tuberculosis* AA sequences, (blank) indicate different amino acid residue between the NTM and *M. bovis/ M. tuberculosis*. Immunogenic epitopes of bovine as described by Vordermeier *et al*., 2003, 2007 are underlined. H, *esxH*; R, *esxR*.

```
                .....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
M. malmesb    MKIEFRK-SV TAAGVAAAAI LTVSACSNDT ST-AAPTAAA ESSSTSVAPT
M. bovis      M--KVKN-TI AATSFAAAGL AALAVAV--- ----SPPAAA G---------
M. tubercu    M--KVKN-TI AATSFAAAGL AALAVAV--- ----SPPAAA G---------
M. komanii    MKIDFRK-SV TAAGVAAAAI LTVSACSNDT STSAAPTAAA ESTSTSVAPA
              *           *   ***                    * ***

                .....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90         100
M. malmesb    PMNQSMDPAA GLVGPGCADY AAQNPTGPGS VNGMALDKVT VAAANNPMLT
M. bovis      ----------  DLVGPGCAEY AAANPTGPAS VQGMSQDPVA VAASNNPELT
M. tubercu    ---------- DLVGPGCAEY AAANPTGPAS VQGMSQDPVA VAASNNPELT
M. komanii    PMNQPMDPAA GLVGPGCADY AAQNPTGPGS VNGMALDKVT VAAANNPMLT
                          ******* * ** ***** * * **  * *  ******* **

                .....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
M. malmesb    TLTSALSGRL NPNVNLVETL DGSQFTVFAP TDDAFAKIDP ATIETLKTDS
M. bovis      TLTAALSGQL NPQVNLVDTL NSGQYTVFAP TNAAFSKLPA STIDELKTNS
M. tubercu    TLTAALSGQL NPQVNLVDTL NSGQYTVFAP TNAAFSKLPA STIDELKTNS
M. komanii    TLTAALSGQL NPNVNLVDTL NGSQFTVFAP TDDAFAKIDP ATIETLKTDS
              ***>****>* ** ****>** >  * ***** *  ** *     **  *** *

                .....|....| ....|....| ....|....| ....|....| ....|....|
                         160        170        180        190        200
M. malmesb    ELLTSILTYH VVPGQADPAQ VIGTHKTVQG ADVRVAGGGQ DITVNDAGVV
M. bovis      SLLTSILTYH VVAGQTSPAN VVGTRQTLQG ASVTVTGQGN SLKVGNADVV
M. tubercu    SLLTSILTYH VVAGQTSPAN VVGTRQTLQG ASVTVTGQGN SLKVGNADVV
M. komanii    DLLTSILTYH VVPGQADPAQ VIGTHKTVQG ADVTVAGGGQ DITVDDAGVV
               ********* ** **  **  * **  * ** * *>* * *     *  * **

                .....|....| ....|....| ....
                         210        220
M. malmesb    CGGVRTANAT VYLIDTVLMP PAN
M. bovis A    CGGVSTANAT VYMIDSVLMP PA
--
M. tubercu    CGGVSTANAT VYMIDSVLMP PA
M. komanii    CGGVRTANPT VYLIDTVLMP PAN
              **** ***>* ** ** **** **
```

Fig 4.1d: alignment of MPB70 AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), *M. smegmatis* MC^2 155 (M. smegmat), *M. malmesburii* sp.nov (M. malmesb), *M. komanii*, *M. bovis* and *M. tuberculosis* (M. tubercu). * indicates identical AA sequences in all species, and > indicates same AA residue in at least one of the NTM species and *M. bovis*/ *M. tuberculosis*. Immunodominant epitopes as described by Pollock *et al.*, 2004 are indicated by solid lines.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
M. smegmati   M----SVTDQ YLANNEEYAK TFSGPLPLPP SKHVAVVACM DARLDVYRIL
M. bovis      V----TVTDD YLANNVDYAS GFKGPLPMPP SKHIAIVACM DARLDVYRML
M. tubercu    V----TVTDD YLANNVDYAS GFKGPLPMPP SKHIAIVACM DARLDVYRML
M. nonchro    M----SVTDQ YLANNEVYAT TFSGPLPLPP SKHVAVVACM DARLDVYRIL
M. fortuit    M----SVTDQ YLANNEVYAT TFSGPLPLPP SKHVAVVACM DARLDVYRIL
M. malmesb    M----SVTDE YLKNNEEYAK TFSGPLPLPP SKHVAVLACM DARLDVYRIL
M. komanii    MVDPMSVTDE YLKNNEEYAK TFSGPLPLPP SKHVAVVACM DARLDVYRIL
                    ***  **>**  **   * **** ** *** *  *** ******** *

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          60         70         80         90        100
M.smegmati    GLGDGEAHVI RNAGGVITDD EIRSLAISQR LLGTKEIILI HHTDCGMLTF
M. bovis      GIKEGEAHVI RNAGCVVTDD VIRSLAISQR LLGTREIILL HHTDCGMLTF
M. tubercu    GIKEGEAHVI RNAGCVVTDD VIRSLAISQR LLGTREIILL HHTDCGMLTF
M. nonchro    GLGDGEAHVI RNAGGVITDD EIRSLAISQR LLGTKEIILI HHTDCGMLTF
M. fortuit    GLGDGEAHVI RNAGGVITDD EIRSLAISQR LLGTKEIILI HHTDCGMLTF
M. malmesb    GLQDGEAHVI RNAGGVVTDD EIRSLAISQR LLGTKEIILI HHTDCGMLTF
M. komanii    GLQDGEAHVI RNAGGVVTDD EIRSLAISQR LLGTKEIILI HHTDCGMLTF
              *   ****** ******>***  ********* ********** **********

                 ....|....| ....|....| ....|....| ....|....| ....|....|
                         110        120        130        140        150
M.smegmati    TDDEFKRAIQ GETGIKPEWA AESFTDLEED VRQSLRRIEA SPFVTKHESL
M. bovis      TDDDFKRAIQ DETGIRPTWS PESYPDAVED VRQSLRRIEV NPFVTKHTSL
M. tubercu    TDDDFKRAIQ DETGIRPTWS PESYPDAVED VRQSLRRIEV NPFVTKHTSL
M. nonchro    TDDGFKQQIQ DETGIKPNWA AESFVDLEED VRQSLRRIES SPFVTKHESL
M. fortuit    TDDGFKQQIQ DETGIKPNWA AESFVDLEED VRQSLRRIES SPFVTKHESL
M. malmesb    TDDGFKQQIQ DEIGIKPEWA AESFIDLEVD VRQSLRRIEA SPFVTKHESL
M. komanii    TDDGFKQQIQ DEIGIKPEWA AESFVDLEVD VRQSLRRIEA SPFVTKHESL
              **> **>>** >*>** * *   **  *  >* *********   *********

                 ....|....| ....|...
                         160

M. smegmati   RGFIFDVATG KLAEVTL-
M. bovis      RGFVFDVATG KLNEVTP-
M. tubercu    RGFVFDVATG KLNEVTP-
M. nonchro    RGFIFDVATG KLNEVTL
M. fortuit    RGFIFDVATG KLNEVTL
M. malmesb    RGFIFDVATG RLTEVTP
M. komanii    RGFVFDVATG RLNEVTL
              ***>****** **>*>**
```

Fig 4.1e: Alignment of CanA *(Mb 1315/ Rv1284)* AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), *M. smegmatis* MC^2 155 (M. smegmat), *M. malmesburii* sp.nov (M. malmesb), *M. komanii* sp. nov., *M. nonchromogenicum* (M. nonchro), *M. bovis* and *M. tuberculosis* (M. tubercu). * indicates same AA residue in all species, and > indicates same AA residue in at least one of the NTM species and *M. bovis/ M. tuberculosis*.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50
M. fortuit       MSLLDAHIPQ LIASEAAFGA KAALMRSTIA QAEQAAMSSQ AFHMGEASAA
M. nonchro       MSLLDAHIPQ LIASEAAFGA KAALMRSTIA QAEQAAMSSQ AFHMGEASAA
M. malmesb       MSMLDAHIPQ LVSSEAAFSA KAALMRSTMA QAEQAAQSAQ AFHMGESSAA
M. komanii       MSLLDAHIPQ LVSSEAAFSA KAALMRSTMA QAEQAAQSAQ AFHMGESSAA
M. tubercu       MSLLDAHIPQ LVASQSAFAA KAGLMRHTIG QAEQAAMSAQ AFHQGESSAA
M. bovis         MSLLDAHIPQ LVASQSAFAA KAGLMRHTIG QAEQAAMSAQ AFHQGESSAA
M. smegmat       MSLLDAHIPQ LIASEANFGA KAALMRSTIA QAEQAAMSSQ AFHMGEASAA
                 **>******* *>>*  >* * ****** *>  ******>*>* *** **>***

                 ....|....| ....|....| ....|....| ....|....| ....|..
                          60         70         80         90
M. fortuit       FQAAHARFVE VSAKVNGLLD IAQLNLGDAA GTYVAQDAAA ASTYTSV
M. nonchro       FQAAHARFVE VSAKVNGLLD IAQLNLGDAA GTYVAQDAAA ASTYTSV
M. malmesb       FQAAHARFLE VSAKVNALLD IAQVNLGDAA GTYVAQDAAA ASTYTGI
M. komanii       FQAAHARFLE VSAKVNALLD IAQVNLGDAA GTYVSQDAAA ASTYTGI
M. tubercu       FQAAHARFVA AAAKVNTLLD VAQANLGEAA GTYVAADAAA ASTYTGF
M. bovis         FQAAHARFVA AAAKVNTLLD VAQANLGEAA GTYVAADAAA ASTYTGF
M. smegmat       FQAAHARFVE VSAKVNALLD IAQLNIGDAA SSYVAQDAAA ASTYTGI
                 ********** ****** ***  ** *>* ** >>**> **** *****>
```

Fig 4.1f: Alignment of EsxG AA sequences of *M. fortuitum* ATCC 6841 (M. fortuit), *M. smegmatis* MC^2 155 (M. smegmat), *M. malmesburii* sp.nov (M. malmesb), *M. komanii* sp. nov., *M. nonchromogenicum* (M. nonchro), *M. bovis* and *M. tuberculosis* (M. tubercu). * indicates same AA residue in all species, and > indicates same AA residue in at least one of the NTM species and *M. bovis/ M. tuberculosis*.

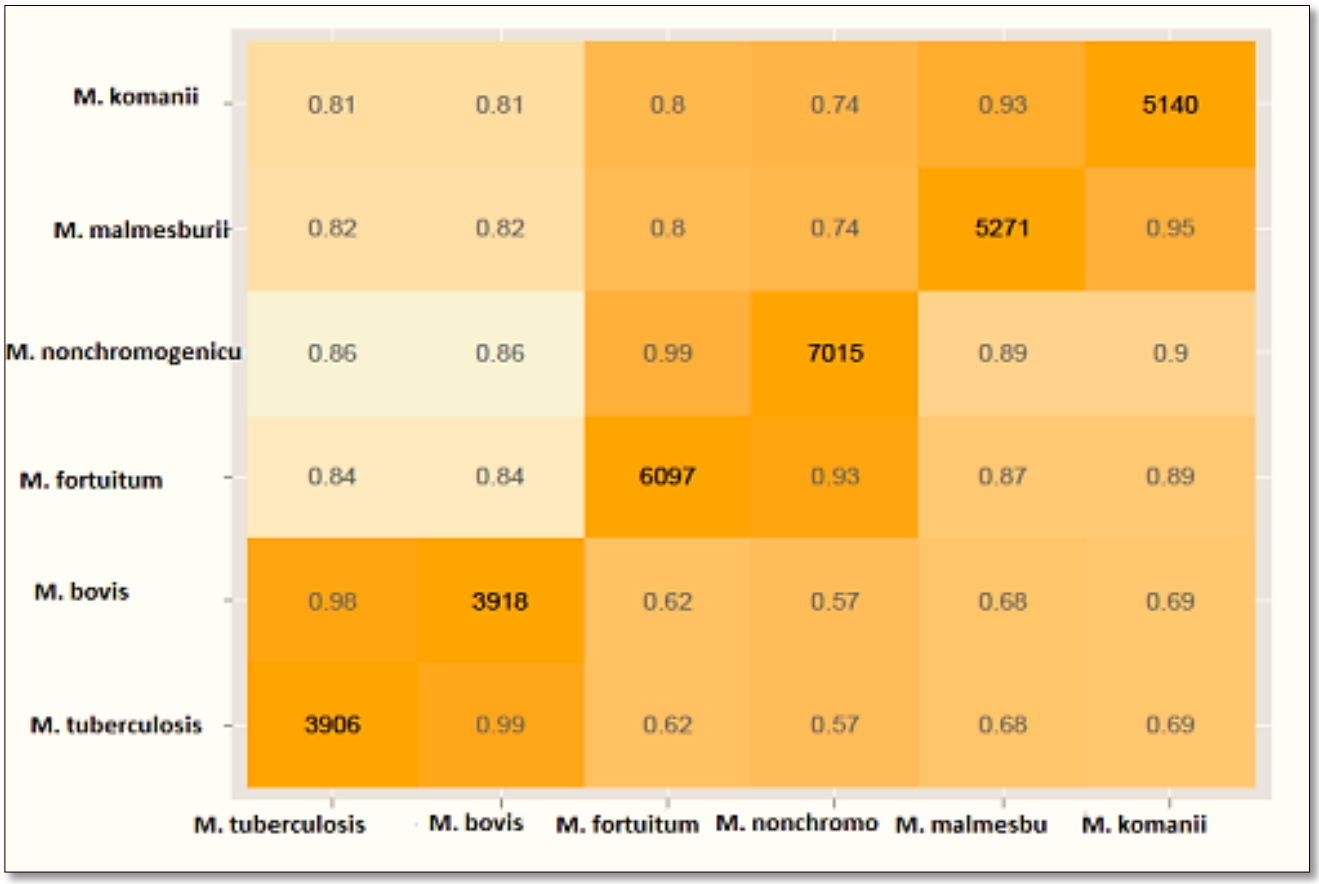

Figure 4.2 a: Genome similarity matrix, showing proportion of protein-coding genes shared (color intensity and numbers in grey). E.g., the first column shows how many genes in *M. tuberculosis* have homologues in each of the other strains. The black numbers along the diagonal represent the number of all genes identified.

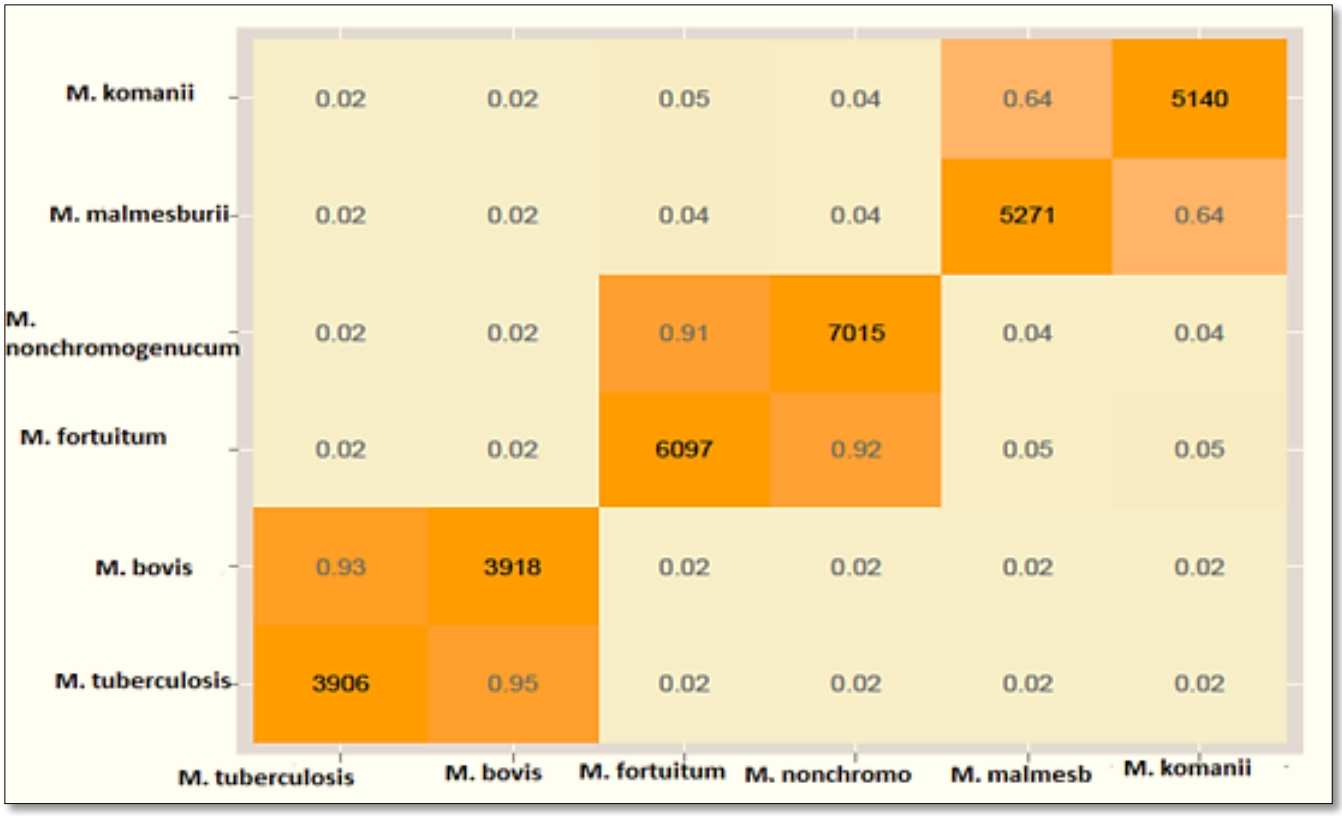

Figure 4. 2b: Genome similarity matrix, showing proportion of protein-coding genes shared (color intensity and numbers in grey). E.g., the first column shows how many genes in *M. tuberculosis* have homologues in each of the other strains. The black numbers along the diagonal represent the number of genes with ≥ 90% sequence identity aligned along ≥ 50% of their length. M. malmes, *M. malmesburii* sp. nov.; M. nonchromo, *M. nonchromogenicum*

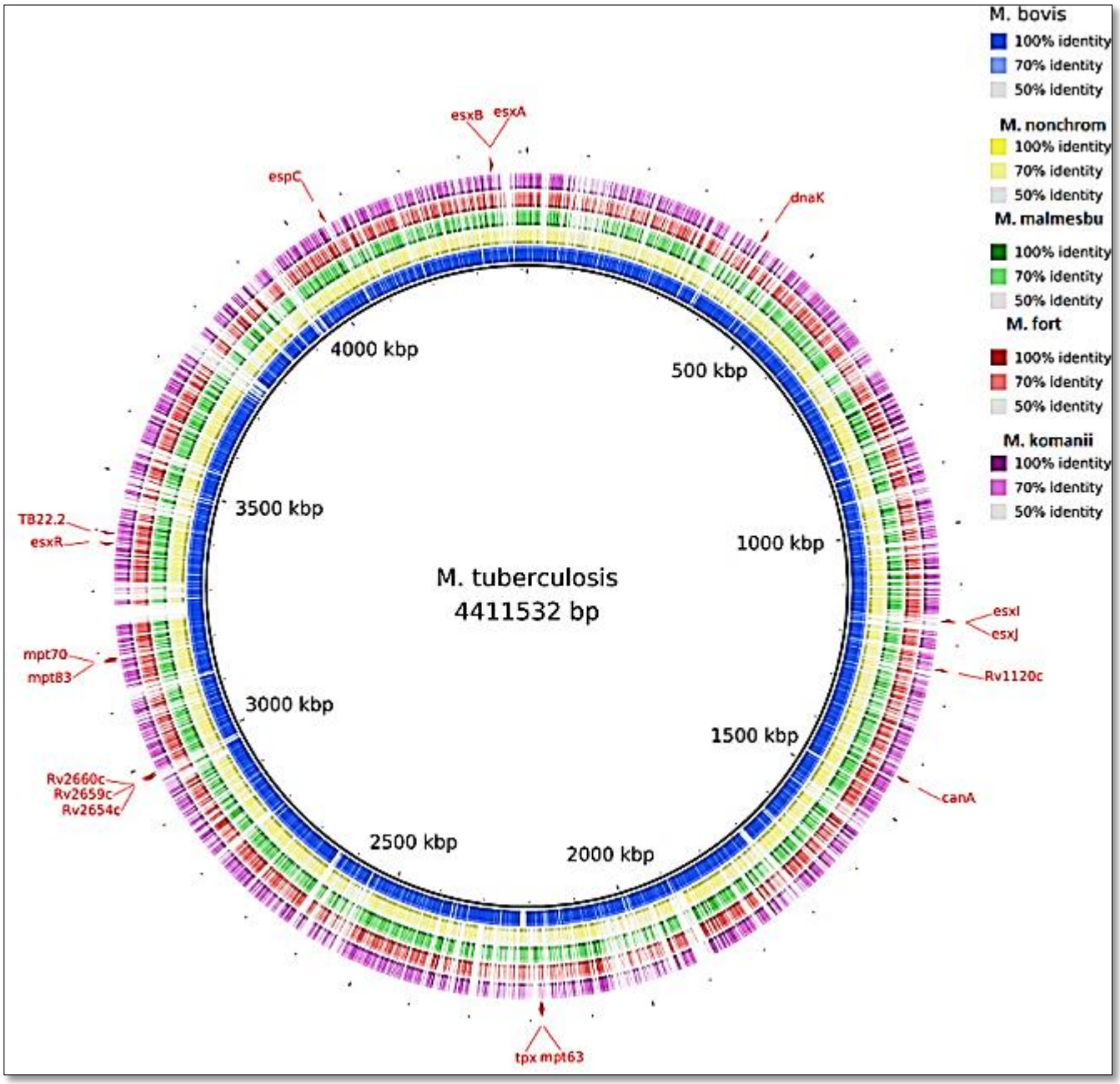

Figure 4.3: Alignment of *M. bovis* and NTM predicted CDS regions to those of *M. tuberculosis* visualised using BRIG. Immunogenic proteins of interest are highlighted in red (see Table 4.1).

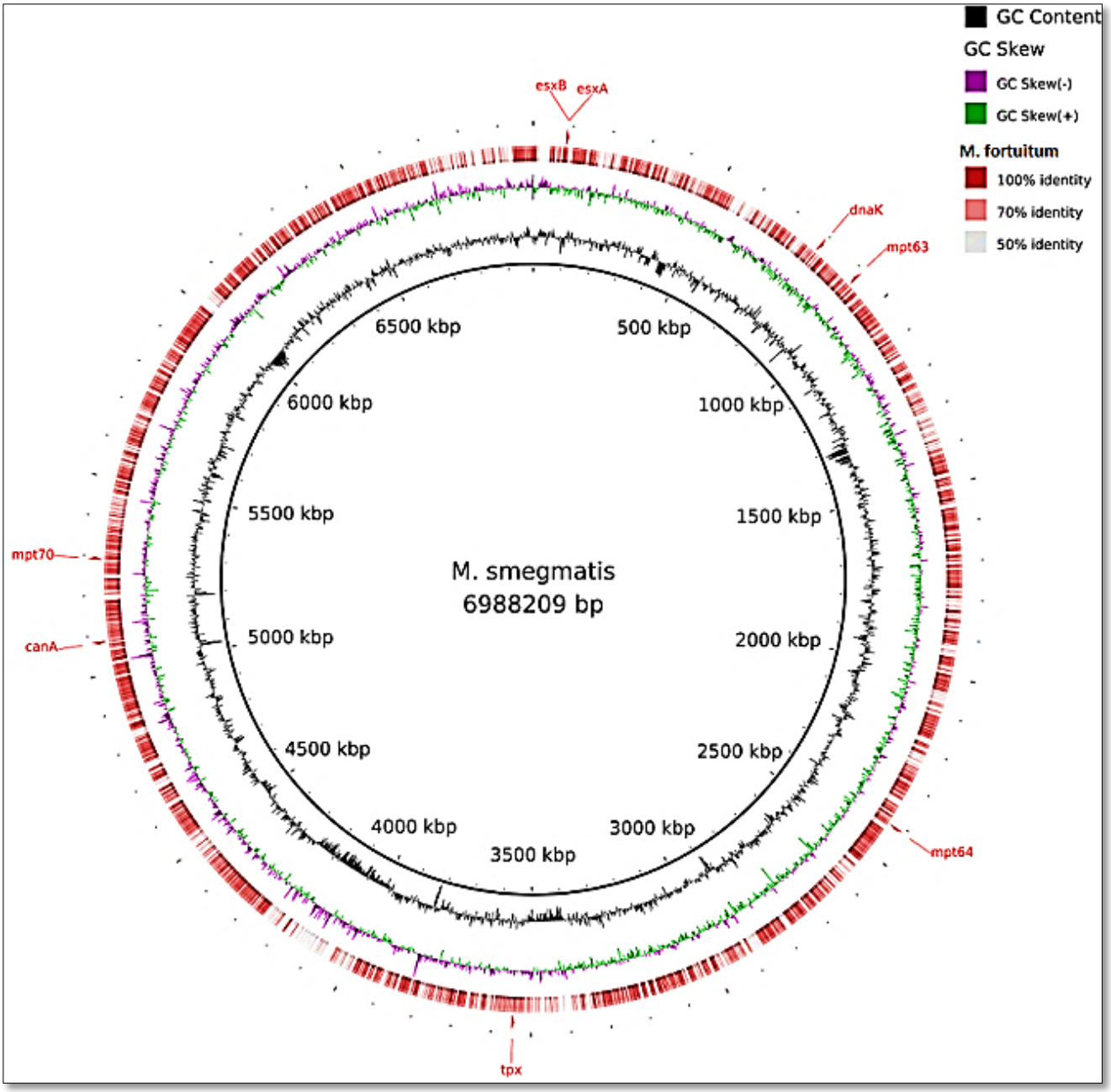

Figure 4.4 a: Alignment of *M. fortuitum* predicted CDS regions to those of *M. smegmatis* MC^2 155 (CP001663.1) using BRIG. Immunogenic proteins of interest identified in M. smegmatis are highlighted in red (esxB: locus tag MSMEG_0065, product hypothetical protein; esxA: locus tagMSMEG_0066, product early secretory antigenic target, 6 kDa; dnaK: locus tag MSMEG_0709, product of chaperone protein DnaK; mpt63: locus tag MSMEG_0828, product immunogenic protein MPT63; mpt64: locus tagMSMEG_2331, product immunogenic protein MPB64/MPT64; mpt70: locus tag MSMEG_5196, product fasciclin domain-containing protein; canA: locus tag MSMEG_4985, product carbonic anhydrase; tpx: locus tagMSMEG_3479, product thiol peroxidase).

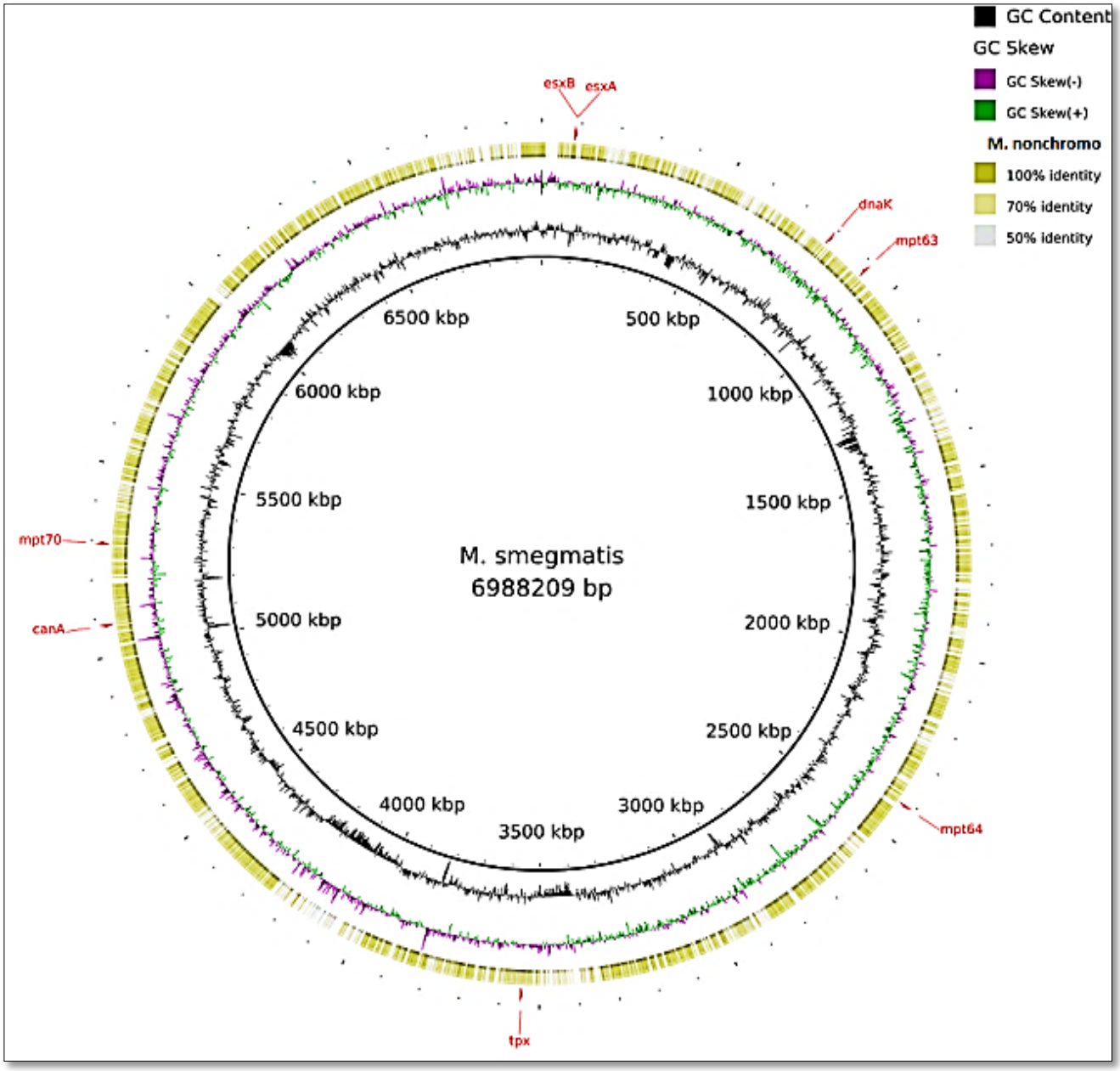

Figure 4.4 b: Alignment of *M. nonchromogenicum* predicted CDS regions to those of *M. smegmatis* MC^2 155 (CP001663.1) using BRIG. Immunogenic proteins of interest identified in M. smegmatis are highlighted in red (esxB: locus tag MSMEG_0065, product hypothetical protein; esxA: locus tagMSMEG_0066, product early secretory antigenic target, 6 kDa; dnaK: locus tag MSMEG_0709, product of chaperone protein DnaK; mpt63: locus tag MSMEG_0828, product immunogenic protein MPT63; mpt64: locus tagMSMEG_2331, product immunogenic protein MPB64/MPT64; mpt70: locus tag MSMEG_5196, product fasciclin domain-containing protein; canA: locus tag MSMEG_4985, product carbonic anhydrase; tpx: locus tagMSMEG_3479, product thiol peroxidase).

