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**Design, development and validation of a series of multiplex real-time PCR diagnostics assays for the rapid and accurate detection and differentiation of the *Mycobacterium tuberculosis* complex**

A thesis submitted to the National University of Ireland for the degree of Doctor of Philosophy by:

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**TABLE OF CONTENTS**

**Acknowledgements**

**Abstract**

**Chapter 1: General Introduction**

**1.0 Introduction** **10**

**1.1 A brief history of the tubercle bacilli** **10**

**1.2 Tuberculosis today** **11**

**1.3 Members of the Mycobacterium tuberculosis complex** **15**

*1.3.1 Mycobacterium tuberculosis* **15**

*1.3.2 Mycobacterium africanum* **16**

*1.3.3 Mycobacterium canettii* **17**

*1.3.4 Mycobacterium bovis* **17**

*1.3.5 Mycobacterium bovis* BCG **18**

*1.3.6 Mycobacterium caprae* **19**

*1.3.7 Mycobacterium microti* **19**

*1.3.8 Mycobacterium pinnipedii* **20**

*1.3.9 Epidemiology of the Mycobacterium tuberculosis complex* **21**

**1.4 The TB Diagnostics landscape** **22**

*1.4.1 Sputum smear microscopy* **22**

*1.4.2 Solid and liquid culture methods* **23**

*1.4.3 Serological tests for Tuberculosis* **24**

*1.4.4 Commercially available nucleic acid amplifications tests for Tuberculosis* **25**

**1.5 Differentiation of the *Mycobacterium tuberculosis* complex** **26**

*1.5.1 DNA Fingerprinting* **27**

*1.5.2 Polymerase Chain Reaction* **27**

*1.5.3 Spoligotyping* **29**

*1.5.4 Mycobacterial interspersed repetitive unit- variable number tandem repeats (MIRU-VNTR)* **30**

*1.5.5 Long sequence polymorphisms and single nucleotide polymorphisms* **31**

*1.5.6 Real-time PCR* **32**

*1.5.6.1 DNA-binding agents* **33**

*1.5.6.2 Hybridisation probes* **34**

*1.5.6.3 Hydrolysis probes* **35**

**1.6 Scope of this Thesis** **36**

**1.7 References** **39**

**Chapter 2: Biodiscovery- Diagnostics target discovery, development and validation**

**2.1 Biodiscovery- Diagnostics target selection** **50**

**2.2 Mycobacterial genome divergence database** **50**

**2.3 Comparative genomics- webACT** **50**

**2.4 Criteria for Go / No Go for further verification** **52**

**2.5 References** **55**

**Chapter 3: A novel multiplex real-time PCR diagnostics assay for the identification and differentiation of *Mycobacterium tuberculosis, Mycobacterium canettii* and the *Mycobacterium tuberculosis* complex**

**Abstract** **57**

**3.1 Introduction** **58**

**3.2 Materials and Methods** **60**

*3.2.1 Diagnostics target identification* **60**

*3.2.2 Bacterial strains, culture media and growth conditions* **61**

*3.2.3 DNA isolation and quantification* **62**

*3.2.4 Conventional and real-time PCR primers and hydrolysis probe design* **63**

*3.2.5 Conventional PCR* **64**

*3.2.6 Sequencing* **64**

*3.2.7 Development of an IAC for real-time PCR* **65**

*3.2.8 Real-time PCR* **65**

*3.2.9 Nucleotide sequence accession numbers* **66**

**3.3 Results** **67**

*3.3.1 Diagnostics targets identification* **67**

*3.3.2 Assay design and development* **68**

*3.3.3 Internal amplification control* **68**

*3.3.4 Specificity of the diagnostic assays* **68**

*3.3.5 Sensitivity of the assays* **69**

**3.4 Discussion** **70**

**3.5 References** **82**

**Chapter 4: A Novel Multiplex Real-Time PCR for the Identification of Mycobacteria Associated with Zoonotic Tuberculosis**

**Abstract** **90**

**4.1 Introduction** **91**

**4.2 Materials and Methods** **93**

*4.2.1 Diagnostics target identification* **93**

*4.2.2 Bacterial strains, culture media and growth conditions* **94**

*4.2.3 DNA isolation and quantification* **94**

*4.2.4 Conventional and real-time PCR primers and hydrolysis probe design* **95**

*4.2.5 Conventional PCR* **96**

*4.2.6 Sequencing* **97**

*4.2.7 Development of an IAC for real-time PCR* **97**

*4.2.8 Real-time PCR* **97**

**4.3 Results** **98**

*4.3.1 Diagnostics target identification* **98**

*4.3.2 Assay design and development* **99**

*4.3.3 Internal amplification control* **100**

*4.3.4 Specificity of the diagnostic assays* **100**

*4.3.5 Sensitivity of the assays* **101**

**4.4 Discussion** **102**

**4.5 References** **114**

**Chapter 5: *Seek*TB- A two stage multiplex real-time PCR based method for the differentiation of the *Mycobacterium tuberculosis* complex**

**Abstract** **123**

**5.1 Introduction** **124**

**5.2 Materials and Methods** **125**

*5.2.1 Ethics statement* **125**

*5.2.2 Bacterial isolates used in this study* **125**

*5.2.3 PCR primers and hydrolysis probe design* **126**

*5.2.4 Conventional PCR and sequencing* **126**

*5.2.5 Real-time PCR* **126**

*5.2.6 Evaluation of SeekTB performance in clinical cultures* **127**

**5.3 Results** **127**

*5.3.1 Diagnostics target identification* **127**

*5.3.2 Specificity of the diagnostic assays* **128**

*5.3.3 Sensitivity of the assays* **129**

*5.3.4 Results interpretation* **129**

*5.3.5 Evaluation of SeekTB performance in clinical cultures* **130**

**5.4 Discussion** **130**

**5.5 References** **146**

**Chapter 6: Concluding remarks and future directions**

**6.1 General Conclusions** **151**

**6.2 Current application of *Seek*TB** **155**

**6.3 Future work** **157**

**6.5 References** **160**

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## Abstract

Tuberculosis (TB) remains a major health concern both in developed and developing countries due to the high rates of morbidity and mortality associated with disease. In humans, TB is caused by members of the *Mycobacterium tuberculosis* complex (MTC) namely *M. tuberculosis*, *M. canettii*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii* and the attenuated *M. bovis* BCG vaccine strain. This group of microorganisms are ~99% similar on a nucleotide sequence level and have a wide range of natural hosts.

While *M. tuberculosis* is responsible for the majority of cases of human TB, accurate identification of other members of the MTC causing infection is not routinely performed. As a result, the global frequency and distribution of each member of the MTC remains largely unknown with some studies suggesting that TB caused by members of the MTC other than *M. tuberculosis* are in fact underreported. Therefore, the capability to accurately identify each member of the MTC causing human TB infection would be desirable. This would enable unambiguous TB epidemiological studies and monitoring of human to human and/or zoonotic TB transmission. Equally, differentiation of the MTC is clinically important for treatment management decisions due to the inherent natural resistance of some members of the complex to the first line anti-TB drug pyrazinamide (PZA).

Currently there is only one commercially available diagnostics assay for differentiation of the MTC, the Genotype MTBC kit. This diagnostics kit is limited by its inability to accurately identify *M. tuberculosis*, *M. canettii*, *M. africanum* and *M. pinnipedii*. There are a number of molecular based assays for MTC differentiation described in the literature which are also limited by an inability to differentiate all members of the MTC and most require post amplification processing increasing method complexity, analysis time and potential contamination.

The overall aim of this study was to design, develop, optimise and validate a robust, internally controlled, multiplex real-time PCR based method for the rapid and accurate identification of all members of the MTC. This was achieved using a sequential experimental design consisting of three main studies.

In the first study, a multiplex real-time PCR diagnostics assay using novel molecular targets was designed to identify the MTC while simultaneously differentiating between *M. tuberculosis* and *M. canettii*. In the second study, a multiplex real-time PCR assay was developed for the identification and simultaneous differentiation of *M. bovis*, *M. bovis* BCG and *M. caprae* in one internally controlled reaction. In the third study two additional biomarkers were incorporated into the previous two multiplex assays specific for the two clades of *M. africanum*. By incorporation of these two additional targets, it was possible to devise a diagnostic algorithm to differentiate all 8 members of the MTC. The specificity of this method was optimised and validated against a panel of 119 MTC isolates which had been previously characterised using methods such as spoligotyping, mycobacterial interspersed repetitive units - variable number tandem repeats (MIRU-VNTR), IS*6110*-based typing methods, RD analysis, biochemical testing in addition to morphological examination. Specificity was also demonstrated against 44 Non Tuberculosis Mycobacteria (NTM) and 17 other bacterial species. Analytical sensitivities of less than 100 genome equivalents were determined for each diagnostics assay developed in multiplex format.

Further evaluation of *Seek*TB was performed by blindly testing 125 Mycobacteria Growth Indicator Tube (MGIT) positive cultures. The results of *Seek*TB were compared to those obtained using the commercially available GenoType MTBC and TBc ID tests. *Seek*TB and GenoType MTBC test results were 100% concordant identifying 84 isolates as *M. tuberculosis* and 41 as non MTC. Nine discordant results were observed between the molecular methods and the TBc ID culture confirmation test, however, nucleotide sequencing of the discordant isolates confirmed the results obtained with *Seek*TB and GenoType MTBC tests

In summary, *Seek*TB, the diagnostic method developed in this study, is the first description of an internally controlled multiplex real-time PCR based diagnostics method for the accurate identification of all eight members of the MTC. This method, designed for use on cultured patient samples, is specific, sensitive and rapid with a turnaround time to results of approximately 1.5 to 3.5 h, depending on which, if any, member of the MTC is present.

# Chapter 1:

# General Introduction

# 1.0 Introduction

## 1.1 A brief history of the tubercle bacilli

At a monthly evening meeting of the Berlin Physiological Society in March 1882, Robert Koch announced his discovery of the tubercle bacilli (89). While his experimental detail was clearly described, this suggestion was met with strong opposition as it was perceived, that owing to the chronic nature of the disease, it was an inherited condition (59). In an effort to prove his theory Koch developed a series of postulates (now known as Koch's Postulates) which remain central in medical microbiology today (89). These postulates include:

1) *The microorganism must be present in diseased individuals and absent in healthy individuals.*
2) *The microorganism must be isolated and grown in pure culture.*
3) *Similar disease must be caused in animals after inoculation with microorganism from pure culture.*
4) *The microorganism must be reisolated and cultured from the diseased animal model and be shown to be the same as the original microorganism.*

It was a significant achievement to identify mycobacteria due to the high concentration of the lipid mycolic acid in their walls which makes Gram staining difficult. Koch developed a staining method using a combination of alkaline methylene blue in conjunction with a bismarck brown stain. This allowed for visualisation of bright blue rod shaped colonies on tissues which remained light brown. To grow the microorganism in pure culture proved a challenge due to the slow growth rate and nutritional requirements of mycobacteria. However, Koch persisted and was able to grow the organism and isolate pure cultures from a medium containing coagulated blood serum. Subsequently, using the pure cultures Koch inoculated healthy guinea pigs with *M. tuberculosis* and they succumbed to disease. Finally, Koch reisolated and cultured the microorganism from infected guinea pigs.

By fulfilling these postulates Koch concluded that “the bacilli present in the tuberculosis lesions do not only accompany tuberculosis but rather cause it. These bacilli are the true agents of consumption”(59).

## 1.2 Tuberculosis today

Since its discovery by Robert Koch in the 1880’s, the tubercle bacillus has remained a formidable pathogen responsible for causing human tuberculosis (TB). TB in humans is a highly contagious infectious disease typically transmitted through cough droplets of infected patients. It is estimated that one third of the global population are infected with TB, however only 10% of these people will develop active TB. Despite advances in the diagnosis and treatment of TB, in 2010 it was estimated that there were 8.8 million new cases of TB and 1.7 million associated death (114).

**Figure 1.1: (Figure has been removed due to Copyright restrictions)**

While a number of working groups including and not limited to the World Health Organisation (WHO), the Centres for Disease Control and Prevention (CDC),

StopTB partnership and Foundation for Innovative New Diagnostics (FIND) have put in place strategies to reduce the global burden of TB, a number of factors may contribute to its persistence;

- Drug resistance

Since the discovery and use of the early mono therapy anti TB drug streptomycin, drug resistance was observed in treatment of TB (21, 40). This has lead to the commonly used multi drug therapeutic approach used today. While the particular combinations of anti-TB drugs utilised have changed over the years, the multi drug approach remains. Currently, the standard short course TB treatment for patients consists of the first line anti-TB drugs isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) for the initial two months, followed by a combination of INH and RIF for a subsequent four months (7, 16).

While this multi drug approach has been successful in TB management, multi drug resistant (MDR-TB) and extreme drug resistant (XDR-TB) isolates now exist which make the control of TB more difficult. MDR-TB is defined as TB whereby the bacilli is resistant to at least INH and RIF, whereas in XDR-TB, the bacilli is resistant to INH, RIF and is also resistant to any fluoroquinolone and to at least one of the following injectable drugs; amikacin, capreomycin and or kanamycin (61, 118).

Both MDR-TB and XDR-TB are attributed to poor patient compliance with anti-TB therapy; inadequate anti-TB drug prescription by health care professionals; inadequate availability of anti-TB drugs; or malabsorption of anti-TB drugs (16, 109). Once a strain becomes resistant it can then be transmitted to other humans, furthering the problem of MDR and XDR TB worldwide. In 2010, it was estimated that there was approximately 650,000 cases of MDR TB globally, however only 46,000 patients were enrolled for MDR TB treatment (114). While no global estimate was made for XDR, at least one strain has now been reported in 58 countries worldwide (115).

- HIV epidemic

The current human immunodeficiency virus (HIV) epidemic has further confounded the problem of TB control. People infected with HIV are more prone to reactivation of latent infection (80-100 fold) and are also are more susceptible to developing active TB than individuals whom are HIV negative (19, 90). In certain countries in Africa, 82% of people with TB are also coinfected with HIV (114). TB is the most common cause of mortality in HIV positive patients (19). Of the 8.8 million new cases of TB in 2010, an estimated 1.1 million new cases and approximately 350,000 deaths were attributed to HIV positive patients (113, 114).

Regardless of CD4 cell counts, the WHO now recommends that all HIV positive patients presenting with active TB should receive antiretroviral treatment (ART) to reduce transmission, morbidity and mortality associated with TB and HIV (113). However, in 2010 only 46 % of HIV positive patients were receiving ART globally (114). To further reduce the burden of TB, the WHO also recommends that all TB patients should be tested for HIV; that an intensified case finding of TB in HIV positive patients should be addressed; and finally that HIV positive patients whom do not have TB should receive isoniazid preventive therapy (IPT) (114).

- Directly Observed Therapy Short-course

The Directly Observed Therapy Short-course (DOTS) strategy was first introduced by the WHO in the early 1990's in an effort to control the spread of TB by breaking the cycle of transmission (67). This strategy initially consisted of 5 requirements:

1) Commitments from governments to provide and sustain TB control activities;
2) Improve case detection using sputum smear microscopy;
3) A standardised treatment regimen of 6-8 months for all sputum smear positive cases for which direct observed therapy is to be conducted for at least the first 2 months;
4) A regular supply of anti-TB drugs;
5) A standardised reporting and recording system to monitor the control programme.

The aims of this strategy were to improve case detection to approximately 70% of sputum smear positive patients in which a cure rate of 85% would be achieved in this cohort (123). While over 100 countries globally (including all 22 high burden countries) have committed to the DOTS strategy some countries failed to realise the aim of reducing TB to these levels. Some reasons for this include poor health care infrastructure; DOTS not being implemented country wide; and both the worsening HIV coinfection and the emergence of drug resistant TB isolates.

Subsequently, the Stop TB partnership in collaboration with the WHO and various working groups published the Stop TB strategy highlighting the advances achieved through the DOTS strategy, but also made further recommendations, as listed below, to aid in the control of TB (120) :

1) Continuation, enhancement and expansion of the DOTS strategy to ensure treatment of all people, particularly the poor;
2) To address the HIV/ TB and drug resistance issues, a scale up of DOTS and other joint ventures;
3) Improvements in health systems and services to aid in control;
4) Scale up of public-private mix (government, non public and private health care providers) to ensure the international standards of TB care are adhered to;
5) Engage people with TB and affected communities to demand, and contribute to, effective care;
6) To ensure that research and development of new diagnostics, anti-TB drugs and vaccines can occur.

By implementing these strategies it is hoped that the case detection and cure rates of 70% and 85% respectively will be maintained and possibly improved upon by 2015. It is also envisaged that the global burden and mortality rates from TB will be reduced by 50% in 2015 (relative to 1990 levels) with the removal of TB as a public health concern by 2050 (less than 1 case per million population) (120). However,

many recent reports suggest that without more novel interventions and significant investments in TB research these targets remain an unrealistic goal (1, 66, 68).

- Causative agent of Tuberculosis

While *M. tuberculosis* is the prominent infectious agent of human TB, there is a group of microorganisms, namely the *Mycobacterium tuberculosis complex* (MTC) responsible for human infection. While the members of the MTC differ in host preference and geographical distribution, they also have different natural responses to anti-TB drugs, therefore require modified therapeutic regimens for patients. Traditional culture based tests and biochemical tests are limited in their scope to for differentiating between the species of the MTC. While in developed countries a number of methods for differentiating between members of the MTC are available and are routinely performed, and described in subsequent sections of this thesis, in high burden countries this is not routinely performed.

## 1.3 Members of the *Mycobacterium tuberculosis* complex

TB in humans is caused by 8 microorganisms collectively known as the *Mycobacterium tuberculosis* complex (MTC). The members of the complex include *M. tuberculosis*, *M. africanum* (West African 1 and West African 2), *M. canettii*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii* and the attenuated for vaccine purpose *M. bovis* BCG. All members of the MTC are considered acid-alcohol fast, non-spore forming, non-motile slow growing bacterium. However some morphological and biochemical differences are present which can be useful for identifying specific members of the MTC. There are also differences in host preferences, intrinsic drug resistances and geographical regions where members of the MTC are encountered.

### *1.3.1 Mycobacterium tuberculosis*

*M. tuberculosis* is the prominent agent of TB in humans and is thought to account for up to 95% of infections (24). Prior to genome sequencing, it was commonly perceived that *M. tuberculosis* arose from a zoonotic transmission of *M. bovis* from cattle to humans. One reason for this perception was that *M. bovis* appeared to have

an ability to infect a wide host range, whereas *M. tuberculosis* nearly exclusively infected humans (13). However, genome nucleotide sequencing revealed that *M. bovis* has a smaller genome size than *M. tuberculosis* and suggests that *M. bovis* has evolved from *M. tuberculosis*. While great diversity is observed globally in the particular strains present in different geographic locations, the species *M. tuberculosis* is present on all continents. The standard short course treatment of INH, RIF, PZA and EMB for the initial 2 months, followed by a combination of INH and RIF for a subsequent 4 months should be administered to patients infected with *M. tuberculosis* (11).

### *1.3.2 Mycobacterium africanum*

*M. africanum* was first identified by Castets *et al.,* in 1968 and was described as an intermediate between *M. tuberculosis* and *M. bovis* (14). From its early description, on the basis of biochemical testing and limited genetic analysis, significant heterogeneity between isolates of this species were observed and two subgroups were suggested. The first subgroup, namely African 1 appeared to be more common in West African countries and showed *M. bovis* like characteristics (*M. africanum* subgroup 1). The second subgroup, African 2 appeared to be common in East African countries and demonstrated *M. tuberculosis* like characteristics (*M. africanum* subgroup 2) (17, 37). On the basis of further genetic studies *M. africanum* subgroup 2 was subsequently reclassified as *M. tuberculosis* sensu stricto (*M. tuberculosis* Uganda type) (13, 50, 70). More recently, *M. africanum* subgroup 1 has been further classified as *M. africanum* West African 1 and *M. africanum* West African 2, with robust specific molecular markers identified for each clade (26, 38, 107). This member of the complex is thought have a host preference of humans and is a prominent cause of TB in humans in Africa (25, 55, 107). While uncommon, cases of human TB caused by *M. africanum* in Europe and the United States have been reported (29, 64). Accurate identification of this member of the MTC is important for epidemiological studies. The standard short course treatment of INH, RIF, PZA and EMB for the initial 2 months, followed by a combination of INH and RIF for a subsequent 4 months should be administered to patients infected with *M. africanum* (11).

### *1.3.3 Mycobacterium canettii*

*M. canettii* was first isolated from a French farmer in 1969 and was defined as *M. tuberculosis* subsp. *canetti*. An additional two cases were described in the subsequent 20 years and the isolates phenotypically characterised with an unusual smooth glossy colony when grown on agar plates (82, 104). This unusual smooth glossy colony morphology is thought to be a result of the high number of lipooligosaccharides in its cell wall (23). Interestingly, this smooth morphology also has the ability to revert to the typical rough colony morphology observed in other members of the MTC (104). Until recently, *M. canettii* was considered a rare cause of TB infection in humans but authors have continuously speculated that the true level of infection may be under represented (92). A significant increase in human TB cases has been attributed to this member of the MTC in recent years, particularly in the horn of Africa. In the Republic of Djibouti, a retrospective study of the causative agent of infections between 1998 and 2001 was performed and revealed that 11.2% of human TB cases were caused by *M. canettii* (62). It has been proposed that the MTC has evolved from *M. canettii* or an *M. canettii* like organism (13, 51). Accurate identification of *M. canettii* is important in a clinical setting as it is intrinsically resistant to PZA. In instances where PZA resistance is observed the treatment regimen recommended for patients consists of INH, RIF, and EMB for the initial 2 months, followed by INH and RIF for a subsequent 7 months (11).

### *1.3.4 Mycobacterium bovis*

While many reports were published in the late 1890's, *M. bovis* was first officially recognised as a bacilli different to *M. tuberculosis* by Karlson and Lessel in 1970 (58). The specific differences were based on the dysgonic growth of colonies observed as opposed to the eugonic colony morphology associated with *M. tuberculosis*. A number of biochemical tests revealed further differences such as an inability to reduce nitrate or accumulate niacin and importantly a resistance to PZA. (58). *M. bovis* is the natural cause of TB in cattle, but has been identified in a large number of animals. It is also responsible for zoonotic transmission of TB to humans.

Prior to the introduction of pasteurisation and eradication measures the rate of bovine TB in humans was as high as 30 % (48, 65). However, this has decreased to negligible levels in regions of the Unites States and many European countries in recent years (87, 91). Unfortunately, the same cannot be said for many developing countries where a prevalence of up to 15% of human TB cases are attributable to *M. bovis* (28). While there are a number of studies specifically associated with bovine TB in humans, a larger number of molecular based studies are required to gain a true reflection of the epidemiology, global frequency and distribution of this member of the MTC. Equally there is a clinical need to specifically identify this member of the MTC as it is intrinsically resistant to PZA. The treatment regimen recommended for patients infected with an *M. bovis* strain consists of INH, RIF, and EMB for the initial 2 months of treatment, followed by INH and RIF for a subsequent 7 months (11).

### *1.3.5 Mycobacterium bovis* BCG

*M. bovis* BCG, which is considered a member of the MTC, is currently used as an attenuated strain for vaccination purposes. This vaccine was developed by Calmette and Guérin after 230 *in vitro* passages on potato bile of a virulent *M. bovis* strain between 1908 - 1921. In 1921, BCG was first used as a vaccine strain to prevent TB (33). As freeze drying techniques were not yet invented, strains were maintained by repetitive subculturing often using different media until the lyophilisation of stocks in the 1960's (8, 77). While this vaccine is amongst the most widely used vaccine on a global scale, it has a reported efficacy of between 0-80 % (33, 83). Some reasons for this high variation in efficacy have been proposed including differences in the particular strain of BCG used for vaccination, the method of delivery of the vaccine or as a result of immune status of a patient (33, 88). In immune competent individuals adverse reactions are not commonly encountered, however BCG has been shown to cause disseminated disease in immunosuppressed patients and children (47, 98). In 2007, the WHO changed its guidelines in relation to BCG vaccination, in that it no longer recommends vaccination of HIV positive children (119). Accurate identification of *M. bovis* BCG strains is important in a clinical setting as like *M. bovis* and *M. canettii* all strains are intrinsically resistant to PZA.

The treatment regimen recommended for patients infected with an *M. bovis* BCG strain consists of INH, RIF, and EMB for the initial 2 months of treatment, followed by INH and RIF for a subsequent 7 months (11).

### *1.3.6 Mycobacterium caprae*

*Mycobacterium tuberculosis* subsp. *caprae* was first described by Aranaz *et al.,* as a causative agent of TB in goats (6). It was subsequently transferred to the *M. bovis* species (*Mycobacterium bovis* subsp. *caprae*) in 2002 and finally elevated to rank of species *M. caprae* in 2003 (5, 75). Since its discovery *M. caprae* has been found in a wide range of animal hosts including goats, cattle and red deer (76, 85). Zoonotic transmission to humans has also been identified in a number of studies and *M. caprae* was responsible for one third of *M. bovis* associated human TB cases in Germany between 1999-2001 (22, 65, 84). While phylogenetically closely related to *M. bovis*, *M. caprae* is considered sensitive to PZA (75). The standard short course treatment of INH, RIF, PZA and EMB for the initial 2 months, followed by a combination of INH and RIF for a subsequent 4 months should be administered to patients infected with *M. caprae* (11).

### *1.3.7 Mycobacterium microti*

*M. microti* was first identified as a causative agent of TB in a wild voles in the late 1930's (108). In subsequent studies it was also shown to be the causative agent of TB in a number of mammals (106). *M. microti* was thought to be avirulent in humans and was used as a vaccine strain in the United Kingdom and the Czech Republic around the 1950's without serious complications (44, 95). However, in 1998 it was discovered that *M. microti* was a causative agent of TB in both immunocompromised and immunocompetent individuals in the Netherlands (105). Since then, *M. microti* has been associated with cases of human TB in a number of studies (27, 34, 36, 39, 49, 53, 74, 79, 125). *M. microti* are extremely slow growing mycobacteria, often requiring longer incubation times than the 8 weeks routinely used in clinical laboratories (27). When visible colonies are observed *M. microti*

isolates can be morphologically characterised by an S-shaped-curved-rod cell, however this characteristic can be lost upon subculturing (105). Using biochemical tests it is also difficult to differentiate *M. microti* from other members of the MTC (86, 105). As a result of these features a number of studies have suggested that the true level of human TB attributed to *M. microti* may be under represented (27, 74, 105). Accurate identification of this member of the MTC using molecular based methods is important to answer this question. The standard short course treatment of INH, RIF, PZA and EMB for the initial 2 months, followed by a combination of INH and RIF for a subsequent 4 months should be administered to patients infected with *M. microti* (11).

### *1.3.8 Mycobacterium pinnipedii*

*M. pinnipedii*, the seal bacillus, was first described as a causative agent of TB in seals and a seal trainer in 1993 (99). From its early description this member of the MTC was thought to be an intermediate between *M. tuberculosis* and *M. bovis*. For example, like *M. bovis*, it was unable to reduce nitrate or accumulate niacin but like *M. tuberculosis*, it was sensitive to PZA (78). Based on phenotypic and genotypic analysis the seal bacillus was elevated to the rank of species *M. pinnipedii* in 2003 (20). This member of the MTC has been identified in a number of marine mammals and has been empirically demonstrated to have the ability to cause TB in guinea pigs and rabbits (10, 20). Also, the ability of *M. pinnipedii* to cause zoonotic TB in humans was demonstrated in a number of studies (60, 99). The standard short course treatment of INH, RIF, PZA and EMB for the initial 2 months, followed by a combination of INH and RIF for a subsequent 4 months should be administered to patients infected with *M. pinnipedii* (20).

**Figure 1.2: (Figure has been removed due to Copyright restrictions)**

*1.3.9 Epidemiology of the Mycobacterium tuberculosis complex*

Human TB caused by each member of the MTC has been reported. However, as differentiation of the MTC is not routinely performed, particularly in low resource settings, the true level of TB in humans caused by members of the MTC other than *M. tuberculosis* remains largely unknown. It has been suggested that the prevalence of human TB caused by members of the MTC other than *M. tuberculosis* is underrepresented (74, 93).