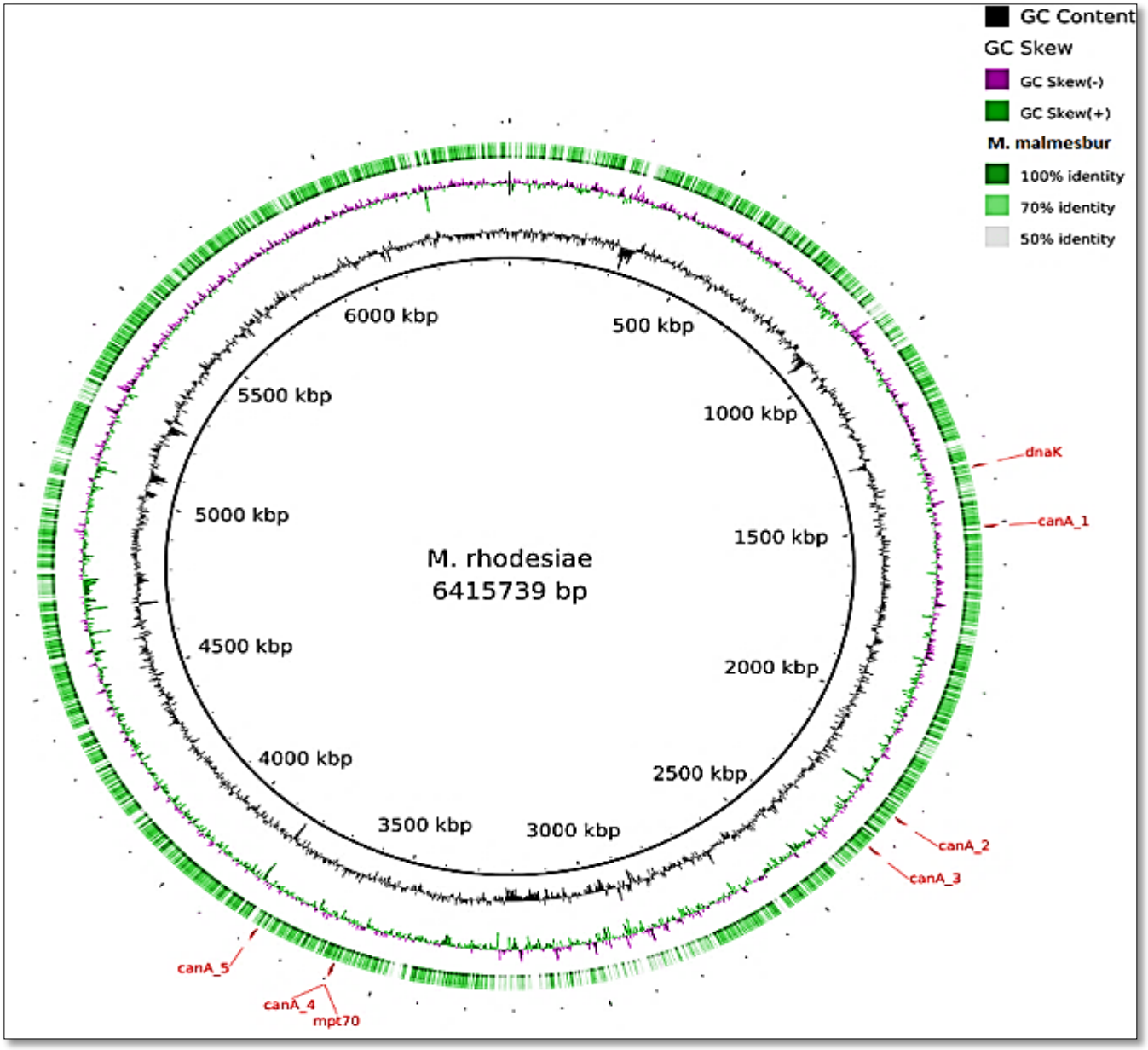

Figure 4.5: Alignment of *M. malmesburii* sp.nov. predicted CDS regions to those of *M. rhodesiae* NBB3 (CP003169.1) using BRIG. Immunogenic proteins of interest identified in M. rhodesiae are highlighted in red (dnaK: locus tagMycrhN_1341, product chaperone protein DnaK; mpt70: locus tag MycrhN_3596, product secreted/surface protein with fasciclin-like repeats; canA_1: locus tag MycrhN_1479, product sulfate permease-like transporter, MFS superfamily; canA_2: locus tag MycrhN_2217, product carbonic anhydrase; canA_3: locus tagMycrhN_2307, product isoleucine patch superfamily enzyme, carbonic anhydrase/acetyltransferase; canA_4: locus tag MycrhN_3599, product isoleucine patch superfamily enzyme, carbonic anhydrase/acetyltransferase; canA_5: locus tag MycrhN_3776, product carbonic anhydrase. Carbonic anhydrase genes have been numbered according to genomic position).

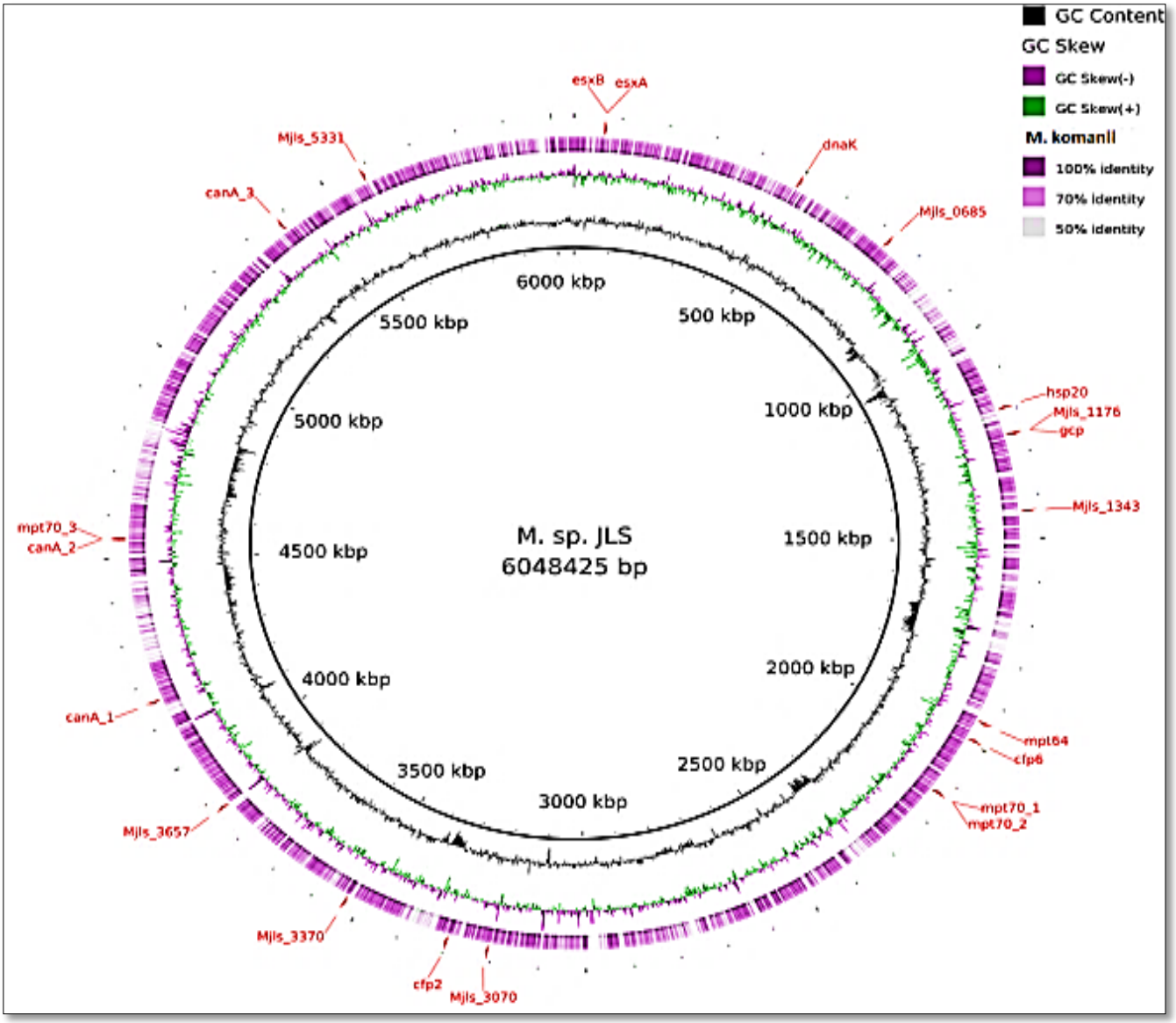

Figure 4.6: Alignment of *M. komanii* sp.nov. predicted CDS regions to those of M. sp. JLS (CP000580.1) using BRIG. Immunogenic proteins of interest identified in M. sp. JLS are highlighted in red (esxB: locus tag Mjls_0060,product hypothetical protein; esxA: locus tag Mjls_0061, product 6 kDa early secretory antigenic targetEsaT6; hsp20: locus tag Mjls_1109, product heat shock protein Hsp20; Mjls_1343: locus tag Mjls_1343, product of polysaccharide biosynthesis protein; cfp6: locus tag Mjls_1885, product low molecular weight protein antigen 6; cfp2: locus tag Mjls_3145, product low molecular weight antigen; Mjls_5331: locus tag Mjls_5331,product lipoprotein antigen family protein; dnaK: locus tag Mjls_0449, product molecular chaperone DnaK;mpt64: locus tag Mjls_1842, product immunogenic protein MPB64/MPT64; mpt70_1: locus tag Mjls_2023,product beta-Ig-H3/fasciclin; mpt70_2: locus tag Mjls_2024, product beta-Ig-H3/fasciclin; mpt70_3: locus tagMjls_4307, product beta-Ig-H3/fasciclin; Mjls_1176: locus tag Mjls_1176, product peptidase M22, glycoprotease; gcp: locus tag Mjls_1178, product putative DNA-binding/iron metalloprotein/AP endonuclease; canA_1: locus tag Mjls_3936, product carbonic anhydrase; canA_2: locus tag Mjls_4306, product carbonic anhydrase;canA_3: locus tag Mjls_5131, product carbonic anhydrase; Mjls_0685: locus tag Mjls_0685, product alkyl hydro peroxide reductase/ Thiol specific antioxidant/ Mal allergen; Mjls_3070: locus tag Mjls_3070, product alkyl hydro peroxide reductase; Mjls_3370: locus tag Mjls_3370, product alkyl hydro peroxide reductase; Mjls_3657: locus tag Mjls_3657, product alkyl hydro peroxide reductase).

CHAPTER 5. Comparative proteome analysis of purified protein derivatives (PPDs) of four abundant NTM species, and the commercial tuberculins, PPD- B and PPD- A

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CONTENTS OF CHAPTER 5 and CHAPTER 4 WERE SUBMITTED FOR PUBLICATION.

5.1 Abstract

Purified protein derivatives (PPDs) of *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Mycobacterium avium* have been used for more than 60 years as antigens in the diagnosis of human and bovine TB based on the delayed type hypersensitivity (skin) reaction they are able to induce.

Very little is known about the composition of PPDs and their active components. Despite specificity constraints, associated with cross-reactivity due to exposure of animals and humans to NTM and *M. bovis* BCG vaccine, PPDs are still widely used. In this study, we elucidated the proteomes of commercial PPD-B and PPD-A (Prionics-Lelystad, The Netherlands) as well as our own PPD preparations from four NTM species previously found to be abundant in South Africa using mass spectrometry. The NTM included *Mycobacterium fortuitum* ATCC 6841 (PPD-F), *Mycobacterium nonchromogenicum* (PPD-N), *Mycobacterium malmesburii* sp.nov (PPD-M) and *Mycobacterium kansasii* ATCC 12478 (PPD-K). Two of these *(M. fortuitum* and *M. nonchromogenicum*) were also found to be abundant in other parts of the world. In total we identified 561 proteins in the six PPD preparations. In our analysis a total of 17 proteins were identified as shared between PPD-B and the NTM PPDs. Of these shared proteins several have been described as immunogenic in the literature before and they include CFP 10 (ESXB) identified in PPD-B and PPD-M, the 10 kDA chaperonin (GroES) protein (identified in all the PPDs except PPD-M), DnaK chaperonin (identified in all PPDs except PPD-M), the 60 kDA chaperonin (GroEL) (identified in all the PPDs) as well as antigen Ag85B and Ag85C (both identified in PPD-A and PPD-B). Identification of these shared proteins in NTM PPDs warrants further investigation of their ability to cause cross reactive immune responses with *M. bovis* antigens. Apart from 111 unique PPD-B proteins, 434 proteins were identified as either specific for some NTM preparations or shared between certain

NTM species. The identification of these proteins, unique in (some) NTM PPDs is expected to contribute towards investigation of antigens that may allow specific diagnosis of NTM infections.

Key words: PPD, NTM, M. bovis cross-reactive immune responses

5.2 Introduction

Purified protein derivatives (PPDs) also known as tuberculins have been used for more than 60 years as antigens in the diagnosis of human and bovine TB, as well as other mycobacterial diseases like leprosy and bovine paratuberculosis, based on the delayed hypersensitivity (skin) reaction they induce. The first preparation of PPD was introduced by Robert Koch in 1890, where a boiled crude extract of Mycobacterium in glycerin, referred to as 'old tuberculin', was prepared as a potential vaccine against tuberculosis in humans. This tuberculin was not properly standardized, impure and toxic, and therefore could not be used in humans but formed the foundation for the modern PPD preparations (Burke, 1993). Later Von Pirquote introduced the tuberculin skin test (TST) for TB diagnosis using Koch's old tuberculin (Von Pirqoute, 1909). In the early 1940's Florence Seiberb replaced Koch's tuberculin by introducing a standardized PPD (Borsuk *et al.,* 2009). Tuberculins derived from *Mycobacterium tuberculosis* (PPD-T), *Mycobacterium bovis* (PPD-B), and *Mycobacterium avium* (PPD-A) have since been used in immunological assays for B (TB) diagnosis. The PPD is prepared by heat sterilization of *Mycobacterium* cultures grown in liquid growth medium, followed by filter sterilization and protein concentration (Landi, 1963).

Diagnostic testing for BTB by tuberculin skin test (TST) and interferon gamma assay (both based on cell mediated immune responsiveness (CMI)) has proven to be more effective for detection of subclinical infections compared to serological tests (Schiller *et al.,* 2010). The major drawback to the tuberculin based immunological assays is the reduced specificity presumably associated with cross-reactive immune responses due to exposure of animals and humans to NTM and *M. bovis* BCG vaccine. These cross-reactive immune responses are most likely due to the presence of immunogenic proteins conserved across

the *Mycobacterium* genus as major components of PPD-B (Schiller *et al.,* 2010). Some studies have compared protein composition of different commercial PPD preparations *i.e*. PPD-B, PPD- A and PPD-T respectively and noted differences (Borsuk *et al.*, 2009; Prasad *et al.*, 2013). In addition, discrepancies in immunological test results from different PPD preparations of the same Mycobacterium species have also been reported (Lifson *et al.,* 1993; Rupp *et al.*, 1994), which could be attributed to the differences in their protein composition. Increased specificity through replacement of PPD-B with defined antigens or inclusion of NTM derived PPD like PPD-A in single intradermal comparative cervical test (SICCT) and the BOVIGAM assay (Prionics) as well as PPD-F in South Africa added in the modified BOVIGAM assay, has been shown (Vordermeier *et al.,* 2007; 2009; Michel *et al.*, 2011). Currently in many countries PPD-B and PPD-A (Brazil, Netherlands, and the United Kingdom) preparations are used for diagnosis of BTB by delayed hypersensitivity test (skin) as recommended by the OIE (OIE Terrestrial Manual, 2014). PPD-F is included in the BOVIGAM assay in South Africa as an additional non MAC control antigen representative to mitigate background responses induced by environmental mycobacterial antigens (Michel *et al.,* 2011). A handful of studies have determined the proteome composition of different PPD-B and PPD-A preparations (Borsuk *et al.,* 2009; Santema *et al.,* 2009); however protein composition of PPD-F was never studied in more detail. From these studies it was observed that some of the antigens that have been tested as candidates for BTB diagnosis in animals to differentiate between infected and vaccinated individuals as well as vaccine targets are also present in PPD-B (Borsuk *et al.,* 2009; Vordermeier *et al.,* 2007; 2009). Despite its specificity constraints PPD is still widely used.

One of the limitations of the use of defined antigens as markers for BTB is the exposure to NTM since some of immunogenic proteins *e.g.* ESAT-6 and CFP 10 have been identified in pathogenic NTM like *Mycobacterium kansasii* and *Mycobacterium marinum* (Vordermeier *et al*., 2007; 2009). NTM orthologs of genes encoding for these proteins have also been identified in various non-pathogenic NTM like

Mycobacterium smegmatis, *Mycobacterium flavescens*, *Mycobacterium* sp. JLS, *Mycobacterium rhodesiae* and *M. fortuitum* (Gey van Pittius *et al.*, 2001; Gcebe *unpublished* data). Where these NTM species are endemic, BTB diagnosis may still be faced with a lack of specificity despite the introduction of the new generation antigens.

Knowledge of the protein composition of PPDs derived from prevalent NTM species in a particular region compared to PPD-B proteome could allow for effective elucidation of PPD-B associated specificity constraints arising from exposure of animals to environmental mycobacteria and henceforth the rational design of BTB diagnostic assays. The information on NTM PPD composition would also help researchers in making informed decisions on inclusion of NTM PPD in the CMI based diagnostic tests which could improve their specificity.

With the advent of high-resolution mass spectrometry it is now possible to analyze complex protein mixtures by comparing the patterns obtained to what is possible in the genome and quickly identify many of the proteins. Proteins are typically digested in-gel or in-solution using proteolytic enzymes (Habermann *et al.*, 2004). The resulting peptides are then analysed by peptide mass mapping and/or tandem mass spectrometry (Liska and Shevchenko, 2003). Database search of the peptides is done for protein identification using algorithms like basic local alignment (BLAST) or Fast-all (FASTA). The process involves matching masses of intact proteins (protein mass mapping) or their fragments (tandem mass spectrometry) to the corresponding masses of peptides and/or peptide fragments obtained by *in silico* processing of protein sequences from database entries (Liska and Shevchenko, 2003). This process requires either a complete genome and/or a substantial number of cDNA sequences to be available in a database. The advantage of having a complete genome sequence is the increased specificity and the speed of database searching, but restricts the reach of proteomics down to a handful of sequenced species (Habermann, 2004). For organisms with unsequenced genomes, cross species protein identification using

MS and available protein sequences can be achieved by partial aligning an analyzed protein from an organism with the unsequenced genome to a database sequence from the related organism (Liska and Shevchenko, 2003). Sequence identity of at least 80% between the reference database entry and the subject protein was proposed for identifying a protein (Liska and Shevchenko, 2003). Peptide mass mapping allows for cross- species protein identification in some cases because only a subset of all peptides from a protein digest need to be recognized. Several database searching approaches like mass spectrometry driven BLAST (MS –BLAST) have also been designed to identify unknown proteins using sequence similarity to homologous proteins of other organisms available in the database (Habermann *et al.,* 2004). De Novo interpretation of the tandem mass spectra can also be used to identify proteins that are not available in the databases. This method employs the peptide fragment mass of a protein and assigns an amino acid. Resulting sequences are used to design degenerate probes and the related gene is cloned. The approach has its inherent limitation mainly of inaccuracy as other amino acids such as leucine and isoleucine share identical masses (Habermann *et al.*, 2004). Another approach is to interpret tandem mass spectra of peptides using specialized software that creates amino acid sequences *de novo* (Liska and Shevchenko, 2003).

For more accurate proteome annotation, the genomic data is therefore essential. The availability of the genomes of several Mycobacterium species makes it possible to elucidate their proteomes and cross-species protein identification is achievable for unsequenced organisms that are phylogenetically closely related to the sequenced genomes.

The key aim of this study was to carry out comparative proteomic analysis of the commercial PPD- B and PPD-A (Prionics-The Netherlands), and PPDs derived from NTM species (NTM PPDs) and produced in-house in order to assess the potential of these NTM to prime cross-reactive immune

responses to *M. bovis* antigens. This study is expected to set a foundation in identification of PPD-B antigens that may be used in more specific BTB tests.

5.3 Materials and Methods

5.3.1 Bacterial cultures

Mycobacterium nonchromogenicum, a soil sample isolate, *M. malmesburii* sp. nov. isolated from a bovine nasal swab, *M. fortuitum* ATCC 6841 and *M. kansasii* ATCC 12478 were cultured on either Middlebrooks 7H11 agar plates (Becton Dickinson, USA) supplemented with 0.1% OADC (Merck Chemicals, South Africa) and glycerol, as well as on Löwenstein Jensen (LJ) slopes supplemented with glycerol and an antibiotic cocktail of PolymyxinB, Amphotericin B, Carbenicillin and Trimethoprim (PACT) (Becton Dickinson, USA). For PPD production, liquid cultures were prepared in Middlebrook 7H9 media (Becton Dickinson, USA) supplemented with 0.1% OADC and glycerol, incubated under continued shaking at 200 *g* at 37 ºC for four weeks for the rapid growing mycobacteria and six weeks for the slow growing NTM or until turbid growth was observed. The liquid cultures were screened for contaminants before PPDs were prepared by spread plating each culture on two nutrient agar plates. The plates were then incubated at 25 ºC and 37 ºC respectively, and evaluated after 2 days and 5 days for fungal or any growth not typical of mycobacteria. Ziehl Neelson staining was done on typical Mycobacterium colony cultures.

5.3.2 PPD production from NTM

PPDs were prepared from *M. nonchromogenicum* (PPD- N), *M. malmesburii* sp. nov. (PPD-M), *M. fortuitum* ATCC 6841 (PPD-F), and *M. kansasii* ATCC 12478 (PPD-K) following the modified protocol by Land, 1963. Briefly, the cultures were inactivated by steaming at 121ºC for 20 min. and filter-sterilized using the Whatman 40 filter paper and a vacuum pump. Each filtrate was then precipitated by adding 40% trichloroacetic acid (TCA) to a final concentration of 4% v/v) and left for at least 12 hours at 4-8 ºC. Afterwards the precipitated filtrates were mixed manually by shaking and centrifuged at room temperature for 20 min at 3900 *g*. The supernatants were discarded and the pellets washed twice by suspending them in 1% TCA and careful mixing, followed by centrifugation at 3900 *g* for 20 min at room temperature. The supernatants were discarded and the pellets suspended in 10% NaCl, then centrifuged for 20 min at 3900 *g*. After discarding the supernatant, the pellet was harvested by turning the tube upside down, on a piece of sterile filter paper and allowed to dry, weighed, diluted with 0.005% tuberculin buffer (0.005% Tween 80 in PBS: PH= 7.38) and stored at 4-8ºC until peptide digestion (see section 5.3.3).

The purified protein derivatives from *M. bovis* (PPD-B) and *M. avium* (PPD-A) were obtained from Prionics at the Netherlands.

5.3.3 In-Solution Digest with trypsin (IAA)

Trypsin digestion was done at the Centre for proteomics and genomics research (CPGR, South Africa). All reagents used were analytical grade or equivalent. Twenty microgram of protein was aliquoted into

a 1.5 ml centrifuge tube and the volume adjusted to 10 µl with 50 mM triethylammonium bicarbonate (TEAB; Sigma). Protein was reduced by adding 1 µl of 100 mM tris (2-carboxyethyl) phosphine (Sigma) prepared in 50 mM TEAB, and the tubes were incubated at 60 ºC for 1 hour. Samples were cooled to room temperature and then protein was alkylated by adding 1 µl of 200 mM iodoacetamide (Sigma) prepared in 50 mM TEAB. Samples were incubated at room temperature in the dark for 30 min. The sample volume was adjusted to 50 µl with 50 mM TEAB and then 5 µl of 1 µg/µl trypsin (Promega) prepared in MilliPore water was added and digestion was allowed to take place at 37 ºC for 18 hours, followed by vacuum centrifugation.

5.3.4 Mass spectrometry

LC MS/MS analysis

LC−MS/MS analysis was conducted with a Q-Exactive quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, USA) coupled with a Dionex Ultimate 3000 nano-HPLC system at CPGR. The mobile phases consisted of solvent A (0.1% formic acid in water) and solvent B (80% ACN, 10% water, and 0.1% formic acid). The peptides (as an estimate 500 ng for each sample) were re-suspended in sample loading buffer (95% water, 5% Acetonitrile, 0.05% TFA) and loaded on a C18 trap column (100 µm × 20 mm ×5 µm). Chromatographic separation was performed with a C18 column (75 µm ×1 50 mm ×3 µm). The gradient was delivered at 300 nl /min and consisted of a linear gradient of mobile phase B initiating from solvent B, 6–60% over 156 min. The mass spectrometer was operated in positive ion mode with a capillary temperature of 250 °C. The applied electrospray voltage was 1.95 kV. Details of data acquisition are listed in table 5.1.

Table 5.1: Data acquisition

Full Scan	
Resolution	70,000 (@ *m/z* 200)
AGC target value	3e6
Scan range	320-2000 m/z
Maximal injection time (ms)	100
Data-dependent MS/MS	
Resolution	17,500 (@ *m/z* 200)
AGC target value	2e5
Maximal injection time (ms)	50
Isolation window width (Da)	3
NCE (%)	27
Data-dependent Settings	
Underfill ratio (%)	1
Charge exclusion	Charge states 1,6-8,>8
Peptide match	preferred
Exclusion isotopes	on
Dynamic exclusion (s)	60

5.3.5 Data analysis

Database searching

All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1). Mascot was set up to search the Mycobacterium database (derived from UniProtKB, 843597 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a subset of the Mycobacterium database including *M. avium* strain 104, *M avium* subp *avium* 10-9275, *M. colombiense, M. avium* subsp *sylvaticum* ATCC 49884, *M. avium* subsp *hominissuis* TH135, *M. avium* subsp *paratuberculosis* S5, *M. avium* subsp *paratuberculosis*

S397 , *M. fortuitum* subsp *fortuitum* DSM 46621, *M. bovis* AF2122/97, *M. bovis* BCG Pasteur 1173P2, *M. bovis* BCG strain Korea 1168P,*M. kansasii* ATCC 12478, *M. smegmatis* mc^2155ATCC (ATCC 700084), *M. smegmatis* J5623, *M. gastri* 'Wayne', *M. parascrofulaceum, M. tuberculosis* KZN strain1435, *M. tuberculosis* Haarlem strain, *M. tuberculosis* H37Ra (ATCC 25177), *M. ulcererans* strain Agy99, *M. szulgai, M. africanum* k85, *M. marinum* ATCC BAA-535, *M. marinum* strain Europe, *M. canettii* CIP 140070017, *M vaccae* ATCC 25954 and *M. rhodesiae* J560 also assuming trypsin. Mascot and X! Tandem were searched with a fragment ion mass tolerance of 0.020 Da and a parent ion tolerance of 10.0 PPM. Carbamidomethyl of cysteine were specified in Mascot and X! Tandem as fixed modifications. Gln->pyro-Glu of the n-terminus, deamidation of asparagine and glutamine, oxidation of methionine were specified in Mascot and X! Tandem as variable modifications.

Criteria for protein identification

Scaffold (version Scaffold_4.3.4, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 9.0% probability to achieve an FDR less than 0.1% by the Scaffold Local FDR algorithm (Keller *et al.*, 2002). Protein identifications were accepted if they could be established at greater than 100.0% probability to achieve an FDR less than 1.0% and contained at least four identified peptides (Keller *et al.*, 2002). Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii *et al.*, 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

5.4 Results

5.4.1. Identification of proteins present in all PPD preparations

Five hundred and sixty one different proteins were identified in all the PPD preparations combined. One hundred and twenty six were identified in PPD-B. Among the NTM PPDs, PPD-A showed the showed the highest number of proteins (n= 222) followed by PPD-F (n=189), PPD-N (n=159), while only 21 and 20 proteins were identified in PPD-M and PPD-K, respectively. The molecular mass of the majority of the proteins was found to be in the range of 10-50 kDA. The list of the identified proteins is presented in Appendix A. Unique as well as shared proteins were identified in each PPD preparations as illustrated in the Venn diagrams in fig 5.1 a-d.

5.4.2. Shared proteins between PPD-B and NTM PPDs and their functions

Seventeen proteins were identified as shared between the NTM PPD preparations and PPD-B. Identities and functions of these proteins are listed in table 5.2. The Venn diagrams in figures 5.1 a-d illustrate shared proteins between PPD-B, PPD-A and different NTM PPDs. There was a higher degree of protein overlap between PPD-A and PPD-B (13/17 shared proteins) than between the other NTM PPDs and PPD-B. Six proteins were shared between PPD-B and PPD-K, PPD-B and PPD-F as well as PPD-B and PPD-N while only four proteins were shared between PPD-B and PPD-M. Most of the shared proteins have been described as immuno-dominant in *M. bovis* and *M. tuberculosis* or have been analysed for their potential as B (TB) diagnostic markers. These include CFP 10 /ESXB (Vordermeier *et al.*, 2009), the 10 kDA chaperonin (GroES) (Prasad *et al.*, 2013), DnaK chaperonin (Das Gupta *et al.*, 2002), the 60

kDA chaperonin (GroEL) (Prasad *et al.*, 2013), Ag85B and Ag85C (Vordermeier *et al.*, 2006; Prasad *et al.*, 2013) and EsxN (Prasad *et al.*, 2013).

Table 5.2: Common proteins identified among the six PPD preparations

Identified Proteins (16/561)	Accession Number	**Mol** Weight	**PPD-A**	**PPD-B**	**PPD-F**	**PPD-K**	**PPD-M**	**PPD-N**	**Function**	**Ref**
10 kDa culture fíltrate antigen EsxB , M**.** tuberculosis	R4MBZ5_MYCTX	11 kDa		+			+		immunodominant antigen involved in secretion, complex formation, immunogenicity and virulence	Brodin et al., 2005
10 kDa chaperonin groS , M. tuberculosis	A2VPK5_MYCTX	11 kDa	+	+	+	+		+	immunodominant protein, ATP binding	Uniprot, Prasad *et al.*, 2013
60 kDa chaperonin groEL , M. tuberculosis	R4LUN2_MYCTX	57 kDa	+	+	+	+	+	+	immunodominant protein	Uniprot, Prasad *et al.*, 2013
50S ribosomal protein L7/L12, M. parascrofulaceum	D5PC72_9MYCO	13 kDa	+	+			+		Involved in interaction with translocation factors	Gudkov, 2008
Elongation factor Tu, tuf, M. fortuitum	K0VK30_MYCFO	44 kDa	+	+	+	+	+	+	GTPase activity	Uniprot
DivIVA domain containing protein, M. avium subsp paratuberculosis	F7P246_MYCPC	28 kDa	+	+	+	+			Cell shape maintenance	He and Buck 2010
Chaperone protein DnaK , M. tuberculosis	A2VF50_MYCTX	67 kDa	+	+	+	+		+	Highly antigenic and act as co-repressor for heat shock protein transcription repressor (hspR)	Das Gupta *et al.*, 2008

Aconitate hydratase , M. sylvalticum	V7KF43_MYCAV	102 kDa	+	+					Unknown function	Uniprot
Elongation factor Tu ,Mycobacterium avium ,	EFTU_MYCA1	44 kDa	+	+					GTPase activity	Uniprot
Secreted antigen 85-c fbpC , ***M. bovis***	A1KEV0_MYCBP	37 kDa	+	+					Proteins of antigen 85 are responsible high affinity of mycobacteria	Uniprot
Malate synthétase, Mycobacterium avium	MASZ_MYCA1	80 kDa	+	+					Enzyme of glyoxylate shunt	Kumar and Bhakuni, 2010
Diacylglycerol acyltransferase/mycolyltransferase Ag85B M. *smegmatis*	A85B_MYCS2	35 kDa	+	+					Proteins of antigen 85 are responsible high affinity of mycobacteria	Uniprot
Probable glyceraldehyde 3-phosphate dehydrogenase , M. *bovis* BCG	A1KIM5_MYCBP	36 kDa	+	+					Phases of glycolysis	Uniprot
Iron-dependent repressor IdeR , M. avium	A0QIP3_MYCA1	25 kDa	+	+					DNA binding transcription factor	Uniprot
Putative ESAT-6 like protein 5 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) , esxN	A1KJK3_MYCBP	10 kDa		+		+			Reported to induce T-cell response	Deng *et al.*, 2014
DNA-directed RNA polymerase subunit beta' OS=Mycobacterium tuberculosis , rpoC P	S5ES53_MYCTX	147 kDa		+				+	Catalyzes the transcription of DNA into RNA	Uniprot
30S ribosomal protein, S4 OS, =Mycobacterium fortuitum subsp fortuitum DSM 46621	KOV712_MYCFO	53 kDA		+	+			+	Binds directly to 16R rRNA, Essential in invitro growth of Mycobacterium tuberculosis	Griffin *et al.*, 2010

+, proteins identified in the respective PPDs; Uniprot, Uniprot database (www.uniprot.org)

5.4.3 Proteins shared among NTM PPD preparations

This analysis included proteins only present in NTM PPD preparations and those shared among these PPDs including the commercial PPD-A. Four hundred and thirty four proteins were identified (table 5.3a and 5.3b). Proteins unique to the commercial PPD-A were 196. No proteins were shared across all NTM PPDs. Some of the proteins described as immunogenic in *M. bovis*/ *M. tuberculosis* were also detected in the NTM database entries and include most notably: *M. fortuitum* MPB63 (identified in PPD-F and PPD-N), *M. fortuitum* Ag85C (identified in PPD-F, PPD-M and PPD-N), *M. fortuitum* Ag85A (identified in PPD-F and PPD-N), *M. kansasii* Ag85B (identified in PPD-K), *M. avium* MPB64 (identified in PPD-A), *M. avium* Ag85A and Ag85B (both identified in PPD-A).