This is particularly true in certain geographical regions for example, it has been suggested that *M. africanum* was responsible for up to 50 % of human TB in certain regions in Africa (54). While human TB caused by *M. canettii* is considered rare, it was responsible for 11.2 % of TB cases in the Republic of Djibouti (62).The rates of bovine TB in humans has decreased dramatically in low burden countries, however it has been suggested that in high burden countries *M. bovis* could account for up to 15 % of human TB cases (28). Also, in Germany *M. caprae* was responsible for one third of human *M. bovis* associated TB cases between 1999-2001.

To determine the true epidemiological impact of each members of the MTC and to monitor for human to human or zoonotic TB transmission both retrospective and prospective studies are required.

Also, due to the varying natural resistances observed between members of this complex there is a clinical need to rapidly and accurately determine the causative agent of TB infection. To this end a number of diagnostics methods have been developed in the last 20 years.

## 1.4 The TB Diagnostics landscape

### *1.4.1 Sputum smear microscopy*

In most developed countries diagnosis of TB has improved greatly with the advent of liquid culture and more importantly nucleic acid amplification techniques (NAAT) which are useful for genotyping of strains and drug susceptibility testing (DST).

However, in resource poor regions of the world, where the highest levels of TB are reported, such advances have not been routinely used. In these settings, diagnosis of TB still relies on the clinical presentation of the patient or laboratory diagnosis of Acid Fast Bacilli (AFB) using sputum smear microscopy.

For smear microscopy, a sputum sample is typically directly smeared onto a slide, subjected to Ziehl-Neelsen staining and analysed using a conventional light microscope. While sputum smear microscopy is both a rapid and inexpensive way to diagnose TB it is notoriously insensitive (5000-10000 bacilli/ ML of sputum is required) and is not specific in incidences where Non Tuberculosis Mycobacteria (NTM) are prevalent (43, 124, 126).

Also, the use of sputum for diagnosis of TB in children is difficult as children often cannot produce (cough up) enough sputum for subsequent laboratory analysis. As a high number of patients in low resource settings present with HIV co-infection, this complicates the use of smear microscopy further owing to the lower bacillary loads typically seen in these patients (81).

Currently the WHO recommends that two sputum samples are taken from a patient for analysis, one sample on presentation and a further one sample the following morning (116). For many patients it is not practical or affordable to return to centres on multiple occasions, therefore the dropout rates for diagnosis and treatment of TB represent a serious problem (81).

In addition, it has been proposed that Light Emitting Diode Fluorescence Microscopy (LED FM) should be phased in as an alternative to Ziehl-Neelsen microscopy (117). However, while faster to perform, recent studies show that the sensitivity is only modestly if at all improved (2, 12).

### *1.4.2 Solid and liquid culture methods*

Culturing of bacterial isolates as opposed to AFB sputum smear microscopy is a more sensitive method of detection (10-100 bacilli/ ML of sputum). While the sensitivity of culture methods is superior to sputum smear microscopy, a significant limitation of this approach is the time to result owing  to the slow growth rate of mycobacterium (35). On solid culture it can take up to 6 weeks to culture some members of the MTC (52).

In low resource settings, LJ egg-based solid medium is most frequently used for culturing of MTC isolates as it can be prepared locally and has a good shelf life. Using solid culture media, such as LJ egg-based solid medium, also allows for DST to be performed to determine antibiotic resistances (81). Also biochemical tests can be performed on solid agar, which can be useful in ascertaining which member of the MTC is present. However, the interpretations of these tests can be cumbersome to perform and the results are often subjective and unreliable (50).

More recently, the WHO has recommended the use of liquid culture in high burden settings which, if implemented, could potentially greatly improve the diagnosis rate of TB (121). In 2007, the WHO Strategic and Technical Advisory Group (WHO-STAG) endorsed the use of Mycobacterial Growth Indicator Tubes (MGIT) developed by Becton Dickenson. MGIT contain enriched culture media which allow growth of mycobacteria in 10-14 days (52). They contain chemicals which fluoresce

if oxygen is consumed by microorganisms which allows for either manual or automated detection. MGIT can also be used for DST by comparing the growth rates of bacteria in the presence of anti-TB drugs.

However, MGIT positive growth alone does not allow for identification of the MTC as other NTM and aerobic actinomycetes can also grow under these conditions. Alternative liquid based culture tests include the MB/BacT alert system (bioMerieux, France) and the VersaTREK system (Thermo Fisher Scientific, Waltham, United States). However, like the MGIT system NTM and other aerobic actinomycetes can also be cultured in these systems (81).

### *1.4.3 Serological tests for tuberculosis*

Serology based tests have become increasingly used if the field of infectious disease and are considered simple to perform and inexpensive. A large number of serological tests have become available for the rapid diagnosis of TB (81). Serological tests typically rely on the antibody recognition of antigens of *M. tuberculosis* by the humoral immune response. In 2010 the WHO conducted a meta analysis study comprising of 67 individual pulmonary and 25 extrapulmonary TB studies which specifically used serological tests to evaluate the specificity and sensitivity achieved (111). In this study the sensitivity ranged from 0-100% and the specificity from 31-100% in patients with pulmonary TB. In patients with extrapulmonary TB the specificity and sensitivities ranged from 0-100% and 59-100% respectively (111).

Based on the highly variable, imprecise results achieved using serological tests, in July 2011 the WHO announced ‘negative’ policy and strongly recommended that these commercial tests not be used for the diagnosis of pulmonary and extra-pulmonary TB (112). This was the first ‘negative’ policy recommendation that the WHO have issued in relation to TB diagnostics.

### *1.4.4 Commercially available nucleic acid amplifications tests for Tuberculosis*

Owing to the speed, specificity and sensitivity which can be achieved using *in-vitro* nucleic acid amplification methods, a number of commercially available tests are routinely used in the field of infectious disease (31). They could demonstrate advantages in TB diagnostics as results can be achieved in a number of hours, as opposed to weeks required for culturing. This can result in earlier initiation of treatment; improved patient outcome; increased opportunities to interpret transmission patterns; and provide more effective public health interventions (15).

Currently, there is only one Food and Drug Administration (FDA) approved commercially available TB test for smear positive/negative samples namely the Amplified MTD assay (Transcription Mediated Amplification (TMA) in a single tube, Genprobe). This test has the ability to detect the presence of a member of the MTC, but cannot identify which particular species. Other commercially available tests include the Amplicor PCR (Roche) which utilises Polymerase Chain Reaction (PCR) and the BD ProbeTec (BD) which utilises strand displacement amplification. While these tests a reported overall sensitivity of 90-100 % they only detect the presence of the MTC (81).

Hain Lifesciences have a wide range of line probe assays in the field of mycobacteria which utilise conventional PCR followed by reverse line hybridisation techniques. These include the GenoType®MTBDR*plus* (WHO endorsed 2008) and GenoType®MTBDRsl which can be used directly on clinical samples. These tests can identify the presence of the MTC and also provide information on drug resistance. The disadvantages of these tests are they cannot identify the specific member of the MTC and also they require post amplification handling of samples which can allow for the introduction of contamination.

An additional test for the detection of the MTC and RIF resistance is the INNO-LiPA Rif (Innogentics) test which again is based on PCR and reverse line hybridisation.

In 2010, the WHO endorsed the Xpert® MTB/RIF test (Cepheid) which must be considered state of the art in molecular diagnostics. This test is a real-time PCR based assay and has the ability to detect the MTC and RIF resistance in a simple 'sample in answer out' platform in approximately 2 hours. As the sputum sample is

loaded directly onto a cartridge, which can be disposed of directly after the test, the risk to health care providers is greatly reduced. In collaboration with FIND, Cepheid are making available and distributing at a reduced cost this technology to almost 150 low income and endemic disease countries. Early studies on the specificity and sensitivity of the test are promising and this test could demonstrate great potential in the global effort to control TB. However, a limitation of this test is it cannot identify the member of the MTC causing human infection.

While great progress is being made in the development of NAAT's for TB diagnostics, there are very a limited number of studies, referred to in chapters 2-4, specifically developing multiplex real-time PCR diagnostics assays for differentiating the species of the MTC. There is currently only one commercially available test, the GenoType MTBC (Hain Lifesciences) which attempts to differentiate the species of the MTC. This test (based on SNP's in the *gyrB* gene and a region of RD1) requires conventional PCR followed by reverse line hybridisation. While useful for identifying certain species of the MTC, this test cannot accurately identify *M. tuberculosis*, *M. canettii*, *M. africanum* or *M. pinnipedii*. Further disadvantages of this test are it requires post amplification handling of samples and is not suitable for direct use on sputum smear positive samples.

### 1.5 Differentiation of the *Mycobacterium tuberculosis* complex

A final recommendation by the WHO, that will be the focus of this thesis, is that all mycobacterial isolates should be characterised to the species level or at least that it is classified as MTC positive and specifically differentiated from the NTM (121). There are currently three well characterised and recognised methods for genotyping mycobacterial strains which can also be used for differentiation of the MTC to the species level namely, DNA fingerprinting, spacer oligotyping (spoligotyping) and mycobacterial interspersed repetitive unit- variable number tandem repeats. Each of these methods are well studied, have known advantages and disadvantages, and are discussed below. On the other hand real-time PCR, particularly multiplex real-time PCR is becoming more routinely used both for the diagnosis of TB and for differentiation of the MTC.

### *1.5.1 DNA Fingerprinting*

The most commonly used, standardised method for differentiating between strains and species of the MTC is restriction fragment length polymorphism (RFLP) analysis of the insertion sequence *6110* (IS*6110*) (57, 100, 103). Briefly, this method involves culturing of an organism, extraction and purification genomic of DNA, followed by digestion with a restriction enzyme (*PvuII*) and separation on an agarose gel. After separation, the restriction fragments are transferred to a membrane and a peroxidase-labelled complimentary IS*6110* probe and hybridisation buffer are added. Following hybridisation, a chemiluminescent signal is observed on a light sensitive film (102). In a study performed by Kremer *et al.,* it was shown that IS*6110* RFLP is highly specific, discriminatory and reproducible (64). However, there are also disadvantages to this method namely, IS*6110* RFLP can take weeks to achieve results as the organisms need to be cultured, it is labor intensive and is hindered in some MTC isolates that have few or no IS*6110* copies (63). RFLP analysis of IS*6110* is predominantly used for differentiating between stains of *M. tuberculosis* but can be useful for identifying potential cases of *M. bovis* infection which typically have low copy numbers of IS*6110*.

### *1.5.2 Polymerase Chain Reaction*

Since the introduction of Polymerase Chain Reaction (PCR) in 1986, it has become a widely used in all molecular applications (72). Briefly, PCR is an *in vitro* amplification method which allows for the amplification of a specific region of DNA using oligonucleotide primers which flank the region of interest and a heat stable DNA polymerase, typically derived from *Thermus aquaticaus*. Double stranded DNA is separated by heating (95 °C) and the primers anneal to the single strands upon cooling (temperature dependant on that of the primers). In the presence of magnesium chloride ($MgCl_2$), deoxyribonucleotide triphosphates (dNTP's) and reaction buffer, primer extension occurs at the optimal temperature of the DNA polymerase (72 °C). This results in newly synthesized copies of target which can then act as target for subsequent rounds of synthesis. As such, the amount of DNA target generated increases exponentially making this a highly sensitive method in

molecular biology (Figure 1.3). Upon completion of the PCR, products are typically subjected to electrophoresis on an agarose gel stained with ethidium bromide and the results are visualised under ultraviolet light (72).

In the area of genotyping of MTC isolates, PCR has recently been used directly and has proven a powerful tool for differentiation of the MTC with long sequence polymorphisms. PCR is also a critical component prior to spoligotyping and mycobacterial interspersed repetitive unit- variable number tandem repeats. However, a significant limitation of PCR is the requirement for post-amplification processing of samples which increases the possibility of introducing contamination.

**Figure 1.3: (Figure has been removed due to Copyright restrictions)**

*1.5.3 Spacer Oligotyping (Spoligotyping)*

Spoligotyping is another commonly used molecular based method which is used for genotyping *M. tuberculosis* strains. It can also be used for differentiation of species of the MTC. This technique, first developed by Kamberbeek *et al.*, targets polymorphisms present in the direct repeat (DR) locus region, present only in members of the MTC (46, 56). This locus consists of DRs of 36 bp separated by non repetitive spacers varying between 35-41 bp (46). Typically 43 spacers are utilised during spoligotyping, in which certain spacer regions are lost in specific members and strains of the MTC. When using spoligotyping the complete DR locus is PCR amplified allowing for amplification of all spacers in this region. PCR products are then reverse hybridised to a membrane containing complimentary spacer oligonucleotides. After hybridisation the membranes are washed and the results are read by chemiluminiscent signal. Based on the presence and absence of certain spacers, identification of particular strains and species of the MTC can be detetrmined, as seen in Figure 1.4 (71, 102). However a disadvantage of this method is the requirement for post amplification handling of samples, which increases the possibility of introducing contamination.

**Figure 1.4: (Figure has been removed due to Copyright restrictions)**

### *1.5.4 Mycobacterial interspersed repetitive unit- variable number tandem repeats (MIRU-VNTR)*

*In vitro* amplification methods of minisatellites that contain variable number of tandem repeats have been proven useful for identification of strains and species of the MTC. These minisatellite regions termed mycobacterial interspersed repetitive unit (MIRU) were first identified by Supply *et al.,* (97). Of the 41 MIRU loci identified in *M. tuberculosis*, the use of 12 loci (Figure 1.5) can give the same discriminatory power as IS*6110*-RFLP analysis (69). When using this method a standardised protocol has been proposed consisting of amplification of multiple loci using specific primer pairs targeting regions which flank the repeat locus region. Subsequently, the amplicons are analysed either using gel electrophoresis or automated capillary based systems to determine the number of MIRU-VNTR copies present (4, 96). Recently, a freely accessible web based resource detailing the MIRU-VNTR signature of 186 well characterised species and strains of the MTC has become available (3, 110).

**Figure1. 5: (Figure has been removed due to Copyright restrictions)**

*1.5.5 Long sequence polymorphisms and single nucleotide polymorphisms*

Despite the high level of nucleotide sequence similarity observed between members of the MTC, whole genome sequencing, bacterial artificial chromosome arrays and DNA microarrays have identified 20 regions ranging from ~2 - 12.7 kb that are deleted in specific members of the MTC (9, 42). These regions are specifically referred to as Long Sequence Polymorphisms (LSP) or as Regions of Difference (RD). In mycobacteria, RD's represent regions of the genome which are present in *M. tuberculosis* and subsequently deleted in other members of the MTC. As deletion events are considered stable and irreversible, the use of RD analysis allows for the construction of a robust phylogenetic tree which cannot be achieved using the other genotyping methods described. A number of studies have shown the suitability of RD's for diagnostics assays and for differentiation of the MTC to the species level using conventional PCR (13, 50, 51, 80). In these studies, PCR primers either flanking regions of RD's or internal in the RD's were used. Upon completion of the PCR products were run on an agarose gel and species identified based on the presence or absence of specific size bands (Figure 1.6). The main disadvantages of using conventional PCR for differentiation of the MTC include; the requirement for multiple reactions which increases method complexity; and the requirement for post amplification handling of samples which increases the possibility of introducing contamination.

**Figure 1.6: (Figure has been removed due to Copyright restrictions)**

Members of the MTC are considered genetically monomorphic, meaning the occurrence of single nucleotide polymorphisms (SNP) (18). It has been demonstrated that the number of SNP's present between members of the MTC occurs at a lower frequency than many other pathogenic bacteria (94). As no horizontal gene transfer has been identified in members of the MTC and convergence within SNP's are rare, they have proven useful for phylogenetic studies and as species specific diagnostics markers [examples include *gyrB* (*M. microti*, *M. caprae*), *pncA* (*M. bovis*, *M. canettii*), *hsp65* (*M. canettii*) (32, 41, 73, 93)] Additionally, SNP's are important indicators of both intrinsic or acquired drug resistance [*pncA* (PZA)*; rpoB (RIF)*, *inhA, katG, ahpC* (INH)*; rpsL, rrs* (SM) (101)].

### *1.5.6 Real-time PCR*

While the methods described above are well established and commonly used for genotyping of MTC isolates, real-time PCR offers a new rapid and specific alternative approach which remains in its infancy for differentiation of the MTC to the species level. Real-time PCR is a method based on the detection and quantitation of PCR products as they accumulate. This occurs by monitoring of a fluorescent signal in real time as PCR products accumulate. The signal which is emitted increases at a rate which is directly proportional to the amount of PCR product present in the reaction. A detectable increase in fluorescence above a baseline value indicates PCR product is being accumulated. The exact point at which this occurs is known as the Cycle threshold ($C_T$) value (30). The concentration of starting template is reflected by this value such that if a high concentration of starting template is used an early $C_T$ value will be observed. Conversely, if a low concentration of initial template is used a much later $C_T$ will be observed. This property of real-time PCR allows for the absolute quantification of an unknown sample, provided a standard curve of known concentrations of targets is designed and incorporated into a real-time PCR run. By monitoring of the fluorescent signal in real time, there is no requirement for post amplification handling of samples, which reduces the possibility of introducing contamination (30). There are currently 3 main fluorescence systems used for monitoring of DNA amplification, namely; DNA-binding agents, hybridisation probes and hydrolysis probes.

#### *1.5.6.1 DNA-binding agents*

Double-stranded DNA binding dye chemistry, quantitates the amplicon production by the use of a non-sequence specific fluorescent intercalating agent most commonly SYBR green, which  does not bind to ssDNA. SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Figure 1.7). While SYBR green is a cheaper alternative to hydrolysis or hybridisation probes discussed below, an inherent disadvantage is the requirement for extensive optimisation to

ensure non-specific amplification or primer dimers interfere with the detection and quantification of products which can lead to the reporting of false positives (30).

**Figure 1.7: (Figure has been removed due to Copyright restrictions)**

### *1.5.6.2 Hybridisation probes*

Hybridisation probes, commonly referred to as FRET probes or LightCycler probes, consist of a donor probe (3' labelled with a donor fluorophore, excited by light) and an acceptor probe (5' labelled with an acceptor fluorophore, not excited by light) which are typically located 1-5bp apart from each other. During the primer annealing phase of the PCR, both probes hybridise to their sequence specific target. Due to the close proximiety of the probes, the excitation energy is transferred by Fluorescence Resonanse Energy Transfer (FRET) from the donor to the acceptor fluorophore (Figure 1.8) (122). This results in a fluorescent signal which can be read by a real-time PCR instrument.

**Figure 1.8: (Figure has been removed due to Copyright restrictions)**

*1.5.6.3 Hydrolysis probes*

Hydrolysis probes (also known as Taqman probes) utilise the 5' exonuclease activity of *Taq* DNA polymerase (45). These probes are typically 20-30 bp in length, have a melting temperatuire 5-10 °C higher than the primers and contain a fluorescent signal (reporter) on the 5' end (eg: FAM, HEX, ROX, Cy5) and a dark quencher on the 3' end [eg. Black Hole Quencher (BHQ)]. When the reporter fluorophore is excited by an outside light source, the fluorescence of the reporter is quenched by the nearby quenching dye and no reporter fluorescence is observed. During PCR, when the Taq polymerase encounters the bound probe it digests the probe which releases the fluorophore from the adjacent quencher (30). When the fluorophore is released it emits a fluorescent signal which can then be monitored (Figure 1.9). This signal increases as the numbers of cycles are performed.

**Figure 1.9: (Figure has been removed due to Copyright restrictions)**

## 1.6 Scope of this Thesis

The main objective of this study was to design, develop and optimise a robust unidirectional hydrolysis probe based multiplex real-time PCR based method for the detection and accurate identification of each member of the MTC, using the Molecular Diagnostics Research Group (MDRG) at NUI, Galway clinical diagnostics assay development strategy (Figure 1.10):

- **Comparative genomics/Bio discovery** Identification of novel nucleic acid based diagnostics targets
- **Design** of specific diagnostics assays
- **Assembly** of a panel of well characterised, geographically relevant species/strains
- **Evaluation** of assay performance- multiplexing optimisation and validation
- Small scale **clinical investigation**

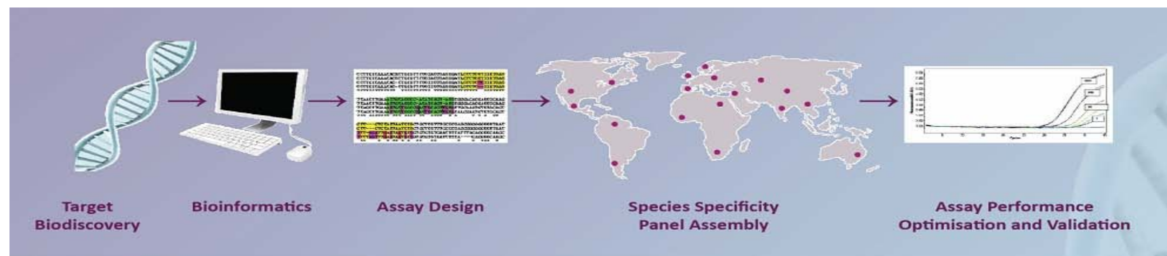


**Figure 1.10 MDRG clinical diagnostics assay development strategy**

Firstly, **Chapter 2** describes the various *in silico* approaches utilised for the identification of putative MTC species specific diagnostics targets. Subsequently, the criteria for demonstrating the suitability of each target identified, and the final optimisation and validation of species specific targets is discussed.

**In Chapter 3**, an internally controlled multiplex real-time PCR diagnostics assay was developed for the detection of the MTC and the simultaneous identification and specific differentiation of *M. tuberculosis* and *M. canettii* in approximately 1.5hr post DNA extraction. All diagnostics targets utilised in this test were novel and identified during this study. This chapter also describes a novel method for genomic DNA extraction and purification from mycobacteria. The diagnostics assay was tested against a panel of well characterised, geographically relevant MTC isolates, NTM and other bacterial species. The diagnostics assay proved to be a rapid, specific, easy to interpret and a sensitive method.

In **Chapter 4**, as before, an internally controlled multiplex real-time PCR diagnostics assay was developed for the detection and identification of the members of the MTC most commonly associated with zoonotic TB, namely *M. bovis* and *M. caprae* using novel diagnostics targets. This test also allowed for the unambiguous identification of *M. bovis* BCG by incorporation of an assay targeting RD1. This diagnostics assay could be used as an independent test where zoonotic TB was suspected or alternatively could be used as a follow on test from that described in **Chapter 3**, allowing for identification of 5 out of 8 members of the MTC. This test was evaluated on the same panel of organisms as tested for in **Chapter 3**, with some additional *M. caprae* and *M. pinnipedii* strains.

**In Chapter 5**, a two stage internally controlled multiplex real time PCR based method, *Seek*TB, is described for the accurate detection and differentiation of each member of the MTC, including differentiation of the 2 clades of *M. africanum*. This method was tested against a much larger panel of MTC isolates than the previous 2 chapters. Upon validation of the method, a biobank of uncharacterised MTC isolates from Africa were blindly tested to show the method developed was robust and suitable for use in the field. The results of the TBcID test were compared to the only commercially available test (GenoType MTBC) and the *Seek*TB method. While some discordance was observed in this study, both molecular methods showed 100% concordance. Nucleotide sequencing of the discordant results showed that the results of the molecular methods were correct.

The principal conclusions of this research along with potential future research directions are discussed in **Chapter 6**.

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# Chapter 2:

# Biodiscovery: Diagnostics target discovery, development and validation

## 2.1 Biodiscovery: diagnostics target selection

The initial stage of the work performed in this thesis was biodiscovery and identification of MTC species specific nucleic acid based diagnostics targets. To identify a panel of putative nucleic acid diagnostics targets two *in silico* approaches were undertaken;

- Identification of biomarkers using an online repository of genetic differences between *M. tuberculosis, M. bovis* and *M. bovis* BCG strains.
- Whole genome comparative genomics

## 2.2 Mycobacterial Genome Divergence Database

Insertions, repeat regions, divergent regions and SNP's were identified using the Mycobacterial Genome Divergence Database (MGDD) (2). This database is a user friendly online repository which contains previously identified genetic differences between 4 strains of *M. tuberculosis* and 1 strain of *M. bovis* and *M. bovis* BCG respectively. While this approach was useful, as a starting point, it was limited to identifying previously known genetic differences which were deemed unsuitable for identifying specific members of the MTC. Subsequently, it was decided to perform whole mycobacteria genome comparative genomics in order to identify suitable diagnostics targets.

## 2.3 Comparative genomics- WebACT

In this study comparative genomics were performed by aligning the publicly available genome sequences for *M. tuberculosis* H37RV and *M. bovis* AF2122/97 using a web based version of the Artemis Comparison Tool (webACT) (1). Approximately 3,000 annotated genes, representing coding regions of these genomes, were analysed to identify novel insertions, deletions and SNP's between species. Putative diagnostics targets selected by this approach were subsequently BLAST analysed (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure they were

specific for their intended purpose *in silico* against additional publicly available nucleotide sequence for MTC members and close relatives. Nucleotide sequences were also retrieved from the Welcome Trust Sanger Institute database (http://www.sanger.ac.uk/resources/downloads/bacteria/mycobacterium.html), which at the time, were sequencing *M. canettii*, *M. africanum* and *M. microti*, and alignments performed using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/). For the remaining members of the MTC for which no nucleotide sequence was available, namely *M. caprae* and *M. pinnipedii*, specificities of the selected diagnostics targets were demonstrated experimentally and subsequently validated through nucleotide sequencing. Initially 41 putative diagnostics targets, specific for members of the MTC, were identified *in silico* but after experimental work was carried out on these putative targets, 31 targets (Table 2.3) were deemed unsuitable for the purpose of this project. The following outlines general observations by which we deemed the 31 putative targets as unsuitable for this study and are further discussed in section 2.4 Criteria for Go / No Go for further verification below;

- The genetic difference identified *in silico* was not present in any of the MTC strains held in our collection.
- The genetic difference identified *in silico* was not present in all MTC strains in our collection.
- Based on the final format of the test and the algorithm developed for results interpretation, the diagnostics assay developed was not required (as was the case for identification of *M. microti*). Also, as it is currently only possible to detect five specific analytes when using hydrolysis probe based assays in a multiplex format, it was not possible to incorporate this target.

## 2.4 Criteria for Go / No Go for further verification

Each putative diagnostics target was evaluated separately and sequentially in accordance with the seven steps outlined below. Upon completion of the target discovery phase of this study 10 diagnostics targets were deemed suitable for incorporation into the diagnostics assays.