Table 5.3a: Proteins unique and shared among NTM PPDs

Protein and Mycobacterium species	Accession number	PPD -A	PPD-F	PPD-K	PPD-M	PPD-N
Bacterioferritin OS=Mycobacterium avium	V7J3W1_MYCAV	+	+			+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UMT4_MYCFO		+		+	+
Acyl carrier protein OS=Mycobacterium avium (strain 104)	A0QER4_MYCA1	+			+	+
Electron transfer protein, beta subunit OS=Mycobacterium avium (strain 104)	A0QJF0_MYCA1	+	+			
Mannose-binding lectin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UPC2_MYCFO		+			+
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VA79_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UX16_MYCFO		+			+
Chaperone protein DnaK OS=Mycobacterium avium (strain 104)	DNAK_MYCA1	+	+			
Diacylglycerol acyltransferase/mycolyltransferase Ag85B OS=Mycobacterium kansasii	A85B_MYCKA			+		
Glutamate binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V4Q6_MYCFO		+			+
Antigen 85-A OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3Z5_MYCFO		+			+
Immunogenic protein MPT63 (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0US52_MYCFO		**+**			**+**
Bacterioferritin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VCV8_MYCFO		**+**			**+**
Electron transfer protein, beta subunit OS=Mycobacterium avium (strain 104)	A0QJF0_MYCA1	+	+			
Mannose-binding lectin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UPC2_MYCFO		+			+
Low molecular weight antigen MTB12 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VWZ9_MYCFO		+			+
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VA79_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM	K0UX16_MYCFO		+			+
ATP synthase subunit alpha OS=Mycobacterium avium (strain 104)	ATPA_MYCA1	+	+			+
Phosphate-binding protein PstS OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZG6_MYCFO		+			+
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7H4_MYCFO		+			+
Fructose-bisphosphate aldolase class-I OS=Mycobacterium avium (strain 104)	A0QN95_MYCA1	+	+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8M4_MYCFO		+			+
ATP synthase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UJQ0_MYCFO	+	+	+		+

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Integration host factor OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155)	A0QWS8_MYCS2	+	+		+	+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WVT2_MYCKA			+		
DNA-directed RNA polymerase subunit alpha OS=Mycobacterium smegmatis	L0IRX4_MYCSM	+	+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V769_MYCFO		+			1
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAQ7_MYCFO		+			+
Elongation factor Ts OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UW15_MYCFO		+			+
Fasciclin domain-containing protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UME3_MYCFO		+			+
60 kDa chaperonin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8R4_MYCFO		+		+	+
Acetyl/propionyl-CoA carboxylase subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VB51_MYCFO		+			+
Enolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UMN7_MYCFO	+	+			+
Probable glyceraldehyde 3-phosphate dehydrogenase gap OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KIM5_MYCBP	+				
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V0B7_MYCFO		+			+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V526_MYCFO		+			+
Porin M1 OS=Mycobacterium fortuitum subsp. fortuitum	A1ING8_MYCFO		+			+
Phosphoglycerate kinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8M9_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZS8_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9J9_MYCFO		+			+
Single-stranded DNA-binding protein OS=Mycobacterium avium subsp. hominissuis TH135	T2GMI5_MYCAV	+	+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UND9_MYCFO		+			+
60 kDa chaperonin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=groL	K0VKG7_MYCFO		+			+
Dihydrolipoyl dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UR46_MYCFO		+			+
30S ribosomal protein S6 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3Q6_MYCFO		+			+
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UQD6_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3D4_MYCFO		+			
Ribonucleoside-diphosphate reductase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VW47_MYCFO		+			+
Aconitate hydratase 1 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZ04_MYCFO		+			+
DNA-directed RNA polymerase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8E8_MYCFO		+		+	+

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Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN	K0VBZ4_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UTU9_MYCFO					+
60 kDa chaperonin OS=Mycobacterium gastri 'Wayne'	W4HVY4_MYCGS	+	+	+		
PadR family transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UVP1_MYCFO					+
Zn-dependent alcohol dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VMI7_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VCR8_MYCFO		+			+
Lipoprotein, ATP binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VE69_MYCFO		+			+
Trypsin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V994_MYCFO		+			+
Polyribonucleotide nucleotidyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VIG7_MYCFO		+			+
Transketolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UJQ7_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UND2_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UWR7_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VQB7_MYCFO		+			+
Lipoprotein Lpps OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V6V4_MYCFO		+			+
Beta-1,3-glucanase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9N4_MYCFO		+			+
ATP synthase subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8M3_MYCFO		+			+
Chaperone ClpB OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UGP7_MYCFO		+			+
Amino acid ABC transporter OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V755_MYCFO		+			+
Fatty acid desaturase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UKU9_MYCFO		+			+
Succinyl-CoA ligase [ADP-forming] subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UVN8_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3W0_MYCFO		+			+
LprG protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VCH2_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UM24_MYCFO		+			+
Acyl carrier protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VG08_MYCFO		+			+
Heparin-binding hemagglutinin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UQ98_MYCFO		+			+
Cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V6X9_MYCFO		+			+
Branched-chain amino acid ABC transporter substrate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UIH3_MYCFO		+			

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Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UY81_MYCFO		+			
Fatty acid desaturase, type 2 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UW52_MYCFO		+			+
Transcription termination/antitermination protein NusG OS=Mycobacterium smegmatis JS623	L0IPB6_MYCSM		+			+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VH76_MYCFO		+			+
Polyketide synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3Z1_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VDE3_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VB17_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V1X8_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3J8_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V4Y4_MYCFO		+			+
F420-dependent glucose-6-phosphate dehydrogenase OS=Mycobacterium fortuitum	FGD_MYCFO	+	+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VEG0_MYCFO		+			+
Esterase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UPB9_MYCFO		+			+
Putative esterase OS=Mycobacterium vaccae ATCC 25954	K0UUC4_MYCVA				+	
Transaldolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UPX8_MYCFO		+			+
Isocitrate lyase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UV53_MYCFO		+			
Thiosulfate sulfurtransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V0H1_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9L0_MYCFO		+			
Cutinase Cfp21 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VHN0_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VTN4_MYCFO		+			
Elongation factor G OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V804_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UQ34_MYCFO		+			+
3-hydroxyacyl-CoA dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V597_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9Y7_MYCFO		+			
Metallopeptidase, zinc binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UYS2_MYCFO		+			
Protein RecA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VHA3_MYCFO					+
2,5-diketo-D-gluconic acid reductase OS=Mycobacterium kansasii ATCC 12478	U5WVJ4_MYCKA			+		

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DivIVA protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAA8_MYCFO		+			
Glyoxalase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VE80_MYCFO		+			+
Thioredoxin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UWY7_MYCFO					+
Short-chain dehydrogenase/reductase SDR OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V488_MYCFO		+			+
Putative esterase OS=Mycobacterium phlei	I0RQ96_MYCPH				+	
Catalase-peroxidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0USD3_MYCFO		+			+
Fructose-1,6-bisphosphate aldolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZU8_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3A6_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9C1_MYCFO		+			+
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VRV3_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAE7_MYCFO		+			
Putative esterase OS=Mycobacterium phlei	I0RCH3_MYCPH				+	
Fatty acid desaturase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VCH0_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7Z6_MYCFO		+			+
Sulfate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VPE1_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VA15_MYCFO		+			+
Acetyl-CoA acetyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UKM1_MYCFO		+			+
Methyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0URJ0_MYCFO		+			+
LpqE protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UTS6_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VXS8_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0URR7_MYCFO					+
ATP synthase epsilon chain OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UKU8_MYCFO		+			+
Cell division protein FtsZ OS=Mycobacterium kansasii	FTSZ_MYCKA		+			+
Electron transfer flavoprotein subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V934_MYCFO		+			+
Cutinase OS=Mycobacterium kansasii ATCC 12478	U5WWB6_MYCKA			+		
ATP-binding protein OS=Mycobacterium kansasii ATCC 12478	U5WTT7_MYCKA			+		
F0F1 ATP synthase subunit delta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UQV8_MYCFO		+			+

Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZV6_MYCFO		+			+
Isocitrate dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VQD7_MYCFO		+			+
Aminopeptidase N OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VWN2_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7R0_MYCFO					+
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UNK4_MYCFO					+
Thioredoxin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UYZ7_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WUN6_MYCKA			+		
Bacterioferritin OS=Mycobacterium avium	V7J3W1_MYCAV	+	+			+
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VBV3_MYCFO					+
Superoxide dismutase [Cu-Zn] OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V1E6_MYCFO		+			
Trigger factor OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V9T0_MYCFO		+			+
Peptidase S1 and S6, chymotrypsin/Hap OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V088_MYCFO		+			+
Malate synthase G OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7X3_MYCFO		+			
Serine hydroxymethyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZ83_MYCFO		+			+
3-oxoacyl-(Acyl carrier protein) synthase II OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VR41_MYCFO					+
Propionyl-CoA carboxylase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V743_MYCFO		+			+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V881_MYCFO		+			
[NADP+] succinate-semialdehyde dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UXD1_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V2K8_MYCFO		+			
Fumarate hydratase class II OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3W5_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WPP2_MYCKA			+		
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7D3_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VSD7_MYCFO		+			
Formamidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAV4_MYCFO	+	+			
30S ribosomal protein S4 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UTQ0_MYCFO		+			
Arylsulfatase, AslA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UNS5_MYCFO		+			
Alpha/beta hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V1Z3_MYCFO		+			+

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Cyclophilin type peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V6T7_MYCFO		+			
Proteasome subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VA96_MYCFO		+			+
Beta-lactamase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAM4_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UH77_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UTZ4_MYCFO		+			
Probable cutinase cut2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KKZ3_MYCBP					
Monooxygenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UT08_MYCFO		+			+
ATP-dependent Clp protease proteolytic subunit OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 G	K0VDN3		+			
Cyclopropane-fatty-acyl-phospholipid synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7V6_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VE63_MYCFO		+			+
Metallopeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VPU0_MYCFO					+
Alkyl hydroperoxide reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VEF0_MYCFO					+
Trypsin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UXD3_MYCFO		+			
Fructose-2,6-bisphosphatase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VEH0_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5X1U5_MYCKA			+		
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0URE6_MYCFO		+			
Proteasome-associated ATPase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VLY9_MYCFO		+			
6-phosphogluconate dehydrogenase, decarboxylating OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V2V2_MYCFO		+			+
Cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UTQ3_MYCFO		+			
DNA gyrase subunit A OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UVA8_MYCFO					+
Arginine biosynthesis bifunctional protein ArgJ OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V230_MYCFO		+			
Two component transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V6I9_MYCFO		+			+
D-3-phosphoglycerate dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VAG8_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3S4_MYCFO		+			+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WRT3_MYCKA			+		
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5X3E8_MYCKA			+		
50S ribosomal protein L10 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7X9_MYCFO		+			

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Transmembrane transport protein MmpL10 OS=Mycobacterium tuberculosis	R4LWQ9_MYCTX				+	
Hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V5Z0_MYCFO		+			
Acyl carrier protein OS=Mycobacterium vanbaalenii (strain DSM 7251 / PYR-1)	A1TBI7_MYCVP					+
6-phosphogluconate dehydrogenase, decarboxylating (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V658_MYCFO		+			
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VIB0_MYCFO		+			
Ribonucleoside-diphosphate reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VL43_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V753_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0ULC0_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UMJ9_MYCFO		+			
Universal stress protein UspA-like protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V1J0_MYCFO					+
Phosphoserine aminotransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V2M1_MYCFO					+
GntR family transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V4G5_MYCFO		+			
FHA domain-containing protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VD97_MYCFO		+			
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZ85_MYCFO					+
Molybdenum ABC transporter periplasmic molybdate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VIY0_MYCFO		+			
Signal-transduction protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UNL7_MYCFO					+
Ferredoxin sulfite reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VF31_MYCFO					+
Pyridoxamine 5''-phosphate oxidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VQG9_MYCFO					+
Alpha-ketoglutarate decarboxylase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UZY0_MYCFO					+
Glycosyl hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UFN0_MYCFO		+			
Acetyl-CoA acetyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UL52_MYCFO		+			
Alpha/beta hydrolase fold protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VSA3_MYCFO		+			+
Ribose-phosphate pyrophosphokinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V2H2_MYCFO		+			
Meromycolate extension acyl carrier protein OS=Mycobacterium aurum	ACPM_MYCAU					+
D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V1M2_MYCFO		+			
R4V0A1|R4V0A1_MYCAB-DECOY	R4V0A1|R4V0A1				+	
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WNW8_MYCKA			+		

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Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VFF0_MYCFO		+			
Anti-sigma factor antagonist OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UNP9_MYCFO		+			
NAD-dependent malic enzyme (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UBD0_MYCFO		+			
B1MMN6|B1MMN6_MYCA9-DECOY	B1MMN6|B1MMN6				+	
Polyketide-type polyunsaturated fatty acid synthase PfaA OS=Mycobacterium rhodesiae JS60	G4I5E7_MYCRH				+	
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V7T7_MYCFO		+			+
Succinate dehydrogenase flavoprotein subunit OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V2T1_MYCFO					+
Methylmalonate-semialdehyde dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UJ90_MYCFO		+			
I6ZHP2|I6ZHP2_MYCAB-DECOY	I6ZHP2|I6ZHP2				+	
Dihydrolipoamide acetyltransferase (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UNN7_MYCFO					+
Pyruvate synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 T	K0UP66_MYCFO					+
Pyruvate kinase OS=Mycobacterium rhodesiae JS60	G4I2V3_MYCRH				+	
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478	U5WYC5_MYCKA			+		
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8N7_MYCFO		+			
Pyruvate kinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V8Y4_MYCFO					+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UYS8_MYCFO		+			
Methyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V5W4_MYCFO					+
D-alanyl-D-alanine carboxypeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V3L8_MYCFO		+			
I4BR24|I4BR24_MYCCN-DECOY	I4BR24|I4BR24				+	
MmpL protein OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155)	A0R1G2_MYCS2				+	
Outer membrane protein OmpA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VN20_MYCFO		+			
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0V4C0_MYCFO		+			
Cystathionine gamma-synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0UYN9_MYCFO		+			
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0URT2_MYCFO		+			
Ribose-5-phosphate isomerase B OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621	K0VA03_MYCFO		+			

Table 5.3b: Proteins unique to PPD-A

Protein and Mycobacterium species name	Accession number
Antigen 85-B OS=Mycobacterium avium (strain 104) GN=MAV_2816 PE=4 SV=1	A0QGG5_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4695 PE=4 SV=1	A0QLN4_MYCA1
Antigen 85-B OS=Mycobacterium avium (strain 104) GN=MAV_2816 PE=4 SV=1	A0QGG5_MYCA1
Putative acetyltransferase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_11260 PE=4 SV=1	F7PBB8_MYCPC
Uncharacterized protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_23545 PE=4 SV=1	V7KKI2_MYCAV
Transglycosylase OS=Mycobacterium avium (strain 104) GN=MAV_0446 PE=4 SV=1	A0Q9Z0_MYCA1
Serine protease OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_01225 PE=4 SV=1	V7KYG3_MYCAV
Antigen 85-A (Mycolyl transferase) (Fragment) OS=Mycobacterium paratuberculosis GN=fbpa PE=4 SV=1	Q70E87_MYCPC
Protease OS=Mycobacterium avium (strain 104) GN=MAV_1096 PE=4 SV=1	A0QBR1_MYCA1
Immunogenic protein MPT64 OS=Mycobacterium avium (strain 104) GN=MAV_4130 PE=4 SV=1	A0QK41_MYCA1
Probable cutinase Cut3 OS=Mycobacterium avium (strain 104) GN=MAV_4283 PE=4 SV=1	A0QKI4_MYCA1
60 kDa chaperonin 2 OS=Mycobacterium avium (strain 104) GN=groL2 PE=3 SV=1	CH602_MYCA1
35kd antigen OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3039 PE=4 SV=1	T2GU78_MYCAV
Transaldolase OS=Mycobacterium avium (strain 104) GN=tal PE=3 SV=1	A0QHX2_MYCA1
Uncharacterized protein OS=Mycobacterium colombiense CECT 3035 GN=MCOL_V201805 PE=4 SV=1	J5ESB1_9MYCO
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2763 PE=4 SV=1	A0QGB4_MYCA1
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26634 PE=4 SV=1	K0UX16_MYCFO
Signal peptide protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_23515 PE=4 SV=1	V7K1R1_MYCAV
N-acetylmuramoyl-L-alanine amidase OS=Mycobacterium avium subsp. paratuberculosis 10-8425 GN=O976_20900 PE=4 SV=1	V7NKM8_MYCPC
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_28750 PE=4 SV=1	F7P319_MYCPC
LpqE protein OS=Mycobacterium avium (strain 104) GN=MAV_0569 PE=4 SV=1	A0QAB1_MYCA1
Antigen 85-C OS=Mycobacterium avium (strain 104) GN=MAV_0215 PE=4 SV=1	A0Q9C1_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_09420 PE=4 SV=1	F7PAT6_MYCPC
Hydroxymethylglutaryl-CoA lyase OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=P863_03630 PE=4 SV=1	V7KWT7_MYCAV
Probable thiol peroxidase OS=Mycobacterium avium (strain 104) GN=tpx PE=3 SV=1	A0QGC1_MYCA1
Malate dehydrogenase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=mdh PE=3 SV=1	F7PBF5_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0628 PE=4 SV=1	A0QAH0_MYCA1
Electron transfer flavoprotein, alpha subunit OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_06000 PE=4 SV=1	F7P9V0_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0239 PE=4 SV=1	A0Q9E4_MYCA1
Uncharacterized protein OS=Mycobacterium avium 05-4293 GN=O984_06270 PE=4 SV=1	V7J9T0_MYCAV
60 kDa chaperonin OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=groEL PE=3 SV=1	V7JZC8_MYCAV
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_08920 PE=4 SV=1	F7PAP0_MYCPC

Phosphate-binding protein PstS OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_31960 PE=3 SV=1	F7P3Y4_MYCPC
Carbohydrate degrading enzyme OS=Mycobacterium avium (strain 104) GN=MAV_1218 PE=4 SV=1	A0QC26_MYCA1
Catalase-peroxidase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=katG PE=3 SV=1	F7PEL7_MYCPC
Peroxisomal multifunctional enzyme type 2 OS=Mycobacterium avium (strain 104) GN=MAV_5146 PE=3 SV=1	A0QMX5_MYCA1
O-methyltransferase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_00660 PE=4 SV=1	V7LLA4_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4742 PE=4 SV=1	A0QLT1_MYCA1
Adenylate kinase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=adk PE=3 SV=1	F7P5P8_MYCPC
Acyl-CoA dehydrogenase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4027 PE=3 SV=1	A0QJU2_MYCA1
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0904 PE=4 SV=1	I3NIF2_MYCPA
Trigger factor OS=Mycobacterium avium 05-4293 GN=tig PE=3 SV=1	V7J4X3_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1314 PE=4 SV=1	A0QCC1_MYCA1
ATP synthase subunit beta OS=Mycobacterium avium subsp. hominissuis 10-5606 GN=atpD PE=3 SV=1	V7N8Q0_MYCAV
Bacteriocin OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_17350 PE=4 SV=1	V7KBQ7_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2808 PE=4 SV=1	A0QGF7_MYCA1
Universal stress protein UspA-like protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2160 PE=4 SV=1	T2GU96_MYCAV
Uncharacterized protein OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=P863_11575 PE=4 SV=1	V7KKF2_MYCAV
Homogentisate 1,2-dioxygenase OS=Mycobacterium avium subsp. hominissuis TH135 GN=hmgA PE=4 SV=1	T2GVD0_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4234 PE=4 SV=1	A0QKD7_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1445 PE=4 SV=1	A0QCQ0_MYCA1
Citrate lyase beta chain citrase beta chain family protein OS=Mycobacterium avium subsp. hominissuis 10-4249 GN=O971_12205 PE=4 SV=1	V7M5M1_MYCAV
Putative acyl-CoA transferase/carnitine dehydratase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_04540 PE=4 SV=1	F7P9F6_MYCPC
Cutinase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23050 PE=4 SV=1	F7PEM9_MYCPC
Aminopeptidase N OS=Mycobacterium avium (strain 104) GN=pepN PE=4 SV=1	A0QDE6_MYCA1
Uncharacterized protein OS=Mycobacterium sp. MOTT36Y GN=W7S_13055 PE=4 SV=1	I2AE52_9MYCO
Trypsin OS=Mycobacterium avium (strain 104) GN=MAV_1366 PE=4 SV=1	A0QCH2_MYCA1
50S ribosomal protein L29 OS=Mycobacterium avium subsp. paratuberculosis S397 GN=rpmC PE=3 SV=1	F7P5S9_MYCPC
Inorganic pyrophosphatase OS=Mycobacterium avium (strain 104) GN=ppa PE=3 SV=1	A0QA70_MYCA1
Metallopeptidase, zinc binding OS=Mycobacterium avium (strain 104) GN=MAV_3451 PE=4 SV=1	A0QI94_MYCA1
Antibiotic biosynthesis monooxygenase domain protein OS=Mycobacterium avium (strain 104) GN=MAV_0562 PE=4 SV=1	A0QAA4_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=P863_19285 PE=4 SV=1	V7K6T2_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3557 PE=4 SV=1	A0QIJ7_MYCA1
60 kDa chaperonin OS=Mycobacterium gastri 'Wayne' GN=groEL PE=3 SV=1	W4HVY4_MYCGS
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2866 PE=4 SV=1	A0QGL4_MYCA1
Phosphoenolpyruvate carboxykinase [GTP] OS=Mycobacterium avium (strain 104) GN=pckG PE=3 SV=1	A0QME6_MYCA1

D-3-phosphoglycerate dehydrogenase OS=Mycobacterium avium (strain 104) GN=serA PE=3 SV=1	A0QJC3_MYCA1
Forkhead-associated protein OS=Mycobacterium avium (strain 104) GN=MAV_2888 PE=4 SV=1	A0QGN6_MYCA1
Glucose-6-phosphate isomerase OS=Mycobacterium avium subsp. avium 10-9275 GN=pgi PE=3 SV=1	V7KYA2_MYCAV
Acetyl-CoA acetyltransferase OS=Mycobacterium avium (strain 104) GN=MAV_1198 PE=3 SV=1	A0QC06_MYCA1
Biotin carboxyl carrier protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_01960 PE=4 SV=1	V7LGX8_MYCAV
Enolase OS=Mycobacterium avium (strain 104) GN=eno PE=3 SV=1	ENO_MYCA1
Phosphotriesterase-like protein OS=Mycobacterium avium (strain 104) GN=MAV_4940 PE=4 SV=1	A0QMC3_MYCA1
6-phosphogluconolactonase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_16990 PE=4 SV=1	F7PCY3_MYCPC
Uncharacterized protein OS=Mycobacterium avium 05-4293 GN=O984_01620 PE=4 SV=1	V7JCA2_MYCAV
Phosphoglycerate kinase OS=Mycobacterium avium 10-5581 GN=pgk PE=3 SV=1	V7JEF1_MYCAV
Serine esterase cutinase OS=Mycobacterium avium (strain 104) GN=MAV_2169 PE=4 SV=1	A0QEP1_MYCA1
UPF0234 protein OCU_44330 OS=Mycobacterium intracellulare (strain ATCC 13950 / DSM 43223 / JCM 6384 / NCTC 13025 / 3600) GN=OCU_44330 PE=3 SV=1	H8ISK7_MYCIA
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3546 PE=4 SV=1	T2GWX9_MYCAV
Uncharacterized protein OS=Mycobacterium intracellulare (strain ATCC 13950 / DSM 43223 / JCM 6384 / NCTC 13025 / 3600) GN=OCU_01440 PE=4 SV=1	H8IJI5_MYCIA
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_4287 PE=3 SV=1	T2GZ63_MYCAV
2,5-diketo-D-gluconic acid reductase OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=dkgA PE=4 SV=1	V7KCT7_MYCAV
Thioredoxin domain protein OS=Mycobacterium avium (strain 104) GN=MAV_1545 PE=4 SV=1	A0QCZ3_MYCA1
Membrane protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_06195 PE=4 SV=1	V7KQI0_MYCAV
Putative molybdenum cofactor synthesis protein OS=Mycobacterium avium (strain 104) GN=MAV_1097 PE=4 SV=1	A0QBR2_MYCA1
Succinate dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_4910 PE=4 SV=1	A0QM93_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2865 PE=4 SV=1	A0QGL3_MYCA1
Serine/threonine protein kinase OS=Mycobacterium avium (strain 104) GN=MAV_0017 PE=4 SV=1	A0Q8T1_MYCA1
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0196c PE=4 SV=1	Q745I9_MYCPA
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S5 GN=D522_22373 PE=4 SV=1	L7DEQ1_MYCPC
Protein GrpE OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=grpE PE=3 SV=1	V7K3P4_MYCAV
NAD-dependent aldehyde dehydrogenase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_44030 PE=3 SV=1	F7P7B3_MYCPC
NADH-quinone oxidoreductase subunit C OS=Mycobacterium avium subsp. paratuberculosis S397 GN=nuoC PE=3 SV=1	F7P9E5_MYCPC
Elongation factor Ts OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=tsf PE=3 SV=1	EFTS_MYCPA
Phosphoribosylformylglycinamidine synthase, PurS protein OS=Mycobacterium avium (strain 104) GN=purS PE=4 SV=1	A0QAS2_MYCA1
Major membrane protein 1 OS=Mycobacterium avium (strain 104) GN=MAV_2054 PE=4 SV=1	A0QED3_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_40010 PE=4 SV=1	F7P667_MYCPC
Adenosylhomocysteinase OS=Mycobacterium avium subsp. paratuberculosis S5 GN=ahcY PE=3 SV=1	L7DDN5_MYCPC
Glyceraldehyde-3-phosphate dehydrogenase, type I OS=Mycobacterium avium (strain 104) GN=gap PE=3 SV=1	A0QHY5_MYCA1
Immunogenic protein MPB64/MPT64 OS=Mycobacterium avium (strain 104) GN=MAV_3901 PE=4 SV=1	A0QJH5_MYCA1

Glycosyl hydrolase family 16 OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_42630 PE=4 SV=1	F7P6X5_MYCPC
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0262 PE=4 SV=1	Q745Q4_MYCPA
Heparin binding hemagglutinin hbha OS=Mycobacterium avium (strain 104) GN=MAV_4675 PE=4 SV=1	A0QLL5_MYCA1
Phosphate-binding protein PstS (Fragment) OS=Mycobacterium avium 09-5983 GN=O983_17710 PE=3 SV=1	V7LKA4_MYCAV
Phosphorylase OS=Mycobacterium avium (strain 104) GN=MAV_1549 PE=3 SV=1	A0QCZ5_MYCA1
Hydrolase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_10695 PE=4 SV=1	V7L2H8_MYCAV
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S5 GN=D522_16748 PE=4 SV=1	L7DHI8_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2964 PE=4 SV=1	A0QGW2_MYCA1
GatB/Yqey domain protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_19940 PE=4 SV=1	V7KTY5_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2908 PE=4 SV=1	A0QGQ6_MYCA1
NlpC/P60 family protein OS=Mycobacterium avium (strain 104) GN=MAV_3208 PE=4 SV=1	A0QHK2_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1705 PE=4 SV=1	A0QDE5_MYCA1
Hydrolase OS=Mycobacterium avium (strain 104) GN=MAV_3654 PE=4 SV=1	A0QIU2_MYCA1
Uncharacterized protein (Fragment) OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=P863_23660 PE=4 SV=1	V7JXY4_MYCAV
Putative thiosulfate sulfurtransferase OS=Mycobacterium avium (strain 104) GN=MAV_4253 PE=4 SV=1	A0QKF6_MYCA1
Uncharacterized protein OS=Mycobacterium xenopi RIVM700367 GN=MXEN_15035 PE=4 SV=1	I0RL64_MYCXE
Chaperone protein HtpG OS=Mycobacterium avium subsp. paratuberculosis S397 GN=htpG PE=3 SV=1	F7P2R7_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3912 PE=4 SV=1	A0QJI6_MYCA1
Anti-sigma factor antagonist OS=Mycobacterium avium (strain 104) GN=MAV_0435 PE=3 SV=1	A0Q9Y1_MYCA1
Zn-dependent hydrolase OS=Mycobacterium avium 05-4293 GN=O984_14515 PE=4 SV=1	V7J0S9_MYCAV
Prokaryotic ubiquitin-like protein Pup OS=Mycobacterium avium 10-5581 GN=pup PE=3 SV=1	V7JJI1_MYCAV
Glyoxalase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4702 PE=4 SV=1	A0QLP1_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_43810 PE=3 SV=1	F7P791_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3591 PE=4 SV=1	A0QIN0_MYCA1
Sulfonate binding protein OS=Mycobacterium avium (strain 104) GN=MAV_0141 PE=4 SV=1	A0Q948_MYCA1
Serine esterase, cutinase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4394 PE=4 SV=1	A0QKU1_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2933 PE=4 SV=1	A0QGT1_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2920 PE=4 SV=1	A0QGR8_MYCA1
Transketolase OS=Mycobacterium avium (strain 104) GN=tkt PE=3 SV=1	A0QHX1_MYCA1
cAMP-binding protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_36470 PE=4 SV=1	F7P565_MYCPC
ATP synthase gamma chain OS=Mycobacterium gastri 'Wayne' GN=atpG PE=3 SV=1	W4I0N8_MYCGS
3-hydroxyisobutyryl-CoA hydrolase OS=Mycobacterium avium subsp. hominissuis TH135 GN=echA9 PE=4 SV=1	T2GNN5_MYCAV
Peptidase, M28 family protein OS=Mycobacterium avium (strain 104) GN=MAV_4738 PE=4 SV=1	A0QLS7_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0450 PE=4 SV=1	A0Q9Z4_MYCA1

NADH-quinone oxidoreductase subunit I OS=Mycobacterium avium subsp. paratuberculosis S397 GN=nuoI PE=3 SV=1	F7P9D9_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3785 PE=4 SV=1	A0QJ69_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_10610 PE=4 SV=1	V7L6G1_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2351 PE=4 SV=1	A0QF66_MYCA1
Glyoxalase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_06050 PE=4 SV=1	V7LCD1_MYCAV
Enoyl-CoA hydratase OS=Mycobacterium avium (strain 104) GN=MAV_3689 PE=1 SV=1	A0QIX8_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3320 PE=4 SV=1	T2GXK2_MYCAV
Putative acyl-CoA dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_3935 PE=3 SV=1	A0QJK8_MYCA1
Peptidase family protein M13 OS=Mycobacterium avium (strain 104) GN=MAV_4977 PE=4 SV=1	A0QMG0_MYCA1
Aldehyde dehydrogenase family protein OS=Mycobacterium avium (strain 104) GN=MAV_2812 PE=3 SV=1	A0QGG1_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4907 PE=4 SV=1	A0QM90_MYCA1
Cutinase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_08300 PE=4 SV=1	V7L8Q7_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4701 PE=4 SV=1	A0QLP0_MYCA1
Transcription elongation factor GreA OS=Mycobacterium avium subsp. paratuberculosis S397 GN=greA PE=3 SV=1	F7P3H3_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4288 PE=4 SV=1	A0QKI9_MYCA1
Dioxygenase OS=Mycobacterium avium (strain 104) GN=MAV_0540 PE=4 SV=1	A0QA82_MYCA1
Glutamine synthetase OS=Mycobacterium avium (strain 104) GN=glnA PE=3 SV=1	A0QEY3_MYCA1
ATP-dependent Clp protease proteolytic subunit OS=Mycobacterium avium subsp. paratuberculosis S5 GN=clpP PE=3 SV=1	L7DK09_MYCPC
Superoxide dismutase OS=Mycobacterium avium (strain 104) GN=MAV_0182 PE=3 SV=1	A0Q988_MYCA1
Nucleoside diphosphate kinase regulator OS=Mycobacterium avium (strain 104) GN=MAV_0234 PE=4 SV=1	A0Q9D9_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2567 PE=4 SV=1	T2GUG0_MYCAV
ErfK/YbiS/YcfS/YnhG family protein OS=Mycobacterium avium (strain 104) GN=MAV_4986 PE=4 SV=1	A0QMG9_MYCA1
Serine esterase cutinase OS=Mycobacterium avium (strain 104) GN=MAV_0369 PE=4 SV=1	A0Q9R7_MYCA1
HIT family hydrolase, diadenosine tetraphosphate hydrolase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_13800 PE=4 SV=1	F7PC21_MYCPC
O-methyltransferase OS=Mycobacterium avium 05-4293 GN=O984_01700 PE=4 SV=1	V7JEA2_MYCAV
Uncharacterized protein, probably involved in trehalose biosynthesis OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_45790 PE=4 SV=1	F7P7T7_MYCPC
Cellobiohydrolase A (1,4-beta-cellobiosidase A) OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23360 PE=4 SV=1	F7PER0_MYCPC
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3218 PE=4 SV=1	A0QHL2_MYCA1
Eptc-inducible aldehyde dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_4691 PE=3 SV=1	A0QLN1_MYCA1
Enoyl-CoA hydratase OS=Mycobacterium avium (strain 104) GN=MAV_4534 PE=4 SV=1	A0QL77_MYCA1
Periplasmic binding protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3333 PE=4 SV=1	T2GUZ7_MYCAV
Short chain dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_0895 PE=3 SV=1	A0QB71_MYCA1
Naphthoate synthase OS=Mycobacterium avium (strain 104) GN=menB PE=4 SV=1	A0QLD7_MYCA1

Nitroreductase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4334 PE=4 SV=1	A0QKN2_MYCA1
Ribosome-recycling factor OS=Mycobacterium avium subsp. paratuberculosis S397 GN=frr PE=3 SV=1	F7PA73_MYCPC
D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase OS=Mycobacterium avium (strain 104) GN=dacB PE=4 SV=1	A0QA71_MYCA1
Probable cytosol aminopeptidase OS=Mycobacterium avium (strain 104) GN=pepA PE=3 SV=1	A0QEZ5_MYCA1
2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase OS=Mycobacterium avium (strain 104) GN=sucB PE=3 SV=1	A0QEY9_MYCA1
6-phosphogluconate dehydrogenase, decarboxylating OS=Mycobacterium avium (strain 104) GN=gnd PE=4 SV=1	A0QC60_MYCA1
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_0266 PE=3 SV=1	T2GMN3_MYCAV
Chaperone protein ClpB OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=clpB PE=3 SV=1	CLPB_MYCPA
Proteasome subunit beta OS=Mycobacterium avium subsp. avium 11-4751 GN=O973_11070 PE=3 SV=1	V7LGT8_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2810 PE=4 SV=1	A0QGF9_MYCA1
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1706 PE=4 SV=1	A0QDE7_MYCA1
Aspartate transaminase OS=Mycobacterium avium (strain 104) GN=MAV_0381 PE=4 SV=1	A0Q9S8_MYCA1
Glycerophosphoryl diester phosphodiesterase family protein OS=Mycobacterium avium (strain 104) GN=MAV_0576 PE=4 SV=1	A0QAB7_MYCA1
R3H domain-containing protein OS=Mycobacterium avium (strain 104) GN=MAV_5309 PE=4 SV=1	A0QND1_MYCA1
Methyltransferase, putative, TIGR00027 family OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_01730 PE=3 SV=1	F7P8M6_MYCPC
Acetyl-CoA acetyltransferase OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_08545 PE=3 SV=1	V7KNS5_MYCAV
Endonuclease IV OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3909 PE=3 SV=1	T2GWR1_MYCAV
Acyl carrier protein OS=Mycobacterium vaccae ATCC 25954 GN=acpP PE=3 SV=1	K0UD35_MYCVA
Phosphoserine aminotransferase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=serC PE=3 SV=1	F7P437_MYCPC
NAD(P)H nitroreductase OS=Mycobacterium avium 05-4293 GN=O984_12220 PE=4 SV=1	V7J5B3_MYCAV
Hydrolase, peptidase M42 family protein OS=Mycobacterium avium (strain 104) GN=MAV_2729 PE=4 SV=1	A0QG80_MYCA1
PE family protein (Fragment) OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_38890 PE=4 SV=1	F7P5V6_MYCPC
Cyanate hydratase OS=Mycobacterium avium (strain 104) GN=cynS PE=3 SV=1	CYNS_MYCA1
Thioredoxin OS=Mycobacterium avium (strain 104) GN=trx PE=3 SV=1	A0QNC4_MYCA1
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23220 PE=4 SV=1	F7PEP6_MYCPC
Alanine and proline rich secreted protein apa OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2430 PE=4 SV=1	T2GV03_MYCAV
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3362 PE=4 SV=1	A0QI06_MYCA1
P40 protein OS=Mycobacterium avium PE=4 SV=1	Q9AIQ0_MYCAV
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23480 PE=4 SV=1	F7PES2

5.4.4 Proteins identified exclusively in PPD-B

Proteins identified as unique to PPD-B are listed in table 5.4. One hundred and eleven of these were detected. Some of these proteins have been investigated as candidate antigens for B (TB) diagnosis. These include most notably the PPE family proteins (Akhter *et al.*, 2012), MPB83 (Wiker 2009), TpX (Prasad *et al.*, 2013), MPB70 (Wiker, 2009), EspC (Millington *et al.*, 2013), ESAT-6 (Vordermeier *et al.*, 2007), HspX (Marongui *et al.*, 2013), MPB64 (Elhay *et al.*, 1998; Prasad *et al.*, 2013). Other proteins identified have been found in other studies to be associated with human host immune response in cases with active tuberculosis. These include malate synthetase G and Aconitate hydratase and were identified in PPD-B. (Prasad *et al.*, 2013).

Table 5.4: List of proteins present in PPD-B only

Protein	Accession number
Cell surface lipoprotein mpt83 (Lipoprotein P23) OS=Mycobacterium tuberculosis	A2VLK6_MYCTX
Putative esat-6 like protein OS=Mycobacterium tuberculosis (strain ATCC 25177)	A5U544_MYCTA
10 kDa culture filtrate protein OS=Mycobacterium szulgai GN=cfp-10	B5TV80
10 kDa culture filtrate antigen EsxB_1 OS=Mycobacterium marinum (strain ATCC BAA-535 / M) GN=esxB_1	B2HJ18
Alanine and proline rich secreted protein apa OS=Mycobacterium tuberculosis	A2VIX6_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KJN9_MYCBP
Acyl carrier protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KKT7_MYCBP
6 kDa early secretory antigenic target OS=Mycobacterium marinum	S7RG91_MYCMR
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase OS=Mycobacterium tuberculosis	A4KG62_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KLX8_MYCBP
Adenosylhomocysteinase OS=Mycobacterium tuberculosis	D5YJL6_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KPV0_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KKI8_MYCBP

PPE family protein OS=Mycobacterium africanum K85	D6FR36_9MYCO
Putative BACTERIOFERRITIN BFRB OS=Mycobacterium tuberculosis 7199-99	LONZH9MYCTX
Serine protease pepA OS=Mycobacterium tuberculosis (strain KZN 1435 / MDR)	C6DQZ0_MYCTK
Iron-regulated conserved protein OS=Mycobacterium ulcerans (strain Agy99)	A0PP51_MYCUA
Conserved protein OS=Mycobacterium marinum (strain ATCC BAA-535 / M)	B2HS57_MYCMM
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KLQ5_MYCBP
DNA-directed RNA polymerase subunit beta' OS=Mycobacterium tuberculosis	S5ES53_MYCTX
Thioredoxin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KEF8_MYCBP
Ribosome-recycling factor OS=Mycobacterium tuberculosis	A2VLL5_MYCTX
0 kDa chaperonin OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=groEL	R4ML4
6 kDa early secretory antigenic target OS=Mycobacterium szulgai	B5A908_MYCSZ
Putative lipoprotein lprA OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KI57_MYCBP
Probable ferredoxin fdxC OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KHW8_MYCBP
Transcription elongation factor GreA OS=Mycobacterium tuberculosis str. Haarlem	A4KG15_MYCTX
Major secreted immunogenic protein mpb70 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KMM0_MYCB
14 kDa antigen OS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97) GN=hspX	14KD_MYCBO
Esat-6 like protein esxL (Esat-6 like protein 4) OS=Mycobacterium tuberculosis	A2VHA0_MYCTX
Immunogenic protein mpt64 OS=Mycobacterium tuberculosis	A2VJ88_MYCTX
Esterase, , antigen 85-A OS=Mycobacterium bovis BCG str. Korea 1168P	M1IRK4_MYCBI
Cfp10 (Fragment) OS=Mycobacterium riyadhense	B2CXA0_9MYCO
10 kDa culture filtrate antigen CFP-10 OS=Mycobacterium sp. 012931	S7QYG9_9MYCO
Succinate dehydrogenase flavoprotein subunit OS=Mycobacterium xenopi	I0RNQ2_MYCXE
35 kDa protein OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra)	35KD_MYCTA
Low molecular weight antigen cfp2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KL66_MYCBP
Ketol-acid reductoisomerase OS=Mycobacterium tuberculosis (strain KZN 1435 / MDR)	C6DW46_MYCTK
Iron-regulated heparin binding hemagglutinin hbhA OS=Mycobacterium tuberculosis	R4M5A7_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KNJ2_MYCBP
Conserved protein with fha domain, fhaa OS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97)	Q7U303_MYCBO
Uncharacterized protein OS=Mycobacterium tuberculosis	H8HY34_MYCTX
Glutamine synthetase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KKR3_MYCBP
Cutinase cfp21 OS=Mycobacterium tuberculosis	A2VJ93_MYCTX
Pyruvate dehydrogenase E1 component OS=Mycobacterium tuberculosis str. Haarlem	R4LZF6_MYCTX
S-adenosylmethionine synthase OS=Mycobacterium tuberculosis str. Haarlem	R4M544_MYCTX
Response regulator OS=Mycobacterium marinum str. Europe	S7RIY0_MYCMR
Lipoprotein lprG OS=Mycobacterium tuberculosis	A2VHU1_MYCTX

Bacterioferritin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KJT8_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KG46_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KNS1_MYCBP
Uncharacterized protein OS=Mycobacterium canettii CIPT 140070017	L0QTC8_9MYCO
Uncharacterized protein OS=Mycobacterium tuberculosis CAS/NITR204	R4MJV0_MYCTX
Electron transfer flavoprotein (Alpha-subunit) fixB OS=Mycobacterium tuberculosis	A2VNJ5_MYCTX
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KEF0_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KGT4_MYCBP
Probable conserved secreted protein tb22.2 oS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97)	Q7TXE4_MYCBO
Probable cutinase cut5 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KQ56_MYCBP
Lipoprotein OS=Mycobacterium tuberculosis (strain ATCC 35801 / TMC 107 / Erdman)	H8EWC8_MYCTE
Putative uncharacterized protein OS=Mycobacterium tuberculosis	A2VNB6_MYCTX
Probable cutinase cut2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KKZ3_MYCBP
Polyribonucleotide nucleotidyltransferase OS=Mycobacterium canettii CIPT 140060008	L0PYM0_9MYCO
Putative lipoprotein lprF OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KIF8_MYCBP
Possible oxidoreductase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KEN1_MYCBP
Putative pterin-4-alpha-carbinolamine dehydratase OS=Mycobacterium tuberculosis str. Haarlem	A4KG87_MYCTX
Serine protease htrA OS=Mycobacterium tuberculosis T17	D5ZEX8_MYCTX
Secreted antigen 85-B fbpB (Fibronectin-binding protein B) OS=Mycobacterium tuberculosis C	A2VJ01
Immunogenic protein MPT63 (Antigen MPT63/MPB63) (16 kDa immunoprotective extracellular protein) OS=Mycobacterium tuberculosis str. Haarlem/NITR20**2**	R4M6J4
60 kDa chaperonin OS=Mycobacterium tuberculosis str. Haarlem GN=groL	A4KEC8
Fatty oxidation protein fadB OS=Mycobacterium tuberculosis	A2VGF6_MYCTX
Malate synthase G OS=Mycobacterium tuberculosis str. Haarlem	R4M7L1_MYCTX
Serine hydroxymethyltransferase OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra)	A5U1E0_MYCTA
Uncharacterized protein OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra)	A5U862_MYCTA
Probable serine protease OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KHB9_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KJV7_MYCBP
Probable acetyl-CoA acyltransferase fadA2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KF64_MYCBP
10 kDa culture filtrate antigen esxB (Cfp10) OS=Mycobacterium tuberculosis C	A2VMP9
Putative ESAT-6 like protein 5 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=esxN	A1KJK3
Putative uncharacterized protein OS=Mycobacterium tuberculosis EAS054	D5YMQ2_MYCTX
Adenylate kinase OS=Mycobacterium tuberculosis	A2VG38_MYCTX
Pyruvate dehydrogenase (E2 component) sucB OS=Mycobacterium tuberculosis	A2VJV6_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KF71_MYCBP

Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem	R4M6G6_MYCTX
Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem	R4LUQ9_MYCTX
Polyketide synthase pks13 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KQD4_MYCBP
Aconitate hydratase OS=Mycobacterium tuberculosis (strain CCDC5079)	F7WJA8_MYCTC
Catalase-peroxidase OS=Mycobacterium tuberculosis	M1JUT6_MYCTX
Possible thioredoxin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KIB4_MYCBP
Probable thiosulfate sulfurtransferase sseA OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KNT5_MYCBP
Esat6 OS=Mycobacterium riyadhense GN=esat6	B2CX99
ESX-1 secretion-associated protein EspA OS=Mycobacterium canettii CIPT 140070017 GN=BN45_100126 PE=4 SV=1	L0QZY1_9MYCO
Possible conserved transmembrane alanine and glycine rich protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KM59_MYCBP
Probable thiol peroxidase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KJZ7_MYCBP
Probable fructose-bisphosphate aldolase fba OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KFI4_MYCBP
6 kDa early secretory antigenic target esxA (Esat-6) OS=Mycobacterium tuberculosis C	A2VMQ0
Cytoplasmic peptidase OS=Mycobacterium tuberculosis (strain ATCC 35801 / TMC 107 / Erdman)	H8EU58_MYCTE
Probable acyl-CoA dehydrogenase fadE35 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KQD1_MYCBP
Bifunctional acetyl-/propionyl-coenzyme A carboxylase subunit alpha accA3 OS=Mycobacterium africanum	D6FRI7_9MYCO
Probable enoyl-CoA hydratase echa16 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KMH4_MYCBP
ATP synthase subunit alpha OS=Mycobacterium tuberculosis SUMu004	E2TXC6_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KG27_MYCBP
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KJE9_MYCBP
Probable NADP-dependent alcohol dehydrogenase adhC OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KN42_MYCBP
Trigger factor OS=Mycobacterium canettii CIPT 140070008	L0Q9X7_9MYCO
Enoyl-CoA hydratase echA21 OS=Mycobacterium tuberculosis SUMu012 GN	E2WNJ1_MYCTX
Uncharacterized protein OS=Mycobacterium bovis BCG str. Korea 1168P	M1IP81_MYCBI
Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem	R4MBZ4_MYCTX
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KN43_MYCBP
Gamma-glutamyl phosphate reductase OS=Mycobacterium canettii CIPT 140070017	L0QY66_9MYCO
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2)	A1KK78_MYCBP
Uncharacterized protein OS=Mycobacterium canettii CIPT 140060008	L0PUB7_9MYCO

5.5 Discussion

Despite the identification of a dozen antigens for development of next generation cocktails for TB diagnosis, it is challenging to replace PPD-B in spite of its specificity constraints, presumably associated with exposure to NTM. It is not known whether immune responses are elicited only when infection persists or whether colonization without disease may also lead to immune responses (Vordermeier *et al.,* 2007). Some of these next generation antigens are probably represented in PPDs but extended knowledge of the composition of PPDs, including NTM PPD preparations is crucial for the development of specific BTB assays. Knowledge of contribution of individual antigens in TB immunodiagnostic assays would allow researchers to select a combination of proteins specific to *M. bovis*/ *M. tuberculosis*. The key aim of this study was to compare the proteomes of the commercial PPDs (PPD-A and PPD-B) and those prepared from NTM field isolates or commercially available NTM strains (*eg* ATCC), in order to assess the potential of these NTM to elicit cross- reactive immune responses against *M. bovis* antigens. We have used LC-MS/MS, which is at best a semi quantitative tool. If a protein is detected it is present in the sample, but the converse is not true depending on the abundance of the protein, the level of degradation that may occur during PPD preparation as well as the efficiency of the trypsin digest (Borsuk *et al.,* 2009). Since a combination of sequenced and unsequenced mycobacteria were investigated, a Mycobacterium database derived from all Mycobacterium sequences in the UniProtKB database was searched for protein identification, to avoid biases of this analysis towards certain species. It was possible to detect as many as 561 proteins in the combined six PPD preparations by trypsin in- solution digestion, LC-MS/MS, and the database search strategy applied. Clearly, although the proteins are largely degraded during PPD preparation (Borsuk *et al.,* 2009), degradation was incomplete so that

several peptides remain intact for identification by LC-MS/MS. We detected 222 and 126 in the commercial PPD-A and PPD-B, respectively. In our NTM PPD preparations most proteins were detected in PPD-F (n=189) and PPD-N (n=159). However, only a few proteins were identified in PPD-K (n=20) and PPD-M (n=21), most likely due to degradation or lack of detection because of low abundance or expression. In case of PPD-M it could also be due to the fact that this species' genome is not available in the public database. The previously characterized immuno-dominant *M. tuberculosis/ M.bovis* antigens including: ESAT-6, CFP 10, MPB70, MBP83, EspC, MPB64, MPB63, HspX, Ag85A, Ag85C, GroES, DnaK, EspC, TpX, EsxN were detected as either unique to PPD-B or shared with the NTM PPDs (Cob and Frothingham, 1999; Vordermeier *et al.*, 2007; Wiker, 2009; Millington *et al.*, 2011; Tsolaki *et al.*, 2013, Morangui *et al.*, 2013). Identification of these proteins in PPD-B correlates with findings from previous studies (Borsuk *et al.*, 2009). The immunogenic proteins shared with NTM include the *M. tuberculosis* CFP 10 (shared between PPD-M and PPD-B), the GroEL and GroES (shared between PPD-B and all the other NTM PPDs except PPD-M, for GroES). *M. bovis* Ag85C (shared between PPD-A and PPD-B), and *M. bovis* BCG EsxN (shared between PPD-B and PPD-K). Some of the proteins described as immunogenic in *M. bovis/ M. tuberculosis* were also available in the NTM database entries and identified in the NTM PPDs. These include *M. fortuitum* MPB63 (identified in PPD- F, PPD-M and PPD- N), *M. avium* MPB64 (identified in *M. avium*), *M. fortuitum* Ag85C (identified in PPD-F, PPD-M and PPD-N), *M. fortuitum* Ag85A (identified in PPD-F and PPD-N), *M. kansasii* Ag85B (identified in PPD-K), *M. avium* MPB64 (identified in PPD-A), *M. avium* Ag85A and Ag85B (both identified in PPD-A). Identification of *M. bovis/ M. tuberculosis* immunogenic proteins in NTM PPDs warrants further investigation of these proteins as candidate BTB markers taking into serious consideration their occurrence in NTM. Of course the impact of these NTM on the host immune responsiveness will depend on the level of T-cell recognition of their 'antigens'.

Immune responsiveness induced by these NTM proteins therefore need to be evaluated in animal experiments in order to unequivocally determine their cross-reactivity. The occurrence in NTM PPDs especially in *M. fortuitum*, of these proteins described as immunogenic in *M. bovis* could explain the cross-reactive immune responses observed against PPD-F and PPD-B in the modified BOVIGAM assay in South Africa (Michel *et al.*, 2011; Michel *et al., unpublished*).

The elucidation and annotation of the genomes of NTM species may also improve investigations of NTM PPD proteomes. Draft genomes of *M. fortuitum* ATCC 6841, *M. malmesburii* sp.nov., *M. non chromogenicum* species reported in the previous chapter of this thesis, have indicated the presence of orthologs of some of the genes encoding the expressed immunogenic proteins (Gcebe *et al., unpublished*). These include *esxB* gene encoding for CFP 10 (identified in *M. malmesburii* /PPD-M), *dnaK* (Identified in *M. fortuitum-/* PPD-F, *M. malmesburii/* PPD-M and *M. nonchromogenicum/* PPD-N) and *mpb63* (identified in *M. fortuitum/* PPD-F and *M. nonchromogenicum/* PPD-N). Depending on the level of cross-recognition by T-cells, exposure to the NTM expressing these immunogenic proteins may lead to cross- reactive immune responses with *M. bovis* antigens. On the other hand, even though a large number of proteins were identified in PPD-F as compared to others among our NTM PPD preparations, some of the genes encoding immunogenic proteins that were detected in the draft genomes (Chapter 4 of this thesis) were not represented as proteins in the PPDs and these include notably ESAT-6, CFP 10 of *M. fortuitum* and *M. nonchromomogenicum*, and MPB64. This could be due to degradation of these proteins during PPD preparation, lack of expression or low abundance of expression due to which they could not be detected. Likewise, immunogenic proteins of *M. kansasii* that have been reported to be recognized bovine T-cell, like MPB70, MPB83, MPB64, ESAT-6 and CFP 10 were not detected in PPD-K, probably due to degradation during PPD preparation or strain differences leading to differences in protein

expression profiles (Woolford *et al.*, 1997; Skjøt *et al.*, 2002; Vordermeier *et al.*, 2007). The absence of these immunogenic proteins in PPD-K could lead to reduced potency of the PPD making it invaluable if it were to be used as a diagnostic marker for background NTM responses.

The identification of immunogenic proteins unique in PPD-B confirms and strengthens their use as BTB diagnostic markers. These include most notably MPB83 and EspC which were not been detected in any of the NTM PPD preparation including PPD-A in this study (Borsuk *et al.*, 2009; Wiker *et al.*, 2009; Millington *et al.*, 2011). To the best of our knowledge genes of these proteins have neither been detected in nonpathogenic NTM species, but in pathogenic *M. kansasii* and *M. marinum* (Wiker *et al.*, 2009; Millington *et al.*, 2011). Therefore serological and immunological assays employing MPB83 and EspC as antigens will probably not display any cross -reactivity due to exposure to non-pathogenic mycobacteria.

Identification of proteins unique to NTM will contribute towards investigations of NTM specific antigens as diagnostic candidates for NTM infections. Immunogenicity of these proteins is as yet unknown. Checkley *et al.*, 2010 had identified Mce family proteins as the most shared proteins among NTM, using genomics and a bio-informatics approach. These proteins were, however, not identified in the NTM PPDs in this study.

In conclusion, we have for the first time elucidated the proteome of PPDs prepared from *M. fortuitum* (PPD-F) which is used as an additional non-MAC NTM representative in the modified BOVIGAM assay. We also deciphered proteomes of other NTM species that are abundant in South Africa and other countries as well, *viz M. nonchromogenicum* (PPD-N) and *M. malmesburii* sp. nov. (PPD-M) (known to be abundant in South Africa only). We identified in these NTM, proteins that are recognized as immunogenic in *M. bovis* explaining the cross-reactive immune responses against PPD-F and PPD-B seen in the BOVIGAM assays in South

Africa. Since PPD-F has already been shown to induce immune responses (Michel *et al.*, 2011; Michel *unpublished*), and since several of the *M. bovis* immunogenic proteins detected in PPD-F are also shared with PPD-N and PPD-M, the follow up to this study will be to investigate the individual contribution of the PPD-F 'immunogenic' proteins on a T-cell recognition level.

In general the presence of immunogenic proteins in NTM PPDs warrants re-consideration of their use as diagnostic markers for BTB diagnosis, until these NTM proteins are shown not to play any role in induction of cross- reactive immune responses against bovine antigens. This study has built a foundation for such investigations.

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5.6 References

AKHTER Y, EHEBAUER MT, MUKHOPADHYAY S, HASNAIN S (2012). The PE/ PPE multigene family codes for virulence factors and is possible source of mycobacterial antigenic variation: Perhaps more. *Biochimie* **94:** 110-116.

BORSUK S, NEWCOMBE J, MENDUM TA, DELLAGOSTIN OA, MCFADDEN J (2009). Identification of proteins from tuberculin purified protein derivative (PPD) by LC-MS/MS. *Tuberculosis* 89: 423-430.

BURKE DS (1993). Of postulates and peccadilloes: Robert Koch and vaccine (tuberculin) therapy for tuberculosis. *Vaccine* **11**: 795-804.

CHECKELY AM, WYLLIE DH, SCRIBA TJ, GOLUBCHIK T, HILL AVS, HANEKON WA, MCSHANE H (2010). Identification of Antigens specific to non- tuberculous mycobacteria: The Mce family of proteins as a target of T cell immune responses. *Public Library of Science*: *One* **6 (10)**: e26434.doi:10.1371/journal.pone.0026434

COB AJ, FROTHINGHAM R (1999). The GroES antigens of *Mycobacterium avium* and *Mycobacterium parafortuitum. Veterinary Microbiology* **67**: 31-35.

DENG W, XIANG X, XIE J (2014). Comparative genomic and proteomic anatomy of mycobacterium ubiquitous esx family proteins: Implications in pathogenicity and virulence. *Current Microbiology* **68:** 558-567.

ELHAY MJ, OETTINGER T, ANDERSON P (1998). Delayed type hypersensitivity responses to ESAT-6 and MPT64 from Mycobacterium tuberculosis in guinea pig. *Infection and Immunity* **66:** 3454-3456.

GCEBE N, GEY VAN PITTIUS NC, RUTTEN V, MICHEL A (2013). Prevalence and distribution of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their environments in South Africa. *Transboundary and Emerging Diseases* **60**: 74-84.

HABERMANN B, OEGEMA J, SUNAEV S, SHEVCHENKO A (2004). The power and the limitations of cross-species protein identification by mass spectrometry-driven sequence similarity searches. *Molecular and Cellular Proteomics* doi 10.1074/mcp.M300073-MCP200.

HARBOE M, WIKER SG, LACHMAN PJ (1990). Carrier effect of concanavalin A- reactive and non- reactive material in tuberculin PPD. *Scandinavian Journal of Immunology* **32**: 263-271.

HE Z, DE BUCK J (2010). Localisation of proteins in the cell wall of *Mycobacterium avium* subsp *paratuberculosis* K10 by proteomic analysis. *Proteome Science* **8**: 21.

KELER A, NESVIZHSKII AI, KOLKER E, AEBERSOLD R (2002). Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Analytical Chemistry* **74:** 5383-5392.

KUMAR R, BHAKUNI V (2011). Comparative analysis of malate synthase G from Mycobacterium tuberculosis and *E. coli*: role of ionic interaction in modulation of structural and functional properties. *International Journal of Biological Macromolecules* **49:** 917-922.

LANDI S (1963). Preparation, purification and stability of tuberculin. *Applied Microbiology* **11**: 408-412.

LIFSON AR, WATERS JK, THOMPSON S, CRANE CM, WISE F (1993). Discrepancies in tuberculin skin test results with two commercial products in a population of intravenous drug users. *Journal of Infectious Diseases* **168**: 1048-1051.

LISKA AJ, SHEVCHENKO A (2003). Expanding the organismal scope of proteomics: Cross-species protein identification by mass spectrometry and its applications. *Proteomics* **3:** 19-28.

MARONGUI L, DONINI M, TOFFALI L, ZENARO E, DUSI S (2013). ESAT-6 and HspX improve effectiveness of BCG to induce human dendric cells- dependant Th1 and NK cells activation. *Public Library of Science*: *One* **8**: e75684.doi:10.1371/journal.pone.0075684.

MICHEL AL, COOPER D, JOOSTE J, DE KLERK LM, JOLLES A (2011). Approaches towards optimizing the gamma interferon assay for diagnosing *Mycobacterium bovis* infection in African buffalo (*Syncerus caffer*). *Preventive Veterinary Medicine* **98**: 142-151.

MILLINGTON KA, FORTUNE SM, LOW J, GARDES A, HINGLEY-WILSON SM, WICKREMASINGHE M, KON, OM, LALVANI A (2011). Rv3615 is a highly immunodominant RD1 Region of difference_ -dependent secreted antigen specific for *Mycobacterium tuberculosis* infection. *Proceedings of National Academy of Science* **108**: 5730-5735.

NESVIZHSKII AL, KELLER A, KOLKER E, AEBERSOLD R (2`003). A statistical model for identifying proteins by Tandem Mass spectrometry. *Anal Chem* **75**:4646-4658.

OIE Terrestrial Manual 2014. Bovine Tuberculosis. Chapter 2.4.7. Online at: http://www.oie.int.

PRASAD TSK, VERMA R, KUMAR S, NIRUJOGI RS, SATHE GJ, MADUGUNDU AK, SHARMA J, PUTTAMALLESHVN, GANJIWALE A, MYNEEDU VP, CHATTERJEE A, PANDEY A, HARSHA HC, NARAYANA J (2013). Proteomic analysis of purified protein derivative of *Mycobacterium tuberculosis*. *Clinical Proteomics* **10**: 8

RUPP ME, SCHULTZ AW, DAVIS JC (1994). Discordance between tuberculin skin test results with two commercial purified protein derivative preparations. *Journal of Infectious Diseases* **169**: 1174-1175.

SKJØT RLV, BROCK I, AREND SM, MUNK ME, THEISEN M, OTTENHOFF THM, ANDERSON P (2002). Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB12.9, which constitute a subfamily of the esat-6 gene family. *Infection and Immunity* **70**: 5446-5453.

TSOLAKI AG, NAGY J, LEIVA S, KISHORE U, ROSENKRANDS I, ROBERTSON (2013). *Mycobacterium tuberculosis* antigen 85B and ESAT-6 expressed as a recombinant fusion protein in *Mycobacterium smegmatis* elicits cell-mediated immune response in a murine vaccination model. *Molecular Immunology* **54**: 278-283.

VORDERMEIER HM, BROWN J, COCKLE PJ, FRANKEN WPJ, AREND SM, OTTENHOFF THM, JAHANS K, HEWINSON RG (2007). Assessment of cross - reactivity

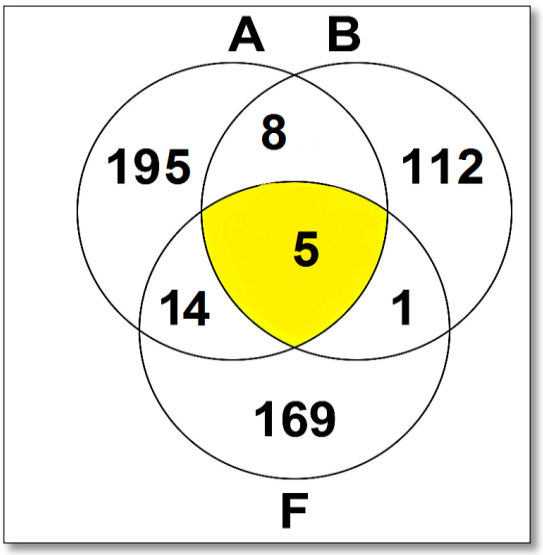
between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP 10 at the T-cell epitope level. *Clinical Vaccine and Immunology* **14:**1203-1209.