1. Identify species specific target *in-silico* – 3000 analysed: **41 Go**
2. Design sequencing primers to span region of interest: **41 Go**
3. Generate nucleotide sequence for 2-4 representative strains- If genetic difference identified is present proceed to step 5: **21 Go**
4. If genetic difference identified was not present return to step 1: **20 No Go**
5. Design real-time PCR assay and test on full panel of MTC isolates to ensure target is specific. If specific continue to step 7:**11 Go**
6. If target was not specific for a particular species **or** was not present in all MTC strains of interest return to step 1:**10 No Go**.
7. Optimise and validate the assay in a multiplex real-time PCR format:10 Go

Table 2.1 Putative diagnostics targets disregarded after experimental validation (No Go Targets)

| Gene Name/Proposed Function | Biomarker Specific For | Comment |
|---|---|---|
| *cut*5A (Rv3724) | *M. tuberculosis* | 1bp insertion *in silico,* not present in all *M. tuberculosis* strains tested |
| *hddA* (Rv0115) | *M. tuberculosis* | 1bp deletion *in silico*, not present in all *M. tuberculosis* strains tested |
| *glgB* (Rv1326c) | *M. tuberculosis* | 2 SNP's in probe region *in silico*, not present in all *M. tuberculosis* strains tested |
| *ppdK* (Rv1227) | *M. tuberculosis* | 2SNP's in probe region *in silico*, not present in all *M. tuberculosis* strains tested |
| *moeB1* (Rv3206c) | *M. bovis* | 3bp deletion *in silico*, not present in any *M. bovis*strains tested |
| Transcriptional regulator (Rv2779c) | *M. bovis* | 24bp deletion *in silico*, not present in any *M. bovis* strains tested |
| *sseB* (Rv2291) | *M. bovis* | 2bp deletion *in silico*, not present in any *M. bovis* strains tested |
| *ddlA (*Rv2981c) | *M. bovis* | 9bp deletion *in silico*, not present in any *M. bovis*strains tested |
| *pknH* (Rv1266c) | *M. bovis* | 2 SNP's in probe region *in silico*, not present in any *M. bovis* strains tested |
| Ketoacyl reductase (Rv1544) | *M. bovis BCG* | 10bp deletion *in silico*, not present in any *M. bovis* BCG strains tested |
| *sugI* (Rv3331) | *M. bovis BCG* | 15bp deletion *in silico*- not present in all *M. bovis* BCG strains tested |
| *lepA* (Rv2404c) | *M. canettii* | 2SNP's in probe region *in silico*, not present in *M. canettii* strains tested |
| Aldehyde dehydrogenase (Rv0223c) | *M. africanum* | 12 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| transcriptional regulatory protein (Rv1773c) | *M. africanum* | 6 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| Hypothetical protein (Rv1995) | *M. africanum* | 7 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| Hypothetical protein (Rv3122) | *M. africanum* | 4 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| Hypothetical protein (Rv1507c) | *M. africanum* | 4 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| Hypothetical protein (Rv2529) | *M. africanum* | 3 bp deletion *in silico*, not present in any *M. africanum* strains tested |
| *rpoB* (Rv0667) | *M. microti* | 6 bp deletion *in silico*, not present in all *M. microti* strains tested |
| *mmr* (Rv3065) | *M. microti* | 7 bp insertion *in silico*, not present in any *M. microti* strains tested |
| *gnd1* (Rv1844c) | *M. microti* | 3 bp insertion *in silico*, not present in any *M. microti* strains tested |
| Probable monooxygenase (Rv3049c) | *M. microti* | 1 bp insertion *in silico*, not present in any *M. microti* strains tested |
| *fas* (Rv2524c) | *M. microti* | 6 bp deletion *in silico*, not present in any *M. microti* strains tested |
| Conserved hypothetical protein (Rv3365c) | *M. microti* | 2 bp deletion *in silico*, not present in any *M. microti* strains tested |
| *ephB* (Rv1938) | *M. microti* | 3 bp deletion *in silico*, not present in all *M. microti* strains tested |
| ephB (Rv1938) (Different region of gene) | *M. microti* | 1 bp deletion *in silico*, not present in all *M. microti* strains tested |
| Conserved membrane protein (Rv0996) | *M. microti* | 6 bp deletion *in silico*, not present any *M. microti* strains tested |
| *far* (Rv0855)* | *M. microti** | 6 bp deletion *in silico*, present in all *M. microti* strains tested* |
| Conserved hypothetical protein (Rv1894c) | *M. microti* | 3 bp deletion *in silico*, not present in any *M. microti* strains tested |
| Hydrolase (Rv3677c) | *M. microti* | 3 bp deletion *in silico*, not present in all *M. microti* strains tested |
| *devR* (Rv3133c) | *M. microti* | 3 bp deletion *in silico*, not present in all *M. microti* strains tested |

* This diagnostics target was specific for the *M. microti* strains in our collection, however owing to the results algorithm used in *Seek*TB the assay developed was not required in the final format of the test. Additionally owing to the current limitations of real-time PCR technologies it was not possible to incorporate this target.

*2.4.1 Rationale for not using putative diagnostics targets*

While a rationale is outlined in the table 2.1 above 3 examples are given as to why some diagnostics targets were deemed unsuitable

1. *cut*5A (Rv3724) 1bp insertion *in silico* specific for *M. tuberculosis*

- **Experimental analysis**: Initially, nucleotide sequence was generated for a region of the *cut5*A gene in 4 *M. tuberculosis* isolates and the 1bp insertion was present therefore a real-time PCR diagnostics assay was designed and tested. When 20 *M. tuberculosis* isolates were tested with this assay, 18 *M. tuberculosis* contained the insertion however 2 *M. tuberculosis* strains did not. As this region was not present in all isolates tested it was deemed unsuitable as an *M. tuberculosis* specific diagnostics target. This may be due to inter strain variation and therefore: **No Go**

2. *sugI* (Rv3331): 15bp deletion *in silico* specific for *M. bovis BCG*

- **Experimental analysis:** Nucleotide sequence was generated for a region of the *sugI* gene in 5 *M. bovis* BCG strains and the 15bp deletion was not present in any isolates tested. This may be an artifact of sequencing or represent inter strain variation therefore: **No Go**

3. *far* (Rv0855): 6 bp deletion *in silico* specific for *M. microti*

- **Experimental analysis:** Nucleotide sequence was generated for a region of the *far* gene and the 6 bp deletion was present in all *M. microti* strains tested. A real-time PCR diagnostics assay was designed, tested and was specific for *M. microti*. However, this target was not required based on the final algorithm developed.

- It was also beyond the limitations of current multiplex real-time PCR capabilities to incorporate this diagnostics target therefore: **No Go**

Table 2.2 Diagnostics targets used (Go Targets)

| Gene Name/Proposed Function | Biomarker Specific For | Comment |
|---|---|---|
| *lepA* (Rv 2404c) | MTC | Suitable for MTC detection |
| *wbbl1* (Rv3265c) | *M. tuberculosis/M. canettii /M. africanum WA1* | 12 bp deleted in all other members of the MTC |
| RD1[canettii1] | *M. canettii* | 2869 bp region specific for *M. canettii* |
| RD713 | *M. africanum WA1* | 1450 bp deletion specific for *M. africanum WA1* |
| *lpqT* (Rv1016c) | *M. bovis/M.bovis*BCG/*M. caprae* | 5 bp deletion specific for *M. bovis/M.bovis*BCG/*M. caprae* |
| *lepA* (Rv 2404c) | *M. caprae* | SNP specific for *M. caprae* |
| RD1 (Rv3876) | *M. bovis/M. caprae* | not present in *M. bovis* BCG, allowed for discrimination of *M. bovis/M. caprae* |
| RD701 | *M. africanum* WA2 | 2545 bp deletion specific for *M. africanum* WA2 |
| *lepA* (Rv 2404c) | *M. smegmatis* | Sufficient nucleotide sequence heterogeneity to develop a competitive IAC |
| MSMEG_0660 | *M. smegmatis* | Not present in any other MTC/ NTM sequenced to date allowed for generation of a non competitive IAC |

## 2.5 References

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# Chapter 3:

## A novel multiplex real-time PCR diagnostics assay for the identification and differentiation of *Mycobacterium tuberculosis, Mycobacterium canettii* and the *Mycobacterium tuberculosis* complex

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## Abstract

Tuberculosis (TB) in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). Rapid detection of the MTC is necessary for the timely initiation of antibiotic treatment, while differentiation between members of the complex may be important to guide the appropriate antibiotic treatment and provide epidemiological information.

In this study, a multiplex real-time PCR diagnostics assay using novel molecular targets was designed to identify the MTC while simultaneously differentiating between *M. tuberculosis* and *M. canettii*. The *lepA* gene was targeted for the detection of members of the MTC, the *wbbl1* gene was used for the differentiation of *M. tuberculosis* and *M. canettii* from the remainder of the complex and a unique region of the *M. canettii* genome, a possible novel "region of difference" (RD), was targeted for the specific identification of *M. canettii*.

The multiplex real-time PCR assay was tested using 125 bacterial strains (64 MTC isolates, 44 non-tuberculosis mycobacteria (NTM) and 17 other bacteria). The assay was determined to be 100% specific for the mycobacteria tested. Limits of detection of 2.2, 2.17 and 0.73 cell equivalents were determined for *M. tuberculosis*/*M. canettii*, the MTC and *M. canettii*, respectively, using Probit regression analysis.

Further validation of this diagnostics assay, using clinical samples, should demonstrate its potential for the rapid, accurate and sensitive diagnosis of TB caused by *M. tuberculosis*, *M. canettii* and the other members of the MTC.

## 3.1 Introduction

Tuberculosis (TB) is the leading cause of death worldwide from an infectious agent (13), with the WHO estimating that one third of the global population are infected with TB. In a global report from the WHO (2009), it was estimated that there was 9.27 million cases of TB in 2007, with 2 million associated deaths. TB in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). The eight closely related species of the MTC have a wide range of natural hosts including humans hosts (*M. tuberculosis*, *M. africanum*, *M. canettii*), bovine hosts (*M. bovis*), caprine hosts (*M. caprae*), rodent hosts (*M. microti*) and pinniped hosts (*M. pinnipedii*), along with the attenuated *M. bovis* strain BCG (*Bacillus Calmette-Guérin)*, the commonly used vaccine strain. While there are a number of natural hosts, each species of the MTC has been implicated in human infection (6, 20).

Traditionally, diagnosis of TB relies on smear microscopy and culture techniques in combination with a battery of biochemical tests which are time consuming, labour intensive and often yield unreliable results (18). Nucleic Acid Diagnostics (NAD) techniques, in particular real-time PCR, offer a rapid, reliable and highly sensitive alternative diagnostic tool for many infectious agents (25, 42). Advances in real-time PCR such as the availability of multiple fluorophores, along with the development of non-fluorescent quenchers has facilitated multiplexing, allowing for the simultaneous detection and differentiation of multiple targets, along with internal controls, in one reaction (3).

While significant advances have been made in the diagnosis of TB using NAD techniques (19), the differentiation of members of the MTC to the species level is not routinely performed. Commercially available real-time PCR kits for the diagnosis of TB generally identify the MTC but not individual species. The high degree of nucleotide sequence homology between members of the complex makes species differentiation challenging (31). Comparative genomics revealed that *M. tuberculosis*

and *M. bovis* genomes are 99.95 % similar at the nucleotide level (14), with whole genome DNA microarrays identifying 16 regions of difference (RD 1-16) (4). These RD's represent regions of the genome deleted in *M. bovis BCG* which are present in *M. tuberculosis* and have been used for the differentiation of members of the MTC. One RD commonly targeted for the specific detection of *M. tuberculosis* is RD9 (31), however, this RD is also present in *M. canettii* (7). There is currently no real-time PCR test which can diagnose TB, while differentiating between *M. tuberculosis* and *M. canettii*, as the causative agent of infection.

*M. tuberculosis* is the most important human pathogen in the MTC and is thought to be responsible for 95% of human cases of TB, yet rarely causes disease in other mammals (1, 7, 9). While drug resistant strains of *M. tuberculosis* are emerging, it is considered sensitive to anti-tuberculosis drugs such as pyrazinamide (PZA), a first line antibiotic that reduces patient treatment time from 9 months to 6 months (27, 35). However, *M. canettii*, which has been reported to cause TB in humans, is intrinsically resistant to PZA, therefore; the ability to differentiate it from *M. tuberculosis* is important for indicating the therapeutic regimen necessary for patient treatment (34).

*M. canettii* is considered to be the most phenotypically distinct member of the MTC and is considered the species from which other members of the complex may have evolved (6). *M. canettii* is phenotypically characterised by its smooth glossy white colonies; however a small number of these colonies have been shown to revert to rough colony variants when individual colonies are replated (37). Smooth colonies are uncharacteristic of the MTC and are due to the presence of large amounts of lipooligosaccharides in the *M. canettii* cell wall (30). Like *M. tuberculosis*, *M. canettii* contains all the RD's with the exception of RD12 *canetti* ($RD12^{can}$) which has been targeted for the specific detection of *M. canettii* in a complex conventional PCR methodology (18).

While infection with *M. canettii* is thought to be rare and confined to eastern African countries, there is a lack of rapid diagnostic tests available to differentiate between it and *M. tuberculosis*. Cases of human TB caused by *M. canettii* have now been reported in Europe and America (12). In addition, recent reports have suggested that the number of true cases of TB caused by *M. canettii* may in fact be underrepresented (15, 34). Therefore, an ability to differentiate *M. tuberculosis* and *M. canettii* is not only important from a patient treatment perspective but will also provide important epidemiological information for clinicians.

We report the design, development and testing of a multiplex real-time PCR assay using novel nucleic acid diagnostics targets to detect the MTC while simultaneously detecting and differentiating between *M. tuberculosis* and *M. canettii* in one reaction.

## 3.2 Materials and Methods

### *3.2.1 Diagnostics target identification*

The diagnostics target genes used in this study were identified using a number of approaches. In order to identify a target for collective detection of the MTC, a number of housekeeping genes, which are highly conserved throughout the *Mycobacterium* genus, were evaluated. To identify novel targets for the detection of *M. tuberculosis,* approximately 3000 genes were evaluated based on regions deleted in other members of the MTC but present in *M. tuberculosis* or alternatively, present in other members of the MTC but deleted in *M. tuberculosis*. These potential target regions were identified using the Mycobacterial Genome Divergence Database (MGDD) (http://mirna.jnu.ac.in/mgdd/), which allowed for identification of insertions, deletions and single nucleotide polymorphisms between *M. tuberculosis*, *M. bovis* and *M. bovis BCG*. Potential target regions were also identified using the web based version of the Artemis comparison tool, WebACT (http://www.webact.org/WebACT/home). Nucleotide sequence information was retrieved from the *M. africanum* and *M. microti* genomes (currently being sequenced

by the Welcome Trust Sanger Institute) to determine, *in silico,* if the candidate diagnostics target nucleotide sequences for *M. tuberculosis* identification were specific. For the remaining members of the complex, namely, *M. canettii*, *M. caprae* and *M. pinnipedii*, for which no nucleotide sequence information was available at the time of this study, the specificity of potential targets for these species was determined empirically and further validated through sequencing.

As the diagnostics target used in this study for the specific detection of *M. tuberculosis*, was experimentally determined to also detect *M. canettii*, a novel diagnostics target specific for *M. canettii* was also required. When nucleotide sequence information became publicly available on the Sanger website for *M. canettii*, a number of large regions of nucleotide sequence were evaluated using BLAST for putative species specific nucleotide sequence diagnostics motifs.

For each putative diagnostics target identified, alignments were carried out using clustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html), from which real-time PCR primers and probes were designed (Table 3.1).

*3.2.2 Bacterial strains, culture media and growth conditions*

Sixty four MTC isolates (26 *M. tuberculosis*, 11 *M. bovis*, 7 *M. bovis BCG*, 5 *M. canettii*, 2 *M. caprae*, 5 *M. africanum*, 5 *M. microti* and 3 *M. pinnipedii*), 44 non tuberculosis mycobacteria (NTM) and 17 other bacterial species were used in this study (Tables 3.2 and 3.3). Of the 64 MTC isolates, 36 strains, previously characterised by a variety of methods as described in the literature (12, 22, 23, 26, 30, 36-38), were provided by Dr. Dick van Soolingen (RIVM, Bilthoven, Netherlands). All other MTC strains, provided by Professor Mario Vaneechoutte (University of Ghent, Ghent, Belgium) were clinical isolates collected over a ten year period from reference laboratories in Belgium and the Netherlands. These

isolates were characterised based on custom techniques available at the time. Twenty eight NTM were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ) and grown on Middlebook agar/broth at either 30 °C or 37 °C. "Fast growing" mycobacteria were cultured for 3-6 days, and "slow growing" mycobacteria were incubated for six weeks, or until sufficient growth was visible. All media were purchased from BD Biosciences (Oxford, United Kingdom). For the 16 remaining NTM, in addition to 4 of the 5 *Norcardia* strains used in this study, DNA was supplied by Professor Vaneechoutte, which had been characterised using techniques previously described (10, 40, 41)

### *3.2.3 DNA isolation and quantification*

Genomic DNA from NTM and 2 *M. bovis BCG* cultures was isolated from 1 ml of culture (Middlebrook 7H9 broth, Becton Dickinson), using a modified procedure combining mechanical lysis (IDI lysiskit, GeneOhm, Quebec, Canada) and purification using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany). Briefly, 1 ml of culture was centrifuged in a bench-top centrifuge (Microcentrifuge 5415, Eppendorf) at 13,000 g for 3 min. The supernatant was discarded and the pellet resuspended in 250 µl GeneOhm sample buffer. The suspension was transferred to a GeneOhm lysis tube and bead beaten (Mini-Bead-Beater-16™, Stratech, UK) for 3 min. After bead-beating, 200 µl was transferred to a sterile microcentrifuge tube and steps 3-8 of the "purification of total DNA from animal tissue" procedure in the Qiagen DNeasy Blood and Tissue kit were followed according to the manufacturer's instructions. Total genomic DNA samples provided by the RIVM were extracted using methods previously described (39), while genomic DNA from Professor Vaneechoutte was extracted as described in a study by De Baere *et al.* (10). For all other bacterial species tested for, DNA was provided from stocks held within this laboratory. DNA concentrations for all NTM and members of the MTC used in this study were determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon, USA) and the TBS-

380 mini-fluorometer (Invitrogen Corporation, California, USA). All DNA samples were stored at -20 °C before use.

### *3.2.4 Conventional and real-time PCR primers and hydrolysis probe design*

Oligonucleotide primers and hydrolysis probes were designed in accordance with general recommendations and guidelines (11, 33), following alignments of each of the nucleic acid diagnostics target genes identified in this study. All primers and probes (Table 3.1) used in this study were supplied by MWG-BIOTECH AG (Essenberg, Germany). The primers used in this study were designed to have a melting temperature (Tm) of 58-61 °C, with all probes designed to have a Tm of 4-7 °C degrees higher. Hydrolysis probes were designed to be specific for each target following published design guidelines (11). These parameters were adhered to during the design of monoplex assays so that the assays could be easily multiplexed after specificity and sensitivity testing was complete.

For the MTC and Internal Amplification Control (IAC) assay, PCR primers MTC_IAC Fw and MTC_IAC Rv (Table 3.1), were designed to amplify a 155 bp fragment of the *lepA* gene for all members of the MTC and *M. smegmatis*. The MTC_IAC Fw primer was located at positions 618-634 bp and the MTC_IAC Rv primer located at positions 754-772 bp of the *M. tuberculosis* H37RV *lepA* gene. For the *M. tuberculosis* and *M. canettii* assay, *wbbl1*_Fw and *wbbl1*_Rv were designed to amplify a 146 bp fragment of the *wbbl1* gene. The *wbbl1*_Fw primer was located at positions 15-34 bp and the *wbbl1*_Rv primer located at positions 141-159 bp of the *M. tuberculosis* H37RV *wbbl1* gene. Finally, the *M. canettii* specific assay was designed to amplify a 128 bp fragment of a 2869 bp region of the genome identified in this study as specific to *M. canettii* (an *M. canettii* specific assay could potentially be designed anywhere in this region). This 2869 bp region of the genome has been mapped to the *M. tuberculosis* H37Rv genome and is inserted between Rv0150c (hypothetical protein) and Rv0151c (gene name *PE1*, a PE family protein) at position 177,445 bp on the *M. tuberculosis* H37Rv genome.

All real-time PCR assays were initially tested for in a monoplex format, evaluating their specificity and sensitivity, using probes labelled with FAM and Black Hole Quencher 1 (BHQ1). After the monoplex real-time PCR assays were optimised, three of the four assay probes were labelled with different fluorescent dyes to allow for multiplex real-time PCR. The MTC probe was labelled with HEX and BHQ1, the *M. canettii* specific probe with ROX and BHQ2 and the internal amplification control (IAC) probe with Cy5 and BHQ2.

### *3.2.5 Conventional PCR*

Conventional PCR was performed using the sequencing primers outlined in Table 3.1 on the iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., California, USA). All reactions were carried out in a final volume of 50 µl, containing 5 µl 10X buffer (15mM $MgCl_2$), forward and reverse primers (0.2 mM final conc.), 2 µl Taq DNA polymerase (1 U/ µl, Roche Diagnostics, Basel, Switzerland), 1 µl dNTP mix (10 mM: deoxynucleoside triphosphate set (Roche Diagnostics), 2 µl of template DNA, 38 µl Nuclease free water (Applied Biosystems/Ambion, Texas, USA). The cycling parameters consisted of initial denaturation at 95 °C for 5 mins, followed by 35 cycles of denaturation at 95 °C (1 min), amplification at 55 °C (1 min), and extension at 72 °C (1 min), followed by a final elongation at 72 °C for 10 min.

### *3.2.6 Sequencing*

Nucleotide sequence data for real-time PCR assay design was generated in this study or was obtained from either the National Centre for Biotechnology Information (NCBI), or the Sanger website (where partial nucleotide sequences for *M. canettii*, *M. africanum* and *M. microti* were available). The primers used for the real-time PCR assays were also used in conventional PCR to generate nucleotide sequence information for each of the assays developed. In addition, sequencing primers were designed to span the full 2869 bp *M. canettii* specific nucleotide sequence identified,

to evaluate if this region is conserved in all *M. canettii* strains tested in this study and to identify potential diagnostics target nucleotide sequences for probe design.

PCR products were generated according to section 3.2.5, followed by purification using the High Pure PCR product purification kit (Roche Diagnostics). The purified PCR products were sequenced externally (Sequiserve, Vaterstetten, Germany).

### *3.2.7 Development of an IAC for real-time PCR*

An IAC targeting the *M. smegmatis lepA* gene was developed for the multiplex real-time PCR. The IAC and MTC targets were both amplified using the same primer set, however the IAC probe, targeting *M. smegmatis*, was labelled with Cy5, while the MTC probe was labelled with HEX (Table 3.1). Titrations of MTC and IAC DNA were performed to determine the optimum concentration of IAC target per reaction such that it was always detected without impacting on detection of the primary MTC target (17). An IAC concentration of 500 genome equivalents per reaction allowed for the positive detection of the IAC at low concentrations or in the absence of primary target.

The *lepA* gene was chosen as the target for the IAC because sufficient sequence heterogeneity exists between the *M. smegmatis* and MTC *lepA* gene sequences for the design of independent, specific probes. There was also enough sequence homology, flanking these probe regions, to design one set of primers to amplify both MTC and IAC targets. This resulted in three rather than four primer pairs in the multiplex PCR, reducing assay complexity.

### *3.2.8 Real-time PCR*

Monoplex real-time PCR was performed on the LightCycler 2.0 Instrument (Roche Diagnostics) using the LightCycler® TaqMan® Master kit (Roche Diagnostics). A final volume of 20 µl was used in each reaction, containing 5X master mix, forward

and reverse primers (0.5 mM final conc.), FAM labelled probe (0.2 mM final conc.), template DNA (2 µl) and the volume adjusted to 20 µl with the addition of nuclease free $dH_2O$. The cycling parameters consisted of incubation for 10 min at 95 °C to activate enzymes and denature DNA followed by 50 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a cooling step at 40 °C for 10 s. The temperature transition rate for all cycling steps was 20 °C/s.

Multiplex real-time PCR reactions were carried out on the LightCycler 480 using LightCycler® 480 Probes Master kit (Roche Diagnostics). A final volume of 40 µl was used for each multiplex reaction. The optimised master mix contained 2X LightCycler 480 Probes Master (6.4 mM $MgCl_2$), forward and reverse primer (0.5mM final conc.), FAM labelled probe (0.4 µM final conc.), HEX, ROX and CY5 labelled probes (0.2 µM final conc.), template DNA (MTC 2 µl, IAC 2 µl, NTM 10 µl) and the volume adjusted to 40 µl with the addition of nuclease free $dH_2O$. The internal control DNA was diluted to contain 500 genome equivalents per 2 µl and the NTM contained ~$10^4$ genome equivalents per 10 µl.

The cycling parameters used were the same as those used on the LightCycler 2.0. The temperature transition rate, referred to as the ramp rate on the LightCycler 480 was 4.4 °C/s while heating and 2.2 °C/s while cooling. Prior to experimental analysis on the LightCycler 480, a colour compensation file was generated using the technical note outlined in the Advanced Software Functionalities of the operator manual (2)

*3.2.9 Nucleotide sequence accession numbers*

Partial *wbbl1* gene nucleotide sequences generated in this study for the five *M. canettii* strains (Table 3.2) were deposited in GenBank with the following accession numbers: HQ625205 through to HQ625209. Nucleotide sequences generated in this study for the proposed novel $RD^{canettii1}$ were also deposited to Genbank with the following accession numbers: HQ625200 through to HQ625204.

## 3.3. Results

### *3.3.1 Diagnostics targets identification*

The diagnostics nucleotide sequence target identified in this study for detection of the MTC in combination with detection of the IAC was *lepA* (Rv2404c). LepA is an elongation factor required for accurate and efficient protein synthesis capable of inducing back-translocation of mistranslocated tRNA's. The *lepA* gene is present in all bacteria sequenced to date and codes for one of the most conserved proteins in bacteria (55-68 % amino acid sequence similarity between bacteria), with a homologue (Guf1) found in higher organisms (32).

For the specific detection of *M. tuberculosis* and *M. canettii,* a *wbbl1* gene nucleotide sequence target (Rv3265c) was identified. The *wbbl1* gene encodes rhamnosyl transferase, which inserts rhamnose into the cell wall and is thought to be essential for mycobacterial viability (24). Nucleotide sequence analysis of members of the MTC revealed a 12 base pair region of the *wbbl1* gene present only in *M. tuberculosis* and *M. canettii*, which has been deleted in all other members of the MTC.

The *M. canettii* specific diagnostics nucleotide sequence target identified in this study is a region of the genome that appears to be deleted in all other members of the MTC. This 2869 bp region was discovered while mapping RD12$^{can}$ to the unfinished genome sequence of *M. canettii* available on the Sanger website. This region appears to be a novel RD specific for *M. canettii*. To date, the *M. canettii* genome is not annotated, therefore, the function of the gene/s in this region are unknown. The *M. canettii* diagnostics target region identified was BLAST analysed and revealed similarity to a putative ATP binding protein gene in *Nocardioides species* (Query coverage 52%, max indent 73%). This region was sequenced using the primers listed in Table 3.1 for the *M. canettii* strains used in this study. Sequence analysis revealed

100% similarity between the 5 strains and the sequence available on the Sanger website.

### *3.3.2 Assay design and development*

While the guidelines for primer and probe design were adhered to as closely as possible, the high GC content (60-65%) of the *Mycobacterium* species did have an impact on assay design. The *wbbl1* specific probe was based on a region present in *M. tuberculosis* and *M. canettii* and deleted in other MTC, that is very G/C rich making probe design difficult. This probe was labelled with FAM and double the standard probe concentration (0.4µM/reaction) was used to improve the endpoint fluorescence, sensitivity and robustness of the assay.

### *3.3.3 Internal amplification control*

In order for a result to be considered valid using the multipex real-time PCR assay developed in this study, a positive signal must be obtained in at least one of the four detection channels on the LightCycler 480. If none of the assay targets or the IAC are detected, the result is considered invalid and must be repeated (17, 28). In this study, *M. smegmatis* DNA is spiked into the PCR master mix to act as an internal control target. Equally *M. smegmatis* cells could also be used as a process control, when spiked into patient samples before total genomic DNA extraction. This could then act as a control for both DNA extraction and PCR efficiency when patient samples are tested for.

### *3.3.4 Specificity of the diagnostic assays*

The specificity of each real-time PCR assay was confirmed both in monoplex and multiplex formats using the specificity panel listed in Tables 3.2 and 3.3. The *wbbl1* assay was specific for the detection of the 26 *M. tuberculosis* and the 5 *M. canettii* strains. Figure 3.1 A shows the detection of 6 *M. tuberculosis* strains (circles) and 5

*M. canettii* strains (triangles). The remaining members of the MTC, the NTM and other bacteria were not detected. The 64 MTC strains were all detected in the MTC assay (Figure 3.1 B) and 44 NTM and 17 other bacteria were not detected. The *M. canettii* assay was specific for the 5 *M. canettii* isolates (Figure 3.1 C) and did not cross-react with the specificity panel. The specificity of the IAC assay was tested using the MTC panel and was specific for *M. smegmatis* DNA. As the MTC and IAC assays are competitive, the IAC is only detected at low concentrations or in the absence of primary target. In Figure 3.1 B, the *M. africanum* 3 sample tested in the assay is at a lower concentration than the other members of the MTC, hence a stronger amplification signal is observed in the Cy 5 channel (IAC) with this sample (Figure 3.1 D, *M. africanum* 3 represented by diamonds). A simple interpretation of the results that can be obtained from the multiplex real-time PCR developed is outlined in Table 3.4.

*3.3.5 Sensitivity of the assays*

The limit of detection (LOD) of each assay was evaluated in a monoplex real-time PCR format. Genomic DNA was quantified and serial dilutions were prepared from 200,000 to 2 genome equivalents based on the genome size of *M. canettii* (4,525,000 bp), with equates to approximately 4.9 fg DNA per cell. *M. canettii* was chosen for sensitivity testing of the multiplex assay as it is detected in each of the primary target assays (*M. tuberculosis*/ *M. canettii*, MTC and *M. canettii* specific).

In a monoplex format, the dilution series was run in duplicate and a sensitivity of 2-20 *M. canettii* genome equivalents was determined for each assay. In multiplex format the lower limit of detection was established using probit regression analysis. In this analysis, 12 replicates of each of 20, 15, 12, 10, 7.5, 4, 2, 0.2 genome equivalents were evaluated. LOD's of 2.17, 2.20, 0.73 genome equivalents for the *M. canettii/M. tuberculosis*, MTC and *M. canettii* specific assays respectively were determined with 95 % probability. The IAC, at a concentration of 500 genome equivalents per reaction, was detected in all samples tested.