WIKER HG (2004). Unmatched sequence in public databases- exemplified by tuberculin-active protein. *Scandinavian Journal of Immunology* **59**: 607-608.

WIKER HG (2009). MPB70 and MPB83- major antigens of *Mycobacterium bovis. Scandinavian Journal of Immunology* **69:** 492-499.

WOOLFORD AJ, HEWINSON RG, WOODWARD M, DALE JW (1997). Sequence heterogeneity of an mpb70 gene analogue in *Mycobacterium kansasii*. *FEMS Microbiology Letters* **148**: 43-48.

WOWER IK, JAHAN N, ZWIEB C, WOWER J (2013). Ribosomal protein S1: An important trans-translation factor. *Biochemistry and Physiology* S2:001.doi:10.4172/2168-9652.S2-001.



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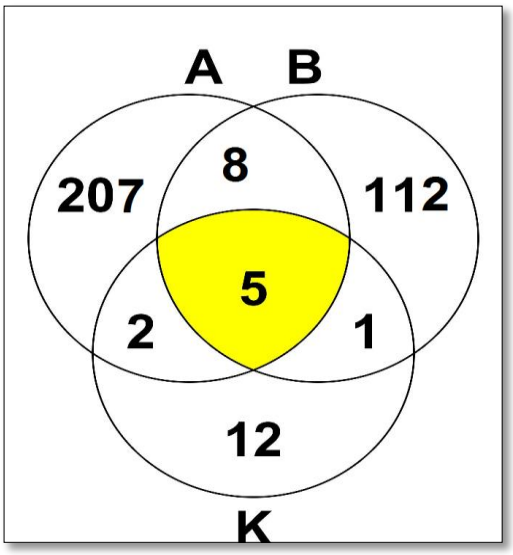

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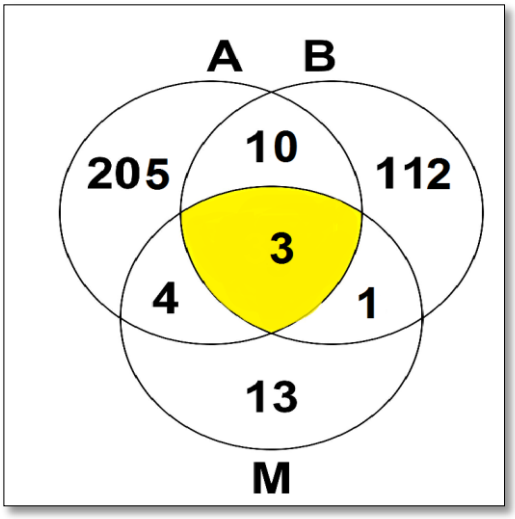

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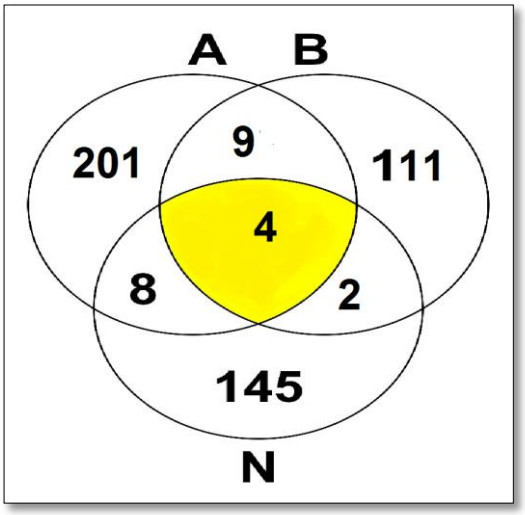

d

Fig 5.1: Venn diagrams illustrating overlap of proteins identified among a) PPDA-PPD-B, PPD-F, b) PPD-A, PPD-B, PPD-K, c) PPD-A, PPD-B, PPD-M, d) PPD-A, PPD-B and PPD-N

Chapter 6. Genes encoding highly immunogenic WXG 100 family proteins present in the genomes of non-tuberculous Mycobacterium (NTM) species

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6.1 Abstract

The Esx family proteins of *M. bovis* and *M. tuberculosis* have been evaluated as candidates for TB diagnosis in both humans and animals. The immunodominant ESAT-6 and CFP 10 proteins encoded by *esxA* and *esxB* genes respectively have been shown to be absent in *M. bovis* BCG vaccine and also lack sequence homology with other members of the Esx family as a consequence they have been used to differentiate between infected and vaccinated individuals. The presence of homologues of proteins of EsxA, EsxB and other members of the Esx family proteins in NTM (both pathogenic and non -pathogenic) and *M. leprae* pose limitations to their use for specific diagnosis of TB. In this study we evaluated various rapidly growing NTM species for the presence of *esxA* and *esxB* genes using primers whose sequences were derived from the *M. smegmatis* MC^2155 orthologs. Among the rapidly growing mycobacteria (RGM) *M. fortuitum* was the only species in addition to *M. smegmatis* that we could demonstrate to have sequences of both *esxA* and *esxB*. The *esxA* and *esxB* orthologs were also detected in two isolates belonging to *M. septicum-peregrinum* group (*esxA*) and *M. mageritense* (*esxB*), respectively. Either no amplification or non-specific amplification of both gene fragments was demonstrated in the following RGM: *M. acapulcensis, M. paraffinicum, M. chitae, M. confluentis, M. vaccae/ M. vanbaalenii, M. parafortuitum, M. austroafricanum, M. neoaurum, M. elephantis, M. engbackii* and *M. moriokaense*. These results confirm the presence of *esxA* and *esxB* in non-pathogenic NTM, and *M. fortuitum, M. mageritense* and *M. septicum/ M. peregrinum* stood out among the rest. Their presence in NTM may pose limitations to their use in diagnostic assays for tuberculosis.

Key words: esxA, esxB, non-tuberculous mycobacteria

6.2 Introduction

The elucidation of the genomes of *M. bovis* and *M. tuberculosis* revealed 23 genes (11 gene pairs and singleton, esxQ) named *EsxA* to *esxW*, related to the WXG100 family, which is characterized by a size of ~100 amino acids and the presence of a Trp-Xaa-Gly (W-X-G) motif (Pallen *et al.*, 2002). Proteins encoded by these genes are among the most immuno-dominant antigens recognised by the bovine immune system (Brodin *et al.*, 2005). RD1 encoded ESX-1 locus proteins (ESAT-6 encoded by *esxA* and CFP 10 encoded by *esxB*) are the most widely investigated for immunodiagnosis of TB in both humans and animals (Vordermeier *et al.*, 2007; Ganguly *et al.*, 2008). ESAT-6 and CFP 10 have been shown to lack sequence homology with other Esx family proteins of *M. bovis* and *M. tuberculosis* and they are absent in *M. bovis* BCG, thus they are used to differentiate between TB infection and *M. bovis* BCG vaccination (Mustafa *et al.*, 2013). Genes encoding these proteins have been described in both pathogenic and non-pathogenic NTM but so far investigations of immunodominance of the NTM homologues of these proteins have mainly focussed on pathogenic NTM that are phylogenetically related to *Mycobacterium tuberculosis* complex like *M. kansasii* and *M. marinum* (Vordermeier *et al.*, 2007) . This is despite the occurrence of these genes in non-pathogenic mycobacteria like *M. smegmatis* (Gey van Pittius *et al.*, 2001), *M. riyadhense* (van Ingen *et al.*, 2009), *M. gastri* (Congalel *et al.*, 2005), *M. fortuitum*, *M. malmesburii* sp. nov., *M. komanii* sp. nov., *Mycobacterium.* sp. JLS, *M. farcinogenes* and *M. vulneris* (Gcebe *et al.*, unpublished). Contrasting results regarding the occurrence of *esxA* and *esxB* in *M. flavescens* were also reported (Harboe *et al.*, 1996; van Ingen *et al.*, 2009), which were probably due to technical reasons like the difference in primer sequences used for screening. The *M. leprae* homologues of ESAT-6 and CFP 10 have sequence homologies of 32% and 40% to the *M. bovis* protein counterparts, and have been shown to induce significant immune responses that

may be used for diagnosis of leprosy (Geluk *et al.*, 2002, Geluk *et al.*, 2004). The presence of ESAT-6 (*esxA*) and CFP 10 (*esxB*) in NTM as well as in *M. leprae* pose potential limitations for their use for specific diagnosis of bovine tuberculosis (BTB).

It is very apparent that the ESX-1 locus is a characteristic of both pathogenic and non-pathogenic mycobacteria and therefore extensive investigation of the protein products of this locus is needed taking into consideration their occurrence in NTM.

This prompted us to investigate the presence of *esx* genes of this locus (*esxA* and *esxB*) in the genomes of NTM species available in our laboratory database. Other studies assessing NTM for the presence of *esxA* and *esxB* have used primers designed from the *M. bovis/ M. tuberculosis* sequences of these genes (Hughes *et al.*, 2005; van Ingen *et al.*, 2009). We reasoned that the *esxA* and *esxB* genes sequences from rapidly growing NTM species by virtue of them being phylogenetically distant from the *M. bovis/M. tuberculosis* will have sequence differences to the *M. bovis* and *M. tuberculosis* orthologs. Alignment of the *esxA* and *esxB* nucleotide sequences of *M. smegmatis* MC^2155 and *M. tuberculosis/ M. bovis* showed sequence differences as illustrated in figures 1A and 1B. We hypothesised that primer sequences derived from a rapidly growing Mycobacterium (RGM) species will have a greater chance to anneal and amplify gene sequences of other RGM. Using primers designed from the *M. smegmatis* MC^2155 sequences of the two genes, isolates belonging to different NTM species available in our laboratory collection were screened for the presence of *esxA* and *esxB* sequences by PCR and sequencing. This study is aimed to contribute towards the investigation on NTM cross-reactivity with *M. bovis* antigens and is therefore believed to assist in the development of BTB diagnostic assays with increased specificity in the long term.

6.3 Materials and Methods

6.3.1 Origin of mycobacterial strains

With the exception of the ATCC strains all the NTM isolates were derived from different sources and were previously identified to species level (Gcebe *et al.*, 2013).

6.3.2 Invitro amplification and sequencing

***Primer design and evaluation*:**

Primers were designed from the *M. smegmatis* nucleotide sequences of the different genes using NCBI primer BLAST. The *M. smegmatis* sequences of *esxA* and *esxB* were retrieved from Smegmalist database (http://mycobrowser.epfl.ch/smegmalist.html). The primer sequences for the respective genes are illustrated in table 6.1 and figures 1A and 1B respectively. The primers were first evaluated on *M. smegmatis* ATCC 14468 by PCR and sequencing.

Table 6.1: Primer sequences used in the study

Gene and size (bp)	Primer sequences	Position in the gene	Expected product size in *M. smegmatis* (bp)
esxA	Forward:5' aatttcgccggtatcgaggg 3' Reverse: 5' caggcaaacattcccgtgac 3'	19-38 287-268	269
esxB	Forward: 5' gcgaatttcgagcgcatctc 3' Reverse: 5' gatgttcatcgacgacgcaag 3'	46-65 300-280	255

Polymerase Chain Reaction (PCR):

Boiled culture suspensions were prepared from colonies as DNA template for amplification. The following PCR conditions were used for amplification of the two gene fragments: A 50 µl PCR mixture was prepared, containing 28.5 µl de-ionised water, 3 µl $MgCl_2$ (25 mM), 1 µl dNTP mix (10 mM), 4.75 µl of 10x PCR buffer (160 mM) (Tris -HCl, $MgCl_2$, Tween 20, $(NH_4)_2$, SO_4), 0.75 µl Taq DNA Polymerase (5 U/ µl) (Supertherm ^{TM}), 1 µl of each forward and reverse primers (50 pmol) and 10 µl of DNA template. The PCR cycling parameters were as follows: Fourty five cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and elongation at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were ran on a 1.5% agarose gel and visualised under ultra violet light (UV).

Sequencing and sequence analysis:

Sequencing of the PCR products was done at Inqaba Biotechnologies (Pretoria, South Africa). Sequencing was performed in both directions using the forward and reverse primers sequences

that were initially used for amplification. Sequences from both strands were edited manually and pairwise alignments undertaken using the BioEdit Sequence alignment editor (version 7.1.9) and Molecular Evolutionary Genetics Analysis (MEGA) platform (www.megasoftware.net) (version 6) (Tamura *et al.*, 2013). The resulting consensus sequences were analysed on the NCBI platform for gene sequence identity/ similarity using Basic local alignment tool (BLAST) (www.blast.ncbi.nlm.nih.gov/Blast.cgi).

6.4 Results

6.4.1 Evaluation of the primers

Amplification of the *esxA* and *esxB* gene fragments was demonstrated in *M. smegmatis* ATCC 14468 strain, (yielding products of between 250bp -300bp). BLAST search results for the respective gene fragment sequences indicated that the amplified sequence using *esxB* primers was 100% identical to *M. smegmatis* INHR2 strain (CP009496.1), position 87041-87295 of the genome sequence, and the amplified sequence when *esxA* primers were used was also *M. smegmatis* INHR2 strain (CP009496.1), position 87348-87617 of the genome sequence.

6.4.2 Screening of NTM for the presence of *esxA* and *esxB*

Among the RGM as shown in table 6.2 , *M. fortuitum* ATCC 6481 is the only species that amplified both e*sxA* (~269bp) and *esxB* (~255bp) in PCR, while an isolate belonging to the *M. septicum-peregrinum* group amplified for *esxA* (~265bp) and another that belongs to *M. mageritense* amplified for *esxB* (~256bp) PCR . No amplification was demonstrated in isolates

belonging to *M. acapulcensis* (n=3), *M. paraffinicum* (n=2), *M. chitae* (n=1), *M. confluentis* (n=1), *M. vaccae/ M. vanbaalenii* (n=5), *M. parafortuitum* (n=3), *M. engbackii* (n=3), *M. austroafricanum* (n=1), *M. madagascariense* (n=2) and *M. moriokaense* ATCC 43059 strain (n=1). Isolates belonging to *M. neoaurum*, yielded the expected band size for *esxA*, but ambiguous sequences were obtained, while non-specific amplifications (~400bp) and ambiguous sequences were obtained for isolates belonging to *M. moriokaense* and *M. elephantis*. PCR for all the isolates that did not amplify for both *esxA* and *esxB* PCR (n=23) was repeated and yielded the same results. Integrity of DNA of all the isolates that yielded negative results for *esxA* and *esxB* was tested by amplification of their *hsp65* gene which amplified in all cases.

Table 6.2: Screening of RGM for *esxA* and *esxB* by PCR

Isolate ID	**RGM species**	**PCR and sequencing results**	
		esxA	*esxB*
Ladybrand S2	***M. septicum/ peregrinum complex***	**positive**	**negative**
Lucingweni swab4	*M. moriokaense*	negative	negative
M. moriokaense ATCC 43059	*M. moriokaense* ATCC 43059	negative	negative
TB 6772-O	***M. mageritense***	positive	negative
***M. fortuitum* ATCC 6481**	***M. fortuitum* ATCC 6481**	**positive**	**positive**
Smithfield H2O	*M. moriokaense*	negative	negative
Welgensra West	*M. austroafricanum*	negative	negative

M. smegmatis	***M. smegmatis ATCC* 14468**	**positive**	**positive**
Trigaarspoort swab 03027	*M. komanii* sp.nov.	negative	negative
W. cape swap 5	*M. malmesburii* sp.nov.	negative	negative
C4	*M. malmesburii* sp.nov.	negative	negative
Xhongora swab 5	*M. vaccae/ M. vanbaalenii*	negative	negative
Bothasrust swab 3	*M. vaccae/ M. vanbaalenii*	negative	negative
Tb6902	*M. vaccae/ M. vanbaalenii*	negative	negative
Knoppies swab 1	*M. vaccae/ M. vanbaalenii*	negative	negative
Kabisa s3	*M. vaccae/ M. vanbaalenii*	negative	negative
Ladybrand swab3	*M. madagascariense*	negative	negative
Virginia swab 3	*M. madagascariense*	negative	negative
Villanora swab 1	*M. acapulcensis*	negative	negative
Kanaland swab 2	*M. acapulcensis*	negative	negative
Honing S4	*M. acapulcensis*	negative	negative
Rustenburg kraal swab 1	*M. elephantis*	negative	negative
Rustenburg Welgevonden swab3	*M. elephantis*	negative	negative
Honing r116 (yellow)	*M. chitae*	negative	negative
Jakkalsfarm S2	*M. confluentis*	negative	negative
Molabedju H_2O	*M. paraffinicum*	negative	negative
Langkul S1	*M. paraffinicum*	negative	negative

Mahlamvu swab 2	*M. neoaurum*	negative	negative
Xhongorha swab 4	*M. neoaurum*	negative	negative
Xhongorha swab 4	*M. neoaurum*	negative	negative
Xhongorha S1	*M. engbackii*	negative	negative
Kabisa S1	*M. engbackii*	negative	negative
Stanop S1	*M. engbackii*	negative	negative
Ncambele swab4	*M. parafortuitum*	negative	negative
Cacadu swab3	*M. parafortuitum*	negative	negative
Ncambele swab4	*M. parafortuitum*	negative	negative

6.4.3 Sequence analysis

***EsxB*:**

BLAST search results of each of the sequences are summarised in table 6. 3a.

Table 6.3a: BLAST results of sequences obtained from *esxB* PCR

Mycobacterium species /isolate ID	Highest BLAST hit (accession number)	Sequence position in the genome of the highest hit	Identity (%)	E-value
M. smegmatis* MC^{2}155 *esxB* (255 bases)	***M. smegmatis* INHR2 (CP009496.1)**	**87041-87295**	**100**	**1e-129**
M. smegmatis ATCC 14468	*M. smegmatis* INHR2 (CP009496.1)	87041-87295	99	7e-128
M. fortuitum ATCC 6841	*M. smegmatis* INHR2 (CP009496.1)	87041-87295	85%	1e-74
TB6772 –O (*M. mageritense*)	*Mycobacterium* sp KMS (CP000518.1)	90818-91065	95%	5e-105

*, *esxB* fragment sequence of *M. smegmatis* retrieved from the Smegmalist database (http://mycobrowser.epfl.ch/smegmalist.html).

EsxA:

BLAST search results of each of the sequences is summarised in table 6.3b.

Table 6.3b: BLAST results of sequences obtained from *esxA* PCR

Mycobacterium species	Highest BLAST hit (accession number)	Sequence position in the genome of the highest hit	Identity (%)	E-value
M.smegmatis* MC^{2}155 *esxA* (288 bases)	***M.smegmatis* INHR2/ *M. smegmatis* MC^{2}155 (CP009496.1/ CP009495.1)**	**87330-87617**	**100**	**4e-144**
M. smegmatis ATCC 14468	*M. smegmatis* INHR2 /*M. smegmatis* MC^{2}155 (CP009496.1/ CP009495.1)	87348-87617	99	5e-130
M. fortuitum ATCC 6841	*M. smegmatis* INHR2 (CP009496.1)	87348-87617	89	2e-29
(*M. septicum /peregrinum*) (Ladybrand S2)	*M. smegmatis* INHR2/ *M. smegmatis* MC^{2}155 (CP000518.1)	87348-87617	88%	2e-89

*****, *esxA* fragment sequence of *M. smegmatis* retrieved from the Smegmalist database (http://mycobrowser.epfl.ch/smegmalist.html).

6.5 Discussion

To improve the current understanding of the role of NTM, in particular non-pathogenic RGM in host immune responsiveness, we set out experiments to screen NTM isolates belonging to different RGM species for the presence of *esxA* and *esxB*. Other investigators have used primers designed from *M. tuberculosis/ M. bovis* and possibly specific to pathogenic mycobacteria to screen both slow growing mycobacteria (SGM) and as well as RGM (van Ingen *et al.*, 2009). In this study we used PCR primers derived from *M. smegmatis* sequences of these genes in a PCR- sequencing assay as we reasoned that such primers would be more specific to RGM as they are phylogenetically closely related to each other (Devulder *et al.*, 2005). We demonstrated the presence of both these gene fragments in *Mycobacterium fortuitum* ATCC 6841 whose nucleotide sequences were 89% and 85% identical to the *M. smegmatis esxA* and *esxB*, respectively. The *M. septicum/ peregrinum* complex isolate showed the presence of *esxA* and the *M. mageritense* isolate's DNA amplified with the *esxB* primers and the resulting sequence was 95% similar to *Mycobacterium* sp KMS *esxB* at nucleotide level . It is possible that the *esxB* and *esxA* gene sequences did not amplify in the two species, respectively, as well as in *M. malmesburii* sp. nov. and *M. komanii* sp. nov. even though these species (*M. komanii* sp.nov. and *M. malmesburii* sp.nov.) were reported in Chapter 4 of this thesis to harbor both gene orthologs in their genomes, due to the fact that the primer sequences used were not specific to these NTM. Therefore a combination of primers derived from different species may be necessary for these investigations. Identification of the *esx* genes of the ESX-1 locus genes (*esxA* and *esxB*) in *M. fortuitum* and one of each gene fragment in *M. septicum/ peregrinum* and *M. mageritense*, respectively, confirms that the *esx* genes of this locus do occur in non-pathogenic RGM species other than *M. smegmatis*, *M. flavescens* and *Mycobacterium.* sp. JLS but with sequence differences (Harboe *et al.*, 1996; Gey van Pittius *et al.*, 2001; Gcebe *et al.*,

unpublished). Identification of e*sxA* and *esxB* genes in these non-pathogenic NTM is a major concern as their protein products are widely used as specific markers for BTB diagnosis in the BOVIGAM assay. The presence and thus recognition of these NTM antigens by T-cell theoretically lowers the specificity of the BOVIGAM assay as experienced previously in African buffalo (Michel, 2008). This investigation will be extended to other NTM isolates available in our laboratory collection. However, more robust tools like use of a combination of primers designed from both non-pathogenic and pathogenic mycobacteria may be useful in screening both the RGM and SGM for the presence of these genes. This approach will be a more cost effective way of characterizing a number of NTM for the presence of *esxA* and *esxB* since only a handful of Mycobacterium genomes have been sequenced, probably due to the cost involved in whole genome sequencing studies.

In conclusion we have demonstrated occurrence of *esxA* and e*sxB* in *Mycobacterium fortuitum* which potentially may have an effect for their use as markers for TB diagnosis. In *M. septicum/ peregrinum* complex and *M. mageritense, esxA* and *esxB* genes were found to occur as singleton respectively. The occurrence of these two genes in NTM should be a subject of investigation when designing immunodiagnostic assays for TB. Characterization of NTM for the presence of genes like *esxA* and *esxB* that play part in immune responses should form part for future studies describing new species.

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6.6 References

BRODIN P, DE JONGE MI, MAJLESSI L, LECLERC C, NILGES M, COLE ST, BROSCH R (2005). Functional analysis of early secreted antigenic target-6, the dominant T-cell antigen of *Mycobacterium tuberculosis,* reveals key residues involved in secretion, virulence and immunogenicity. *Journal Biology and Chemistry* **280**: 33953-33959.

COLANGELI R, SPENCER JS, BIFANI P, WILLIAMS A, LYASHCHENKO K, KEEN MA, HILL PJ, BELISLEJ, GENNARO ML (2000). MTSA-10, the product of the Rv3874 gene of *Mycobacterium tuberculosis,* elicits tuberculosis specific delayed type hypersensitivity in guinea pigs. *Infection and Immunity* **68**: 990-993.

GCEBE N, GEY VAN PITTIUS NC, RUTTEN V, MICHEL A (2013). Prevalence and distribution of non-tuberculous mycobacteria (NTM) in cattle, African buffaloes (*Syncerus caffer*) and their environments in South Africa. *Transboundary and Emerging Disease* **60**: 74-84.

GELUK A, VAN MEIJGAARDEN KE, FRANKEN KLMC, SUBRONTO YW, WIELES B, AREND SM, SAMPIAIO EP, DE BOER T, FABER WR, NAAFS B, OTTENHOGFF THM (2002). Identification and characterisation ESAT-6 homologue of *Mycobacterium leprae* and T-cell cross reactivity with *Mycobacterium tuberculosis*. *Infection and Immunity* **70**: 2544-2548.

GELUK A, VAN MEIJGAARDEN E, FRANKEN KL, WIELES B, AREND SM, FABER WR, NAAFS B, OTTENFOFF TH (2004). Immunological cross-reactivity of the *Mycobacterium leprae* CFP-10 with its homologue in *Mycobacterium tuberculosis. Scandinavian Journal of Immunology* **59:** 66-70**.**

GEY VAN PITTIUS NC, GAMIELDIEN J, HIDE W, BROWN GD, SIEZEN RJ, BEYERS A (2001). The ESAT-6 gene cluster of *Mycobacterium* and other high GC Gram-positive bacteria. *Genome Biology* **2**: research0044.1-0044.18

HARBOE M, OETTINGER T, WIKER HG, ROSENKRANDS I, ANDERSEN P (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infection and Immunity* **64**: 16-22.

HLOKWE TN, VAN HELDEN P, MICHEL AL (2014). Evidence of increasing intra and inter-specifies transmission of *Mycobacterium bovis* in South Africa: Are we losing the battle? *Preventative Veterinary Medicine* **115:** 10-17.

HUGHES MS, BALL NW, MCCARROLL J, ERSKINE M, TAYLOR MJ, POLLOCK JM, SKUCE RA, NEILL SD (2005), Molecular analysis of mycobacteria other than the *M. tuberculosis* complex isolated from Northern Ireland cattle. *Veterinary Microbiology* **108**: 101-112.

MICHEL A (2008). *Mycobacterium fortuitum* infection interference with *Mycobacterium bovis* diagnostics: natural infection cases and a pilot experimental Infection. *Journal of Veterinary Diagnostic Investigation.* **20**:501-503.

PALLEN MJ (2002). The ESAT-6/ WCG 100 superfamily- and a new Gram positive secretion system? *Trends Microbiol* **10**: 209-212.

TAMURA, K., STECHER, G., PETERSON, D., FILIPSKI, A., KUMAR, S. (2013). MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution* 30: 2725-2729.

THOMPSOM, J.D., HIGGINS, D.G., GIBSON, T.J (1994). CLUSTAW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic acids Research* **22**:4673-4680.

VAN INGEN J, DE ZWAAN R, DEKHUIJZEN R, BOEREE M, VAN SOOLINGEN D (2009). Region of difference 1 in non- tuberculous *Mycobacterium* species adds a phylogenetic and taxonomic character. *Journal of Bacteriology* **191**: 5865-5867.

VORDERMEIER HM, BROWN J, COCKLE PJ, FRANKEN WPJ, AREND SM, OTTENHOFF THM, JAHANS K, HEWINSON RG (2007). Assessment of cross - reactivity

between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP 10 at the T-cell epitope level. *Clinical Vaccine and Immunol* **14:** 1203-1209.

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
M. smegmat      ATGACAGAAC AGGTATGGAA TTTCGCCGGT ATCGAGGGCG GCGCGTCGGA
M. tubercu      ATGACAGAGC AGCAGTGGAA TTTCGCGGGT ATCGAGGCCG CGGCAAGCGC
                ********** ***  ***** ****** *** ******* **   **    *

                ....|....| ....|....| ....|....| ....|....| ....|....|
                         60         70         80         90        100
M. smegmat      GATCCACGGC GCCGTGTCCA CCACGGCCGG TCTGCTCGAC GAGGGCAAGG
M. tubercu      AATCCAGGGA AATGTCACGT CCATTCATTC CCTCCTTGAC GAGGGGAAGC
                 ***** **     **      ***          **  * *** ***** ***

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        110        120        130        140        150
M. smegmat      CCTCGCTGAC CACTCTCGCC TCGGCGTGGG GCGGCACCGG TTCGGAGGCC
M. tubercu      AGTCCCTGAC CAAGCTCGCA GCGGCCTGGG GCGGTAGCGG TTCGGAGGCG
                  ** ***** **  *****   ********* **** *  ** **********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        160        170        180        190        200
M. smegmat      TACCAGGCCG TCCAGGCCCG TTGGGACTCC ACCTCCAACG AGCTGAACCT
M. tubercu      TACCAGGGTG TCCAGCAAAA ATGGGACGCC ACGGCTACCG AGCTGAACAA
                *******  * ******      ****** ** **  * * ** ********

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        210        220        230        240        250
M. smegmat      GGCACTGCAG AACCTCGCCC AGACCATCAG CGAGGCGGGC CAGACCATGG
M. tubercu      CGCGCTGCAG AACCTGGCGC GGACGATCAG CGAAGCCGGT CAGGCAATGG
                 ** ****** ***** ** *  *** ***** *** *****  *** *  ***

                ....|....| ....|....| ....|....| ....|...
                        260        270        280
M. smegmat      CGCAGACCGA GGCCGGCGTC ACGGGAATGT TTGCCTGA
M. tubercu      CTTCGACCGA AGGCAACGTC ACTGGGATGT TCGCATAG
                *   ******  * *  **** ** ** **** * ** *
```

Fig 6.1a: Alignment of *esxA* nucleotide sequences of *M. smegmatis* MC^{2}155 (M. smegmat) *and M. tuberculosis* H37Rv (M. tubercu). *, represents identical sequences, and primers sequences used are underlined.

```
                ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50
M. smegmat      ATGGCAGCAA TGAATACAGA TGCCGCCGTC CTCGCCAAGG AGGCGGCGAA
M. tubercu      ATGGCAGAGA TGAAGACCGA TGCCGCTACC CTCGCGCAGG AGGCAGGTAA
                *******  * **** ** ** ******   * *****  *** **** *  **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                        60         70         80         90        100
M. smegmat      TTTCGAGCGC ATCTCCGGCG AGCTCAAGGG CGTCATCGCG CAGGTTGAGT
M. tubercu      TTTCGAGCGG ATCTCCGGCG ACCTGAAAAC CCAGATCGAC CAGGTGGAGT
                *********  ********** * ** **    *   ****   ***** ****

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       110        120        130        140        150
M. smegmat      CCACGGGCTC CGCTCTGGCC GCTCAGATGG TCGGCCAGGC AGGCACCGCC
M. tubercu      CGACGGCAGG TTCGTTGCAG GGCCAGTGGC GCGGCGCGGC GGGGACGGCC
                * ****          **    *  ***  *   ****  ***  ** ** ***

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       160        170        180        190        200
M. smegmat      GCGCAGGCCG CGCTGGCTCG GTTCCACGAG GCCGCCGCCA AGCAGGTTCA
M. tubercu      GCCCAGGCCG CGGTGGTGCG CTTCCAAGAA GCAGCCAATA AGCAGAAGCA
                ** ******* ** ***  **  ***** **  ** ***   * *****   **

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       210        220        230        240        250
M. smegmat      GGAGTTGAAC GAGATCTCGG CCAACATCCA CACCTCGGGC ACGCAGTACA
M. tubercu      GGAACTCGAC GAGATCTCGA CGAATATTCG TCAGGCCGGC GTCCAATACT
                ***  *  ** *********  * ** ** *       * ***    ** ***

                ....|....| ....|....| ....|....| ....|....| ....|....|
                       260        270        280        290        300
M. smegmat      CCTCGACCGA CGAGGACCAG GCGGGCACGC TTGCGTCGTC GATGAACATC
M. tubercu      CGAGGGCCGA CGAGGAGCAG CAGCAGGCGC TGTCCTCGCA AATGGGCTTC
                *   * **** ****** ***   *    *** *  * ***     **  * **

                ...