## 3.4 Discussion

In 1998 the 'Stop TB' initiative was established with the ultimate goal of obtaining a world free of TB. Within this initiative a core group, the New Diagnostics Working Group (NDWG), was founded in an effort to establish new diagnostics for TB. This working group, along with a number of collaborators, such as FIND (Foundation for Innovative New Diagnostics), have launched a new web resource 'Evidence-based Tuberculosis Diagnosis' which highlights the importance of new diagnostics for the rapid and cost effective detection of TB (29)

At present, literature describing TB NAD assays, in addition to commercially available TB NAD kits (5, 8, 16), is limited, and the scope and capacity of these assays to differentiate the MTC to the species level are also limited. For example, the GenoType MTBC kit (Hain Lifescience GmbH, Nehren, Germany), which claims to differentiate the MTC, does not in fact differentiate between *M. tuberculosis* and *M. canettii* nor between *M. africanum* and *M. pinnipedii* (21). Recently a study by Pinsky and Banaei (2008), which describes a real-time PCR for differentiation of the MTC, fails to differentiate *M. tuberculosis* and *M. canettii*, and is also unable to differentiate between *M. africanum*, *M. microti*, *M. pinnipedii* and *M. caprae*. A capability to identify the specific MTC species causing infection is important for determining the appropriate therapeutic regimen for the patient (34).

The multiplex real-time PCR assay described in this study uses novel nucleic acid diagnostics targets for the identification of the MTC, *M. tuberculosis* and *M. canettii*. The first novel target identified was a *lepA* gene nucleotide sequence. This target was used to detect the MTC and to develop the IAC for the real-time PCR diagnostics assay. The second novel molecular target identified and evaluated in this study was a *wbbl1* gene nucleotide sequence target which enables the simultaneous detection of *M. tuberculosis* and *M. canettii*, a target with the same diagnostics potential as the widely used RD9 region for *M. tuberculosis* identification. The third novel diagnostics target nucleotide sequence identified, allowed for the differentiation of *M. canettii* from *M. tuberculosis*. As this nucleotide sequence is

present in the 5 *M. canettii* strains used in this study, and is 100 % homologous, we propose this to be a novel RD ($RD^{canettii1}$).

An *M. canettii* RD has previously been described by Huard *et al*. (2003) which represents a region of the genome flanking RD 12 which is deleted in *M. canettii* but present in *M. tuberculosis*. The study uses conventional PCR for differentiation of the MTC including *M. tuberculosis* and *M. canettii*, based on PCR product size. The method requires time consuming multiple reactions and produces results that require detailed interpretation. In this current study, we have identified a putative new RD which is present in *M. canettii* but deleted in *M. tuberculosis* and all other members of the MTC. As this region is only present in *M. canettii*, the interpretation of results is less complex.

The multiplex real-time PCR developed in this study is the first description of a hydrolysis probe based diagnostic tool capable of rapid detection of the MTC, combined with the detection and differentiation of *M. tuberculosis* and *M. canettii,* using novel targets. This rapid, specific and sensitive multiplex real-time PCR assay produces a diagnostic result in less than one hour after DNA extraction. These novel genetic markers will be further optimised and validated against a more extensive range of clinical isolates in addition to clinical samples in the future.

While this current study has focused on identifying novel nucleotide sequence diagnostics targets and the development of a multiplex real-time PCR for differentiation of *M. tuberculosis* and *M. canettii*, work has begun in this laboratory on developing a series of nucleic acid based diagnostics assays with the ability to differentiate all species of the MTC. For example, there is a requirement to differentiate infection caused by both *M. bovis* and *M. bovis BCG* from the remainder of the MTC as they, like *M. canettii*, are intrinsically resistant to PZA (35).

Ultimately, there will be a requirement to transfer this set of assays to a platform with a capability of detecting and differentiating the MTC in a single test diagnostics format. For example, the design of a microarray utilising these novel MTC diagnostics targets, in combination with diagnostics targets for the determination of MTC drug resistance, could be significantly advantageous. This rapid diagnostics approach would provide the clinician with important information as to the optimal therapeutic regimen required for an infected patient, while at the same time providing the clinical laboratory with unambiguous MTC epidemiological data.

Table 3.1: Oligonucleotide primers and probes used in this study

| **Name** | **Function** | **Sequence 5’→3’** |
|---|---|---|
| MTC_IAC Fw | Forward Sequencing primer, forward MTC and internal control real- time PCR assay primer | AGACCGTGCGGATCTTG |
| MTC_IAC Rv | Reverse Sequencing primer, Reverse MTC and internal control real-time PCR assay primer | CATGGAGATCACCCGTGA |
| MTC Probe | MTC probe | HEX-ACGGATTGGTCACCCGGATT-BHQ1 |
| IAC probe | Internal control probe | CY5-ACGACCTTCTCGGAACCGT-BHQ2 |
| *wbbl1*_Fw | Forward sequencing primer, Forward real-time PCR assay primer | TACCAGCTTCAGTTTCCGT |
| *wbbl1*_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | GCACCTATATCTTCTTAGCCG |
| *wbbl1* probe | *wbbl1* probe | FAM-ATGGTGCGCAGTTCACTGC-BHQ1 |
| *M. canetti* sp Fw | Forward *M. canetti* specific primer | ATGTGGTTTCAGTACGACTTC |
| *M. canetti* sp Rv | Reverse *M. canetti* specific primer | GATGGCAGTGTCTTATCCAA |
| *M. canetti* sp probe | *M. canetti specific* probe | ROX-TGAGAGGTGTTGGCACGCAA-BHQ2 |
| *M. canetti* seq 1.a | Forward sequencing primer 1 | TGTCGGCGGCCACGT |
| *M. canett* seq 1.b | Reverse sequencing primer 1 | GAAGTCCAGCATCTTGGCGTT |
| *M. canetti* seq 2.a | Forward sequencing primer 2 | TGTCGGCGGCCACGT |
| *M. canetti* seq 2.b | Reverse sequencing primer 2 | ATCGTGCAGTGCGGCCA |
| *M. canetti* seq 3.a | Forward sequencing primer 3 | GCAGCATTGTGGTTGACCGA |
| *M. canetti* seq 3.b | Reverse sequencing primer 3 | TCCCAGCGTTGCGCCTT |
| *M. canetti* seq 4.a | Forward sequencing primer 4 | TGATGCGGCTGCTCAAGC |
| *M. canetti* seq 4.b | Reverse sequencing primer 4 | TGTCAAGGGACATGGGGAACT |

Table 3.2: *Mycobacterium tuberculosis complex* isolates used in this study

| Species | Strain | Isolated from | Country of Isolation | Origin | Remark |
|---|---|---|---|---|---|
| *M. tuberculosis* | 22 | Human | Mongolia | RIVM | Beijing genotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* | 53 | Human | Argentina | RIVM | Haarlem genotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* | 112 | Human | The Netherlands | RIVM | CAS spoligotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* | 67 | Human | Comoro Islands | RIVM | EAI spoligotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* | 41 | Human | Chile | RIVM | LAM spoligotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* | 103 | Human | China | RIVM | T-family spoligotype, See Kremer *et al.* 2005 |
| *M. tuberculosis* (*19 clinical isolates*) | - | NA[b] | NA[b] | Mario Vaneechoutte | Clinical isolates |
| *M. tuberculosis* H37Rv | H37Rv | NA[b] | NA[b] | DSMZ | Laboratory strain, DNA provided |
| *M. canettii* | 116 | Human | Somalia | RIVM | Smooth growing strain described by van Soolingen *et al.* 1997 |
| *M. canettii* | 1997-1549 | Human | Switzerland | RIVM | Swiss isolate described in Pfyffer *et al.* 1998 |
| *M. canettii* | NLA000701671 | Human | Somalia | RIVM | Characterised on the basis of their spoligotype, IS6110 RFLP type and smooth growth as described by van Soolingen *et al.* 1997. |
| *M. canettii* | NLA000200937 | Human | Eritrea | RIVM | Characterised on the basis of their spoligotype, IS6110 RFLP type and smooth growth as described by van Soolingen *et al.* 1997. |
| *M. canettii* | 1996-46 | Human | France | RIVM | Canetti strain, see also van Soolingen *et al.* 1997 |
| *M. bovis* | 117 | Cattle | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 126 | Cattle | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 73 | Cattle | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 130 | Cattle | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 24 | Orxy | Saudi Arabia | RIVM | Isolated from an oryx, Antelope clade, see also Smith *et al.* 2006 |
| M. bovis (6 isolates) | - | NA[b] | NA[b] | Mario Vaneechoutte | Clinical isolates |
| *M. bovis* BCG | 48 (2) | Vaccine strain | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 71 | Vaccine strain | Japan | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 83 | Vaccine strain | Russia | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 2008-714[a] | Human | NA[b] | RIVM | Identified on basis of characteristic IS6110/IS1081 RFLP patterns according to van Soolingen *et al.* 1992 |
| *M. bovis* BCG | 2008-1601[a] | Human | NA[b] | RIVM | Identified on basis of characteristic IS6110/IS1081 RFLP patterns according to van Soolingen *et al.* 1992 |
| *M. bovis* BCG | DSM 43990 | Vaccine strain | NA[b] | DSMZ | *Mycobacterium bovis* Karlson and Lessel 1970 BCG, Chicago 1 |
| *M. bovis* BCG | DSM 45071 | Vaccine strain | NA[b] | DSMZ | *Mycobacterium bovis* Karlson and Lessel 1970 |
| *M. caprae* | 2006-1960[a] | NA[b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |
| *M. caprae* | 2007-0039[a] | NA[b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |
| *M. microti* | 62 | Vole | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 25 | Vole | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 15274[a] | Vole | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 15912[a] | Vole | Belgium | RIVM | see van Soolingen *et al.* 1998 |

| | | | | | |
|---|---|---|---|---|---|
| *M. microti* | 15911 [a] | Vole | Netherlands | RIVM | see van Soolingen *et al.* 1998 |
| *M. pinnipedii* | 76 | Seal | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. pinnipedii* | 81 | Seal | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. pinnipedii* | 101 | Seal | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. africanum* | 6 | Human | The Netherlands | RIVM | See Mostowy *et al.* 2004 |
| *M. africanum* | 128 (85) | Human | The Netherlands | RIVM | See Mostowy *et al.* 2004 |
| *M. africanum* | 2007-1386 [a] | NA [b] | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. africanum* | 2007-1154 [a] | NA [b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |
| *M. africanum* | 2007-1073 [a] | NA [b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |

[a] Represent RIVM strains not previously described in literature, however have been characterised to the species level using techniques outlined in references supplied as remark.
[b] This information was not available (NA) for this study.

Table 3.3: Non *tuberculosis* mycobacteria and other strains of bacteria used in this study

| **Non *tuberculosis* mycobacteria** | **Strain designation** [a] | **Remark** |
|---|---|---|
| *Mycobacterium aichiense* | DSM 44147 | Type strain, isolated from soil |
| *Mycobacterium alvei* | DSM 44176 | Type strain, isolated from water sample |
| *Mycobacterium arupense* | DSM 44942 | Type strain, isolated from a tendon |
| *Mycobacterium asiaticum* | ITG 8182 | See De Baere *et al.* 2002 |
| *Mycobacterium avium* | ITG 7886 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium boenickei* | DSM 44677 | Type strain, isolated from a leg wound |
| *Mycobacterium branderi* | DSM 44624 | Type strain, isolated from human sputum |
| *Mycobacterium brisbanense* | DSM 44680 | Type strain, isolated from a sinus |
| *Mycobacterium brumae* | DSM 44177 | Type strain, isolated from water sample |
| *Mycobacterium canariasense* | DSM 44828 | Type strain, isolated from human blood |
| *Mycobacterium celatum* | ITG 6147 | See De Baere *et al.* 2002 |
| *Mycobacterium chelonae* | ITG 4975 | NA [b] |
| *Mycobacterium chelonae subsp. abscessus* | DSM 44196 | Type strain |
| *Mycobacterium confluentis* | DSM 44017 | Type strain, isolated from human sputum |
| *Mycobacterium conspicuum* | DSM 44136 | Type strain, isolated from patient with disseminated infection |
| *Mycobacterium flavescens* | VUB A016 | See De Baere *et al.* 2002 |
| *Mycobacterium fortuitum* | ITG 8020 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium genavense* | ITG 97-102 | See De Baere *et al.* 2002 |
| *Mycobacterium gilvum* | DSM 9487 | Isolated from soil |
| *Mycobacterium goodii* | DSM 44492 | Type strain |
| *Mycobacterium gordonae* | ITG 7704 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium heckeshornense* | DSM 44428 | Type strain, isolated from human respiratory tract |
| *Mycobacterium houstonense* | DSM 44676 | Type strain, isolated from a facial abscess |
| *Mycobacterium intracellulare* | DSM 43223 | Type strain |
| *Mycobacterium kansasii* | ITG 7727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium kubiciae* | DSM 44627 | Type strain, isolated from human sputum |
| *Mycobacterium lacus* | DSM 44577 | Type strain, isolated from human elbow |
| *Mycobacterium mageritense* | DSM 44476 | Type strain, isolated from human sputum |
| *Mycobacterium malmoense* | ITG 940611 | See De Baere *et al.* 2002 |
| *Mycobacterium marinum* | ITG 1727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium massiliense* | DSM 45103 | Type strain, isolated from human blood |
| *Mycobacterium moriokaense* | DSM 44221 | Type strain, isolated from soil |
| *Mycobacterium mucogenicum* | DSM 44625 | Type strain, isolated from human cyst |
| *Mycobacterium nebraskense* | DSM 44803 | Type strain, isolated from human sputum |
| *Mycobacterium neworleansense* | DSM 44679 | Type strain, isolated from human scalp |

| | | |
|---|---|---|
| *Mycobacterium paratuberculosis* | ITG 2666 | See De Baere *et al.* 2002 |
| *Mycobacterium scrofulaceum* | DSM 43992 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium shimoidei* | DSM 44152 | Type strain, isolated from sputum of patient with tuberculosis-like disease |
| *Mycobacterium simiae* | ITG 4485 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium smegmatis* | DSM 43756 | Type strain |
| *Mycobacterium szulgai* | ITG 4979 | NA [b] |
| *Mycobacterium tusciae* | DSM 44338 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium ulcerans* | ITG 96-1439 | NA [b] |
| *Mycobacterium xenopi* | ITG 4986 | See De Baere *et al.* 2002 |
| **Other bacteria** | **Strain designation** | **Remark** |
| *Staphylococcus aureus* | DSM 20231 | Type strain, isolated from human pleural fluid |
| *Listeria monocytogenes* | DSM 20600 | Type strain, isolated from a rabbit |
| *Escherichia coli* | DSM 301 | Disinfectant test strain |
| *Klebsiella oxytoca* | ATCC 43086 | |
| *Enterococcus faecalis* | DSM 20371 | Isolated from pleural fluid |
| *Proteus mirabilis* | DSM 4479 | Type strain |
| *Bacillus cereus* | DSM 31 | Type strain |
| *Bordetella pertussis* | CCUG 13475 | Isolated from patient suffering from whooping cough |
| *Streptococcus agalactiae* | DSM 2134 | Type strain |
| *Rhodococcus equi* | DSM 20307 | Type strain, isolated from lung abscess of foal |
| *Streptomyces albidoflavus* | DSM 40455 | Type strain |
| *Nocardioides sp.* | DSM 17401 | Proposed type strain, isolated from marine sediment |
| *Nocardia salmonicida* | DSM 40472 | Type strain, isolated from blueback salmon |
| *Nocardia asiatica* | clinical isolate | Isolated from human wound, see Wauters *et al.* 2005 |
| *Nocardia nova* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |
| *Nocardia cyriacigeorgica* | clinical isolate | Isolated from human bronchial aspirate, see Wauters *et al.* 2005 |
| *Nocardia farcinica* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |

[a] RIVM = National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands; *DSM = The German Collection of Microorganisms; *ATCC = American Type Culture Collection; *ITG = Institute of Tropical Medicine, Antwerp, Germany; *CCUG = Culture Collection, University of Göteborg, Sweden; *VUB = Department of Microbiology, Academic Hospital of the Free University of Brussels, Brussels, Belgium.
[b] This information was not available (NA) for this study.

Table 3.4: Result interpretation table

| | FAM (*Wbbl1* assay) | HEX (MTC assay) | ROX (M. canettii RD assay) | Cy5 (IAC[a]) | Result |
|---|---|---|---|---|---|
| Diagnostics result profile | + ve | + ve | + ve | +/ -ve | *M. canettii* present. |
| Diagnostics result profile | + ve | + ve | -ve | +/ -ve | *M. tuberculosis* present. |
| Diagnostics result profile | -ve | + ve | -ve | +/ -ve | MTC present (other than *M. canettii or M. tuberculosis*). |
| Diagnostics result profile | -ve | -ve | -ve | + ve | Not a member of the MTC. |
| Diagnostics result profile | -ve | -ve | -ve | -ve | Result invalid and test must be repeated. |

[a] The IAC is only detected in the presence of low concentrations of primary target or absence of primary target.

**Figure 3.1 A**

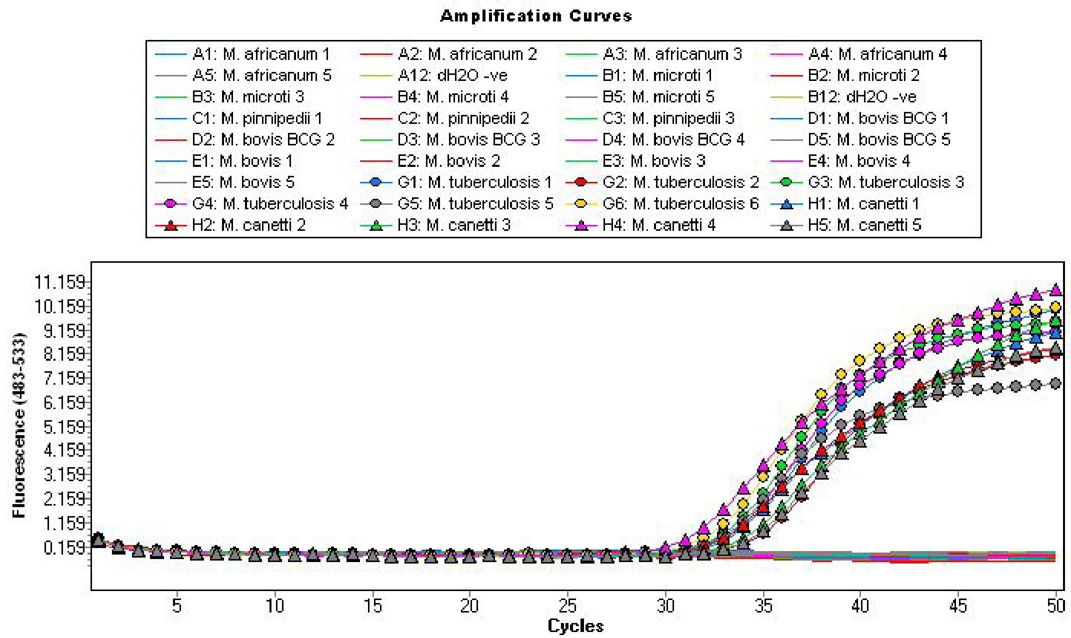


**Figure 3.1 B**

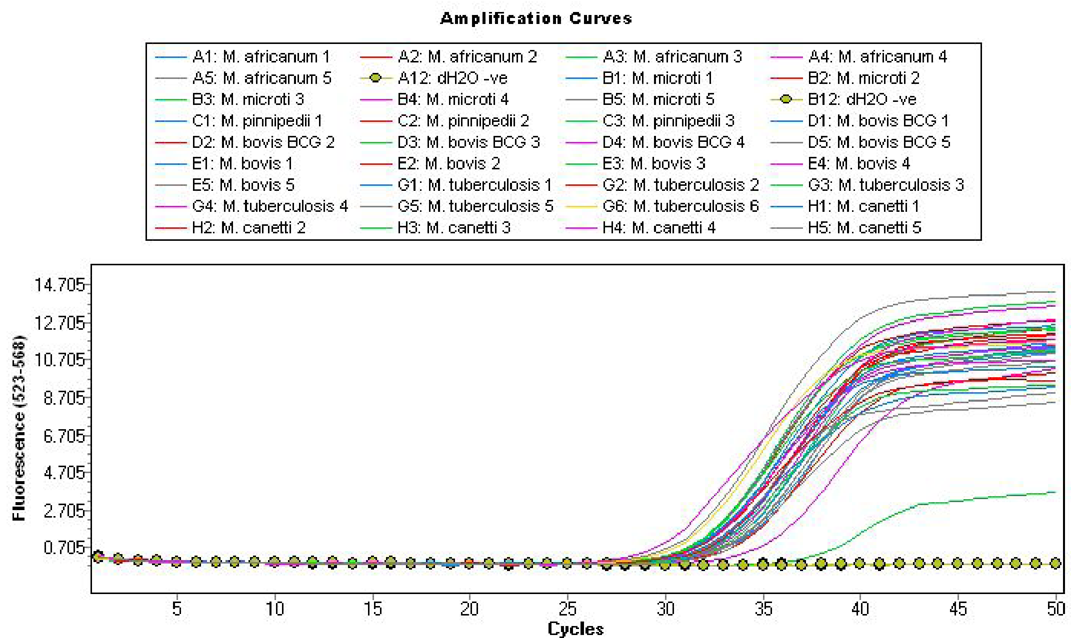

**Figure 3.1 C**

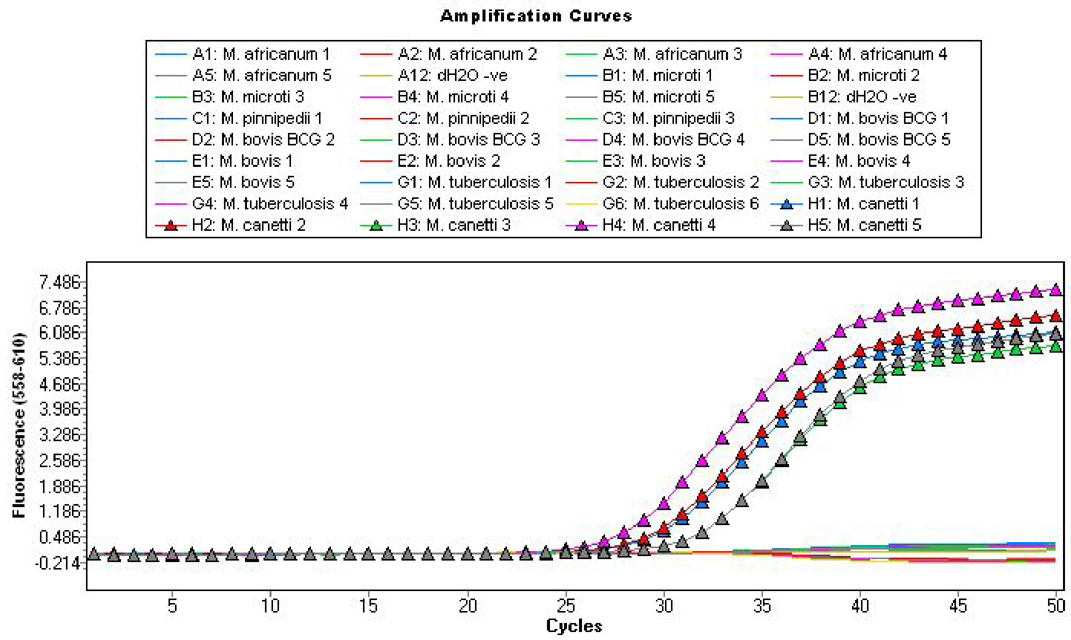


**Figure 3.1 D**

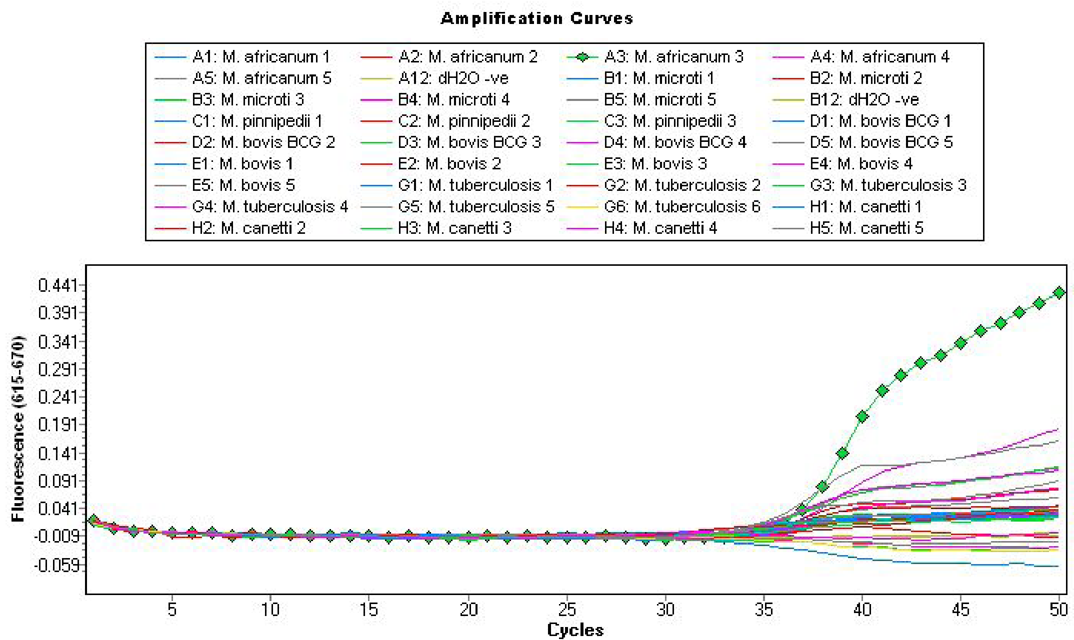

**Figure 3.1 A. Real-time amplification curves for *M. tuberculosis* (circle) and *M. canettii* (triangle) using *wbbl1* gene in FAM channel (438-533). Figure 3.1 B Amplification curves for all MTC using *lepA* gene in HEX channel (523-568), with the non-template control highlighted with circles through line. Figure 3.1 C Amplification curves for *M. canettii* specific assay in ROX channel (558-610), with the 5 *M. canettii* strains depicted with triangles. Figure 3.1 D Amplification curves for IAC in Cy5 channel (615-670) with *M. africanum* 3 highlighted with boxes through amplification curve. The Reduction of the IAC signal is due to competition from high concentrations of primary target, resulting in competition between the MTC and IAC assays.**

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# Chapter 4:

## A Novel Multiplex Real-Time PCR for the Identification of Mycobacteria Associated with Zoonotic Tuberculosis

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### Abstract

**Background:** Tuberculosis (TB) is the leading cause of death worldwide from a single infectious agent. An ability to detect the *Mycobacterium tuberculosis* complex (MTC) in clinical material while simultaneously differentiating its members is considered important. This allows for the gathering of epidemiological information pertaining to the prevalence, transmission and geographical distribution of the MTC, including those MTC members associated with zoonotic TB infection in humans. Also differentiating between members of the MTC provides the clinician with inherent MTC specific drug susceptibility profiles to guide appropriate chemotherapy.

**Methodology/Principal Findings:** The aim of this study was to develop a multiplex real-time PCR assay using novel molecular targets to identify and differentiate between the phylogenetically closely related *M. bovis*, *M. bovis* BCG and *M. caprae*.

The *lpqT* gene was explored for the collective identification of *M. bovis*, *M. bovis* BCG and *M. caprae*, the *lepA* gene was targeted for the specific identification of *M. caprae* and a Region of Difference 1 (RD1) assay was incorporated in the test to differentiate *M. bovis* BCG.

The multiplex real-time PCR assay was evaluated on 133 bacterial strains and was determined to be 100% specific for the members of the MTC targeted.

**Conclusions/Significance:** The multiplex real-time PCR assay developed in this study is the first assay described for the identification and simultaneous differentiation of *M. bovis*, *M. bovis* BCG and *M. caprae* in one internally controlled reaction. Future validation of this multiplex assay should demonstrate its potential in the rapid and accurate diagnosis of TB caused by these three mycobacteria. Furthermore, the developed assay may be used in conjunction with a recently described multiplex real-time PCR assay for identification of the MTC and simultaneous differentiation of *M. tuberculosis*, *M. canettii* resulting in an ability to differentiate five of the eight members of the MTC.

## 4.1 Introduction

Tuberculosis (TB) in humans is mostly caused by members of the *Mycobacterium tuberculosis* complex (MTC), although infections caused by nontuberculous mycobacteria (NTM) can mimic this disease [1]. *M. tuberculosis* is considered to be the primary pathogen of TB in humans [2]. Other members of the MTC associated with human TB infection include *M. canettii* and *M. africanum,* with the highest prevalence in various parts of Africa [3]. The remaining members of the MTC including *M. bovis*, *M. caprae*, *M. microti* and *M. pinnipedii* are considered predominantly animal pathogens, apart from the, for vaccination purposes, attenuated *M. bovis* BCG strain. However, all members of the MTC have been shown to cause human TB infection [4]. For example, the major etiological agents of zoonotic TB in humans are the phylogenetically related *M. bovis* and *M. caprae* [5]. These members of the MTC occur worldwide and there are indications which suggest the true prevalence of zoonotic human TB infection may be underrepresented, especially because of the extrapulmonary manifestation of the disease generally caused by these bacteria [6,7,8]. In developed countries it has been suggested that the burden of bovine TB in humans ranges from 0.5 to 7.2% of TB cases, while in developing countries, where very little data is available, this figure may be as high as 15% [9,10].

The diagnosis of TB in humans in high prevalence settings usually still relies on smear microscopy, although this is insensitive, especially in patients co-infected with HIV [11,12].The gold standard culture method is both time consuming and laborious [13]. Therefore, *in vitro* nucleic acid amplification tests (NAATs) offer a rapid and sensitive alternative to traditional methods for the detection of TB infection, with an increasing number of commercially available TB diagnostics kits becoming available [14,15,16], in addition to NAAT methods described in the literature [17,18]. However, most of these rapid and traditional diagnostics methods detect the presence of the MTC but do not identify the specific etiological agent of infection.

Since the introduction of pasteurisation and bovine TB eradication programmes in developed countries there has been a significant reduction in the number of zoonotic TB cases in humans [10], with the majority of such cases occurring as a result of endogenous reactivation of a previous infection [9,19]. However, recent reports have identified TB in humans caused by *M. bovis* in countries officially free from bovine TB [8] and suggest that the true prevalence of zoonotic TB may be underestimated clinically [7]. In addition, recent reports show that *M. bovis* infection in younger patient groups in the Western world are still common, most likely owing to consumption of raw milk (products) in the country of origin [20]. Moreover, zoonotic TB remains a significant threat to human health in developing countries where its prevalence is currently unknown, as differentiation between the members of the MTC is not routinely performed [9,21].

The accurate differentiation of the MTC is also clinically important, due to varying natural resistances of members of the MTC to anti-TB drugs. [22]. As such, the ability to differentiate between members of the MTC could potentially allow for the monitoring of zoonotic exposure leading to human TB infection, in addition to providing accurate epidemiological information for the clinician, guiding contact tracing and source case finding.

In this study, we report the design, development and testing of a multiplex real-time PCR assay, using novel nucleic acid molecular targets, to identify and simultaneously differentiate between *M. bovis*, *M. bovis* BCG and *M. caprae* in one internally controlled reaction. This assay can be used independently, or in combination with, a recently described multiplex PCR assay for the identification of the MTC, *M. tuberculosis* and *M. canettii* [23].

## 4.2 Materials and Methods

### *4.2.1 Diagnostics target identification*

The diagnostics target genes used in this study were identified using a number of experimental and bioinformatics approaches. Potential target regions were identified using the Mycobacterial Genome Divergence Database (MGDD) (http://mirna.jnu.ac.in/mgdd/), which allowed for identification of insertions, deletions and single nucleotide polymorphisms between *M. tuberculosis*, *M. bovis* and *M. bovis* BCG. Other potential target regions were identified using the web based version of the Artemis comparison tool, WebACT (http://www.webact.org/WebACT/home). Nucleotide sequence information for *M. africanum*, *M. microti* and *M. canettii* was retrieved from the Welcome Trust Sanger Institute website (www.sanger.ac.uk/resources/downloads/bacteria/mycobacterium-currently being sequenced and annotated) to determine, *in silico,* if the candidate diagnostics target sequences for *M. bovis* identification were specific. As there was no nucleotide sequence information available for *M. caprae* or *M. pinnipedii* at the time of this study, specificity of the developed assays against these members of the MTC was determined empirically and further validated by nucleotide sequencing.

Since the diagnostics target used in this study for the differentiation of *M. bovis* from the remaining members of the MTC was experimentally determined to also identify *M. caprae* and *M. bovis* BCG, alternative nucleic acid diagnostics targets were required to differentiate this sub-group of the MTC.

For each putative diagnostics target identified, alignments were performed using clustalW multiple sequence alignment programme (http://www.ebi.ac.uk/Tools/clustalw2/index.html), from which real-time PCR primers and probes were designed (Table 4.1).

*4.2.2 Bacterial strains, culture media and growth conditions*

Seventy two MTC isolates (26 *M. tuberculosis*, 14 *M. bovis*, 7 *M. bovis BCG*, 5 *M. canettii*, 5 *M. caprae*, 5 *M. africanum*, 5 *M. microti* and 5 *M. pinnipedii*), 44 non tuberculosis mycobacteria (NTM) and 17 other bacterial species were used in this study (Table 4.2 and Table 4.3). Of the 72 MTC isolates, 36 strains previously characterised by a variety of methods [24,25,26,27,28] were provided by The National Institute for Public Health and the Environment (RIVM), the Netherlands. Three *M. caprae*, 3 *M. bovis* and 2 *M. pinnipedii* strains were supplied by the National Reference Centre for Mycobacteria, Borstel, Germany and were previously characterised using a variety of methods [29,30]. All other MTC strains, provided by Professor Mario Vaneechoutte (University of Ghent, Ghent, Belgium), were clinical isolates collected over a ten year period from reference laboratories in Belgium and the Netherlands. These isolates were characterised on the basis of custom techniques available at the time. Twenty eight NTM were purchased from the German Collection of Microorganisms and Cell Cultures (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, DSMZ) and grown on Middlebook agar/broth at the appropriate temperature (either 30 °C or 37 °C). Fast growing mycobacteria were cultured for 3-6 days and slow growing mycobacteria were incubated for six weeks, or until sufficient growth was visible. All media were purchased from BD Biosciences (Oxford, United Kingdom). DNA was supplied from the 16 remaining NTM and 4 of the 5 *Norcardia* strains by Professor Vaneechoutte and these species were characterised using techniques previously described [31,32,33].

*4.2.3 DNA isolation and quantification*

Genomic DNA from 28 NTM and 2 *M. bovis BCG* cultures was isolated from 1 ml of culture (Middlebrook 7H9 broth, Becton Dickinson), using a technique previously described in the literature [23]. Total genomic DNA samples provided by the RIVM were extracted according to Van Soolingen *et al.* [34], whereas total genomic DNA samples from the National Reference Centre for Mycobacteria, Borstel were

extracted as described by van Embden *et al.* [35], while genomic DNA provided by Professor Vaneechoutte was extracted as described by De Baere *et al.* [31]. For all other bacterial species tested, DNA was provided from stocks held within this laboratory (Microbiology, NUIG). DNA concentrations for all species and strains used in this study were determined using the PicoGreen dsDNA Quantitation Kit (Molecular Probes, Eugene, Oregon, USA) and the TBS-380 mini-fluorometer (Invitrogen Corporation, California, USA). DNA samples were stored at -20 °C before use.

*4.2.4 Conventional and real-time PCR primers and hydrolysis probe design*

Oligonucleotide primers and hydrolysis probes were designed in accordance with general recommendations and guidelines [36,37], following alignments of each of the nucleic acid diagnostics target genes identified in this study. Primers and probes (Table 4.1) were supplied by MWG-BIOTECH AG (Essenberg, Germany) or TIB MOLBIOL (Berlin, Germany). Primers were designed to have a melting temperature (Tm) of 58-61 °C and the probes were designed to have a Tm 4-7 °C higher. The probe designed to identify *M. bovis*, *M. bovis* BCG and *M. caprae* had a higher Tm (69.8 °C) due to a particularly G/C rich target region. Hydrolysis probes were designed to be specific for each target following published design guidelines [36]. Assay oligonucleotides were designed with similar properties such that monoplex assays could be easily multiplexed after specificity and sensitivity testing was complete [23].

The *M. caprae* specific assay PCR primers, MTC_Fw (position 618-634 bp of the *lepA* gene of *M. tuberculosis* H37Rv) and MTC_Rv (position 754-772 bp), were designed to amplify a 155 bp fragment of the *lepA* gene. lpqT_FW and lpqT_RV were designed to amplify a 141 bp fragment of the *lpqT* gene for the identification of *M. bovis*, *M. bovis* BCG and *M. caprae* (positions 17-34 bp and 146-162 bp of the *M. tuberculosis* H37RV *lpqT* gene). The RD1 assay was designed to amplify a 117 bp region of the Rv3876 gene, a conserved hypothetical protein, part of RD1, absent

in all *M. bovis* BCG strains. The RD1_Fw primer was located at position 1416-1433 bp and the reverse primer RD1_Rv between 1516-1532 bp of the *M. tuberculosis* H37 Rv 3876 gene. The internal amplification control (IAC) PCR primers, IAC_Fw and IAC_Rv, were designed to amplify a 157 bp region of the *M. smegmatis* MSMEG_0660 gene. The IAC_Fw primer was located at positions 497-513 bp and the reverse primer between positions 636-653 bp.

All real-time PCR assays were initially tested for in a monoplex format, evaluating their specificity and sensitivity using probes labelled with FAM and Black Hole Quencher 1 (BHQ1). After optimisation of the monoplex real-time PCR assays, three of the four assay probes were labelled with different fluorescent dyes to allow for incorporation into a multiplex real-time PCR format. The *M. caprae* specific probe was labelled with cyan500 and BBQ, the *M. bovis/M. bovis* BCG*/M. caprae* specific probe with FAM and BHQ1, the *M. bovis/M. caprae* probe with HEX and BHQ2 and the IAC probe with Cy5 and BHQ2.

*4.2.5 Conventional PCR*

Conventional PCR was performed using the sequencing primers outlined in Table 4.1 on the iCycler iQ thermal cycler (Bio-Rad Laboratories Inc., California, USA). All reactions were carried out in a final volume of 50 µl, containing 5 µl 10 × buffer (15mM $MgCl_2$), 1 µl forward and reverse primers (0.2 µM final conc.), 2 µl Taq DNA polymerase (1 U/ µl, Roche Diagnostics, Basel, Switzerland), 1 µl dNTP mix [(10 mM deoxynucleoside triphosphate set (Roche Diagnostics)], 2 µl of template DNA and 38 µl nuclease free water (Applied Biosystems/Ambion, Texas, USA). The cycling parameters consisted of initial denaturation at 95 °C for 5 mins, followed by 35 cycles of denaturation at 95 °C (1 min), annealing at 50 °C (1 min), and extension at 72 °C (1 min), with a final elongation step at 72 °C for 10 min.

*4.2.6 Sequencing*

Nucleotide sequence data for real-time PCR assay design was generated in this study or was obtained from the National Centre for Biotechnology Information (NCBI- http://www.ncbi.nlm.nih.gov/), or the Sanger website (where partial nucleotide sequences for *M. canettii*, *M. africanum* and *M. microti* were available). Real-time PCR assay primers were used to generate nucleotide sequence information for each of the assays developed. Sequencing primers were also designed to span the full gene sequence of *lpqT*, *lepA* and a number of other genes to identify potential candidate nucleic acid diagnostics targets for the specific identification of *M. caprae.*

PCR products were generated according to section 4.2.5, purified using the High Pure PCR Product Purification Kit (Roche Diagnostics) and sequenced commercially (Sequiserve, Vaterstetten, Germany).

*4.2.7 Development of an IAC for real-time PCR*

A non-competitive IAC [38] was designed and incorporated into the multiplex real-time PCR. The *M. smegmatis* MSMEG_0660 gene was chosen as the target as it is specific to *M. smegmatis*, avoiding any possible cross reaction. Titration of IAC DNA was performed to determine the optimum concentration per reaction that could be reliably detected in the presence of MTC DNA, yet not inhibit detection of low concentrations of the primary assay targets. An IAC concentration of 100 genome equivalents per reaction was determined as optimum.

*4.2.8 Real-time PCR*

Monoplex real-time PCR was performed on the LightCycler 2.0 Instrument (Roche Diagnostics) using the LightCycler® TaqMan® Master kit (Roche Diagnostics). Each reaction contained 5 × master mix, forward and reverse primers (0.5 µM final conc.), FAM labelled probe (0.2 µM final conc.), template DNA (2 µl) and nuclease free

$dH_2O$ to a final volume of 20 µl. The cycling parameters consisted of 10 min incubation at 95 °C to activate the Taq, 50 cycles of 95 °C for 10 s and 60 °C for 30 s, followed by a cooling step at 40 °C for 10 s. The temperature transition rate for all cycling steps was 20 °C/s.

Multiplex real-time PCR reactions were carried out on the LightCycler 480 using LightCycler® 480 Probes Master kit (Roche Diagnostics). The optimised PCR mix contained 2 × LightCycler 480 Probes Master (6.4 mM $MgCl_2$), forward and reverse primer (0.5 µM final conc.), Cyan500, FAM, HEX and CY5 labelled probes (0.2 µM final conc.), dimethyl sulfoxide (4%, Sigma-Aldrich Missouri, USA), template DNA (MTC: 2 µl; IAC: 2 µl; NTM: 10 µl) adjusted to a final volume of 40 µl with the addition of nuclease free $dH_2O$. The *M. smegmatis* internal control DNA was diluted to contain 100 genome equivalents per 2 µl and NTM DNA was diluted to contain ~$10^4$ genome equivalents per 10 µl.

The cycling parameters used were the same as those used on the LightCycler 2.0. The temperature transition rate, referred to as the ramp rate on the LightCycler 480 was 4.4 °C/s while heating and 2.2 °C/s while cooling. Prior to experimental analysis on the LightCycler 480, a colour compensation file was generated, to avoid fluorescence leaking from channel to channel, using the technical note outlined in the Advanced Software Functionalities of the operator manual [39].

## 4.3 Results

### *4.3.1 Diagnostics target identification*

The diagnostics target used for the specific identification of *M. caprae* was the *lepA* (Rv 2404c) gene. *In-silico* analysis of nucleotide sequences generated in this study revealed a single nucleotide polymorphorism (SNP) at position 690 bp of the *M. tuberculosis* H37Rv *lepA* gene which was specific for *M. caprae*. LepA is an

essential bacterial elongation factor. The *lepA* gene codes for a highly conserved protein present in all bacteria sequenced to date, with a homologue (Guf1) found in higher organisms [40]. The *M. caprae* specific SNP, identified from nucleotide sequence data generated in this study, was a C to T substitution which was conserved in all 5 strains of *M. caprae* tested.

The diagnostics target identified in this study for collective identification of *M. bovis*, *M. bovis* BCG and *M. caprae* was the *lpqT* (Rv1016c) gene. LpqT belongs to a group of lipoproteins which are present in all bacteria [41] and is thought to be required for optimal growth of mycobacteria *in vivo* [42]. *In-silico* analysis of publicly available *lpqT* nucleotide sequences, performed in this study, revealed a 5 bp region of *lpqT*, deleted in *M. bovis* and *M. bovis* BCG but present in *M. tuberculosis*, *M. canettii*, *M. africanum* and *M. microti*. Sequence information was generated for the *lpqT* gene in *M. caprae* and *M. pinnipedii* which confirmed the deletion was also present in *M. caprae* but not *M. pinnipedii*.

The diagnostics target used to differentiate *M. bovis* BCG from *M. bovis* and *M. caprae* was the Rv3876 gene, which is part of Region of Difference 1 (RD1). Regions of Difference (RD) in the *Mycobacterium tuberculosis* complex refer to regions of the genome present in *M. tuberculosis* but deleted from *M. bovis* BCG [43]. The particular RD targeted in this study (RD1), has been shown to be deleted in all *M. bovis* BCG strains and has been linked to the attenuation of this strain [13]. Any region of RD1 could potentially be incorporated into this multiplex real-time PCR and used with the novel diagnostics targets described above.

*4.3.2 Assay design and development*

While the guidelines for primer and probe design were adhered to as closely as possible, the high G/C content (60-65%) of the *Mycobacterium* species had an impact on assay design. The *lpqT* specific probe was designed spanning the deletion junction of a region deleted in *M. bovis, M. bovis* BCG and *M. caprae* and present in

the other members of the MTC, that was very G/C rich, making probe design difficult. The *lpqT* probe, therefore, had a relatively high Tm, however this did not impact on assay performance. The *M. caprae* specific probe targeted an SNP in the *lepA* gene. Avoiding cross reaction of the *M. caprae* probe with other members of the MTC proved challenging. A number of probes were designed and tested and the optimum probe was chosen empirically based on specificity and sensitivity results. The optimum probe was designed complementary to the + strand of the *lepA* gene as the resulting G/A mismatch, that occurred in the presence of non-target MTC DNA, was more destabilising to the probe than the C/T mismatch, hence improving specificity. The probe was designed to have a Tm of 60.1°C, only slightly above the annealing temperature of the assay (60°C), allowing the probe to hybridise to exactly matched sequence only, therefore maximising the specificity effect of the SNP. This did, however, slightly reduce probe binding efficiency, leading to a small reduction in sensitivity.

#### *4.3.3 Internal amplification control*

In order for a result to be considered valid using this assay, a positive signal must be obtained in the Cy5 detection channel on the LightCycler 480. If the IAC is not detected, the result is considered invalid and must be repeated [38,44]. For the purposes of this study, *M. smegmatis* DNA was spiked into the PCR master mix to act as an internal control target. Alternatively, *M. smegmatis* cultured cells could be used as a process control, spiked into patient samples before genomic DNA isolation. In this design, the *M. smegmatis* cells would serve as both an extraction control and an amplification control and could be used when testing patient samples.

#### *4.3.4 Specificity of the diagnostic assays*

The specificity of each real-time PCR assay was confirmed both in monoplex and multiplex formats using the specificity panel listed in Table 4.2 and Table 4.3. The *lepA* assay was specific for the detection of the 5 *M. caprae* strains in our collection

(Figure 4.1 A). The remaining members of the MTC, NTM and other bacteria tested were not detected. The *M. bovis*, *M. bovis BCG* and *M. caprae* assay was also specific, detecting only target species (Figure 4.1 B). The diagnostics assay targeting RD1 did not detect any of the 7 *M. bovis* BCG strains tested for (Figure 4.1 C) but detected all *M. bovis* and *M. caprae* strains tested for, allowing for the specific identification of *M. bovis* BCG. All remaining members of the MTC (with the exception of the 5 *M. microti* and one *M. canettii*) were detected. None of the NTM or other bacteria on the specificity panel were detected using the RD1 assay. The IAC assay targeted the *M. smegmatis* MSMEG_0660 gene. This assay was tested against all members of the MTC and NTM and was specific for the detection of *M. smegmatis*. One hundred genome equivalents of *M. smegmatis* DNA was added to all samples tested, to control for PCR inhibition, and was detected in all samples (Figure 4.1 D). Table 4.4 describes how to interpret the results of the multiplex real-time PCR assay.

*4.3.5 Sensitivity of the assays*

The limit of detection (LOD) of each assay was evaluated in a monoplex real-time PCR format. Genomic DNA was quantified and serial dilutions were prepared from 200,000 to 2 genome equivalents (with an estimated genome size of 4.5 million base pairs, one *M. caprae* cell contains approximately 5.0 fg DNA [45]). Assay sensitivity testing was performed using *M. caprae* DNA as it is detected in all of the multiplex assays.

In a monoplex format, the dilution series was run in duplicate and a sensitivity of 2-20 *M. caprae* genome equivalents was determined for each assay. In multiplex format the lower limit of detection was established using probit regression analysis. Twelve replicates of each of 20, 15, 12, 10, 7.5, 4, 2 and 0.2 *M. caprae* genome equivalents were tested. LOD's of 3.95 and 3.55 genome equivalents were determined (95 % probability) for the *M. bovis*/*M. bovis* BCG/*M. caprae* and the *M. bovis*/*M. caprae* assays respectively. However, these dilutions were not appropriate

to determine the *M. caprae* specific assay LOD. The analysis was repeated using concentrations of 200, 100, 75, 50, 25, 15, 10 and 5 genome equivalents of *M. caprae* DNA.  An LOD of 14.77 genome equivalents (95 % probability) was determined for the *M. caprae* specific assay when tested as part of the multiplex. The IAC, at a concentration of 100 genome equivalents per reaction, was included in all samples during sensitivity testing and was detected as expected.

## 4.4 Discussion

The WHO [46] defines zoonoses as infections and diseases which can be transmitted naturally from animals to humans. A recent WHO report highlights the difficulties in the diagnosis of such diseases and suggests that the true incidence of many neglected zoonoses, including zoonotic TB, may be greatly underestimated [46]. Zoonotic TB in humans is most commonly caused by *M. bovis* and to a lesser extent *M. caprae* (prevalent in certain regions in Europe [47]). Human TB infection caused by the zoonotic transmission of *M. microti* and *M. pinnipedii* has also been reported [48,49,50]. While bovine TB in humans is considered rare in developed countries, it has been described as an ‘endemic neglected zoonoses’ in developing countries [51]. In these settings, the true incidence of zoonotic TB in humans remains largely unknown due to inabilities in hospital laboratories to distinguish between the causative agent of infection, a scarcity of trained personnel, financial constraints in addition to limited control measures in place [51,52].

*M. bovis* is the predominant member of the MTC associated with bovine TB infection. However, it also has been isolated from and is the causative agent of TB in a wide range of mammalian hosts including humans, goats, dogs, deer and ferrets [10]. Transmission to and infection of human hosts with *M. bovis* can result from zoonotic exposure to TB infected animals or to consumption of unpasteurised dairy products [8]. Recent studies have also shown that human to human transmission of *M. bovis* can also occur, but is considered rare [53].

Since its transfer from *M. tuberculosis* subsp *caprae* to *M. bovis* subsp *caprae* in 2002 [5], this member of the MTC has been recognised as the cause of a number of outbreaks of TB in animals such as goats, cattle and red deer [47]. In addition, *M. caprae* has been associated with outbreaks of TB in humans in a number of European countries [54,55,56] and was shown to be the true causative agent of one third of human *M. bovis* associated TB cases in Germany between 1999-2001 [10].

*M. bovis* BCG, the commonly used vaccine strain, has also been implicated in human disease particularly in immunocompromised patients, such as HIV positive and cancer patients, where it has been shown to cause disseminated disease [57], and in children who have been vaccinated in endemic TB areas [58].

While the members of the MTC discussed above share many phenotypic characteristics, in addition to natural host preference, *M. bovis, M. bovis* BCG and *M. caprae* exhibit a differential intrinsic susceptibility profile to pyrazinamide (PZA), an important first line anti-TB drug responsible for reducing patient treatment time [59]. *M. bovis* and *M. bovis* BCG are intrinsically resistant to PZA whereas *M. caprae* is sensitive to PZA [5]. This highlights the need for a rapid test that can detect the MTC and differentiate between its members so that the clinician can choose the appropriate chemotherapy for the patient. More accurate diagnosis and treatment will protect against the development of drug resistant TB and reduce morbidity and mortality [10]. Furthermore, accurate identification of these organisms allows monitoring of these neglected members of the MTC with respect to prevalence, transmission and global distribution [48]. Also, differentiation of the complex could contribute to the better understanding of the clinical relevance of these members of the MTC.

Currently, there is only one commercially available diagnostics kit for the differentiation of the MTC, the GenoType MTBC line probe assay (Hain Lifesciences). This assay differentiates members of the MTC utilising highly conserved SNP's in the *gyrB* gene [60], however it is limited as it does not differentiate *M. canettii* and *M. pinnipedii*. Literature describing NAAT's for the

complete differentiation of the MTC [50,61,62,63] are hindered by the complexity of the multiple reactions required, the inability to differentiate between some members of the MTC, in addition to the complex interpretation of the results generated. While SNP's have commonly been used for accurate differentiation of members of the MTC [3,50] further validation of the SNP identified in this study for the specific detection of *M. caprae* is required.

The multiplex real-time PCR assay described in this study is the first description of a real-time PCR diagnostics assay for the identification and simultaneous differentiation of *M. bovis*, *M. bovis* BCG and *M. caprae* using novel diagnostics targets in one internally controlled reaction. This diagnostics assay takes approximately one hour to perform after DNA extraction. The assay has been validated against a large panel of well characterised clinical isolates. In its current format, this multiplex real-time PCR assay would be applied to MTC clinical isolates for the identification of the most common causes of zoonotic TB. Monitoring bovine TB in humans is valuable for guiding public health policy and is important for the study of zoonotic TB epidemiology.

The developed assay also has the potential to be used directly in sputum samples following identification of an efficient sputum sample preparation method. If performing direct sputum testing, the assay developed in this study would be used as a follow on test in combination with a real-time PCR assay for identification of the MTC and differentiation of *M. tuberculosis* and *M. canettii,* which was previously developed by this group. More specifically, if a patient sample is identified as positive for the presence of the MTC but *M. tuberculosis* or *M. canettii* negative, the assay developed in this study can then be used to determine if sample contains *M. bovis, M. bovis* BCG or *M. caprae*, the most common causes of zoonotic TB in humans.

The study presented here focuses on accurately differentiating members of the MTC most commonly associated with zoonotic human TB infection. However, there is a need to differentiate between all members of the MTC to supply unambiguous epidemiological data as to the true prevalence of each member of the MTC causing TB infection [3]. Work has begun in this group on the development of a final multiplex real-time PCR diagnostics assay for the accurate identification of *M. africanum*, *M. microti* and *M. pinnipedii*. This will complete a cassette of three multiplex real-time PCR diagnostics assays for the identification and differentiation of all members of the MTC.