M. smegmat      TGA
M. tubercu      TGA
                ***
```

Fig 6.1b: Alignment of *esxB* nucleotide sequences of *M. smegmatis* MC^{2}155 (M. smegmat) and *M. tuberculosis* H37Rv (M. tubercu). *, represents identical sequences, and primers sequences used are underlined.

Chapter 7. Discussion and Conclusions

The aim of this study was to investigate the diversity and prevalence of NTM in cattle, African buffaloes and their environments in South Africa and the potential of these NTM to elicit cross-reactive immune responses in these animal species. In this chapter we present the summary and discussion of the results and conclusions.

Cattle and African buffaloes were our targeted species since they are both maintenance hosts of bovine tuberculosis (BTB) in South Africa. Furthermore, no country-wide NTM survey has ever been conducted in South Africa, so it was necessary for us to first conduct such a study in order to address the question of the likelihood of exposure of cattle and African buffaloes to certain NTM species. Mycobacteria were isolated from 56% of the samples collected during the survey and from retrospective samples that were collected over the years from routine tuberculosis diagnostic samples. Of the 420 isolates that were analysed for species identification, 40 different NTM species were identified from 303 isolates, while 117 were not identified to species level. Of the 117 isolates, 79 isolates were found to be closely related to 23 known NTM species, and 38 isolates were found to be potential novel species that are not currently listed in the RIDOM and NCBI BLAST databases. These results indicated that NTM, which cattle and African buffaloes are exposed to in South Africa, are abundant and diverse. Furthermore, the discovery of several unknown NTM in South Africa suggest the occurrence of a number of as yet uncharacterized *Mycobacterium* species and warranted further characterization of these species. The four NTM species or closely related groups most frequently isolated in this study included *Mycobacterium terrae*, a group of mycobacteria closely related to *Mycobacterium moriokaense* (referred to as *M. moriokaense*-like organisms),

Mycobacterium nonchromogenicum and *Mycobacterium vaccae/ M. vanbaalenii.* Therefore cattle and African buffaloes are most likely to be exposed to the four NTM in South Africa. Some of these NTM have been isolated as the most frequently occurring species in several other studies elsewhere. A study in Ethiopian cattle identified *M. nonchromogenicum* as a predominant NTM species (Berg *et al.,* 2009) and in a study in Chad published in 2006, *M. nonchromogenicum* together with MAC and *M. fortuitum* were found to be common in humans and cattle (Diguimbaye-Djaibé *et al.,* 2006). *M. nonchromogenicum* and *M. terrae* were also identified as among the most abundant in the environment in a study in Zaire (Portaels, 1995). *M. terrae* was isolated as a frequently occurring species in a study in Ethiopian wildlife (Tschopp *et al.,* 2010), and was isolated from cattle in Tanzania (Kazwala, *et al.,* 1998; Cleaveland *et al*, 2005), and in the wildlife environment in South Africa (Michel *et al.*, 2007). In Uganda, Kankya *et al.,* 2011 identified *M. nonchromogenicum, M. fortuitum* complex, *M. avium* complex and *M. gordonae* as most frequent species in pastoral ecosystems. *M. nonchromogenicum* has also been detected in small mammals and cattle in Tanzania (Durnez *et al.,* 2011).This suggests that *M. nonchromogenicum* and *M. terrae* are ubiquitous NTM species in Africa although percentages differ in different countries. *M. nonchromogenicum* have also been isolated from cattle in Great Britain and Ireland (Vordermeier *et al.,* 2007; Hughes *et al.,* 2009). NTM closely related to *M. moriokaense* have been isolated previously in a buffalo and environmental samples in South Africa (Michel *et al.,* 2008). The effects of these NTM species, except *M. fortuitum* in immune responsiveness of animals are unknown, but they can potentially induce cross- reactive immune responses to *M. bovis* antigens and therefore needed to be investigated further (Vordermeier *et al.,* 2007; Hughes *et al.,* 2009; Michel *et al.,* 2011).

The abundance of *M. moriokaense*-like isolates prompted us to further characterize them to species level. Phenotypic characterization, multiple gene sequence characterization, as well as

their phylogenetic analysis revealed that these isolates belong to two novel NTM species that are closely related. We proposed *M. malmesburii* sp. nov. and *M. komanii* sp. nov. as names for these two new species.

To investigate the potential of NTM to induce cross-reactive immune responses to *M. bovis* antigens, we conducted comparative genomics as well as proteomic analysis of the NTM and *M. bovis*. We searched the genomes of four NTM species which were among the abundant in our study in South Africa (*M. nonchromogenicum*, *M. malmesburii* sp. nov., and *M. komanii*) and *M. fortuitum* ATCC 6841 for shared genes encoding proteins that have been described as immunogenic in *M. bovis*. *M fortuitum* ATCC 6841 was included in the study because a PPD prepared from it is included in the modified BOVIGAM assay in South Africa following its isolation from BTB free buffaloes that tested positive on the PPD based BOVIGAM assay (Michel *et al.,* 2008; 2011). Furthermore *M. fortuitum* has been isolated as a frequently occurring species in cattle in several studies. The comparative genomics study revealed that these NTM's closest relatives were other NTM species *viz M. smegmatis, M. rhodesiae* and *Mycobacterium* sp. JLS and very little similarities in their whole genomes were observed between these NTM and *M. bovis* AF2122/97 as well as *M. tuberculosis* H37Rv genomes. However, though there were huge differences between the genomes of NTM and those of *M. bovis* as well as *M. tuberculosis*, orthologs (with amino acid sequence similarity of ≥ 50%) of some of the targeted genes encoding immunogenic proteins as well as immunogenic epitopes existed in NTM. Genes encoding proteins of the Esx- family (*esx* genes), in particular those encoded in the ESX-1 locus (*esxA* and *esxB*) and ESX-3 (*esxH*, *esxG)* as well as *esxR* were present in the NTM genomes. These genes were of interest to us because their proeten products have been extensively investigated for their role in immune response resulting in their application in TB diagnosis for differentiation of infected from vaccinated animals (DIVA) as well as vaccine candidates (Vordermeier *et al.,* 2000; Skjøt *et al.,* 2000; Vordermeier *et al.,*

2007; Hoang *et al.*, 2013). In NTM, it is mainly in pathogenic slow growing NTM that are phylogenetically closely related to MTBC; like *M. kansasii* and *M. marinum* as well as in *M. leprae* that proteins products of the orthologs of *esxA* and *esxB* have been investigated for their role in immune responses (Geluk *et al.*, 2002; Vordermeier *et al*., 2007). Among the NTM sequenced in this study, *M. fortuitum, M. nonchromogenicum, M. malmesburii* sp. nov., and *M. komanii* sp.nov were found to harbor both *esx* genes of the ESX-1 locus, with the *M. fortuitum* and *M. nonchromogenicum* orthologs of both genes showing the highest amino acid sequence similarity to the *M. bovis esxA* (75.79% and 71.58 respectively) and *esxB* (64% for both NTM). All the four NTM annotations were found to have EsxG with *M. fortuitum* and *M. nonchromogenicum* orthologs showing the highest sequence similarities at amino acid level to *M. bovis* EsxG (both 81.5%). *M. malmesburii* sp. nov. and *M. komanii* sp. nov. genomes had the EsxH ortholog while *M. fortuitum* and *M. nonchromogenicum* genomes had EsxR. The occurrence of the *esx* genes of the ESX-1 locus (*esxA* and those of ESX-3 (*esxG, esxH)* as well as *esxR* in non- pathogenic NTM annotations in this study and in other NTM genomes available in the NCBI Genbank database suggests that perhaps, despite sequence differences, the Esx family proteins encoded in these loci are not only characteristics of pathogenic, slow growing mycobacteria, but do occur in several non-pathogenic RGM. This view supports several studies that detected orthologs of these genes in other non-pathogenic RGM like *M. smegmatis, M. flavescens* and *M. mageritense* (Harboe *et al.*, 1996, Colangeli *et al*., 2000; Gey van Pittius *et al*., 2001). Comparative genomics study by Gey van Pittius *et al* 2001 also noted the occurrence of ESX-3 in *M. smegmatis, M. leprae,* and *M. avium*. Expression of the NTM orthologs of the *esx* genes situated in both these loci (ESX-1 and ESX-3) in non-pathogenic RGM has not been investigated, despite their occurrence in several of these species. However; if expressed in these NTM they could induce cross reactive immune responses that interfere with BTB diagnosis.

The identification of CDS for the other *M. bovis* immunogenic proteins like CanA (Rv1315) (identified in all four NTM), HspX (identified in *M. fortuitum* and *M. nonchromogenicum*), MPB70 (identified in *M. malmesburii* sp.nov. and *M. komanii* sp.nov), MPB63 (identified in *M. fortuitum* and *M. nonchromogenicum*), TpX (identified in *M. fortuitum* and *M. nonchromogenicum*), DnaK (identified in all four NTM annotations), Rv1120c (identified in all NTM) and MPB64 (identified in all NTM) in the NTM annotations, including their closely related species, also suggest the potential of these proteins to be expressed and therefore these NTM can potentially cause cross-reactive immune responses against *M. bovis* antigens leading to false positive diagnosis of the disease.

Since we could not predict if the NTM proteins could be recognized by T-cells based on the overall sequence homology to the *M. bovis* homologues, we compared protein sequences at epitopic level. *M. bovis* immunogenic epitopes were found in the NTM homologues, although there were sequence differences except for two EsxR/EsxH epitopes. For sequence homologies of less than 100% we could not unambiguously predict if the NTM homologues could give rise to cross reactive immune responses except for the two EsxR/EsxH epitopes (100% identical to the *M. bovis* EsxR epitopes) which are most likely to induce cross reactive immune responses. Therefore antigen cross- recognition as well as species specificity of these NTM proteins needs to be investigated at individual peptide level recognition by T- cells from infected cattle.

Using the comparative proteomics approach of the NTM PPD preparations from *M. fortuitum* ATCC 6841, *M. malmesburii* sp. nov., *M. nonchromogenicum* and *M. kansasii* ATCC 12478 and the commercial PPD- A and PPD-B we identified shared immunogenic proteins between PPD-B and NTM PPDs suggesting these NTM species could potentially induce cross reactive immune responses against the *M. bovis* antigens depending on the level of T-cell recognition. The shared immunogenic proteins included the *M. tuberculosis* CFP 10 (shared between PPD-M and PPD-B), the GroEL and GroES (shared between PPD-B and all the other NTM PPDs

except PPD-M, for GroES). *M. bovis* Ag85C (shared between PPD-A and PPD-B), and *M. bovis* BCG EsxN (shared between PPD-B and PPD-K). These NTM proteins therefore need to be evaluated in animal experiments in order to unequivocally determine their cross-reactivity. We reasoned that occurrence of these proteins in NTM that are recognized as immunogenic in *M. bovis* explains the immune responses against PPD-F and PPD-B seen in the BOVIGAM assays in South Africa (Michel *et al.,* 2011). Therefore since PPD-F is already been shown to induce immune responses, and since several of these immunogenic proteins are shared among PPD-F, PPD-N and PPD-M , the follow up to this study will be to investigate the individual contribution of its proteins whose homologues are known to be immunogenic in *M. bovis*, on a T-cell recognition level. The identification of PPD-B unique proteins that do not have non-pathogenic NTM homologues further confirms and strengthens their use as BTB diagnostic markers. These include most notably MPB83 and EspC which were only detected in PPD-B and not in any of the NTM PPD preparation including PPD-A (Borsuk *et al.,* 2009; Wiker *et al.,* 2009; Millington *et al.,* 2011).However these two proteins have been previously identified in pathogenic NTM like *M. kansasii* and *M. marinum* (Wiker *et al.,* 2009; Millington *et al.,* 2011)

Screening of NTM isolates available in our laboratory database for the ESX-1 locus genes (*esxA* and *esxB*) was motivated by the fact that this locus seemed to be a characteristic of both pathogenic slow growing and non-pathogenic RGM, and also protein products of these two genes have been investigated as candidate markers for BTB diagnosis and used in DIVA strategies (Vordermeier *et al.,* 2009). Since it was apparent that there were sequence differences between the RGM and MTBC orthologs of *esxA* and *esxB*, we reasoned that using oligonucleotides designed from RGM sequences would increase our chances of detecting these genes in RGM. Using primer sequences designed from *M. smegmatis esxA* and *esxB* we could amplify both these two genes in *M. fortuitum* and each from *M. mageritense* and *M.*

septicum/M. peregrinum, respectively. This approach is a more cost effective way of characterizing a number of NTM for the presence of *esxA* and *esxB*, it will therefore in future be extended to other isolates available in our laboratory collection as well as well as type strains.

In conclusion in this study we determined the diversity and abundance of NTM in cattle, African buffalo and their environments through a country wide survey. We also defined the four most prevalent species or closely related groupings which cattle and African buffaloes are most likely to be exposed. We identified two novel species among the closely related species, and we named them *M. malmesburii* sp.nov. and *M. komanii* sp. nov. respectively (currently under peer review). Using the combined genomics and proteomics approach, we identified NTM homologues of *M. bovis* immunogenic proteins in four NTM species, demonstrating the potential of these NTM species to induce cross-reactive immune responses against *M. bovis* antigens. *M. fortuitum* homologues of these proteins could be responsible for the reactions seen against PPD-F and PPD-B in South Africa. Hence further investigation of this PPD antigens on T-cell recognition level will give insight into individual contribution of these proteins.

Lastly we screened RGM for *esxA* and *esxB* using PCR and found their occurrence in *M. fortuitum, M. septicum/ M. peregrinum* and *M. mageritense* further confirming that the ESX-1 locus is a characteristic of all mycobacteria and therefore use of its protein products as markers for BTB should seriously consider their occurrence in NTM. We also recommend that future studies describing new *Mycobacterium* species should include characterization of *esxA* and *esxB* in those species.

The knowledge gained in this study will hopefully help researchers to make informed decisions when selecting markers for BTB diagnosis. This study has built a foundation for such investigations.

References

ABDALLA AM, SAVAGE ND, VAN ZON M, WILSON L, VANDENBROUKE-GRAULS CM, VAN DER WEL NN, OTTENHOFF TH, BITTER W (2008). The ESX-5 secretion system of Mycobacterium marinum modulates the macrophage response. *Journal of Immunology* **181:** 7166-7175.

BERG S, FIRDESSA R, HABTAMU M, GADISA E, MENGISTU A, et al. (2009). The burden of mycobacterial disease in Ethiopian cattle: Implications for public health. *Public Library of Science: One* **4(4)**: edoi: 10.1371/ journal.pone.0005068.

BUDDLE BM, DENIS M, ALDWELL FE, VORDERMEIER HM, HEWINSON RG, WEDLOCK DN (2008).Vaccination of cattle with *Mycobacterium bovis* BCG by combination of systematic and oral routes. *Tuberculosis* (Edinb) **88:** 595-600.

CLEAVELAND S, MLENGEYA T, KAZWALA RR, MICHEL A, KAARE MT, JONES SL, EBLATE E, SHIRIMA GM, PACKER C (2005). Tuberculosis in Tanzanian wildlife. *Journal of Wildlife Diseases.* **41**: 446-453.

COLANGELI R, SPENCER JS, BIFANI P, WILLIAMS A, LYASHCHENKO K, KEEN MA, HILL PJ, BELISLEJ, GENNARO ML (2000). MTSA-10, the product of the Rv3874 gene of

Mycobacterium tuberculosis, elicits tuberculosis specific delayed type hypersensitivity in guinea pigs. *Infection and Immunity* **68**: 990-993.

DENG W, XIANG X, XIE J (2014). Comparative genomic and proteomic anatomy of Mycobacterium ubiquitous Esx family proteins: Implications in pathogenicity and virulence. *Current Microbiology* **68:** 558-567.

DIGUIMBAYE-DJAIBÉ C, VINCENT V, SHELLING E, HILTY M, NGANDOLO R, *et al.*, (2006). Species identification of non-tuberculous mycobacteria from humans and cattle of Chad. Schweiz Arch Tierheilkd **148**: 251-256.

DURNEZ L, KATAKWEBA A, SADIKI H, KATHOLI CR, KAZWALA RR, MACHANG'U RR, PORTAELS F, LEIRS H. (2011). Mycobacteria in terrestrial small mammals on cattle farms in Tanzania. Veterinary Medicine International. Doi:10.4061/2011/495074.

GELUK A, VAN MEIJGAARDEN KE, FRANKEN KLMC, SUBRONTO YW, WIELES B, AREND SM, SAMPIAIO EP, DE BOER T, FABER WR, NAAFS B, OTTENHOGFF THM (2002). Identification and characterisation ESAT-6 homologue of *Mycobacterium leprae* and T-cell cross reactivity with *Mycobacterium tuberculosis. Infection and. Immunity* **70:** 2544-2548.

GEY VAN PITTIUS NC, GAMIELDIEN J, HIDE W, BROWN GD, SIEZEN RJ, BEYERS A (2001). The ESAT-6 gene cluster of *Mycobacterium* and other high GC Gram-positive bacteria. *Genome Biology* **2**: research0044.1-0044.18.

HARBOE M, OETTINGER T, WIKER HG, ROSENKRANDS I, ANDERSEN P (1996). Evidence for occurrence of the ESAT-6 protein in *Mycobacterium tuberculosis* and virulent *Mycobacterium bovis* and for its absence in *Mycobacterium bovis* BCG. *Infection and Immunity* **64**: 16-22.

HOANG T, AAGAARD C, DIETRICH J, CASSIDY JP, DOLGANOV G, SCHOOLNIK GK, LUNDBERG CV, AGGER EM, ANDERSON P (2013). Esat-6 (esxA) and TB10.4 (esxH) based vaccines for pre and post- exposure tuberculosis vaccination. *Public Library of Science One* **8(12):** e80579.doi:10.137/journal.pone.0080579.

HUGHES MS, BALL NW, MCCARROLL J, ERSKINE M, TAYLOR MJ, POLLOCK JM, SKUCE RA, NEILL SD (2005), Molecula analysis of mycobacteria other than the *M. tuberculosis* complex isolated from Northern Ireland cattle. *Veterinary Microbiology* 108: 101-112.

KAZWALA RR, DABORN CJ, KUSILUKA LJM, JIWA SFH, SHARP JM, KAMBARAGE DM (1998): Isolation of Mycobacterium species from raw milk of pastoral cattle of southern highlands of Tanzania. *Tropical Animal Health Production* **30**: 233–239.

KANKYA C, MUWONGE A, DJØNNE B, MUNYEME M, OPUDA-ASIBO J, SKJERVE E, OLOYA J, EDVARDSEN V, JOHANSEN T (2011). Isolation of non- tuberculous mycobacteria from pastoral ecosystems of Uganda: Public health significance. *Biomed Central: Public Health* **11**: 320.

MILLINGTON KA, FORTUNE SM, LOW J, GARDES A, HINGLEY-WILSON SM, WICKREMASINGHE M, KON, OM, LALVANI A (2011). Rv3615 is a highly immunodominant RD1 Region of difference_ -dependent secreted antigen specific for *Mycobacterium tuberculosis* infection. *Proceedings of National Academy of Science* **108**: 5730-5735.

SCHILLER I, OESCH B, VORDERMEIER HM, PALMER MV, HARRIS BN, ORLOSKI KA, BUDDLE BM, THACKER TC, LYASHCHENKO KP, WATERS WR (2010). Bovine tuberculosis: A review of current and emerging diagnostic techniques in view of their relevance for disease control and eradication. *Transboundary and Emerging Diseases* **57:** 205-220.

SANTEMA W, OVERDIJK M, BARENDS J, KRIJGSVELD J, RUTTEN V, KOETS A (2009). Searching for proteins of *Mycobacterium avium* subspecies *paratuberculosis* with diagnostic potential by comparative proteomic analysis of Mycobacterium tuberculins. *Veterinary Microbiology* **138:** 191-196.

SKJØT, RL, OETTINGER T, ROSENKRANDS I, RAVN P, BROCK I, JACOBSEN S, ANDERSON P (2000). Comparative evaluation of low molecular mass proteins from *Mycobacterium tuberculosis* identifies members of the ESAT-6 family as immunodominant T-cell antigens. *Infection and Immunity* **68:** 214-220.

SKJØT RLV, BROCK I, AREND SM, MUNK ME, THEISEN M, OTTENHOFF THM, ANDERSON P (2002). Epitope mapping of the immunodominant antigen TB10.4 and the two homologous proteins TB10.3 and TB 12.9, which constitute a subfamily of the esat-6 gene family. *Infection and Immunity* **70**: 5446-5453

TSCHOPP , BERG S, ARGAW K, GADISA E, HABTAMU M, SCHELLING E, YOUNG D, ASEFFA A, ZINSSTAG J (2010). Bovine Tuberculosis in Ethiopian wildlife. *Journal of Wildlife Diseases* **46:** 753-762.

TURENNE CY, TSCHETTER L, WOLFE J, KABANI A (2001). Necessity of quality - controlled 16S rRNA gene sequence databases: Identifying non – tuberculous *Mycobacterium* species. *The American Society of Microbiology* **39**: 3637- 3648.

VAN INGEN J, DE ZWAAN R, DEKHUIJZEN R, BOEREE M, VAN SOOLINGEN D (2009). Region of difference 1 in non- tuberculous *Mycobacterium* species adds a phylogenetic and taxonomic character. *Journal of. Bacteriology* **191:** 5865-5867.

VON PIRQUET C (1909). Frequency of tuberculosis in childhood. *The Journal of the American Medical Association* **52:** 675-678.

VORDERMEIER HM, VILLARAEAL-RAMOS B, COCKLE PJ, MCAULAY M, RHODES SG, THACKER T, GILBERT SC, MCSHANE H, HILL AVS, XING A, HEWINSON RG (2009). Viral Booster vaccines improve *Mycobacterium bovis* BCG- induced protection against bovine tuberculosis. *Infection and Immunity* **77:** 3364-3373.

VORDERMEIER HM, BROWN J, COCKLE PJ, FRANKEN WPJ, AREND SM, OTTENHOFF THM, JAHANS K, HEWINSON RG (2007). Assessment of cross - reactivity between *Mycobacterium bovis* and *M. kansasii* ESAT-6 and CFP 10 at the T-cell epitope level. *Clinical Vaccine and Immunology* **14:**1203-1209.

VORDERMEIER HM, COCKLE PC, WHELAN A, RHODES S, HEWINSON RG (2000). Towards the development of diagnostic assays to discriminate between *Mycobacterium bovis* infection and BCG vaccination in cattle. *Clinical and Infectious Diseases* **30:** S291-S298.

WIKER HG (2009). MPB70 and MPB83- major antigens of *Mycobacterium bovis.* *Scandinavian Journal of Immunology* **69:** 492-499

WOOLFORD AJ, HEWINSON RG, WOODWARD M, DALE JW (1997). Sequence heterogeneity of an mpb70 gene analogue in *Mycobacterium kansasii*. *FEMS Microbiology Letters* **148:** 43-48.

APENDIX A

Chapter five

List of proteins identified from the different PPD preparations

		PPD-A	PPD-B	PPD-F	PPD-K	PPD-M	PPD-N
2,5-diketo-D-gluconic acid reductase OS=Mycobacterium avium subsp. sylvalticum ATCC 49884 GN=dkgA PE=4 SV=1	V7KCT7_MYCAV	+	0	0	0	0	0
Fatty acid desaturase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_29479 PE=3 SV=1	K0UKU9_MYCFO	0	0	+	0	0	+
Succinyl-CoA ligase [ADP-forming] subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=sucC PE=3 SV=1	K0UVN8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17993 PE=4 SV=1	K0V3W0_MYCFO	0	0	+	0	0	+
LprG protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_05138 PE=4 SV=1	K0VCH2_MYCFO	0	0	+	0	0	+
Thioredoxin domain protein OS=Mycobacterium avium (strain 104) GN=MAV_1545 PE=4 SV=1	A0QCZ3_MYCA1	+	0	0	0	0	0
DNA-directed RNA polymerase subunit beta' OS=Mycobacterium tuberculosis EAI5 GN=rpoC PE=3 SV=1	S5ES53_MYCTX	**0**	**+**	**0**	**0**	**0**	**+**
Thioredoxin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=trxC_2 PE=3 SV=1	A1KEF8_MYCBP	0	+	0	0	0	0
Membrane protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_06195 PE=4 SV=1	V7KQI0_MYCAV	+	0	0	0	0	0
Ribosome-recycling factor OS=Mycobacterium tuberculosis C GN=frr PE=3 SV=1	A2VLL5_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28714 PE=4 SV=1	K0UM24_MYCFO	0	0	+	0	0	+
Acyl carrier protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=acpP PE=3 SV=1	K0VG08_MYCFO	0	0	+	0	0	+
Putative molybdenum cofactor synthesis protein OS=Mycobacterium avium (strain 104) GN=MAV_1097 PE=4 SV=1	A0QBR2_MYCA1	+	0	0	0	0	0
Heparin-binding hemagglutinin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22870 PE=4 SV=1	K0UQ98_MYCFO	0	0	+	0	0	+
6 kDa early secretory antigenic target OS=Mycobacterium szulgai GN=esat-6 PE=4 SV=1	B5A908_MYCSZ	0	**+**	0	0	0	0
Cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20545 PE=4 SV=1	K0V6X9_MYCFO	0	0	+	0	0	+
Branched-chain amino acid ABC transporter substrate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27109 PE=4 SV=1	K0UIH3_MYCFO	0	0	+	0	0	0
Succinate dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_4910 PE=4 SV=1	A0QM93_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2865 PE=4 SV=1	A0QGL3_MYCA1	+	0	0	0	0	0
Putative lipoprotein lprA OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=lprA PE=4 SV=1	A1KI57_MYCBP	0	+	0	0	0	0
Serine/threonine protein kinase OS=Mycobacterium avium (strain 104) GN=MAV_0017 PE=4 SV=1	A0Q8T1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_25794 PE=4 SV=1	K0UY81_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0196c PE=4 SV=1	Q745I9_MYCPA	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S5 GN=D522_22373 PE=4 SV=1	L7DEQ1_MYCPC	+	0	0	0	0	0
Fatty acid desaturase, type 2 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23050 PE=3 SV=1	K0UW52_MYCFO	0	0	+	0	0	+

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Protein GrpE OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=grpE PE=3 SV=1	V7K3P4_MYCAV	+	0	0	0	0	0
NAD-dependent aldehyde dehydrogenase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_44030 PE=3 SV=1	F7P7B3_MYCPC	+	0	0	0	0	0
Transcription termination/antitermination protein NusG OS=Mycobacterium smegmatis JS623 GN=nusG PE=3 SV=1	L0IPB6_MYCSM	0	0	+	0	0	+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10614 PE=4 SV=1	K0VH76_MYCFO	0	0	+	0	0	+
NADH-quinone oxidoreductase subunit C OS=Mycobacterium avium subsp. paratuberculosis S397 GN=nuoC PE=3 SV=1	F7P9E5_MYCPC	+	0	0	0	0	0
Probable ferredoxin fdxC OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=fdxC PE=4 SV=1	A1KHW8_MYCBP	0	+	0	0	0	0
Polyketide synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11546 PE=4 SV=1	K0V3Z1_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23237 PE=4 SV=1	K0VDE3_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00015 PE=4 SV=1	K0VB17_MYCFO	0	0	+	0	0	+
Elongation factor Ts OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=tsf PE=3 SV=1	EFTS_MYCPA	+	0	0	0	0	0
Phosphoribosylformylglycinamidine synthase, PurS protein OS=Mycobacterium avium (strain 104) GN=purS PE=4 SV=1	A0QAS2_MYCA1	+	0	0	0	0	0
Transcription elongation factor GreA OS=Mycobacterium tuberculosis str. Haarlem GN=greA PE=3 SV=1	A4KG15_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_30474 PE=4 SV=1	K0V1X8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13860 PE=4 SV=1	K0V3J8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17021 PE=4 SV=1	K0V4Y4_MYCFO	0	0	+	0	0	+
Major membrane protein 1 OS=Mycobacterium avium (strain 104) GN=MAV_2054 PE=4 SV=1	A0QED3_MYCA1	+	0	0	0	0	0
35 kDa protein OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra) GN=MRA_2770 PE=3 SV=1	35KD_MYCTA	0	+	0	0	0	0
F420-dependent glucose-6-phosphate dehydrogenase OS=Mycobacterium fortuitum GN=fgd PE=3 SV=1	FGD_MYCFO	+	0	+	0	0	+
Esterase, , antigen 85-A OS=Mycobacterium bovis BCG str. Korea 1168P GN=K60_039460 PE=4 SV=1	M1IRK4_MYCBI	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00599 PE=4 SV=1	K0VEG0_MYCFO	0	0	+	0	0	+
Low molecular weight antigen cfp2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=cfp2 PE=4 SV=1	A1KL66_MYCBP	0	+	0	0	0	0
Cfp10 (Fragment) OS=Mycobacterium riyadhense GN=cfp10 PE=4 SV=1	B2CXA0_9MYCO	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_40010 PE=4 SV=1	F7P667_MYCPC	+	0	0	0	0	0
Ketol-acid reductoisomerase OS=Mycobacterium tuberculosis (strain KZN 1435 / MDR) GN=ilvC PE=3 SV=1	C6DW46_MYCTK	0	+	0	0	0	0
Esterase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23557 PE=4 SV=1	K0UPB9_MYCFO	0	0	+	0	0	+
Adenosylhomocysteinase OS=Mycobacterium avium subsp. paratuberculosis S5 GN=ahcY PE=3 SV=1	L7DDN5_MYCPC	+	0	0	0	0	0