Table 4.1: Oligonucleotide primers and probes used in this study

| **Name** | **Purpose** | **Sequence 5’→3’** |
|---|---|---|
| MTC_Fw | Forward sequencing primer, forward *M. caprae* real- time PCR assay primer | AGACCGTGCGGATCTTG |
| MTC_Rv | Reverse sequencing primer, reverse *M. caprae* real-time PCR assay primer | CATGGAGATCACCCGTGA |
| *M. caprae* specific Probe | *M. caprae* probe | Cyan 500- TATCGGGTACACAAAGACGA -BBQ |
| lpqT_FW | Forward sequencing primer, forward real-time PCR assay primer | ACGAATCCGGCGATGATC |
| lpqT_RV | Reverse sequencing primer, reverse real-time PCR assay primer | CGACTGCACACCTGGAA |
| lpqT Probe | *M. caprae*, *M. bovis*, *M. bovis* BCG probe | FAM-TTGGCCGGCGCCGGTT-BHQ1 |
| RD1_Fw | Forward sequencing primer, forward real-time PCR assay primer | CATCGCTGATGTGCTTGC |
| RD1_Rv | Reverse sequencing primer, reverse real-time PCR assay primer | TGCGCCGAGCTGTATTC |
| RD1 Probe | Absence of *M. bovis* BCG | HEX-ACACTAGCGTCAATGCGGTCA-BHQ1 |
| IAC_Fw | Forward sequencing primer, forward real-time PCR assay primer | TCACCGACCATGTCCAG |
| IAC_RV | Reverse sequencing primer, reverse real-time PCR assay primer | CGTTGCCCAATCCGTATG |
| IAC probe | IAC probe | CY5-CAGCAGTACCATCGCCATCG-BHQ2 |

Table 4.2: *Mycobacterium tuberculosis* complex isolates used in this study

| Species | Strain/ collection number [a] | Country of Isolation | DNA provided by |
|---|---|---|---|
| *M. tuberculosis* | 22 | Mongolia | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* | 53 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* | 112 | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* | 67 | Comoro Islands | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* | 41 | Chile | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* | 103 | China | RIVM, Bilthoven, the Netherlands |
| *M. tuberculosis* (*19 clinical isolates*) | - | NA[c] | Mario Vaneechoutte, University of Ghent, Ghent, Belgium |
| *M. tuberculosis* H37Rv | H37Rv | NA[c] | DSMZ |
| *M. canettii* | 116 | Somalia | RIVM, Bilthoven, the Netherlands |
| *M. canettii* | 1997-1549 | Switzerland | RIVM, Bilthoven, the Netherlands |
| *M. canettii* | NLA000701 671 | Somalia | RIVM, Bilthoven, the Netherlands |
| *M. canettii* | NLA000200 937 | Eritrea | RIVM, Bilthoven, the Netherlands |
| *M. canettii* | 1996-46 | France | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 117 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 126 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 73 | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 130 | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 24 | Saudi Arabia | RIVM, Bilthoven, the Netherlands |
| *M. bovis* | 4258_00 | Germany | Research Center Borstel, Germany |
| *M. bovis* | 751_01 | Germany | Research Center Borstel, Germany |
| *M. bovis* | 7540_01 | Germany | Research Center Borstel, Germany |
| *M. bovis* (6 isolates) | - | NA[c] | Mario Vaneechoutte, University of Ghent, Ghent, Belgium |
| *M. bovis* BCG | 48 (2) | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. bovis* BCG | 71 | Japan | RIVM, Bilthoven, the Netherlands |
| *M. bovis* BCG | 83 | Russia | RIVM, Bilthoven, the Netherlands |
| *M. bovis* BCG | 2008-714 [b] | NA[c] | RIVM, Bilthoven, the Netherlands |
| *M. bovis* BCG | 2008-1601 [b] | NA[c] | RIVM, Bilthoven, the Netherlands |
| *M. bovis* BCG | DSM 43990 | NA[c] | DSMZ |
| *M. bovis* BCG | DSM 45071 | NA[c] | DSMZ |
| *M. caprae* | 2006-1960 [b] | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. caprae* | 2007-0039 [b] | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. caprae* | 1694_00 | Germany | Research Center Borstel, Germany |
| *M. caprae* | 8986_99 | Germany | Research Center Borstel, Germany |
| *M. caprae* | 9577_99 | Germany | Research Center Borstel, Germany |
| *M. microti* | 62 | United Kingdom | RIVM, Bilthoven, the Netherlands |
| *M. microti* | 25 | United Kingdom | RIVM, Bilthoven, the Netherlands |
| *M. microti* | 15274 [b] | United Kingdom | RIVM, Bilthoven, the Netherlands |
| *M. microti* | 15912 [b] | Belgium | RIVM, Bilthoven, the Netherlands |
| *M. microti* | 15911 [b] | Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. pinnipedii* | 76 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. pinnipedii* | 81 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. pinnipedii* | 101 | Argentina | RIVM, Bilthoven, the Netherlands |
| *M. pinnipedii* | 7011_02 | Germany | Research Center Borstel, Germany |
| *M. pinnipedii* | 7739_01 | Germany | Research Center Borstel, Germany |
| *M. africanum* | 6 | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. africanum* | 128 (85) | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. africanum* | 2007-1386 [b] | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. africanum* | 2007-1154 [b] | The Netherlands | RIVM, Bilthoven, the Netherlands |
| *M. africanum* | 2007-1073 [b] | The Netherlands | RIVM, Bilthoven, the Netherlands |

[a] RIVM = National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands; *DSM = The German Collection of Microorganisms; *Research Center Borstel = Germany National Reference Center for Mycobacteria, Forschungszentrum Borstel, Germany
[b] Represent RIVM strains not previously described in literature, however have been characterised to the species level using techniques outlined in references supplied in the manuscript.
[c] This information was not available (NA) for this study.

Table 4.3: Non *tuberculosis* mycobacteria and other strains of bacteria used in this study

| **Non *tuberculosis* mycobacteria** | **Strain designation** [a] | **Remark** |
|---|---|---|
| *Mycobacterium aichiense* | DSM 44147 | Type strain, isolated from soil |
| *Mycobacterium alvei* | DSM 44176 | Type strain, isolated from water sample |
| *Mycobacterium arupense* | DSM 44942 | Type strain, isolated from a tendon |
| *Mycobacterium asiaticum* | ITG 8182 | See De Baere *et al.* 2002 |
| *Mycobacterium avium* | ITG 7886 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium boenickei* | DSM 44677 | Type strain, isolated from a leg wound |
| *Mycobacterium branderi* | DSM 44624 | Type strain, isolated from human sputum |
| *Mycobacterium brisbanense* | DSM 44680 | Type strain, isolated from a sinus |
| *Mycobacterium brumae* | DSM 44177 | Type strain, isolated from water sample |
| *Mycobacterium canariasense* | DSM 44828 | Type strain, isolated from human blood |
| *Mycobacterium celatum* | ITG 6147 | See De Baere *et al.* 2002 |
| *Mycobacterium chelonae* | ITG 4975 | NA [b] |
| *Mycobacterium chelonae subsp. abscessus* | DSM 44196 | Type strain |
| *Mycobacterium confluentis* | DSM 44017 | Type strain, isolated from human sputum |
| *Mycobacterium conspicuum* | DSM 44136 | Type strain, isolated from patient with disseminated infection |
| *Mycobacterium flavescens* | VUB A016 | See De Baere *et al.* 2002 |
| *Mycobacterium fortuitum* | ITG 8020 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium genavense* | ITG 97-102 | See De Baere *et al.* 2002 |
| *Mycobacterium gilvum* | DSM 9487 | Isolated from soil |
| *Mycobacterium goodii* | DSM 44492 | Type strain |
| *Mycobacterium gordonae* | ITG 7704 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium heckeshornense* | DSM 44428 | Type strain, isolated from human respiratory tract |
| *Mycobacterium houstonense* | DSM 44676 | Type strain, isolated from a facial abscess |
| *Mycobacterium intracellulare* | DSM 43223 | Type strain |
| *Mycobacterium kansasii* | ITG 7727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium kubiciae* | DSM 44627 | Type strain, isolated from human sputum |
| *Mycobacterium lacus* | DSM 44577 | Type strain, isolated from human elbow |
| *Mycobacterium mageritense* | DSM 44476 | Type strain, isolated from human sputum |
| *Mycobacterium malmoense* | ITG 940611 | See De Baere *et al.* 2002 |
| *Mycobacterium marinum* | ITG 1727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium massiliense* | DSM 45103 | Type strain, isolated from human blood |
| *Mycobacterium moriokaense* | DSM 44221 | Type strain, isolated from soil |
| *Mycobacterium mucogenicum* | DSM 44625 | Type strain, isolated from human cyst |
| *Mycobacterium nebraskense* | DSM 44803 | Type strain, isolated from human sputum |

| | | |
|---|---|---|
| *Mycobacterium neworleansense* | DSM 44679 | Type strain, isolated from human scalp |
| *Mycobacterium paratuberculosis* | ITG 2666 | See De Baere *et al.* 2002 |
| *Mycobacterium scrofulaceum* | DSM 43992 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium shimoidei* | DSM 44152 | Type strain, isolated from sputum of patient with tuberculosis-like disease |
| *Mycobacterium simiae* | ITG 4485 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium smegmatis* | DSM 43756 | Type strain |
| *Mycobacterium szulgai* | ITG 4979 | NA [b] |
| *Mycobacterium tusciae* | DSM 44338 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium ulcerans* | ITG 96-1439 | NA [b] |
| *Mycobacterium xenopi* | ITG 4986 | See De Baere *et al.* 2002 |

| **Other bacteria** | **Strain designation** | **Remark** |
|---|---|---|
| *Staphylococcus aureus* | DSM 20231 | Type strain, isolated from human pleural fluid |
| *Listeria monocytogenes* | DSM 20600 | Type strain, isolated from a rabbit |
| *Escherichia coli* | DSM 301 | Disinfectant test strain |
| *Klebsiella oxytoca* | ATCC 43086 | |
| *Enterococcus faecalis* | DSM 20371 | Isolated from pleural fluid |
| *Proteus mirabilis* | DSM 4479 | Type strain |
| *Bacillus cereus* | DSM 31 | Type strain |
| *Bordetella pertussis* | CCUG 13475 | Isolated from patient suffering from whooping cough |
| *Streptococcus agalactiae* | DSM 2134 | Type strain |
| *Rhodococcus equi* | DSM 20307 | Type strain, isolated from lung abscess of foal |
| *Streptomyces albidoflavus* | DSM 40455 | Type strain |
| *Nocardioides sp.* | DSM 17401 | Proposed type strain, isolated from marine sediment |
| *Nocardia salmonicida* | DSM 40472 | Type strain, isolated from blueback salmon |
| *Nocardia asiatica* | clinical isolate | Isolated from human wound, see Wauters *et al.* 2005 |
| *Nocardia nova* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |
| *Nocardia cyriacigeorgica* | clinical isolate | Isolated from human bronchial aspirate, see Wauters *et al.* 2005 |
| *Nocardia farcinica* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |

[a] DSM = The German Collection of Microorganisms; *ATCC = American Type Culture Collection; *ITG = Institute of Tropical Medicine, Antwerp, Germany; *CCUG = Culture Collection, University of Göteborg, Sweden; *VUB = Department of Microbiology, Academic Hospital of the Free University of Brussels, Brussels, Belgium.
[b] This information was not available (NA) for this study.

Table 4.4: Result interpretation table

| Analysis channel result | | | | Diagnostics test result |
|---|---|---|---|---|
| Cyan 500<br><br>(+ve indicates presence of *M. caprae*) | FAM<br><br>(+ve indicates presence of *M. bovis*, *M. bovis* BCG or *M. caprae*) | HEX<br><br>(-ve indicates presence of *M. bovis* BCG if positive in FAM channel) | Cy5<br><br>(IAC) | |
| + ve | + ve | + ve | + ve | *M. caprae* present |
| - ve | + ve | + ve | + ve | *M. bovis* present |
| - ve | + ve | - ve | + ve | *M. bovis BCG* present |
| - ve | - ve | + ve | + ve | MTC member other than *M. caprae, M. bovis*, *M. bovis* BCG or *M. microti* |
| - ve | - ve | - ve | - ve | Result invalid and test must be repeated |

**Figure 4.1 A**

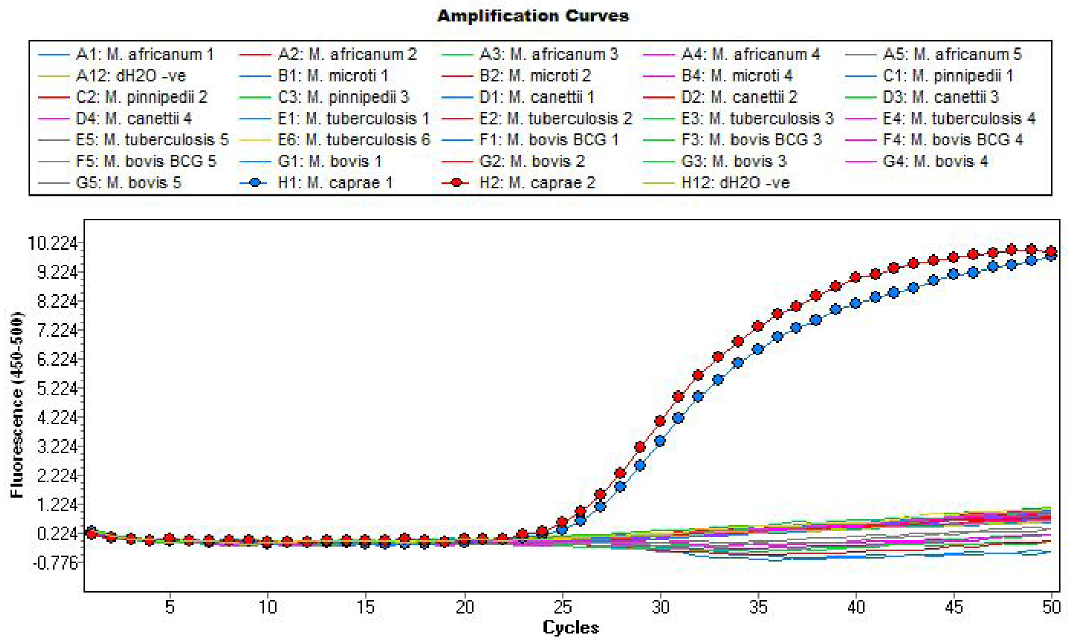


**Figure 4.1 B**

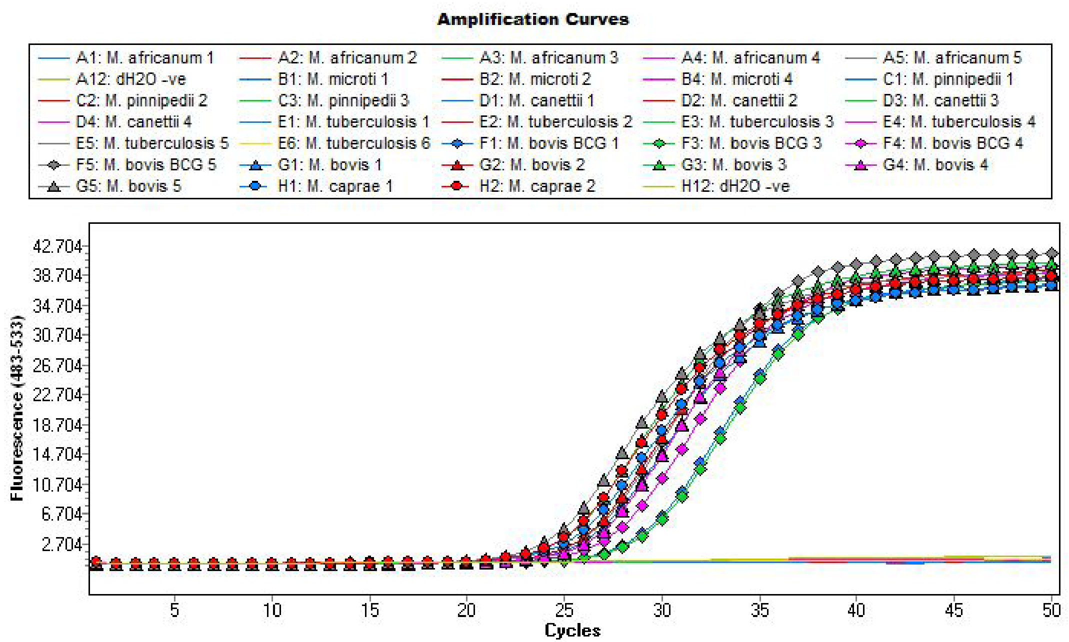

**Figure 4.1 C**

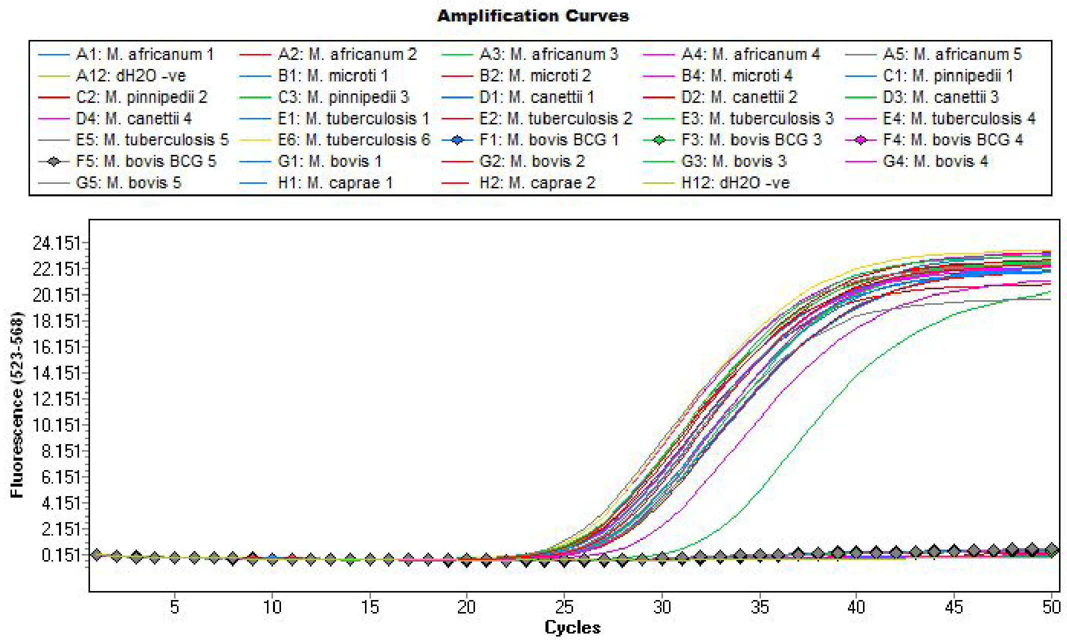


**Figure 4.1 D**

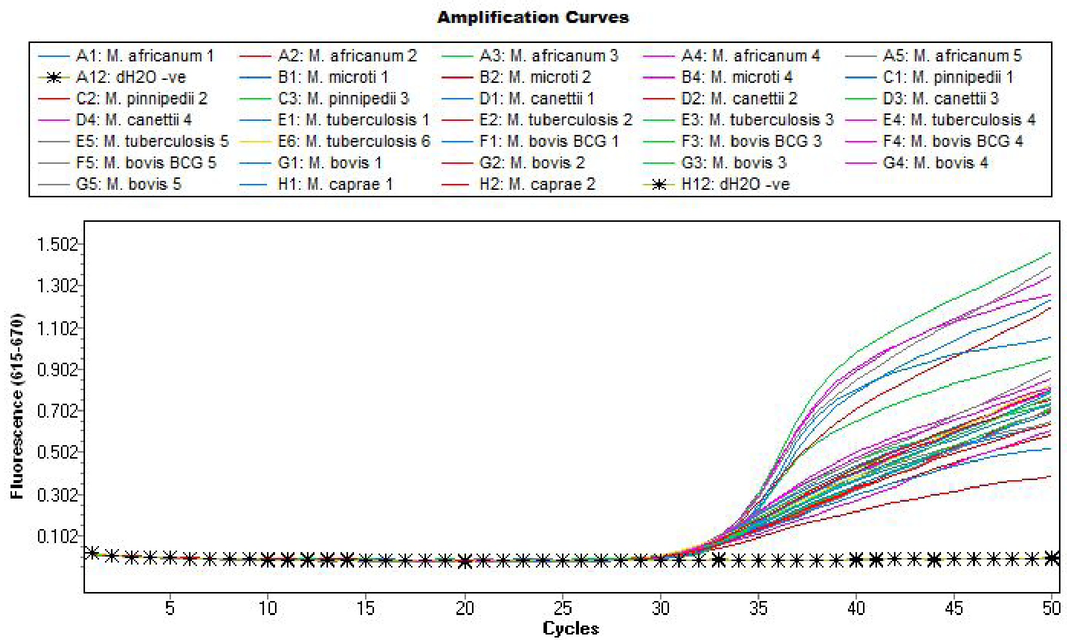

**Figure 4.1 A. Real-time amplification curves for *M. caprae* (circle) using *lepA* gene in Cyan500 channel (450-500). Figure 4.1 B Amplification curves for *M. caprae* (circle), *M. bovis* (triangles) and *M. bovis BCG* (diamonds) using the *lpqT* gene in FAM channel (483-533). Figure 4.1 C Amplification curves for all members of the MTC with the exception of *M. bovis* BCG (diamonds) and *M. microti* in the HEX channel (523-568). Figure 4.1 D Amplification curves for IAC in Cy5 channel (615-670) with the no template control highlighted with stars through amplification curve.**

**4.5 References**

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# Chapter 5:

# *Seek*TB- A two stage multiplex real-time PCR based method for the differentiation of the *Mycobacterium tuberculosis* complex

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## Abstract

Tuberculosis (TB) in humans is caused by members of the *Mycobacterium tuberculosis* complex (MTC). Accurate identification of the MTC member causing human infection is important because: treatment of TB caused by some MTC members requires alteration of the standard drug regimen; it can inform whether transmission is human to human or zoonotic; it enables accurate epidemiology studies that help improve TB control.

In this study, an internally controlled two stage multiplex real-time PCR based method, *Seek*TB, was developed for the accurate identification of all members of the MTC. The method was tested against a panel of well characterised bacterial strains (n=180) and determined to be 100% specific for members of the MTC. Additionally, 125 Mycobacteria Growth Indicator Tube (MGIT) positive cultures were blindly tested using *Seek*TB and results compared to the GenoType MTBC and TBc ID tests. *Seek*TB and GenoType MTBC results were 100% concordant identifying 84 of these isolates as *M. tuberculosis* and 41 as non MTC. Nine discordant results were observed between the molecular methods and the TBc ID culture confirmation test, however, nucleotide sequencing confirmed the results obtained with GenoType MTBC and *Seek*TB.

*Seek*TB is the first description of an internally controlled multiplex real-time PCR diagnostics method for the accurate identification of all eight members of the MTC. This method, designed for use on cultured patient samples, is specific, sensitive and rapid with a turnaround time to results of approximately 1.5 - 3.5 h, depending on which, if any, member of the MTC is present.

## 5.1 Introduction

Tuberculosis (TB) remains a major health concern both in developed and developing countries due to the high rates of morbidity and mortality associated with disease. In 2010 there were an estimated 8.5 - 9.2 million incident cases of TB globally, with an associated 1.5 million deaths, 23% of which were co-infected with HIV (28).

The *Mycobacterium tuberculosis* complex (MTC) comprises eight members (*M. tuberculosis*, *M. canettii*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*, *M. pinnipedii* and the attenuated *M. bovis* BCG vaccine strain), ~99% similar on a nucleotide sequence level, that are responsible for causing TB in humans (5).

While *M. tuberculosis* is responsible for the majority of cases of human TB, accurate identification of other MTC members causing infection is not routinely performed (21). As a result the global frequency and distribution of each MTC member remains largely unknown, with some studies suggesting that TB caused by members of the MTC other than *M. tuberculosis* are more prevalent than previously reported (1, 26). *M. africanum*, for example, has been identified as the causative agent of up to 50% of human TB infections in west African countries (15) and *M. canettii*, was responsible for ~10% of TB cases in a recent study performed in the Republic of Djibouti (17). Furthermore, *M. bovis* remains an important cause of zoonotic TB worldwide and should not be overlooked in a clinical setting (1, 12). In developing countries, where there is a paucity of data, one study suggests the prevalence of bovine TB in humans may be as high as 15% (8).

Some MTC members (*M. bovis*, *M. bovis* BCG and *M. canettii*) are intrinsically resistant to pyrazinamide (PZA), an important first line anti-TB drug (26). Therefore, specific identification of these members of the MTC is clinically important for treatment management decisions. This highlights the need for a rapid diagnostics assay capable of differentiating the members of the MTC, while simultaneously providing information relating to contact and source tracing.

Currently there is only one commercially available diagnostics assay for differentiation of the MTC, namely the Genotype MTBC kit (Hain Lifesciences GmbH, Nehren, Germany). While useful for identifying some members of the MTC,

this kit is limited by its inability to accurately identify *M. tuberculosis*, *M. canettii*, *M. africanum* and *M. pinnipedii* (16). There are also a number of molecular based assays for MTC differentiation described in the literature (9, 11, 13, 20, 21). These methods are also limited by an inability to differentiate all members of the MTC and most require post-amplification processing which increases method complexity, analysis time and potential contamination.

Previously we have reported the design and development of two 4-plex real-time PCR diagnostics assays for the accurate identification of the MTC, *M. tuberculosis*, *M. canettii* and of *M. bovis*, *M. bovis* BCG and *M. caprae* (22, 23). In this study, additional diagnostics targets were incorporated into these 4-plex assays resulting in two 5-plex real-time PCR diagnostics assays. By incorporation of these targets, we now have devised a diagnostics method and algorithm which allows for the accurate identification of the MTC and differentiation of all 8 members, including the capability to differentiate between *M. africanum* West African 1 and *M. africanum* West African 2. This two stage method, *Seek*TB, can be performed in approximately 3.5 hours post DNA extraction.

## 5.2 Materials and Methods

### *5.2.1 Ethics statement*

This study was approved by the research ethics review committee of the University of Zambia School of Medicine, Ridgeway Campus, Nationalist Road, Lusaka, Zambia. All study participants gave written informed consent in accordance with internationally recognized clinical trial standards.

### *5.2.2 Bacterial isolates used in this study*

The panel of MTC isolates, non-Tuberculosis Mycobacteria (NTM) and other bacterial species used in this study (Tables 5.1 and 5.2) was the same as described in a previous study (23) with the addition of 47 MTC isolates provided by the National Reference centre for Mycobacteria, Borstel, Germany (characterised as per Allix-

Béguec *et al.* (2)). All genomic DNA samples used in this study were isolated, quantified and stored as described previously (22).

*5.2.3 PCR primers and hydrolysis probe design*

The novel diagnostics targets used in this study were identified using *in silico* comparative genomic approaches previously described (22, 23). All oligonucleotides used in this study were designed in accordance with previously published guidelines (10, 24). All primers and probes (Table 5.3) were designed with similar properties to facilitate multiplexing (23).

*5.2.4 Conventional PCR and sequencing*

Conventional PCR amplification of the RD713 region (2798bp *M. africanum* West African 1) and RD701 (340bp *M. africanum* West African 2) was performed according to Huard *et al.* (14). Genomic DNA was also amplified for sequencing to confirm the identity of some clinical isolates. The sequencing primers (Table 5.3) were used in conventional PCR reactions as previously described (22, 23). PCR products were purified and sequenced according to Reddington *et al.* (23).

*5.2.5 Real-time PCR*

Multiplex real-time PCR reactions were performed on the LightCycler 480 using the LightCycler® 480 Probes Master kit (Roche Diagnostics). The optimised PCR mix for multiplex 1 contained 2 × LightCycler 480 Probes Master (6.4 mM $MgCl_2$), forward and reverse primer (0.5 µM final conc.), Cyan500, ROX, and CY5 labelled probes (0.2 µM final conc.) in addition to HEX (0.1 µM final conc.) and FAM (0.4 µM final conc.) labelled probes, dimethyl sulfoxide (4%, Sigma-Aldrich Missouri, USA), template DNA (MTC: 2 µl; IAC: 2 µl; NTM: 10 µl) adjusted to a final volume of 40 µl with the addition of nuclease free $dH_2O$. The *M. smegmatis* internal control DNA was diluted to contain 100 genome equivalents per 2 µl and NTM DNA was diluted to contain ~$10^4$ genome equivalents per 10 µl. For multiplex 2 the

optimised PCR mix was the same with the exception of the FAM labelled probe, where 0.2 µM final conc. was sufficient. PCR cycling was performed as previously described (22). To avoid fluorescence leaking from channel to channel a colour compensation file was generated (3) and the noise band was set to 1.2 fluorescence units (multiplex 1) and 1.3 fluorescence units (multiplex 2) in the Cyan 500, FAM, HEX and ROX channels.

*5.2.6 Evaluation of SeekTB performance in clinical cultures*

Genomic DNA from 125 Mycobacteria Growth Indicator Tube (MGIT) positive culture samples isolated at the University Teaching Hospital, Lusaka, Zambia were tested blindly. Positive MGIT cultures were identified as MTC or non MTC using the TBc ID (BD Diagnostics) test as per the manufacturer's instructions before DNA extraction. To ensure that the DNA extracted from MGIT cultures was amplifiable, a 16s universal conventional PCR was performed prior to analysis with the molecular methods. Isolated DNA samples were also tested using the GenoType MTBC kit (Hain Lifesciences) as per manufacturer's instructions.

## 5.3 Results

*5.3.1 Diagnostics targets*

During this study, it was determined that the *wbbl1* assay, outlined previously for the specific detection of *M. tuberculosis*/*M. canettii* (22), also detected *M. africanum* West African 1. Therefore, an additional assay for the specific detection of *M. africanum* West African 1 was necessary. Region of difference (RD) 713 has been previously identified as specific to *M. africanum* West African 1 (7, 19, 27). RD's represent regions of the genome present in *M. tuberculosis* which are deleted in other members of the MTC (4). In members of the MTC with an intact RD 713, this region is 4248 bp, whereas in *M. africanum* West African 1, this region is 2798 bp (14, 27). An assay targeting a 138 bp region of RD713 (primers RD_713Fw and RD_713Rv and RD713 probe (Table 5.3)), was designed for the specific detection *M. africanum*

West African 1 and incorporated into the first multiplex assay. The MTC specific probe was also redesigned to improve specificity (Table 5.3).

The second multiplex real-time PCR diagnostics assay was modified from a previously described multiplex (23) by incorporating an assay for the specific detection of *M. africanum* West African 2. PCR primers RD701_Fw and RD_701 and RD701 probe were designed to amplify a 81 bp region of RD701, an RD specific to *M. africanum* West African 2 (7, 19, 27) (Table 5.3). In members of the MTC with an intact RD 701, this region is 2885 bp, whereas in *M. africanum* West African 2, this region is 340 bp (27).

Incorporation of an additional diagnostics assay to each of the previously described multiplex assays, resulted in two 5-plex assays capable of differentiating all 8 members of the MTC and the 2 *M. africanum* clades (Fig. 5.1 and 5.2).

*5.3.2 Specificity of the diagnostic assays*

The specificities of all monoplex assays were evaluated against a panel of 180 MTC, NTM and other bacteria (Tables 5.1 and 5.2). Subsequently, the specificity of the assays was re-evaluated in multiplex format. All assays were 100% specific for the target organisms in both monoplex and multiplex format.

Using multiplex 1, the RD 713 assay was specific for the 5 *M. africanum* West African 1 isolates tested. The *wbbl1* assay was specific for the 60 *M. tuberculosis*, 8 *M. canettii* and 5 *M. africanum* West African 1 isolates tested. The MTC assay was specific for the 119 MTC isolates tested. The $RD^{canettii1}$ assay was specific for the detection of the 8 *M. canettii* isolates tested. The IAC (targeting MSMEG_0660) was specific for the detection of *M. smegmatis* DNA.

Using multiplex 2, the *lepA* assay was specific for the detection of the 5 *M. caprae* isolates tested. The RD701 assay was specific for the 5 *M. africanum* West African 2 isolates tested. The *lpqT* assay was specific for the 14 *M. bovis*, 7 *M. bovis BCG* and 5 *M. caprae* isolates tested. The diagnostics assay targeting RD1 did not detect any of the 7 *M. bovis* BCG strains tested but detected all *M. bovis* and *M. caprae* strains

tested, allowing for the specific identification of *M. bovis* BCG. The IAC assay was 100% specific.

A typical representation of the amplification curves generated in each of the analysis channels in the two multiplex assays is shown in supplementary data (Fig. 5.1 and 5.2).

### *5.3.3 Sensitivity of the assays*

The lower limit of detection (LOD) of the assays was determined using probit regression analysis (22, 23). LOD's of 9.04, 5.88, 0.4 and 5.08 genome equivalents for the MTC (*lepA*), *M. tuberculosis*/*M.canettii*/*M. africanum* West African 1 (*wbbl1*), *M. canettii* specific ($RD^{canettii1}$) and the *M. africanum* West African 1 specific (RD713) assays respectively were determined.

For multiplex 2, LOD's of 5.66, 6.05, 98.28 and 24.9 genome equivalents for the *M. bovis*/*M. bovis* BCG/*M. caprae* (*lpqT*), *M. bovis*/*M. caprae* (RD1), *M. caprae* (*lepA*) and *M. africanum* West African 2 (RD701) assays respectively were determined. In both multiplex assays the IAC at a concentration of 100 cell equivalents was detected in all samples tested.

### *5.3.4 Results interpretation*

Interpretation of the multiplex assay results was performed as per Table 5.4. When testing isolates, multiplex 1 should always be performed first. If the sample is positive for the MTC but *M. tuberculosis*, *M. canettii* or *M. africanum* West African 1 is not identified; multiplex 2 should subsequently be performed. Multiplex 2 directly identifies *M. bovis*, *M. bovis* BCG, *M. caprae* and *M. africanum* West African 2. To identify the remaining members of the MTC, namely *M. microti* and *M. pinnipedii*, the combined results from both multiplex assays must be taken into account. Assay results can be easily interpreted according to Table 5.4.

*5.3.5 Evaluation of SeekTB performance in clinical cultures*

MGIT positive isolates were blindly tested using two molecular methods, the GenoType MTBC (Hain Lifesciences) line probe assay and *Seek*TB. Both molecular methods demonstrated 100% agreement, identifying 84 isolates as *M. tuberculosis* and 41 isolates as non MTC. Results of TBc ID [reported specificity and sensitivity of 92.4-100% and 94.9-100% respectively (18, 25, 29)] and the molecular methods were concordant for 79 MTC isolates and 37 non MTC isolates. The *ssrA* gene of the 9 discordant samples was sequenced to consolidate results. Nucleotide sequence analysis revealed the molecular tests were correct for 8 out of the 9 discordant isolates. The remaining isolate returned a mixed sequence possibly indicating the presence of a mixed culture containing both MTC and non-MTC species.

## 5.4 Discussion

Accurate differentiation of members of the MTC is necessary to (a) perform epidemiological studies (b) monitor whether TB transmission is human to human or zoonotic (9, 21) and (c) administer appropriate anti-TB treatment, due to some members of the complex displaying natural resistance to PZA (1, 26).

The method described in this study, *Seek*TB, is composed of two 5-plex real-time PCR assays which can be performed sequentially. The first multiplex diagnostics assay has the capability of detecting the presence of the MTC while simultaneously differentiating *M. tuberculosis*, *M. canettii* and *M. africanum* West African 1, taking approximately 1.5 h to complete after DNA extraction. In the majority of TB cases, only the first multiplex assay would have to be performed, as *M. tuberculosis* is thought to be the causative agent of approximately 95% of human infections (6). The second multiplex diagnostics assay would be performed if a member of the MTC was detected in the sample but *M. tuberculosis*, *M. canettii* or *M. africanum* West African 1 were not identified. The second multiplex enables the specific identification of the remaining members of the complex, namely, *M. bovis*, *M. bovis* BCG, *M. caprae*, *M. africanum* West African 2, *M. microti* and *M. pinnipedii*. To sequentially perform both tests takes approximately 3.5 h after DNA extraction.

The specificity of the diagnostics targets used in this study was initially tested using a panel of well defined MTC isolates. These isolates had been previously characterised using methods such as spoligotyping, mycobacterial interspersed repetitive units - variable number tandem repeats (MIRU-VNTR), IS*6110*-based typing methods, RD analysis, biochemical testing in addition to morphological examination. The developed method demonstrated 100% agreement with the previously used methods, correctly identifying all members of the MTC, demonstrating the robustness of the rapid tests developed.

Further evaluation of the *Seek*TB method was performed by blindly testing a panel of uncharacterised positive MGIT cultures and comparing results with those generated using the GenoType MTBC test. The results of both molecular methods demonstrated 100% agreement, however some discordance was observed when these molecular methods were compared to culture results. The concordant molecular method results were indicative of true results in this study as confirmed by nucleotide sequencing.

While characterisation of the MGIT positive culture samples resulted in the identification of *M. tuberculosis* only, it should be noted that this is a relatively small number of isolates from one urban geographical region. MGIT positive culture sample bio-banks with a high likelihood of containing a variety of members of the MTC are required to further validate *Seek*TB (and the novel diagnostics targets used). Additional testing of a larger panel of MGIT positive culture samples from this region and other regions worldwide will be performed in future.

When comparing the two molecular methods tested, the *Seek*TB method offers a number of advantages over the GenoType line probe assay; the developed method is less laborious, requiring less hands on time; there is no need for post-amplification handling of samples which significantly reduces the likelihood of introducing contamination; there is an enhanced sensitivity using the real-time PCR based *Seek*TB method; and *Seek*TB has the ability to accurately identify *M. tuberculosis*, *M. canettii*, *M. pinnipedii* and both clades of *M. africanum*, in addition to the members of the MTC identifiable using the GenoType MTBC test.

In conclusion, the diagnostics method developed in this study, *Seek*TB, is the first description of internally controlled, multiplex PCR based method capable of rapidly and accurately identifying all members of the MTC. This method has been validated on a large panel of well characterised MTC isolates and has been successfully used for culture confirmation of African positive MGIT cultures. Ultimately, *Seek*TB needs to be further tested in the field, both on positive culture samples and directly on clinical samples, such as sputum, to demonstrate its potential and robustness for specific identification and differentiation of the MTC.

Table 5.1: *Mycobacterium tuberculosis complex* isolates used in this study

| Species | Strain | Country of Isolation | Origin [a] | Remark |
|---|---|---|---|---|
| *M. tuberculosis* | 22 | Mongolia | RIVM | Beijing lineage |
| *M. tuberculosis* | 53 | Argentina | RIVM | Haarlem lineage |
| *M. tuberculosis* | 112 | The Netherlands | RIVM | CAS lineage |
| *M. tuberculosis* | 67 | Comoro Islands | RIVM | EAI lineage |
| *M. tuberculosis* | 41 | Chile | RIVM | LAM lineage |
| *M. tuberculosis* | 103 | China | RIVM | T-family lineage |
| *M. tuberculosis* | 12594_02 | Former Soviet Union | Borstel | Beijing lineage |
| *M. tuberculosis* | 1500_03 | Former Soviet Union | Borstel | Beijing lineage |
| *M. tuberculosis* | 1934_03 | Former Soviet Union | Borstel | Beijing lineage |
| *M. tuberculosis* | 1428_02 | Ghana | Borstel | Cameroon lineage |
| *M. tuberculosis* | 5390_02 | Ghana | Borstel | Cameroon lineage |
| *M. tuberculosis* | 5400_02 | Ghana | Borstel | Cameroon lineage |
| *M. tuberculosis* | 2637_02 | Germany | Borstel | Delhi/CAS lineage |
| *M. tuberculosis* | 7936_01 | Germany | Borstel | Delhi/CAS lineage |
| *M. tuberculosis* | 1797_03 | Germany | Borstel | EAI lineage |
| *M. tuberculosis* | 4850_03 | Germany | Borstel | EAI lineage |
| *M. tuberculosis* | 947_01 | Germany | Borstel | EAI lineage |
| *M. tuberculosis* | 2336_02 | Germany | Borstel | Haarlem lineage |
| *M. tuberculosis* | 9532_03 | Germany | Borstel | Haarlem lineage |
| *M. tuberculosis* | 7968_03 | Germany | Borstel | LAM lineage |
| *M. tuberculosis* | 8885_03 | Germany | Borstel | LAM lineage |
| *M. tuberculosis* | 946_03 | Germany | Borstel | LAM lineage |
| *M. tuberculosis* | 2151_03 | Germany | Borstel | S-type lineage |
| *M. tuberculosis* | 2318_06 | Germany | Borstel | S-type lineage |
| *M. tuberculosis* | 10469_01 | NA[c] | Borstel | Ghana lineage |
| *M. tuberculosis* | 10493_01 | NA[c] | Borstel | Ghana lineage |
| *M. tuberculosis* | 2570_02 | NA[c] | Borstel | Ghana lineage |
| *M. tuberculosis* | 2201_99 | Uganda | Borstel | Uganda I lineage |
| *M. tuberculosis* | 2333_99 | Uganda | Borstel | Uganda I lineage |
| *M. tuberculosis* | 2176_99 | Uganda | Borstel | Uganda II lineage |
| *M. tuberculosis* | 2191_99 | Uganda | Borstel | Uganda II lineage |
| *M. tuberculosis* | 4412_04 | Germany | Borstel | X-type lineage |
| *M. tuberculosis* | 9953_04 | Germany | Borstel | X-type lineage |
| *M. tuberculosis* | 11313_03 | Germany | Borstel | Tur lineage |
| *M. tuberculosis* | 1657_03 | Germany | Borstel | Ural lineage |
| *M. tuberculosis* | 10264_03 | Germany | Borstel | Tur lineage |
| *M. tuberculosis* | 10529_03 | Germany | Borstel | Tur lineage |
| *M. tuberculosis* | 8431_03 | Germany | Borstel | Ural lineage |
| *M. tuberculosis* | 3493_07 | | Borstel | Hamburg lineage |
| *M. tuberculosis* | 10707_07 | | Borstel | Hamburg lineage |
| *M. tuberculosis* H37Rv | 9679_00 | NA[c] | Borstel | Laboratory strain ATCC |
| *M. tuberculosis* | - | NA[c] | Mario | Clinical isolates |

| | | | | |
|---|---|---|---|---|
| (*19 clinical isolates*) | | | Vaneechoutte | |
| *M. canettii* | 116 | Somalia | RIVM | Smooth growing strain described by van Soolingen *et al.* 1997 |
| *M. canettii* | 1997-1549 | Switzerland | RIVM | Swiss isolate described in Pfyffer *et al.* 1998 |
| *M. canettii* | NLA000701671 | Somalia | RIVM | Characterised on the basis of their spoligotype, IS6110 RFLP type and smooth growth as |
| *M. canettii* | NLA000200937 | Eritrea | RIVM | Characterised on the basis of their spoligotype, IS6110 RFLP type and smooth growth |
| *M. canettii* | 1996-46 | France | RIVM | Canetti strain |
| *M. canettii* | 3040_99 | The Netherlands | Borstel | |
| *M. canettii* | 3151_08 | NA[c] | Borstel | |
| *M. canettii* | 3041_99 | The Netherlands | Borstel | |
| *M. bovis* | 117 | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 126 | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 73 | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 130 | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* | 24 | Saudi Arabia | RIVM | Isolated from an oryx, Antelope clade, see also Smith *et al.* 2006 |
| *M. bovis* | 4258_00 | Germany | Borstel | |
| *M. bovis* | 751_01 | Germany | Borstel | |
| *M. bovis* | 7540_01 | Germany | Borstel | |
| M. bovis (6 isolates) | - | NA[c] | Mario Vaneechoutte | Clinical isolates |
| *M. bovis* BCG | 48 (2) | The Netherlands | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 71 | Japan | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 83 | Russia | RIVM | See Kremer *et al.* 2005 |
| *M. bovis* BCG | 2008-714 [b] | NA[c] | RIVM | Identified on basis of characteristic IS6110/IS1081 RFLP patterns according to van Soolingen *et al.* 1992 |
| *M. bovis* BCG | 2008-1601 [b] | NA[c] | RIVM | Identified on basis of characteristic IS6110/IS1081 RFLP patterns according to van Soolingen *et al.* 1992 |
| *M. bovis* BCG | DSM 43990 | NA[c] | DSMZ | *Mycobacterium bovis* Karlson and Lessel 1970<br>BCG, Chicago 1 |
| *M. bovis* BCG | DSM 45071 | NA[c] | DSMZ | *Mycobacterium bovis* Karlson and Lessel 1970 |
| *M. caprae* | 2006-1960 [b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |
| *M. caprae* | 2007-0039 [b] | The Netherlands | RIVM | Characterised using Hain genotype MTBC kit |
| *M. caprae* | 1694_00 | Germany | Borstel | |
| *M. caprae* | 8986_99 | Germany | Borstel | |
| *M. caprae* | 9577_99 | Germany | Borstel | |
| *M. microti* | 62 | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |

| | | | | |
|---|---|---|---|---|
| *M. microti* | 25 | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 15274 [b] | United Kingdom | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 15912 [b] | Belgium | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 15911 [b] | Netherlands | RIVM | see van Soolingen *et al.* 1998 |
| *M. microti* | 417/01 | Germany | | Llama lineage |
| *M. pinnipedii* | 76 | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. pinnipedii* | 81 | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. pinnipedii* | 101 | Argentina | RIVM | See Kremer *et al.* 2005 |
| *M. pinnipedii* | 7011_02 | Germany | Borstel | |
| *M. pinnipedii* | 7739_01 | Germany | Borstel | |
| *M. africanum* | 6 | The Netherlands | RIVM | *M. africanum* clade 2 |
| *M. africanum* | 128 (85) | The Netherlands | RIVM | *M. africanum* clade 2 |
| *M. africanum* | 2007-1386 [b] | The Netherlands | RIVM | *M. africanum* clade 2 |
| *M. africanum* | 2007-1154 [b] | The Netherlands | RIVM | *M. africanum* clade 2 |
| *M. africanum* | 2007-1073 [b] | The Netherlands | RIVM | *M. africanum* clade 2 |
| *M. africanum* | 1449_02 | Ghana | Borstel | *M. africanum* clade 1 |
| *M. africanum* | 1473_02 | Ghana | Borstel | *M. africanum* clade 1 |
| *M. africanum* | 10473_01 | Ghana | Borstel | *M. africanum* clade 1 |
| *M. africanum* | 10494_01 | Ghana | Borstel | *M. africanum* clade 1 |
| *M. africanum* | 1443_02 | Ghana | Borstel | *M. africanum* clade 1 |
| *M. africanum* | 10476_01 | Ghana | Borstel | *M. africanum* clade 2 |
| *M. africanum* | 10514_01 | Ghana | Borstel | *M. africanum* clade 2 |
| *M. africanum* | 5468_02 | Ghana | Borstel | *M. africanum* clade 2 |
| *M. africanum* | 9550_99 | Ghana | Borstel | *M. africanum* clade 2 ATCC |

[a] RIVM = National Tuberculosis Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands; *Borstel = National Reference Center for Mycobacteria, Forschungszentrum Borstel, Germany; *DSM = The German Collection of Microorganisms
[b] Represent RIVM strains not previously described in literature, however have been characterised to the species level using techniques outlined in references supplied in the manuscript.
[c] This information was not available (NA) for this study.

Table 5.2: Non *tuberculosis* mycobacteria and other strains of bacteria used in this study

| **Non *tuberculosis* mycobacteria** | **Strain designation** [a] | **Remark** |
|---|---|---|
| *Mycobacterium aichiense* | DSM 44147 | Type strain, isolated from soil |
| *Mycobacterium alvei* | DSM 44176 | Type strain, isolated from water sample |
| *Mycobacterium arupense* | DSM 44942 | Type strain, isolated from a tendon |
| *Mycobacterium asiaticum* | ITG 8182 | See De Baere *et al.* 2002 |
| *Mycobacterium avium* | ITG 7886 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium boenickei* | DSM 44677 | Type strain, isolated from a leg wound |
| *Mycobacterium branderi* | DSM 44624 | Type strain, isolated from human sputum |
| *Mycobacterium brisbanense* | DSM 44680 | Type strain, isolated from a sinus |
| *Mycobacterium brumae* | DSM 44177 | Type strain, isolated from water sample |
| *Mycobacterium canariasense* | DSM 44828 | Type strain, isolated from human blood |
| *Mycobacterium celatum* | ITG 6147 | See De Baere *et al.* 2002 |
| *Mycobacterium chelonae* | ITG 4975 | NA [b] |
| *Mycobacterium chelonae subsp. abscessus* | DSM 44196 | Type strain |
| *Mycobacterium confluentis* | DSM 44017 | Type strain, isolated from human sputum |
| *Mycobacterium conspicuum* | DSM 44136 | Type strain, isolated from patient with disseminated infection |
| *Mycobacterium flavescens* | VUB A016 | See De Baere *et al.* 2002 |
| *Mycobacterium fortuitum* | ITG 8020 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium genavense* | ITG 97-102 | See De Baere *et al.* 2002 |
| *Mycobacterium gilvum* | DSM 9487 | Isolated from soil |
| *Mycobacterium goodii* | DSM 44492 | Type strain |
| *Mycobacterium gordonae* | ITG 7704 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium heckeshornense* | DSM 44428 | Type strain, isolated from human respiratory tract |
| *Mycobacterium houstonense* | DSM 44676 | Type strain, isolated from a facial abscess |
| *Mycobacterium intracellulare* | DSM 43223 | Type strain |
| *Mycobacterium kansasii* | ITG 7727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium kubiciae* | DSM 44627 | Type strain, isolated from human sputum |
| *Mycobacterium lacus* | DSM 44577 | Type strain, isolated from human elbow |
| *Mycobacterium mageritense* | DSM 44476 | Type strain, isolated from human sputum |
| *Mycobacterium malmoense* | ITG 940611 | See De Baere *et al.* 2002 |
| *Mycobacterium marinum* | ITG 1727 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium massiliense* | DSM 45103 | Type strain, isolated from human blood |
| *Mycobacterium moriokaense* | DSM 44221 | Type strain, isolated from soil |
| *Mycobacterium mucogenicum* | DSM 44625 | Type strain, isolated from human cyst |
| *Mycobacterium nebraskense* | DSM 44803 | Type strain, isolated from human sputum |

| | | |
|---|---|---|
| *Mycobacterium neworleansense* | DSM 44679 | Type strain, isolated from human scalp |
| *Mycobacterium paratuberculosis* | ITG 2666 | See De Baere *et al.* 2002 |
| *Mycobacterium scrofulaceum* | DSM 43992 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium shimoidei* | DSM 44152 | Type strain, isolated from sputum of patient with tuberculosis-like disease |
| *Mycobacterium simiae* | ITG 4485 | See Vaneechoutte *et al.* 1993 |
| *Mycobacterium smegmatis* | DSM 43756 | Type strain |
| *Mycobacterium szulgai* | ITG 4979 | NA [b] |
| *Mycobacterium tusciae* | DSM 44338 | Type strain, isolated from human cervical lymph node |
| *Mycobacterium ulcerans* | ITG 96-1439 | NA [b] |
| *Mycobacterium xenopi* | ITG 4986 | See De Baere *et al.* 2002 |
| **Other bacteria** | **Strain designation** | **Remark** |
| *Staphylococcus aureus* | DSM 20231 | Type strain, isolated from human pleural fluid |
| *Listeria monocytogenes* | DSM 20600 | Type strain, isolated from a rabbit |
| *Escherichia coli* | DSM 301 | Disinfectant test strain |
| *Klebsiella oxytoca* | ATCC 43086 | NA [b] |
| *Enterococcus faecalis* | DSM 20371 | Isolated from pleural fluid |
| *Proteus mirabilis* | DSM 4479 | Type strain |
| *Bacillus cereus* | DSM 31 | Type strain |
| *Bordetella pertussis* | CCUG 13475 | Isolated from patient suffering from whooping cough |
| *Streptococcus agalactiae* | DSM 2134 | Type strain |
| *Rhodococcus equi* | DSM 20307 | Type strain, isolated from lung abscess of foal |
| *Streptomyces albidoflavus* | DSM 40455 | Type strain |
| *Nocardioides sp.* | DSM 17401 | Proposed type strain, isolated from marine sediment |
| *Nocardia salmonicida* | DSM 40472 | Type strain, isolated from blueback salmon |
| *Nocardia asiatica* | clinical isolate | Isolated from human wound, see Wauters *et al.* 2005 |
| *Nocardia nova* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |
| *Nocardia cyriacigeorgica* | clinical isolate | Isolated from human bronchial aspirate, see Wauters *et al.* 2005 |
| *Nocardia farcinica* | clinical isolate | Isolated from human abscess, see Wauters *et al.* 2005 |

[a] DSM = The German Collection of Microorganisms; *ITG = Institute of Tropical Medicine, Antwerp; *VUB = Department of Microbiology, Academic Hospital of the Free University of Brussels, Brussels, Belgium Germany; *ATCC = American Type Culture Collection; *CCUG = Culture Collection, University of Göteborg, Sweden.
[b] This information was not available (NA) for this study.

Table 5.3: Oligonucleotide primers and probes used in this study

| **Name** | **Function** | **Sequence 5’→3’** |
|---|---|---|
| MTC_Fw | Forward Sequencing primer, forward MTC real-time PCR assay primer | AGACCGTGCGGATCTTG |
| MTC_Rv | Reverse Sequencing primer, Reverse MTC real-time PCR assay primer | CATGGAGATCACCCGTGA |
| MTC Probe | MTC probe | HEX-ATTGGTCACCCGGATTTCGGT-BHQ1 |
| *wbbl1*_Fw | Forward sequencing primer, Forward real-time PCR assay primer | TACCAGCTTCAGTTTCCGT |
| *wbbl1*_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | GCACCTATATCTTCTTAGCCG |
| *wbbl1* probe | *wbbl1* probe | FAM-ATGGTGCGCAGTTCACTGC |
| *M. canetti* sp Fw | Forward *M. canetti* specific primer | ATGTGGTTTCAGTACGACTTC |
| *M. canetti* sp Rv | Reverse *M. canetti* specific primer | GATGGCAGTGTCTTATCCAA |
| *M. canetti* sp probe | *M. canetti specific* probe | ROX-TGAGAGGTGTTGGCACGCAA-BHQ2 |
| *lpqT* Fw | Forward sequencing primer, Forward real-time PCR assay primer | ACGAATCCGGCGATGATC |
| *lpqT*_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | CGACTGCACACCTGGAA |
| *lpqT* probe | *lpqT* Probe | FAM-TTGGCCGGCGCCGGTT-BHQ1 |
| RD1_Fw | Forward sequencing primer, Forward real-time PCR assay primer | CATCGCTGATGTGCTTGC |
| RD1_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | TGCGCCGAGCTGTATTC |
| RD1_probe | RD1 Probe | ROX-ACACTAGCGTCAATGCGGTCA-BHQ2 |
| *M. caprae lepA*_Fw | Forward sequencing primer, Forward real-time PCR assay primer | AGACCGTGCGGATCTTG |
| *M. caprae lepA*_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | CATGGAGATCACCCGTGA |
| *M. caprae lepA* probe | *M. caprae lepA* Probe | Cyan 500-TATCGGGTACACAAAGACGA – BBQ |
| RD713_Fw S | RD713 Forward sequencing primer | CCATCTGCGCTTTCGGTGCTTCT |
| RD713_RvS | RD 713 Reverse sequencing primer | CTGCCAGTCGTCTTCCCCATTGTG |
| RD713_Fw | Forward real-time PCR assay primer | ACGGAACGGTCAAGAAC |
| RD713_Rv | Reverse real-time PCR assay primer | GCTCAAGAATCGTCGCTA |
| RD713 probe | RD 713 Probe | Cyan 500-ACGTCCTTGTGACCGCGAC- BBQ |
| RD701_FwS | RD 701 Forward sequencing primer | CTGTGCAGGTGGTCGTTTC |
| RD701_RvS | RD 701 Reverse sequencing primer | CGAATTGCTCATCCCGTAAC |
| RD701_Fw | Forward real-time PCR assay primer | AACGGGTCGGATTCTCC |
| RD701_Rv | Reverse real-time PCR assay primer | CCGAAACCCTCGTTGATC |
| RD701 probe | RD 701 Probe | ROX-TCAGCCGCCGGCCAACC-BHQ2 |
| IAC MSMEG_0660_Fw | Forward sequencing primer, Forward real-time PCR assay primer | TCACCGACCATGTCCAG |
| IAC MSMEG_0660_Rv | Reverse sequencing primer, Reverse real-time PCR assay primer | CGTTGCCCAATCCGTATG |
| IAC MSMEG_0660 probe | IAC MSMEG_0660 probe | Cy5-CAGCAGTACCATCGCCATCG-BHQ2 |
| *ssrA*_Fw | *ssrA* Forward sequencing primer | TGGGGCTGAAAGGTTTCGA |
| *ssrA*_Rv | *ssrA* Reverse sequencing primer | TGGAGCTGCCGGGAAT |

Table 5.4 – Result scenario for each member of the MTC

| **Multiplex 1** | **Analysis Channel (Target)** | | | | | **Result Interpretation** |
|---|---|---|---|---|---|---|
| | **Cyan 500 (RD713)** | **FAM (*wbbl1*)** | **HEX (MTC *lepA*)** | **ROX (RD$^{canettii1}$)** | **Cy5 (IAC MSMEG_0660)** | |
| | + | + | + | - | + | ***M. africanum* West African 1** |
| | - | + | + | + | + | ***M. canettii*** |
| | - | + | + | - | + | ***M. tuberculosis*** |
| | - | - | + | - | + | **MTC – perform second multiplex** |
| | - | - | - | - | + | **Not a member of the MTC** |
| | - | - | - | - | - | **Result invalid, test must be repeated** |
| **Multiplex 2** | **Analysis Channel (Target)** | | | | | **Result Interpretation (accounting for multiplex 1 result)** |
| | **Cyan 500 (*M. caprae lepA*)** | **FAM (*lpqT*)** | **HEX (RD1)** | **ROX (RD701)** | **Cy5 (IAC MSMEG_0660)** | |
| | + | + | + | - | + | ***M. caprae*** |
| | - | + | + | - | + | ***M. bovis*** |
| | - | + | - | - | + | ***M. bovis* BCG** |
| | - | - | + | + | + | ***M. africanum* West African2** |
| | - | - | + | - | + | ***M. pinnipedii*** |
| | - | - | - | - | + | ***M. microti*** |
| | - | - | - | - | - | **Result invalid, test must be repeated** |