Glyceraldehyde-3-phosphate dehydrogenase, type I OS=Mycobacterium avium (strain 104) GN=gap PE=3 SV=1	A0QHY5_MYCA1	+	0	0	0	0	0

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Immunogenic protein MPB64/MPT64 OS=Mycobacterium avium (strain 104) GN=MAV_3901 PE=4 SV=1	A0QJH5_MYCA1	+	0	0	0	0	0
Glycosyl hydrolase family 16 OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_42630 PE=4 SV=1	F7P6X5_MYCPC	+	0	0	0	0	0
Putative esterase OS=Mycobacterium vaccae ATCC 25954 GN=MVAC_22785 PE=4 SV=1	K0UUC4_MYCVA	0	0	0	0	+	0
ATP-dependent Clp protease proteolytic subunit OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=clpP PE=3 SV=1	K0VDN3_MYCFO	0	0	+	0	0	0
Transaldolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=tal PE=3 SV=1	K0UPX8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0262 PE=4 SV=1	Q745Q4_MYCPA	+	0	0	0	0	0
Iron-regulated heparin binding hemagglutinin hbhA OS=Mycobacterium tuberculosis CAS/NITR204 GN=J113_03365 PE=4 SV=1	R4M5A7_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_3219c PE=4 SV=1	A1KNJ2_MYCBP	0	+	0	0	0	0
Conserved protein with fha domain, fhaa OS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97) GN=fhaa PE=4 SV=1	Q7U303_MYCBO	0	+	0	0	0	0
10 kDa culture filtrate antigen CFP-10 OS=Mycobacterium sp. 012931 GN=MMSP_0744 PE=4 SV=1	S7QYG9_9MYCO	0	+	0	0	0	0
Isocitrate lyase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23697 PE=4 SV=1	K0UV53_MYCFO	0	0	+	0	0	0
Heparin binding hemagglutinin hbha OS=Mycobacterium avium (strain 104) GN=MAV_4675 PE=4 SV=1	A0QLL5_MYCA1	+	0	0	0	0	0
Thiosulfate sulfurtransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17411 PE=4 SV=1	K0V0H1_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_03396 PE=4 SV=1	K0V9L0_MYCFO	0	0	+	0	0	0
Cutinase Cfp21 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20173 PE=4 SV=1	K0VHN0_MYCFO	0	0	+	0	0	+
Phosphate-binding protein PstS (Fragment) OS=Mycobacterium avium 09-5983 GN=O983_17710 PE=3 SV=1	V7LKA4_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium tuberculosis RGTB423 GN=MRGA423_01200 PE=4 SV=1	H8HY34_MYCTX	0	+	0	0	0	0
Phosphorylase OS=Mycobacterium avium (strain 104) GN=MAV_1549 PE=3 SV=1	A0QCZ5_MYCA1	+	0	0	0	0	0
Hydrolase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_10695 PE=4 SV=1	V7L2H8_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S5 GN=D522_16748 PE=4 SV=1	L7DHI8_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2964 PE=4 SV=1	A0QGW2_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_08336 PE=4 SV=1	K0VTN4_MYCFO	0	0	+	0	0	0
Elongation factor G OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=fusA PE=3 SV=1	K0V804_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_30809 PE=4 SV=1	K0UQ34_MYCFO	0	0	+	0	0	+
3-hydroxyacyl-CoA dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10926 PE=3 SV=1	K0V597_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02889 PE=4 SV=1	K0V9Y7_MYCFO	0	0	+	0	0	0
Metallopeptidase, zinc binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20965 PE=4 SV=1	K0UYS2_MYCFO	0	0	+	0	0	0
Protein RecA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=recA PE=3 SV=1	K0VHA3_MYCFO	0	0	0	0	0	+

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GatB/Yqey domain protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_19940 PE=4 SV=1	V7KTY5_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2908 PE=4 SV=1	A0QGQ6_MYCA1	+	0	0	0	0	0
NlpC/P60 family protein OS=Mycobacterium avium (strain 104) GN=MAV_3208 PE=4 SV=1	A0QHK2_MYCA1	+	0	0	0	0	0
2,5-diketo-D-gluconic acid reductase OS=Mycobacterium kansasii ATCC 12478 GN=dkgA PE=4 SV=1	U5WVJ4_MYCKA	0	0	0	+	0	0
DivIVA protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02086 PE=3 SV=1	K0VAA8_MYCFO	0	0	+	0	0	0
60 kDa chaperonin OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=groEL PE=3 SV=1	R4M2L4_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28534 PE=4 SV=1	K0UFS1_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1705 PE=4 SV=1	A0QDE5_MYCA1	+	0	0	0	0	0
Hydrolase OS=Mycobacterium avium (strain 104) GN=MAV_3654 PE=4 SV=1	A0QIU2_MYCA1	+	0	0	0	0	0
Uncharacterized protein (Fragment) OS=Mycobacterium avium subsp. sylvaticum ATCC 49884 GN=P863_23660 PE=4 SV=1	V7JXY4_MYCAV	+	0	0	0	0	0
Glutamine synthetase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=glnA1 PE=3 SV=1	A1KKR3_MYCBP	0	+	0	0	0	0
Glyoxalase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22715 PE=4 SV=1	K0VE80_MYCFO	0	0	+	0	0	+
Thioredoxin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22065 PE=3 SV=1	K0UWY7_MYCFO	0	0	0	0	0	+
Short-chain dehydrogenase/reductase SDR OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11296 PE=3 SV=1	K0V488_MYCFO	0	0	+	0	0	+
Putative esterase OS=Mycobacterium phlei RIVM601174 GN=MPHLEI_14132 PE=4 SV=1	I0RQ96_MYCPH	0	0	0	0	+	0
Putative thiosulfate sulfurtransferase OS=Mycobacterium avium (strain 104) GN=MAV_4253 PE=4 SV=1	A0QKF6_MYCA1	+	0	0	0	0	0
Cutinase cfp21 OS=Mycobacterium tuberculosis C GN=TBCG_01933 PE=4 SV=1	A2VJ93_MYCTX	0	+	0	0	0	0
Pyruvate dehydrogenase E1 component OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=aceE PE=3 SV=1	R4LZF6_MYCTX	0	+	0	0	0	0
S-adenosylmethionine synthase OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=metK PE=3 SV=1	R4M544_MYCTX	0	+	0	0	0	0
Catalase-peroxidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=katG PE=3 SV=1	K0USD3_MYCFO	0	0	+	0	0	+
Fructose-1,6-bisphosphate aldolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17643 PE=4 SV=1	K0UZU8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_12651 PE=4 SV=1	K0V3A6_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_04181 PE=4 SV=1	K0V9C1_MYCFO	0	0	+	0	0	+
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11656 PE=4 SV=1	K0VRV3_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_25242 PE=4 SV=1	K0VAE7_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium xenopi RIVM700367 GN=MXEN_15035 PE=4 SV=1	I0RL64_MYCXE	+	0	0	0	0	0
Chaperone protein HtpG OS=Mycobacterium avium subsp. paratuberculosis S397 GN=htpG PE=3 SV=1	F7P2R7_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3912 PE=4 SV=1	A0QJI6_MYCA1	+	0	0	0	0	0

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Putative esterase OS=Mycobacterium phlei RIVM601174 GN=MPHLEI_26607 PE=4 SV=1	I0RCH3_MYCPH	0	0	0	0	+	0
Fatty acid desaturase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23927 PE=3 SV=1	K0VCH0_MYCFO	0	0	0	0	0	+
Response regulator OS=Mycobacterium marinum str. Europe GN=MMEU_0581 PE=4 SV=1	S7RIY0_MYCMR	0	+	0	0	0	0
Anti-sigma factor antagonist OS=Mycobacterium avium (strain 104) GN=MAV_0435 PE=3 SV=1	A0Q9Y1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26639 PE=4 SV=1	K0V7Z6_MYCFO	0	0	+	0	0	+
Sulfate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_16029 PE=4 SV=1	K0VPE1_MYCFO	0	0	+	0	0	+
Zn-dependent hydrolase OS=Mycobacterium avium 05-4293 GN=O984_14515 PE=4 SV=1	V7J0S9_MYCAV	+	0	0	0	0	0
Prokaryotic ubiquitin-like protein Pup OS=Mycobacterium avium 10-5581 GN=pup PE=3 SV=1	V7JJI1_MYCAV	+	0	0	0	0	0
Glyoxalase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4702 PE=4 SV=1	A0QLP1_MYCA1	+	0	0	0	0	0
Lipoprotein lprG OS=Mycobacterium tuberculosis C GN=TBCG_01389 PE=4 SV=1	A2VHU1_MYCTX	0	+	0	0	0	0
Succinate dehydrogenase flavoprotein subunit OS=Mycobacterium xenopi RIVM700367 GN=sdhA PE=4 SV=1	I0RNQ2_MYCXE	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_43810 PE=3 SV=1	F7P791_MYCPC	+	0	0	0	0	0
Bacterioferritin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=bfrA PE=3 SV=1	A1KJT8_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02298 PE=4 SV=1	K0VA15_MYCFO	0	0	+	0	0	+
Acetyl-CoA acetyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_25574 PE=3 SV=1	K0UKM1_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3591 PE=4 SV=1	A0QIN0_MYCA1	+	0	0	0	0	0
Methyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_21845 PE=4 SV=1	K0URJ0_MYCFO	0	0	+	0	0	+
LpqE protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20455 PE=4 SV=1	K0UTS6_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00255 PE=4 SV=1	K0VXS8_MYCFO	0	0	+	0	0	0
Sulfonate binding protein OS=Mycobacterium avium (strain 104) GN=MAV_0141 PE=4 SV=1	A0Q948_MYCA1	+	0	0	0	0	0
Serine esterase, cutinase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4394 PE=4 SV=1	A0QKU1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22160 PE=4 SV=1	K0URR7_MYCFO	0	0	0	0	0	+
ATP synthase epsilon chain OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=atpC PE=3 SV=1	K0UKU8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2933 PE=4 SV=1	A0QGT1_MYCA1	+	0	0	0	0	0
Cell division protein FtsZ OS=Mycobacterium kansasii GN=ftsZ PE=3 SV=2	FTSZ_MYCKA	0	0	+	0	0	+
Electron transfer flavoprotein subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_03276 PE=4 SV=1	K0V934_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2920 PE=4 SV=1	A0QGR8_MYCA1	+	0	0	0	0	0
Transketolase OS=Mycobacterium avium (strain 104) GN=tkt PE=3 SV=1	A0QHX1_MYCA1	+	0	0	0	0	0

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Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_0614 PE=4 SV=1	A1KG46_MYCBP	0	+	0	0	0	0
ATP-binding protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_21685 PE=4 SV=1	U5WTT7_MYCKA	0	0	0	+	0	0
cAMP-binding protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_36470 PE=4 SV=1	F7P565_MYCPC	+	0	0	0	0	0
ATP synthase gamma chain OS=Mycobacterium gastri 'Wayne' GN=atpG PE=3 SV=1	W4I0N8_MYCGS	+	0	0	0	0	0
F0F1 ATP synthase subunit delta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26179 PE=3 SV=1	K0UQV8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20128 PE=4 SV=1	K0UZV6_MYCFO	0	0	+	0	0	+
3-hydroxyisobutyryl-CoA hydrolase OS=Mycobacterium avium subsp. hominissuis TH135 GN=echA9 PE=4 SV=1	T2GNN5_MYCAV	+	0	0	0	0	0
Peptidase, M28 family protein OS=Mycobacterium avium (strain 104) GN=MAV_4738 PE=4 SV=1	A0QLS7_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_3298 PE=4 SV=1	A1KNS1_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium canettii CIPT 140070017 GN=BN45_20099 PE=4 SV=1	L0QTC8_9MYCO	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium tuberculosis CAS/NITR204 GN=J113_19405 PE=4 SV=1	R4MJV0_MYCTX	0	+	0	0	0	0
Isocitrate dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14487 PE=3 SV=1	K0VQD7_MYCFO	0	0	+	0	0	+
Aminopeptidase N OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02288 PE=4 SV=1	K0VWN2_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26984 PE=4 SV=1	K0V7R0_MYCFO	0	0	0	0	0	+
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24062 PE=4 SV=1	K0UNK4_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0450 PE=4 SV=1	A0Q9Z4_MYCA1	+	0	0	0	0	0
NADH-quinone oxidoreductase subunit I OS=Mycobacterium avium subsp. paratuberculosis S397 GN=nuoI PE=3 SV=1	F7P9D9_MYCPC	+	0	0	0	0	0
Electron transfer flavoprotein (Alpha-subunit) fixB OS=Mycobacterium tuberculosis C GN=TBCG_02963 PE=4 SV=1	A2VNJ5_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17406 PE=4 SV=1	K0V4V2_MYCFO	0	0	0	0	0	+
Thioredoxin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20665 PE=3 SV=1	K0UYZ7_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3785 PE=4 SV=1	A0QJ69_MYCA1	+	0	0	0	0	0
3-hydroxybutyryl-CoA dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_09710 PE=4 SV=1	K0V627_MYCFO	0	0	+	0	0	0
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_06349 PE=4 SV=1	K0VBV3_MYCFO	0	0	0	0	0	+
Superoxide dismutase [Cu-Zn] OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_15984 PE=3 SV=1	K0V1E6_MYCFO	0	0	+	0	0	0
Trigger factor OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=tig PE=3 SV=1	K0V9T0_MYCFO	0	0	+	0	0	+
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=ppiA_1 PE=3 SV=1	A1KEF0_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_10610 PE=4 SV=1	V7L6G1_MYCAV	+	0	0	0	0	0

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Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2351 PE=4 SV=1	A0QF66_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_0853 PE=4 SV=1	A1KGT4_MYCBP	0	+	0	0	0	0
Glyoxalase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_06050 PE=4 SV=1	V7LCD1_MYCAV	+	0	0	0	0	0
Peptidase S1 and S6, chymotrypsin/Hap OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24657 PE=4 SV=1	K0V088_MYCFO	0	0	+	0	0	+
Malate synthase G OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=glcB PE=3 SV=1	K0V7X3_MYCFO	0	0	+	0	0	0
Serine hydroxymethyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=glyA PE=3 SV=1	K0UZ83_MYCFO	0	0	+	0	0	+
3-oxoacyl-(Acyl carrier protein) synthase II OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_12856 PE=3 SV=1	K0VR41_MYCFO	0	0	0	0	0	+
Propionyl-CoA carboxylase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_07736 PE=4 SV=1	K0V743_MYCFO	0	0	+	0	0	+
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_05814 PE=4 SV=1	K0V881_MYCFO	**0**	**0**	**+**	0	0	0
[NADP+] succinate-semialdehyde dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26309 PE=3 SV=1	K0UXD1_MYCFO	0	0	+	0	0	0
PROBABLE CONSERVED SECRETED PROTEIN TB22.2 OS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97) GN=TB22.2 PE=4 SV=1	Q7TXE4_MYCBO	0	+	0	0	0	0
Probable cutinase cut5 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=cut5 PE=4 SV=1	A1KQ56_MYCBP	0	+	0	0	0	0
Lipoprotein OS=Mycobacterium tuberculosis (strain ATCC 35801 / TMC 107 / Erdman) GN=lppX PE=4 SV=1	H8EWC8_MYCTE	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23232 PE=4 SV=1	K0V2K8_MYCFO	0	0	+	0	0	0
Fumarate hydratase class II OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=fumC PE=3 SV=1	K0V3W5_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_13555 PE=4 SV=1	U5WPP2_MYCKA	0	0	0	+	0	0
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14592 PE=4 SV=1	K0V7D3_MYCFO	0	0	+	0	0	0
Enoyl-CoA hydratase OS=Mycobacterium avium (strain 104) GN=MAV_3689 PE=1 SV=1	A0QIX8_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3320 PE=4 SV=1	T2GXK2_MYCAV	+	0	0	0	0	0
Putative acyl-CoA dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_3935 PE=3 SV=1	A0QJK8_MYCA1	+	0	0	0	0	0
Peptidase family protein M13 OS=Mycobacterium avium (strain 104) GN=MAV_4977 PE=4 SV=1	A0QMG0_MYCA1	+	0	0	0	0	0
Aldehyde dehydrogenase family protein OS=Mycobacterium avium (strain 104) GN=MAV_2812 PE=3 SV=1	A0QGG1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4907 PE=4 SV=1	A0QM90_MYCA1	+	0	0	0	0	0
Cutinase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_08300 PE=4 SV=1	V7L8Q7_MYCAV	+	0	0	0	0	0
Putative uncharacterized protein OS=Mycobacterium tuberculosis C GN=TBCG_00190 PE=4 SV=1	A2VNB6_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4701 PE=4 SV=1	A0QLP0_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10349 PE=4 SV=1	K0VSD7_MYCFO	0	0	+	0	0	0
Formamidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00070 PE=4 SV=1	K0VAV4_MYCFO	0	0	+	0	0	0

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30S ribosomal protein S4 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=rpsD PE=3 SV=1	K0UTQ0_MYCFO	0	0	+	0	0	0
Arylsulfatase, AslA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24432 PE=4 SV=1	K0UNS5_MYCFO	0	0	+	0	0	0
Alpha/beta hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_30354 PE=4 SV=1	K0V1Z3_MYCFO	0	0	+	0	0	+
Cyclophilin type peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_07436 PE=3 SV=1	K0V6T7_MYCFO	0	0	+	0	0	0
Proteasome subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=prcA PE=3 SV=1	K0VA96_MYCFO	0	0	+	0	0	+
Beta-lactamase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_08116 PE=4 SV=1	K0VAM4_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27605 PE=4 SV=1	K0UH77_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28729 PE=4 SV=1	K0UTZ4_MYCFO	0	0	+	0	0	0
Transcription elongation factor GreA OS=Mycobacterium avium subsp. paratuberculosis S397 GN=greA PE=3 SV=1	F7P3H3_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4288 PE=4 SV=1	A0QKI9_MYCA1	+	0	0	0	0	0
Dioxygenase OS=Mycobacterium avium (strain 104) GN=MAV_0540 PE=4 SV=1	A0QA82_MYCA1	+	0	0	0	0	0
Glutamine synthetase OS=Mycobacterium avium (strain 104) GN=glnA PE=3 SV=1	A0QEY3_MYCA1	+	0	0	0	0	0
Probable cutinase cut2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=cut2 PE=4 SV=1	A1KKZ3_MYCBP	0	+	0	0	0	0
Monooxygenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24947 PE=4 SV=1	K0UT08_MYCFO	0	0	+	0	0	+
ATP-dependent Clp protease proteolytic subunit OS=Mycobacterium avium subsp. paratuberculosis S5 GN=clpP PE=3 SV=1	L7DK09_MYCPC	+	0	0	0	0	0
Cyclopropane-fatty-acyl-phospholipid synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_05368 PE=4 SV=1	K0V7V6_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02001 PE=4 SV=1	K0VE63_MYCFO	0	0	+	0	0	+
Polyribonucleotide nucleotidyltransferase OS=Mycobacterium canettii CIPT 140060008 GN=pnp PE=3 SV=1	L0PYM0_9MYCO	0	+	0	0	0	0
Putative lipoprotein lprF OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=lprF PE=4 SV=1	A1KIF8_MYCBP	0	+	0	0	0	0
Metallopeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_15262 PE=4 SV=1	K0VPU0_MYCFO	0	0	0	0	0	+
E**SX-1 secretion-associated protein EspA OS=Mycobacterium canettii CIPT 140070017 GN=BN45_10012**6 PE=4 SV=1	L0QZY1_9MYCO	0	+	0	0	0	0
Superoxide dismutase OS=Mycobacterium avium (strain 104) GN=MAV_0182 PE=3 SV=1	A0Q988_MYCA1	+	0	0	0	0	0
Nucleoside diphosphate kinase regulator OS=Mycobacterium avium (strain 104) GN=MAV_0234 PE=4 SV=1	A0Q9D9_MYCA1	+	0	0	0	0	0
Possible oxidoreductase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_0094 PE=4 SV=1	A1KEN1_MYCBP	0	+	0	0	0	0
Putative pterin-4-alpha-carbinolamine dehydratase OS=Mycobacterium tuberculosis str. Haarlem GN=TBHG_01143 PE=3 SV=1	A4KG87_MYCTX	0	+	0	0	0	0
Alkyl hydroperoxide reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22390 PE=4 SV=1	K0VEF0_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2567 PE=4 SV=1	T2GUG0_MYCAV	+	0	0	0	0	0
ErfK/YbiS/YcfS/YnhG family protein OS=Mycobacterium avium (strain 104) GN=MAV_4986 PE=4 SV=1	A0QMG9_MYCA1	+	0	0	0	0	0

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ATP-dependent Clp protease proteolytic subunit OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=clpP PE=3 SV=1	K0V9Z4_MYCFO	0	0	+	0	0	0
Triosephosphate isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=tpiA PE=3 SV=1	K0V898_MYCFO	0	0	+	0	0	0
Serine protease htrA OS=Mycobacterium tuberculosis T17 GN=TBJG_01731 PE=4 SV=1	D5ZEX8_MYCTX	0	+	0	0	0	0
Trypsin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_21860 PE=4 SV=1	K0UXD3_MYCFO	0	0	+	0	0	0
Fructose-2,6-bisphosphatase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_15377 PE=4 SV=1	K0VEH0_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_10295 PE=4 SV=1	U5X1U5_MYCKA	0	0	0	+	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22475 PE=4 SV=1	K0URE6_MYCFO	0	0	+	0	0	0
Serine esterase cutinase OS=Mycobacterium avium (strain 104) GN=MAV_0369 PE=4 SV=1	A0Q9R7_MYCA1	+	0	0	0	0	0
HIT family hydrolase, diadenosine tetraphosphate hydrolase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_13800 PE=4 SV=1	F7PC21_MYCPC	+	0	0	0	0	0
O-methyltransferase OS=Mycobacterium avium 05-4293 GN=O984_01700 PE=4 SV=1	V7JEA2_MYCAV	+	0	0	0	0	0
Proteasome-associated ATPase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=mpa PE=3 SV=1	K0VLY9_MYCFO	0	0	+	0	0	0
Fatty oxidation protein fadB OS=Mycobacterium tuberculosis C GN=TBCG_00850 PE=3 SV=1	A2VGF6_MYCTX	0	+	0	0	0	0
Malate synthase G OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=glcB PE=3 SV=1	R4M7L1_MYCTX	0	+	0	0	0	0
3-ketoacyl-(Acyl-carrier-protein) reductase (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=fabG PE=3 SV=1	K0UUR3_MYCFO	0	0	0	0	0	+
Serine hydroxymethyltransferase OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra) GN=glyA1 PE=3 SV=1	A5U1E0_MYCTA	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra) GN=MRA_3428A PE=4 SV=1	A5U862_MYCTA	0	+	0	0	0	0
6-phosphogluconate dehydrogenase, decarboxylating OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13435 PE=3 SV=1	K0V2V2_MYCFO	0	0	+	0	0	+
Cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20540 PE=4 SV=1	K0UTQ3_MYCFO	0	0	+	0	0	0
Probable serine protease OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_1038 PE=4 SV=1	A1KHB9_MYCBP	0	+	0	0	0	0
Uncharacterized protein, probably involved in trehalose biosynthesis OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_45790 PE=4 SV=1	F7P7T7_MYCPC	+	0	0	0	0	0
Cellobiohydrolase A (1,4-beta-cellobiosidase A) OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23360 PE=4 SV=1	F7PER0_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3218 PE=4 SV=1	A0QHL2_MYCA1	+	0	0	0	0	0
Lipoprotein, ATP binding protein OS=Mycobacterium vaccae ATCC 25954 GN=MVAC_19346 PE=4 SV=1	K0UKI4_MYCVA	0	0	0	0	0	0
DNA gyrase subunit A OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=gyrA PE=3 SV=1	K0UVA8_MYCFO	0	0	0	0	0	+
Arginine biosynthesis bifunctional protein ArgJ OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=argJ PE=3 SV=1	K0V230_MYCFO	0	0	+	0	0	0
Probable acetyl-CoA acyltransferase fadA2 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=fadA2 PE=3 SV=1	A1KF64_MYCBP	0	+	0	0	0	0
Putative uncharacterized protein OS=Mycobacterium tuberculosis EAS054 GN=TBGG_03363 PE=4 SV=1	D5YMQ2_MYCTX	0	+	0	0	0	0

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Adenylate kinase OS=Mycobacterium tuberculosis C GN=adk PE=3 SV=1	A2VG38_MYCTX	0	+	0	0	0	0
Eptc-inducible aldehyde dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_4691 PE=3 SV=1	A0QLN1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_1931 PE=4 SV=1	A1KJV7_MYCBP	0	+	0	0	0	0
Two component transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_08835 PE=4 SV=1	K0V6I9_MYCFO	0	0	+	0	0	+
D-3-phosphoglycerate dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_08481 PE=3 SV=1	K0VAG8_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13480 PE=4 SV=1	K0V3S4_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_29175 PE=4 SV=1	U5X3E8_MYCKA	0	0	0	+	0	0
50S ribosomal protein L10 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=rplJ PE=3 SV=1	K0V7X9_MYCFO	0	0	+	0	0	0
Transmembrane transport protein MmpL10 OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=I917_08410 PE=4 SV=1	R4LWQ9_MYCTX	0	0	0	0	+	0
Enoyl-CoA hydratase OS=Mycobacterium avium (strain 104) GN=MAV_4534 PE=4 SV=1	A0QL77_MYCA1	+	0	0	0	0	0
Periplasmic binding protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3333 PE=4 SV=1	T2GUZ7_MYCAV	+	0	0	0	0	0
Pyruvate dehydrogenase (E2 component) sucB OS=Mycobacterium tuberculosis C GN=TBCG_02162 PE=3 SV=1	A2VJV6_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_0288c PE=4 SV=1	A1KF71_MYCBP	0	+	0	0	0	0
Short chain dehydrogenase OS=Mycobacterium avium (strain 104) GN=MAV_0895 PE=3 SV=1	A0QB71_MYCA1	+	0	0	0	0	0
Hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10055 PE=4 SV=1	K0V5Z0_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=I917_10075 PE=4 SV=1	R4M6G6_MYCTX	0	+	0	0	0	0
Acyl carrier protein OS=Mycobacterium vanbaalenii (strain DSM 7251 / PYR-1) GN=acpP PE=3 SV=1	A1TBI7_MYCVP	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=I917_10575 PE=4 SV=1	R4LUQ9_MYCTX	0	+	0	0	0	0
6-phosphogluconate dehydrogenase, decarboxylating (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28060 PE=3 SV=1	K0V658_MYCFO	0	0	+	0	0	0
Polyketide synthase pks13 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=pks13 PE=4 SV=1	A1KQD4_MYCBP	0	+	0	0	0	0
Aconitate hydratase OS=Mycobacterium tuberculosis (strain CCDC5079) GN=CCDC5079_1371 PE=4 SV=1	F7WJA8_MYCTC	0	+	0	0	0	0
2,3-bisphosphoglycerate-dependent phosphoglycerate mutase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=gpmA PE=3 SV=1	K0VIB0_MYCFO	0	0	+	0	0	0
Ribonucleoside-diphosphate reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_03506 PE=3 SV=1	K0VL43_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_19851 PE=4 SV=1	K0V753_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_29254 PE=4 SV=1	K0ULC0_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24567 PE=4 SV=1	K0UMJ9_MYCFO	0	0	+	0	0	0
Universal stress protein UspA-like protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24067 PE=4 SV=1	K0V1J0_MYCFO	0	0	0	0	0	+

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Phosphoserine aminotransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=serC PE=3 SV=1	K0V2M1_MYCFO	0	0	0	0	0	+
Naphthoate synthase OS=Mycobacterium avium (strain 104) GN=menB PE=4 SV=1	A0QLD7_MYCA1	+	0	0	0	0	0
Nitroreductase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4334 PE=4 SV=1	A0QKN2_MYCA1	+	0	0	0	0	0
Ribosome-recycling factor OS=Mycobacterium avium subsp. paratuberculosis S397 GN=frr PE=3 SV=1	F7PA73_MYCPC	+	0	0	0	0	0
Catalase-peroxidase OS=Mycobacterium tuberculosis GN=katG PE=3 SV=1	M1JUT6_MYCTX	0	+	0	0	0	0
GntR family transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10764 PE=4 SV=1	K0V4G5_MYCFO	0	0	+	0	0	0
FHA domain-containing protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_16749 PE=4 SV=1	K0VD97_MYCFO	0	0	+	0	0	0
Cyanate hydratase OS=Mycobacterium avium (strain 104) GN=cynS PE=3 SV=1	CYNS_MYCA1	+	0	0	0	0	0
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17998 PE=4 SV=1	K0UZ85_MYCFO	0	0	0	0	0	+
Molybdenum ABC transporter periplasmic molybdate-binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_19604 PE=4 SV=1	K0VIY0_MYCFO	0	0	+	0	0	0
Signal-transduction protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23917 PE=4 SV=1	K0UNL7_MYCFO	0	0	0	0	0	+
Ferredoxin sulfite reductase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00485 PE=4 SV=1	K0VF31_MYCFO	0	0	0	0	0	+
D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase OS=Mycobacterium avium (strain 104) GN=dacB PE=4 SV=1	A0QA71_MYCA1	+	0	0	0	0	0
Possible thioredoxin OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_1386 PE=4 SV=1	A1KIB4_MYCBP	0	+	0	0	0	0
Pyridoxamine 5''-phosphate oxidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13990 PE=4 SV=1	K0VQG9_MYCFO	0	0	0	0	0	+
Probable cytosol aminopeptidase OS=Mycobacterium avium (strain 104) GN=pepA PE=3 SV=1	A0QEZ5_MYCA1	+	0	0	0	0	0
Probable thiosulfate sulfurtransferase sseA OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=sseA PE=4 SV=1	A1KNT5_MYCBP	0	+	0	0	0	0
2-oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase OS=Mycobacterium avium (strain 104) GN=sucB PE=3 SV=1	A0QEY9_MYCA1	+	0	0	0	0	0
Alpha-ketoglutarate decarboxylase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=kgd PE=4 SV=1	K0UZY0_MYCFO	0	0	0	0	0	+
Possible conserved transmembrane alanine and glycine rich protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_2734c PE=4 SV=1	A1KM59_MYCBP	0	+	0	0	0	0
Glycosyl hydrolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28674 PE=4 SV=1	K0UFN0_MYCFO	0	0	+	0	0	0
Acetyl-CoA acetyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_29394 PE=3 SV=1	K0UL52_MYCFO	0	0	+	0	0	0
Alpha/beta hydrolase fold protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10569 PE=4 SV=1	K0VSA3_MYCFO	0	0	+	0	0	+
Ribose-phosphate pyrophosphokinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=prs PE=3 SV=1	K0V2H2_MYCFO	0	0	+	0	0	0
Probable thiol peroxidase OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=tpx PE=3 SV=1	A1KJZ7_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14717 PE=4 SV=1	K0V7E6_MYCFO	0	0	+	0	0	0

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Probable fructose-bisphosphate aldolase fba OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=fba PE=4 SV=1	A1KFI4_MYCBP	0	+	0	0	0	0
6-phosphogluconate dehydrogenase, decarboxylating OS=Mycobacterium avium (strain 104) GN=gnd PE=4 SV=1	A0QC60_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_0266 PE=3 SV=1	T2GMN3_MYCAV	+	0	0	0	0	0
Chaperone protein ClpB OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=clpB PE=3 SV=1	CLPB_MYCPA	+	0	0	0	0	0
Meromycolate extension acyl carrier protein OS=Mycobacterium aurum GN=acpM PE=3 SV=2	ACPM_MYCAU	0	0	0	0	0	+
D-alanyl-D-alanine carboxypeptidase/D-alanyl-D-alanine-endopeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23852 PE=4 SV=1	K0V1M2_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_04170 PE=3 SV=1	U5WNW8_MYCKA	0	0	0	+	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13870 PE=4 SV=1	K0VFF0_MYCFO	0	0	+	0	0	0
Anti-sigma factor antagonist OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23872 PE=3 SV=1	K0UNP9_MYCFO	0	0	+	0	0	0
Cytoplasmic peptidase OS=Mycobacterium tuberculosis (strain ATCC 35801 / TMC 107 / Erdman) GN=pepQ PE=4 SV=1	H8EU58_MYCTE	0	+	0	0	0	0
NAD-dependent malic enzyme (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_31371 PE=3 SV=1	K0UBD0_MYCFO	0	0	+	0	0	0
R4V0A1|R4V0A1_MYCAB-DECOY		0	0	0	0	+	0
Proteasome subunit beta OS=Mycobacterium avium subsp. avium 11-4751 GN=O973_11070 PE=3 SV=1	V7LGT8_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2810 PE=4 SV=1	A0QGF9_MYCA1	+	0	0	0	0	0
B1MMN6|B1MMN6_MYCA9-DECOY	B1MMN6|DECOY	0	0	0	0	+	0
Polyketide-type polyunsaturated fatty acid synthase PfaA OS=Mycobacterium rhodesiae JS60 GN=MycrhDRAFT_5147 PE=4 SV=1	G4I5E7_MYCRH	0	0	0	0	+	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_06631 PE=4 SV=1	K0V7T7_MYCFO	0	0	+	0	0	+
Succinate dehydrogenase flavoprotein subunit OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=sdhA PE=4 SV=1	K0V2T1_MYCFO	0	0	0	0	0	+
Methylmalonate-semialdehyde dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_30399 PE=3 SV=1	K0UJ90_MYCFO	0	0	+	0	0	0
I6ZHP2|I6ZHP2_MYCAB-DECOY	I6ZHP2|I6ZHP2_	0	0	0	0	+	0
Probable acyl-CoA dehydrogenase fadE35 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=fadE35 PE=3 SV=1	A1KQD1_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1706 PE=4 SV=1	A0QDE7_MYCA1	+	0	0	0	0	0
Aspartate transaminase OS=Mycobacterium avium (strain 104) GN=MAV_0381 PE=4 SV=1	A0Q9S8_MYCA1	+	0	0	0	0	0
Bifunctional acetyl-/propionyl-coenzyme A carboxylase subunit alpha accA3 OS=Mycobacterium africanum K85 GN=TBOG_03849 PE=4 SV=1	D6FRI7_9MYCO	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_17890 PE=4 SV=1	U5WRT3_MYCKA	0	0	0	0	0	0
Cutinase OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_20050 PE=4 SV=1	U5WWB6_MYCKA	0	0	0	0	0	0
Probable enoyl-CoA hydratase echa16 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=echA16 PE=4 SV=1	A1KMH4_MYCBP	0	+	0	0	0	0

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Dihydrolipoamide acetyltransferase (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27485 PE=3 SV=1	K0UNN7_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_08035 PE=4 SV=1	U5WYC5_MYCKA	0	0	0	0	0	0
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_19032 PE=4 SV=1	K0V8N7_MYCFO	0	0	+	0	0	0
Pyruvate kinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26049 PE=3 SV=1	K0V8Y4_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20990 PE=4 SV=1	K0UYS8_MYCFO	0	0	+	0	0	0
Methyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_09905 PE=3 SV=1	K0V5W4_MYCFO	0	0	0	0	0	+
ATP synthase subunit alpha OS=Mycobacterium tuberculosis SUMu004 GN=atpA PE=3 SV=1	E2TXC6_MYCTX	0	+	0	0	0	0
Glycerophosphoryl diester phosphodiesterase family protein OS=Mycobacterium avium (strain 104) GN=MAV_0576 PE=4 SV=1	A0QAB7_MYCA1	+	0	0	0	0	0
R3H domain-containing protein OS=Mycobacterium avium (strain 104) GN=MAV_5309 PE=4 SV=1	A0QND1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_0595c PE=4 SV=1	A1KG27_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_1771c PE=4 SV=1	A1KJE9_MYCBP	0	+	0	0	0	0
Probable NADP-dependent alcohol dehydrogenase adhC OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=adhC PE=3 SV=1	A1KN42_MYCBP	0	+	0	0	0	0
Methyltransferase, putative, TIGR00027 family OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_01730 PE=3 SV=1	F7P8M6_MYCPC	+	0	0	0	0	0
Acetyl-CoA acetyltransferase OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_08545 PE=3 SV=1	V7KNS5_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_21780 PE=4 SV=1	U5WUN6_MYCKA	0	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium kansasii ATCC 12478 GN=MKAN_25885 PE=4 SV=1	U5WVT2_MYCKA	0	0	0	0	0	0
D-alanyl-D-alanine carboxypeptidase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_13560 PE=3 SV=1	K0V3L8_MYCFO	0	0	+	0	0	0
Endonuclease IV OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3909 PE=3 SV=1	T2GWR1_MYCAV	+	0	0	0	0	0
MmpL protein OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) GN=MSMEG_4741 PE=4 SV=1	A0R1G2_MYCS2	0	0	0	0	+	0
Trigger factor OS=Mycobacterium canettii CIPT 140070008 GN=tig PE=3 SV=1	L0Q9X7_9MYCO	0	+	0	0	0	0
I4BR24|I4BR24_MYCCN-DECOY		0	0	0	0	+	0
Enoyl-CoA hydratase echA21 OS=Mycobacterium tuberculosis SUMu012 GN=TMLG_00265 PE=4 SV=1	E2WNJ1_MYCTX	0	+	0	0	0	0
Acyl carrier protein OS=Mycobacterium vaccae ATCC 25954 GN=acpP PE=3 SV=1	K0UD35_MYCVA	+	0	0	0	0	0
Outer membrane protein OmpA OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17241 PE=3 SV=1	K0VN20_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11006 PE=4 SV=1	K0V4C0_MYCFO	0	0	+	0	0	0
Cystathionine gamma-synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_25604 PE=3 SV=1	K0UYN9_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium bovis BCG str. Korea 1168P GN=K60_032490 PE=4 SV=1	M1IP81_MYCBI	0	+	0	0	0	0

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Uncharacterized protein OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=I917_27180 PE=4 SV=1	R4MBZ4_MYCTX	0	+	0	0	0	0
Pyruvate synthase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23647 PE=4 SV=1	K0UP66_MYCFO	0	0	0	0	0	+
Phosphoserine aminotransferase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=serC PE=3 SV=1	F7P437_MYCPC	+	0	0	0	0	0
NAD(P)H nitroreductase OS=Mycobacterium avium 05-4293 GN=O984_12220 PE=4 SV=1	V7J5B3_MYCAV	+	0	0	0	0	0
Hydrolase, peptidase M42 family protein OS=Mycobacterium avium (strain 104) GN=MAV_2729 PE=4 SV=1	A0QG80_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_3070c PE=4 SV=1	A1KN43_MYCBP	0	+	0	0	0	0
PE family protein (Fragment) OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_38890 PE=4 SV=1	F7P5V6_MYCPC	+	0	0	0	0	0
Ribose-5-phosphate isomerase B OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02248 PE=4 SV=1	K0VA03_MYCFO	0	0	+	0	0	0
Gamma-glutamyl phosphate reductase OS=Mycobacterium canettii CIPT 140070017 GN=proA PE=3 SV=1	L0QY66_9MYCO	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_2051 PE=4 SV=1	A1KK78_MYCBP	0	+	0	0	0	0
Pyruvate kinase OS=Mycobacterium rhodesiae JS60 GN=MycrhDRAFT_4014 PE=4 SV=1	G4I2V3_MYCRH	0	0	0	0	+	0
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22045 PE=4 SV=1	K0URT2_MYCFO	0	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium canettii CIPT 140060008 GN=cfp PE=4 SV=1	L0PUB7_9MYCO	0	+	0	0	0	0

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kDa early secretory antigenic target esxA (Esat-6) OS=Mycobacterium tuberculosis C GN=TBCG_03802 PE=4 SV=1	A2VMQ0_MYCTX	0	+	0	0	0	0
10 kDa chaperonin OS=Mycobacterium tuberculosis C GN=groS PE=3 SV=1	A2VPK5_MYCTX	0	+	+	+	0	+
Major secreted immunogenic protein mpb70 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=mpb70 PE=4 SV=1	A1KMM0_MYCBP	0	+	0	0	0	0
Bacterioferritin OS=Mycobacterium avium 05-4293 GN=O984_13350 PE=3 SV=1	V7J3W1_MYCAV	+	0	+	0	0	+
10 kDa culture filtrate antigen esxB (Cfp10) OS=Mycobacterium tuberculosis C GN=TBCG_03801 PE=4 SV=1	A2VMP9_MYCTX	0	+	0	0	0	0
Esat6 OS=Mycobacterium riyadhense GN=esat6 PE=4 SV=2	B2CX99_9MYCO	0	+	0	0	0	0
60 kDa chaperonin OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=groEL PE=3 SV=1	R4LUN2_MYCTX	0	+	+	+	+	+
Antigen 85-B OS=Mycobacterium avium (strain 104) GN=MAV_2816 PE=4 SV=1	A0QGG5_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4695 PE=4 SV=1	A0QLN4_MYCA1	+	0	0	0	0	0
Putative ESAT-6 like protein 5 OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=esxN PE=4 SV=1	A1KJK3_MYCBP	0	+	0	+	0	0
Alanine and proline rich secreted protein apa OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2430 PE=4 SV=1	T2GV03_MYCAV	+	0	0	0	0	0
Cell surface lipoprotein mpt83 (Lipoprotein P23) OS=Mycobacterium tuberculosis C GN=TBCG_02811 PE=4 SV=1	A2VLK6_MYCTX	0	+	0	0	0	0
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28224 PE=4 SV=1	K0UMT4_MYCFO	0	0	+	0	+	+
50S ribosomal protein L7/L12 OS=Mycobacterium parascrofulaceum ATCC BAA-614 GN=rplL PE=3 SV=1	D5PC72_9MYCO	+	+	0	0	+	0
Uncharacterized protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_23545 PE=4 SV=1	V7KKI2_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3362 PE=4 SV=1	A0QI06_MYCA1	+	0	0	0	0	0
Acyl carrier protein OS=Mycobacterium avium (strain 104) GN=acpP PE=3 SV=1	A0QER4_MYCA1	+	0	0	0	+	+
P40 protein OS=Mycobacterium avium PE=4 SV=1	Q9AIQ0_MYCAV	+	0	0	0	0	0
Elongation factor Tu OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=tuf PE=3 SV=1	K0VK30_MYCFO	+	+	+	+	+	+
Putative esat-6 like protein OS=Mycobacterium tuberculosis (strain ATCC 25177 / H37Ra) GN=MRA_2375 PE=4 SV=1	A5U544_MYCTA	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_04593 PE=4 SV=1	K0VKN4_MYCFO	0	0	+	0	0	+
Serine protease OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_01225 PE=4 SV=1	V7KYG3_MYCAV	+	0	0	0	0	0

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DivIVA domain containing protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_25430 PE=3 SV=1	F7P246_MYCPC	+	+	+	+	0	0
Chaperone protein DnaK OS=Mycobacterium tuberculosis C GN=dnaK PE=3 SV=1	A2VF50_MYCTX	+	+	+	+	0	+
Antigen 85-A (Mycolyl transferase) (Fragment) OS=Mycobacterium paratuberculosis GN=fbpa PE=4 SV=1	Q70E87_MYCPC	+	0	0	0	0	0
14 kDa antigen OS=Mycobacterium bovis (strain ATCC BAA-935 / AF2122/97) GN=hspX PE=3 SV=2	14KD_MYCBO	0	+	0	0	0	0
Esat-6 like protein esxL (Esat-6 like protein 4) OS=Mycobacterium tuberculosis C GN=TBCG_01180 PE=4 SV=1	A2VHA0_MYCTX	0	+	0	0	0	0
Protease OS=Mycobacterium avium (strain 104) GN=MAV_1096 PE=4 SV=1	A0QBR1_MYCA1	+	0	0	0	0	0
10 kDa culture filtrate antigen EsxB_1 OS=Mycobacterium marinum (strain ATCC BAA-535 / M) GN=esxB_1 PE=4 SV=1	B2HJI8_MYCMM	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_07456 PE=4 SV=1	K0V7A7_MYCFO	0	0	+	0	0	+
Immunogenic protein MPT64 OS=Mycobacterium avium (strain 104) GN=MAV_4130 PE=4 SV=1	A0QK41_MYCA1	+	0	0	0	0	0
Aconitate hydratase OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_14265 PE=4 SV=1	V7KF43_MYCAV	+	+	0	0	0	0
Glutamate binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10289 PE=3 SV=1	K0V4Q6_MYCFO	0	0	+	0	0	+
Immunogenic protein mpt64 OS=Mycobacterium tuberculosis C GN=TBCG_01928 PE=4 SV=1	A2VJ88_MYCTX	0	+	0	0	0	0
Antigen 85-A OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11571 PE=4 SV=1	K0V3Z5_MYCFO	0	0	+	0	0	+
Immunogenic protein MPT63 (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_21420 PE=4 SV=1	K0US52_MYCFO	0	0	+	0	0	+
Bacterioferritin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_04403 PE=3 SV=1	K0VCV8_MYCFO	0	0	+	0	0	+
Probable cutinase Cut3 OS=Mycobacterium avium (strain 104) GN=MAV_4283 PE=4 SV=1	A0QKI4_MYCA1	+	0	0	0	0	0
Alanine and proline rich secreted protein apa OS=Mycobacterium tuberculosis C GN=TBCG_01812 PE=4 SV=1	A2VIX6_MYCTX	0	+	0	0	0	0
Elongation factor Tu OS=Mycobacterium avium (strain 104) GN=tuf PE=3 SV=1	EFTU_MYCA1	+	+	0	0	0	0
60 kDa chaperonin 2 OS=Mycobacterium avium (strain 104) GN=groL2 PE=3 SV=1	CH602_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=cfp17 PE=4 SV=1	A1KJN9_MYCBP	0	+	0	0	0	0
35kd antigen OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3039 PE=4 SV=1	T2GU78_MYCAV	+	0	0	0	0	0
Transaldolase OS=Mycobacterium avium (strain 104) GN=tal PE=3 SV=1	A0QHX2_MYCA1	+	0	0	0	0	0

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Electron transfer protein, beta subunit OS=Mycobacterium avium (strain 104) GN=MAV_3876 PE=4 SV=1	A0QJF0_MYCA1	+	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium colombiense CECT 3035 GN=MCOL_V201805 PE=4 SV=1	J5ESB1_9MYCO	+	0	0	0	0	0
Mannose-binding lectin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27326 PE=4 SV=1	K0UPC2_MYCFO	0	0	+	0	0	+
Low molecular weight antigen MTB12 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_01821 PE=4 SV=1	K0VWZ9_MYCFO	0	0	+	0	0	+
60 kDa chaperonin OS=Mycobacterium tuberculosis str. Haarlem GN=groL PE=3 SV=1	A4KEC8_MYCTX	0	+	0	0	0	0
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_08605 PE=4 SV=1	K0VA79_MYCFO	0	0	+	0	0	+
Acyl carrier protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=acpM PE=3 SV=1	A1KKT7_MYCBP	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2763 PE=4 SV=1	A0QGB4_MYCA1	+	0	0	0	0	0
Secreted antigen 85-c fbpC (85C) OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=fbpC PE=4 SV=1	A1KEV0_MYCBP	+	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26634 PE=4 SV=1	K0UX16_MYCFO	0	0	+	0	0	+
Signal peptide protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_23515 PE=4 SV=1	V7K1R1_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23480 PE=4 SV=1	F7PES2_MYCPC	+	0	0	0	0	0
Chaperone protein DnaK OS=Mycobacterium avium (strain 104) GN=dnaK PE=3 SV=1	DNAK_MYCA1 +	+	0	+	0	0	0
N-acetylmuramoyl-L-alanine amidase OS=Mycobacterium avium subsp. paratuberculosis 10-8425 GN=O976_20900 PE=4 SV=1	V7NKM8_MYCPC	+	0	0	0	0	0
Immunogenic protein MPT63 (Antigen MPT63/MPB63) (16 kDa immunoprotective extracellular protein) OS=Mycobacterium tuberculosis str. Haarlem/NITR202 GN=I917_13650 PE=4 SV=1	R4M6J4_MYCTX	0	**+**	0	0	0	0
Diacylglycerol acyltransferase/mycolyltransferase Ag85B OS=Mycobacterium kansasii GN=fbpB PE=1 SV=1	A85B_MYCKA	0	0	0	+	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_28750 PE=4 SV=1	F7P319_MYCPC	+	0	0	0	0	0
LpqE protein OS=Mycobacterium avium (strain 104) GN=MAV_0569 PE=4 SV=1	A0QAB1_MYCA1	+	0	0	0	0	0
ATP synthase subunit alpha OS=Mycobacterium avium (strain 104) GN=atpA PE=3 SV=1	ATPA_MYCA1	+	0	+	0	0	+
Phosphate-binding protein PstS OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17441 PE=3 SV=1	K0UZG6_MYCFO	0	0	+	0	0	+
Universal stress protein family protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14210 PE=4 SV=1	K0V7H4_MYCFO	0	0	+	0	0	+
Antigen 85-C OS=Mycobacterium avium (strain 104) GN=MAV_0215 PE=4 SV=1	A0Q9C1_MYCA1	+	0	0	0	0	0

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Malate synthase G OS=Mycobacterium avium (strain 104) GN=glcB PE=3 SV=1	MASZ_MYCA1	+	+	0	0	0	0
Fructose-bisphosphate aldolase class-I OS=Mycobacterium avium (strain 104) GN=MAV_5271 PE=4 SV=1	A0QN95_MYCA1	+	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_09420 PE=4 SV=1	F7PAT6_MYCPC	+	0	0	0	0	0
Hydroxymethylglutaryl-CoA lyase OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_03630 PE=4 SV=1	V7KWT7_MYCAV	+	0	0	0	0	0
Probable thiol peroxidase OS=Mycobacterium avium (strain 104) GN=tpx PE=3 SV=1	A0QGC1_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_04338 PE=4 SV=1	K0V8M4_MYCFO	0	0	+	0	0	+
6 kDa early secretory antigenic target OS=Mycobacterium marinum MB2 GN=MMMB2_3804 PE=4 SV=1	S7RG91_MYCMR	0	+	0	0	0	0
5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase OS=Mycobacterium tuberculosis str. Haarlem GN=metE PE=3 SV=2	A4KG62_MYCTX	0	+	0	0	0	0
10 kDa culture filtrate protein OS=Mycobacterium szulgai GN=cfp-10 PE=4 SV=1	B5TV80_MYCSZ	0	**+**	0	0	0	0
ATP synthase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=atpD PE=3 SV=1	K0UJQ0_MYCFO	+	0	+	+	0	+
Malate dehydrogenase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=mdh PE=3 SV=1	F7PBF5_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0628 PE=4 SV=1	A0QAH0_MYCA1	+	0	0	0	0	0
Electron transfer flavoprotein, alpha subunit OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_06000 PE=4 SV=1	F7P9V0_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_0239 PE=4 SV=1	A0Q9E4_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium 05-4293 GN=O984_06270 PE=4 SV=1	V7J9T0_MYCAV	+	0	0	0	0	0
60 kDa chaperonin OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=groEL PE=3 SV=1	V7JZC8_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_08920 PE=4 SV=1	F7PAP0_MYCPC	+	0	0	0	0	0
Putative acetyltransferase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_11260 PE=4 SV=1	F7PBB8_MYCPC	+	0	0	0	0	0
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23220 PE=4 SV=1	F7PEP6_MYCPC	+	0	0	0	0	0
Integration host factor OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) GN=mihF PE=4 SV=1	A0QWS8_MYCS2	+	0	+	0	+	+
DNA-directed RNA polymerase subunit alpha OS=Mycobacterium smegmatis JS623 GN=rpoA PE=3 SV=1	L0IRX4_MYCSM	+	0	+	0	0	+
Transglycosylase OS=Mycobacterium avium (strain 104) GN=MAV_0446 PE=4 SV=1	A0Q9Z0_MYCA1	+	0	0	0	0	0

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Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27321 PE=4 SV=1	K0V769_MYCFO	0	0	+	0	0	+
Serine esterase, cutinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00909 PE=4 SV=1	K0VAQ7_MYCFO	0	0	+	0	0	+
Phosphate-binding protein PstS OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_31960 PE=3 SV=1	F7P3Y4_MYCPC	+	0	0	0	0	0
Carbohydrate degrading enzyme OS=Mycobacterium avium (strain 104) GN=MAV_1218 PE=4 SV=1	A0QC26_MYCA1	+	0	0	0	0	0
Elongation factor Ts OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=tsf PE=3 SV=1	K0UW15_MYCFO	0	0	+	0	0	+
Fasciclin domain-containing protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_25382 PE=4 SV=1	K0UME3_MYCFO	0	0	+	0	0	+
Catalase-peroxidase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=katG PE=3 SV=1	F7PEL7_MYCPC	+	0	0	0	0	0
Diacylglycerol acyltransferase/mycolyltransferase Ag85B OS=Mycobacterium smegmatis (strain ATCC 700084 / mc(2)155) GN=fbpB PE=2 SV=1	A85B_MYCS2	+	+	0	0	0	0
Peroxisomal multifunctional enzyme type 2 OS=Mycobacterium avium (strain 104) GN=MAV_5146 PE=3 SV=1	A0QMX5_MYCA1	+	0	0	0	0	0
O-methyltransferase OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_00660 PE=4 SV=1	V7LLA4_MYCAV	+	0	0	0	0	0
60 kDa chaperonin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=groL PE=3 SV=1	K0V8R4_MYCFO	0	0	+	0	+	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4742 PE=4 SV=1	A0QLT1_MYCA1	+	0	0	0	0	0
Acetyl/propionyl-CoA carboxylase subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_07781 PE=4 SV=1	K0VB51_MYCFO	0	0	+	0	0	+
Enolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=eno PE=3 SV=1	K0UMN7_MYCFO	+	0	+	0	0	+
Adenylate kinase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=adk PE=3 SV=1	F7P5P8_MYCPC	+	0	0	0	0	0
Acyl-CoA dehydrogenase family protein OS=Mycobacterium avium (strain 104) GN=MAV_4027 PE=3 SV=1	A0QJU2_MYCA1	+	0	0	0	0	0
Probable glyceraldehyde 3-phosphate dehydrogenase gap OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=gap PE=3 SV=1	A1KIM5_MYCBP	+	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium paratuberculosis (strain ATCC BAA-968 / K-10) GN=MAP_0904 PE=4 SV=1	I3NIF2_MYCPA	+	0	0	0	0	0
Peptidyl-prolyl cis-trans isomerase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_16694 PE=3 SV=1	K0V0B7_MYCFO	0	0	+	0	0	+
Trigger factor OS=Mycobacterium avium 05-4293 GN=tig PE=3 SV=1	V7J4X3_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1314 PE=4 SV=1	A0QCC1_MYCA1	+	0	0	0	0	0
Antigen 85-C OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_11566 PE=4 SV=1	K0V526_MYCFO	0	0	+	0	0	+

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Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_2653c PE=4 SV=1	A1KLX8_MYCBP	0	+	0	0	0	0
ATP synthase subunit beta OS=Mycobacterium avium subsp. hominissuis 10-5606 GN=atpD PE=3 SV=1	V7N8Q0_MYCAV	+	0	0	0	0	0
Porin M1 OS=Mycobacterium fortuitum subsp. fortuitum GN=porM1 PE=4 SV=1	A1ING8_MYCFO	0	0	+	0	0	+
Phosphoglycerate kinase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=pgk PE=3 SV=1	K0V8M9_MYCFO	0	0	+	0	0	+
Bacteriocin OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_17350 PE=4 SV=1	V7KBQ7_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20470 PE=4 SV=1	K0UZS8_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_03321 PE=4 SV=1	K0V9J9_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2808 PE=4 SV=1	A0QGF7_MYCA1	+	0	0	0	0	0
Universal stress protein UspA-like protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_2160 PE=4 SV=1	T2GU96_MYCAV	+	0	0	0	0	0
Single-stranded DNA-binding protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=ssb PE=4 SV=1	T2GMI5_MYCAV	+	0	+	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27840 PE=4 SV=1	K0UND9_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_11575 PE=4 SV=1	V7KKF2_MYCAV	+	0	0	0	0	0
Homogentisate 1,2-dioxygenase OS=Mycobacterium avium subsp. hominissuis TH135 GN=hmgA PE=4 SV=1	T2GVD0_MYCAV	+	0	0	0	0	0
Adenosylhomocysteinase OS=Mycobacterium tuberculosis EAS054 GN=ahcY PE=3 SV=1	D5YJL6_MYCTX	0	+	0	0	0	0
60 kDa chaperonin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=groL PE=3 SV=1	K0VKG7_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_4234 PE=4 SV=1	A0QKD7_MYCA1	+	0	0	0	0	0
Dihydrolipoyl dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_22940 PE=3 SV=1	K0UR46_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_3679c PE=4 SV=1	A1KPV0_MYCBP	0	+	0	0	0	0
30S ribosomal protein S6 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=rpsF PE=3 SV=1	K0V3Q6_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_1445 PE=4 SV=1	A0QCQ0_MYCA1	+	0	0	0	0	0
Citrate lyase beta chain citrase beta chain family protein OS=Mycobacterium avium subsp. hominissuis 10-4249 GN=O971_12205 PE=4 SV=1	V7M5M1_MYCAV	+	0	0	0	0	0
Putative acyl-CoA transferase/carnitine dehydratase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_04540 PE=4 SV=1	F7P9F6_MYCPC	+	0	0	0	0	0

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Cutinase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_23050 PE=4 SV=1	F7PEM9_MYCPC	+	0	0	0	0	0
Uncharacterized protein (Fragment) OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_30732 PE=4 SV=1	K0UQD6_MYCFO	0	0	+	0	0	+
Aminopeptidase N OS=Mycobacterium avium (strain 104) GN=pepN PE=4 SV=1	A0QDE6_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium sp. MOTT36Y GN=W7S_13055 PE=4 SV=1	I2AE52_9MYCO	+	0	0	0	0	0
Trypsin OS=Mycobacterium avium (strain 104) GN=MAV_1366 PE=4 SV=1	A0QCH2_MYCA1	+	0	0	0	0	0
50S ribosomal protein L29 OS=Mycobacterium avium subsp. paratuberculosis S397 GN=rpmC PE=3 SV=1	F7P5S9_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=wag31 PE=3 SV=1	A1KKI8_MYCBP	0	+	0	0	0	0
PPE family protein OS=Mycobacterium africanum K85 GN=TBOG_01699 PE=4 SV=1	D6FR36_9MYCO	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_18228 PE=4 SV=1	K0V3D4_MYCFO	0	0	+	0	0	0
Inorganic pyrophosphatase OS=Mycobacterium avium (strain 104) GN=ppa PE=3 SV=1	A0QA70_MYCA1	+	0	0	0	0	0
Metallopeptidase, zinc binding OS=Mycobacterium avium (strain 104) GN=MAV_3451 PE=4 SV=1	A0QI94_MYCA1	+	0	0	0	0	0
Ribonucleoside-diphosphate reductase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=nrdF PE=3 SV=1	K0VW47_MYCFO	0	0	+	0	0	+
Aconitate hydratase 1 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_20690 PE=4 SV=1	K0UZ04_MYCFO	0	0	+	0	0	+
Antibiotic biosynthesis monooxygenase domain protein OS=Mycobacterium avium (strain 104) GN=MAV_0562 PE=4 SV=1	A0QAA4_MYCA1	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. silvaticum ATCC 49884 GN=P863_19285 PE=4 SV=1	V7K6T2_MYCAV	+	0	0	0	0	0
DNA-directed RNA polymerase subunit beta OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=rpoB PE=3 SV=1	K0V8E8_MYCFO	0	0	+	0	+	+
Putative BACTERIOFERRITIN BFRB OS=Mycobacterium tuberculosis 7199-99 GN=MT7199_3910 PE=4 SV=1	L0NZH9_MYCTX	0	+	0	0	0	0
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_06029 PE=4 SV=1	K0VBZ4_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_24502 PE=4 SV=1	K0UTU9_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_3557 PE=4 SV=1	A0QIJ7_MYCA1	+	0	0	0	0	0
60 kDa chaperonin OS=Mycobacterium gastri 'Wayne' GN=groEL PE=3 SV=1	W4HVY4_MYCGS	+	0	+	+	0	0
Iron-dependent repressor IdeR OS=Mycobacterium avium (strain 104) GN=MAV_3604 PE=4 SV=1	A0QIP3_MYCA1	+	+	0	0	0	0

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PadR family transcriptional regulator OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_23167 PE=4 SV=1	K0UVP1_MYCFO	0	0	0	0	0	+
Serine protease pepA OS=Mycobacterium tuberculosis (strain KZN 1435 / MDR) GN=TBMG_00126 PE=4 SV=1	C6DQZ0_MYCTK	0	+	0	0	0	0
Zn-dependent alcohol dehydrogenase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_00964 PE=3 SV=1	K0VMI7_MYCFO	0	0	0	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_04883 PE=4 SV=1	K0VCR8_MYCFO	0	0	+	0	0	+
Secreted antigen 85-B fbpB (Fibronectin-binding protein B) OS=Mycobacterium tuberculosis C GN=TBCG_01838 PE=4 SV=1	A2VJ01_MYCTX	0	+	0	0	0	0
Lipoprotein, ATP binding protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02051 PE=4 SV=1	K0VE69_MYCFO	0	0	+	0	0	+
Trypsin OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_10754 PE=4 SV=1	K0V994_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium avium (strain 104) GN=MAV_2866 PE=4 SV=1	A0QGL4_MYCA1	+	0	0	0	0	0
Polyribonucleotide nucleotidyltransferase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=pnp PE=3 SV=1	K0VIG7_MYCFO	0	0	+	0	0	+
Phosphoenolpyruvate carboxykinase [GTP] OS=Mycobacterium avium (strain 104) GN=pckG PE=3 SV=1	A0QME6_MYCA1	+	0	0	0	0	0
D-3-phosphoglycerate dehydrogenase OS=Mycobacterium avium (strain 104) GN=serA PE=3 SV=1	A0QJC3_MYCA1	+	0	0	0	0	0
30S ribosomal protein S1 OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=rpsA PE=3 SV=1	K0V7I2_MYCFO	0	+	+	0	0	+
Transketolase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26874 PE=3 SV=1	K0UJQ7_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_27790 PE=4 SV=1	K0UND2_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_26979 PE=4 SV=1	K0UWR7_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14737 PE=4 SV=1	K0VQB7_MYCFO	0	0	+	0	0	+
Forkhead-associated protein OS=Mycobacterium avium (strain 104) GN=MAV_2888 PE=4 SV=1	A0QGN6_MYCA1 (+20)	+	0	0	0	0	0
Iron-regulated conserved protein OS=Mycobacterium ulcerans (strain Agy99) GN=MUL_1619 PE=4 SV=1	A0PP51_MYCUA (+5)	0	+	0	0	0	0
Lipoprotein Lpps OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_15267 PE=4 SV=1	K0V6V4_MYCFO	0	0	+	0	0	+
Beta-1,3-glucanase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_02769 PE=4 SV=1	K0V9N4_MYCFO	0	0	+	0	0	+
Conserved protein OS=Mycobacterium marinum (strain ATCC BAA-535 / M) GN=MMAR_4227 PE=4 SV=1	B2HS57_MYCMM	0	+	0	0	0	0
Glucose-6-phosphate isomerase OS=Mycobacterium avium subsp. avium 10-9275 GN=pgi PE=3 SV=1	V7KYA2_MYCAV	+	0	0	0	0	0

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Acetyl-CoA acetyltransferase OS=Mycobacterium avium (strain 104) GN=MAV_1198 PE=3 SV=1	A0QC06_MYCA1	+	0	0	0	0	0
Biotin carboxyl carrier protein OS=Mycobacterium avium subsp. avium 10-9275 GN=O972_01960 PE=4 SV=1	V7LGX8_MYCAV	+	0	0	0	0	0
Enolase OS=Mycobacterium avium (strain 104) GN=eno PE=3 SV=1	ENO_MYCA1	+	0	0	0	0	0
ATP synthase subunit alpha OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=atpA PE=3 SV=1	K0V8M3_MYCFO	0	0	+	0	0	+
Acyl-ACP desaturase OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_17978 PE=4 SV=1	K0UYA2_MYCFO	0	0	0	0	0	+
Thioredoxin OS=Mycobacterium avium (strain 104) GN=trx PE=3 SV=1	A0QNC4_MYCA1	+	0	0	0	0	0
Chaperone ClpB OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_28849 PE=3 SV=1	K0UGP7_MYCFO	0	0	+	0	0	+
Phosphotriesterase-like protein OS=Mycobacterium avium (strain 104) GN=MAV_4940 PE=4 SV=1	A0QMC3_MYCA1	+	0	0	0	0	0
6-phosphogluconolactonase OS=Mycobacterium avium subsp. paratuberculosis S397 GN=MAPs_16990 PE=4 SV=1	F7PCY3_MYCPC	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium 05-4293 GN=O984_01620 PE=4 SV=1	V7JCA2_MYCAV	+	0	0	0	0	0
Phosphoglycerate kinase OS=Mycobacterium avium 10-5581 GN=pgk PE=3 SV=1	V7JEF1_MYCAV	+	0	0	0	0	0
Serine esterase cutinase OS=Mycobacterium avium (strain 104) GN=MAV_2169 PE=4 SV=1	A0QEP1_MYCA1	+	0	0	0	0	0
UPF0234 protein OCU_44330 OS=Mycobacterium intracellulare (strain ATCC 13950 / DSM 43223 / JCM 6384 / NCTC 13025 / 3600) GN=OCU_44330 PE=3 SV=1	H8ISK7_MYCIA	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_3546 PE=4 SV=1	T2GWX9_MYCAV	+	0	0	0	0	0
Amino acid ABC transporter OS=Mycobacterium fortuitum subsp. fortuitum DSM 46621 GN=MFORT_14757 PE=4 SV=1	K0V755_MYCFO	0	0	+	0	0	+
Uncharacterized protein OS=Mycobacterium intracellulare (strain ATCC 13950 / DSM 43223 / JCM 6384 / NCTC 13025 / 3600) GN=OCU_01440 PE=4 SV=1	H8IJI5_MYCIA	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium avium subsp. hominissuis TH135 GN=MAH_4287 PE=3 SV=1	T2GZ63_MYCAV	+	0	0	0	0	0
Uncharacterized protein OS=Mycobacterium bovis (strain BCG / Pasteur 1173P2) GN=BCG_2580 PE=4 SV=1	A1KLQ5_MYCBP	0	+	0	0	0	0

+, indicates that the protein was present, and 0 indicates that the protein was not present