+ Positive signal observed; **-** Positive signal not observed

**Figure 5.1 A**

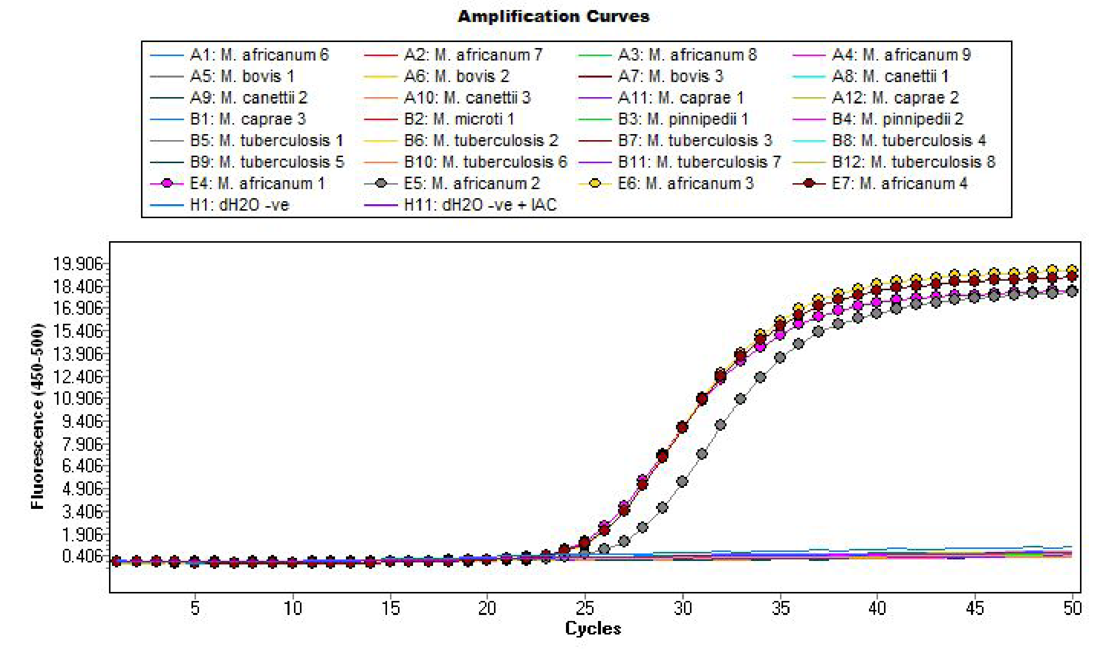


**Figure 5.1 B**

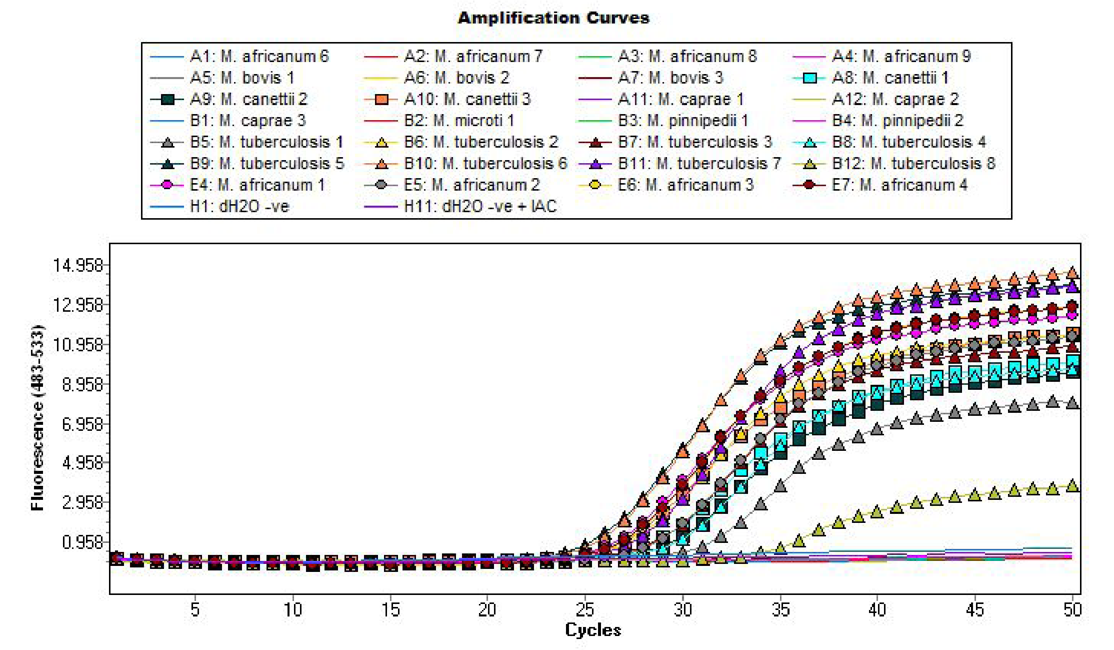

**Figure 5.1 C**

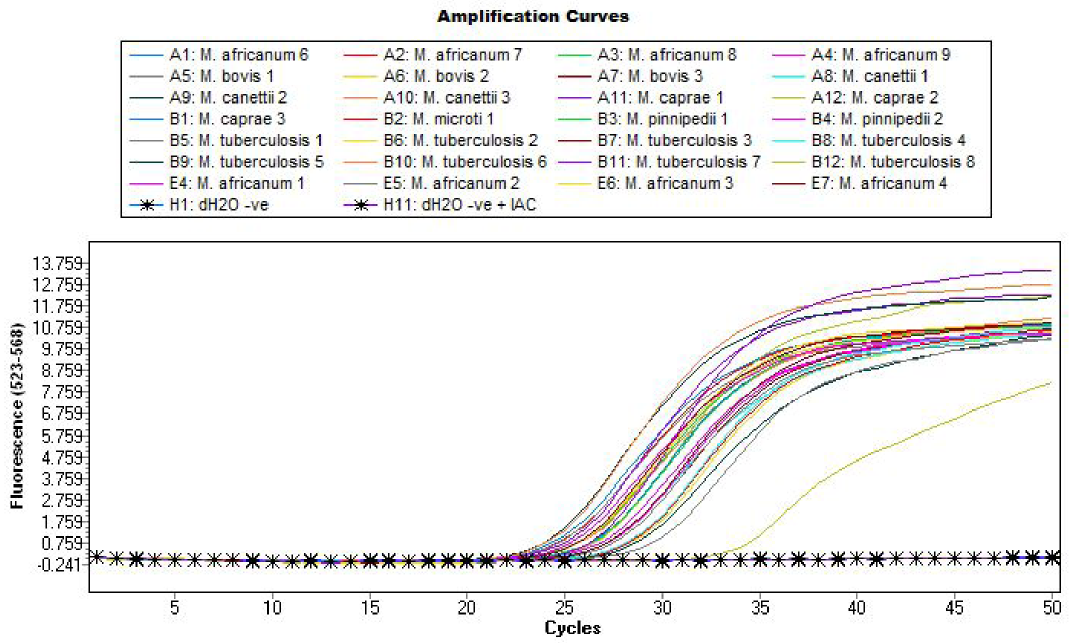


**Figure 5.1 D**

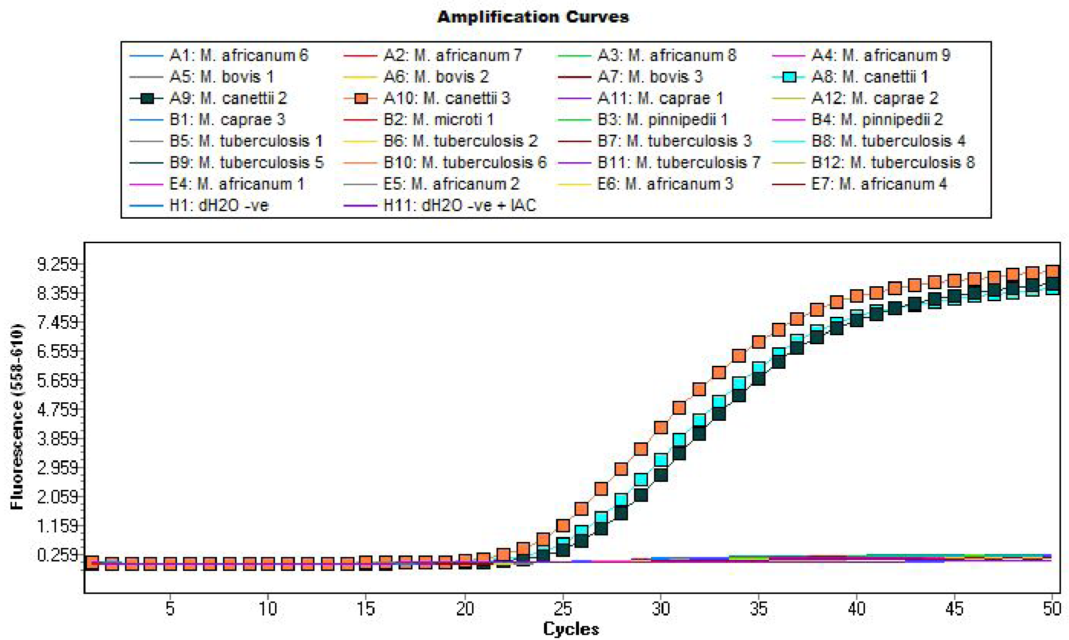

**Figure 5.1 E**

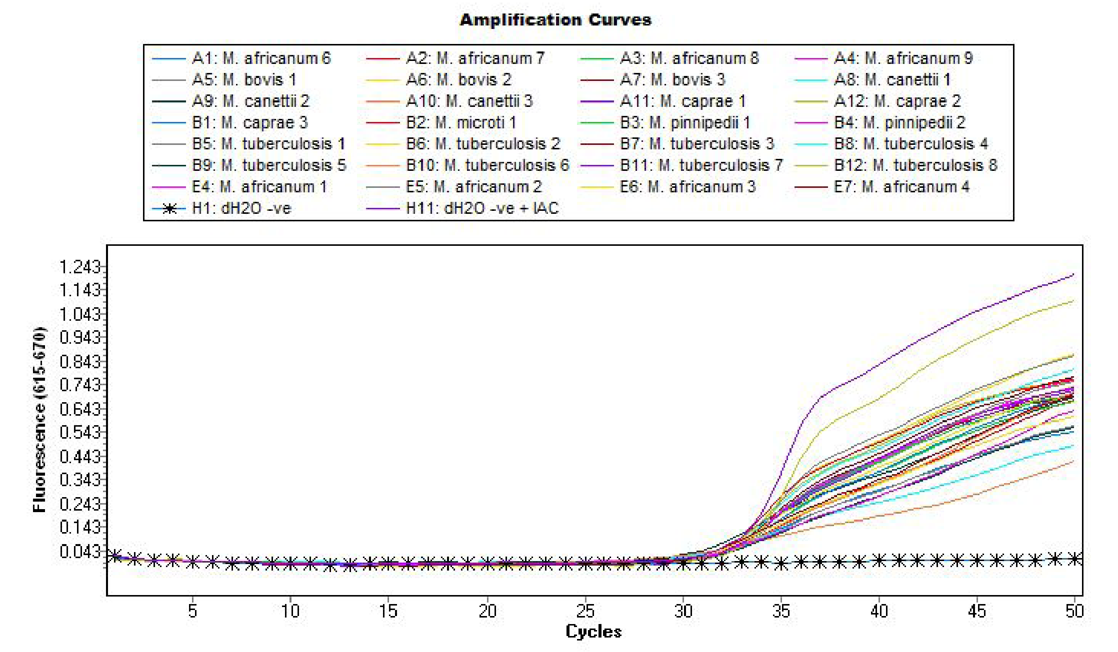


**Figure 5.1 Amplification curves for multiplex 1 real-time PCR assay**

**Figure 5.1 A Real-time amplification curves for *M. africanum* West African 1 (circle) using RD713 in Cyan500 channel (450-500). Figure 5.1 B Amplification curves for *M. africanum* West African 1 (circle), *M. tuberculosis* (triangles) and *M. canettii* (rectangles) using the *wbbl1* gene in FAM channel (483-533). Figure 5.1 C Amplification curves for all members of the MTC using the *lepA* gene, with the no template control highlighted (stars) in the HEX channel (523-568). Figure 5.1 D Amplification curves of *M. canettii* (rectangles) in the ROX channel (558-610) using $RD^{canettii1}$. Figure 5.1 E Amplification curves for IAC in Cy5 channel (615-670) with the no template control highlighted with stars through amplification curve.**

**Figure 5.2 A**

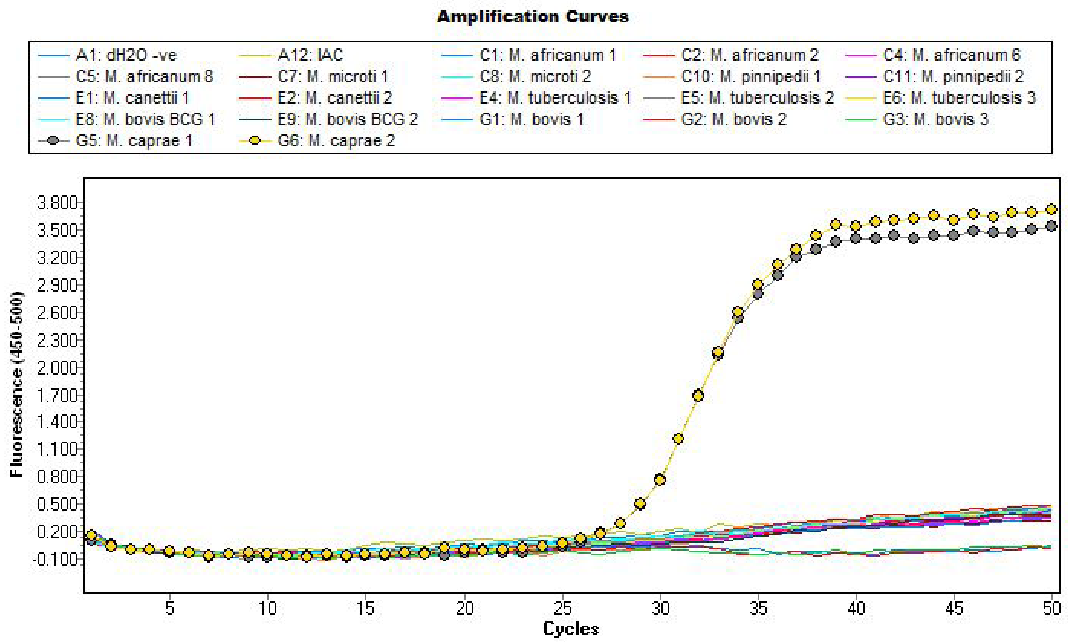


**Figure 5.2 B**

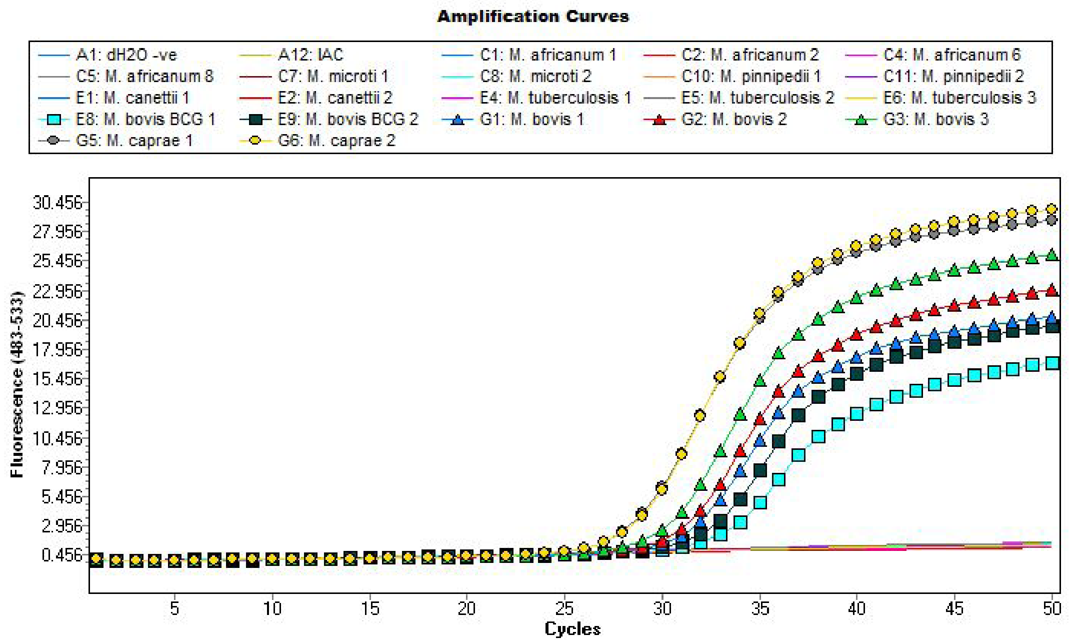

**Figure 5.2 C**

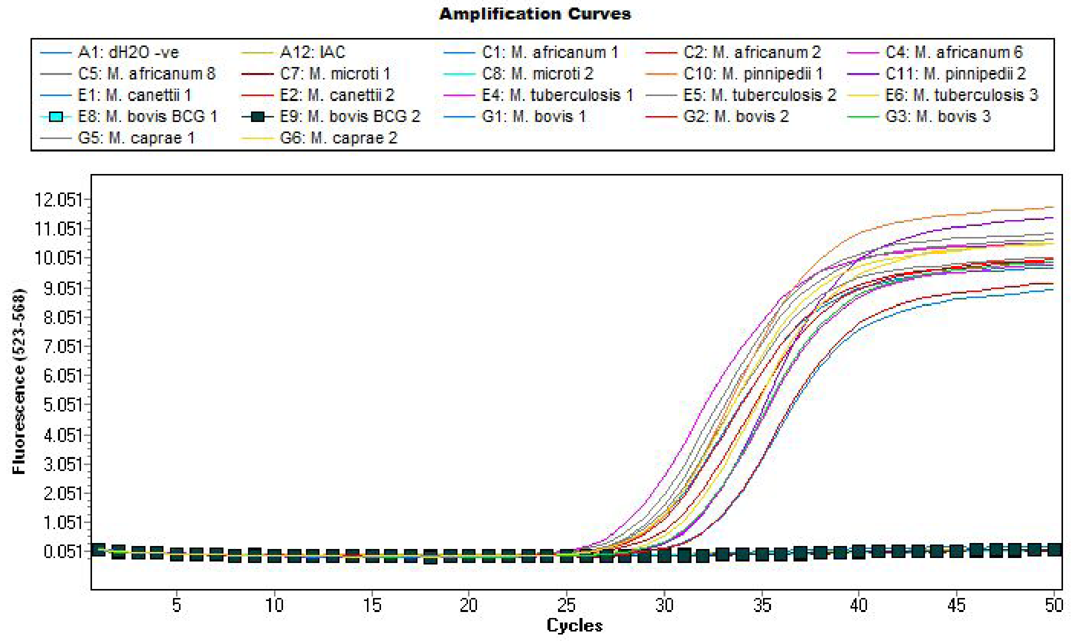


**Figure 5.2 D**

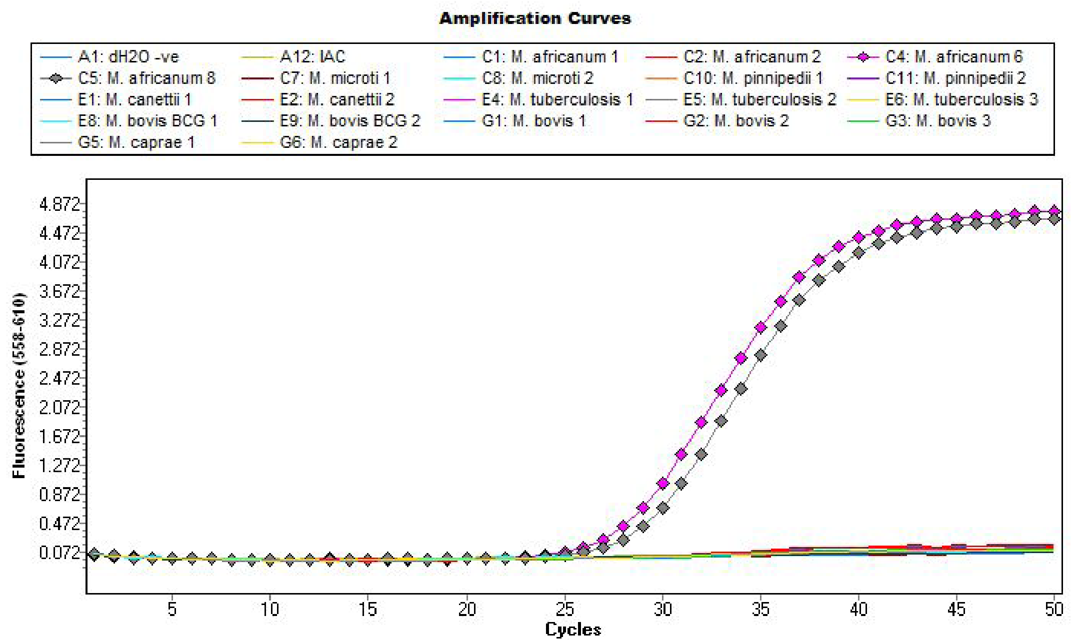

**Figure 5.2 E**

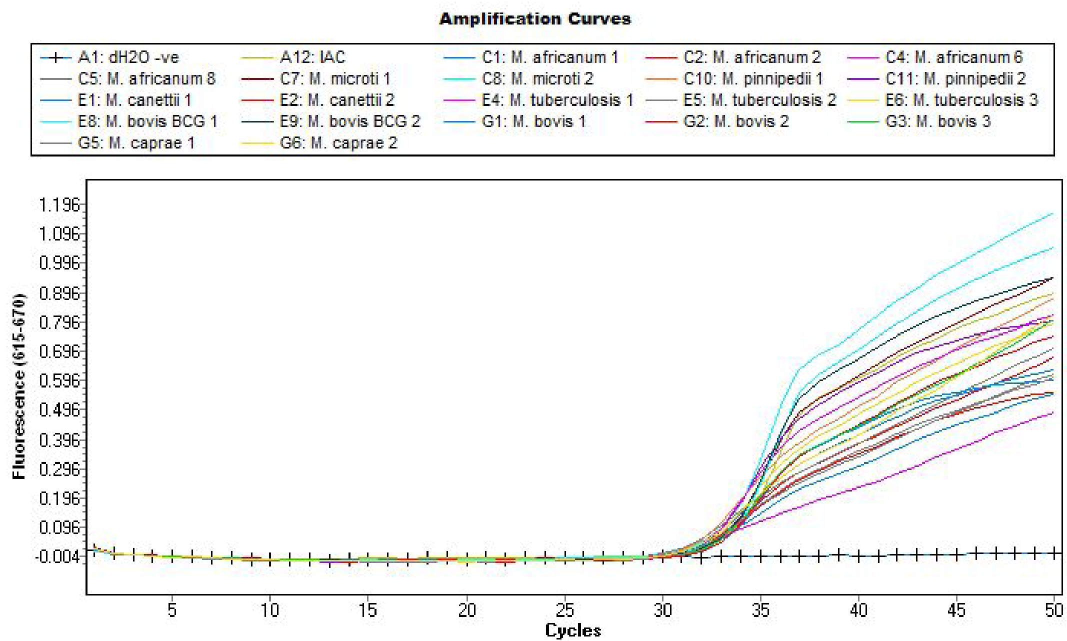


**Figure 5.2: Amplification curves for multiplex 2 real-time PCR assay**

**Figure 5.2A Real-time amplification curves for *M. caprae* (circle) using *lepA* gene in Cyan500 channel (450-500). Figure 5.2B Amplification curves for *M. caprae* (circle), *M. bovis* (triangles) and *M. bovis BCG* (rectangles) using the *lpqT* gene in FAM channel (483-533). Figure 5.2C Amplification curves for all members of the MTC with the exception of *M. bovis* BCG (diamonds) and *M. microti* in the HEX channel (523-568). Figure 5.2D Amplification curves of *M. africanum* West African 2 (diamonds) in the ROX channel (558-610) using RD701. Figure 5.2E Amplification curves for IAC in Cy5 channel (615-670) with the no template control highlighted with stars through amplification curve.**

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# Chapter 6:

# Concluding remarks and future directions

## 6.1 General Conclusions

The overall aim of this thesis was to design, develop, optimise and validate a robust, internally controlled, unidirectional multiplex real-time PCR based method for the rapid and accurate detection and identification of each member of the MTC. This was achieved using a sequential experimental design strategy consisting of three studies (**Chapters 3-5**), each detailing the first description of a hydrolysis probe based detection methodology for identifying specific members of the MTC.

**Chapter 2** describes the *in silico* methodologies utilised to identify putative MTC species specific nucleic acid diagnostics targets. Initially, using the Mycobacterial Genome Divergence Database and a web based version of the Artemis comparison tool, 41 putative diagnostics targets, specific for members of the MTC, were identified. A Go / No Go strategy was developed to validate these diagnostics targets further. Using this Go / No Go strategy the first step involved nucleotide sequencing of target genes to identify if the insertion, deletion or SNP's were present in a representative number of strains held in our collection. If the target still remained specific, based on the nucleotide sequencing results, a real-time PCR diagnostics assay was developed and tested on a full panel of MTC isolates. Using this strategy, 31 putative diagnostics targets were deemed unsuitable as species specific targets. The remaining 10 diagnostics targets were further optimised, validated and incorporated into multiplex real-time PCR assays as described in **Chapters 3-5.**

**Chapter 3** describes a multiplex real-time PCR diagnostics assay for the detection and accurate identification of the MTC with the simultaneous detection, identification and differentiation of the human adapted *M. tuberculosis* and *M. canettii*.

Firstly, an assay targeting a region of the *lepA* gene (Rv2404c) was developed for the detection and identification of the MTC. The *lepA* gene was chosen as sufficient nucleotide heterogeneity existed between members of the MTC and closely related NTM thus allowing for highly specific MTC probe design.

To allow for the simultaneous detection and identification of *M. tuberculosis* and *M. canettii* an assay targeting a region of the *wbbl1* gene (Rv3265c), in which there is a 12bp deletion in all other members of the MTC, was developed. Identification of a region of the genome present in *M. tuberculosis* and *M. canettii* which has been deleted in other members of the MTC has similar properties to the widely used RD9 target for the detection of *M. tuberculosis*. This diagnostics target also supports the proposed evolutionary scenario for the MTC (4).

To specifically differentiate between *M. canettii* and *M. tuberculosis* a region of the *M. canettii* genome which is not present in *M. tuberculosis* (proposed name $RD^{canettii1}$) was identified and targeted in this study. We hypothesise that this may represent a novel RD, specific for *M. canettii*. The discovery of a region of DNA present in *M. canettii* which is deleted in all other members of the MTC also supports the proposed evolutionary scenario for the MTC (4).

A competitive IAC was designed and incorporated targeting the *lepA* gene in *M. smegmatis* (MSMEG_4556). As the same primer combination could be used for the MTC and IAC assays, the complexity of the multiplex was reduced.

All diagnostics targets utilised in this study were novel, specific for their intended purpose and LODs of 2.17, 2.20, and 0.73 genome equivalents for the *M.canettii*/*M. tuberculosis*, MTC, and *M. canettii* specific assays, respectively, were determined with 95% probability.

During this study a novel method for extracting and purifying genomic DNA from mycobacterial cultures was concurrently developed and is described in **Chapter 3**. The optimised method developed combined a mechanical lysis (IDI lysis kit) step followed by DNA purification using the DNeasy blood and tissue kit (Qiagen). Owing to the complex cell wall in mycobacteria, incorporation of a bead beating step prior to purification of DNA was demonstrated to yield a higher recovery of total genomic DNA from mycobacterial cultures.

In **Chapter 4** an optimised, internally controlled multiplex real-time PCR diagnostics assay is described for the detection and accurate identification of the members of the MTC most commonly associated with zoonotic TB in humans and the vaccine strain *M. bovis* BCG.

For the collective detection and identification of *M. bovis*, *M. caprae* and *M. bovis* BCG an assay was designed and incorporated targeting a region of the *lpqT* gene (Rv1016c) in which there is a 5 bp region deleted in *M. bovis*, *M. caprae* and *M.bovis* BCG which is present in all remaining members of the MTC. The discovery of a deletion conserved to these members of the MTC reiterates the close phylogenetic relationship between these species.

The diagnostics target used to differentiate *M. bovis* BCG from *M. bovis* and *M. caprae* was the Rv3876 gene which is part of RD1, a region which is deleted in all *M. bovis* BCG strains.

To differentiate between *M. bovis* and *M. caprae*, a diagnostics assay targeting a novel SNP discovered during this study, in the *lepA* gene specific for *M. caprae* was designed and incorporated.

As there was no diagnostics assay incorporated in this multiplex test to identify all members of the MTC collectively, a non-competitive IAC was required to avoid the possible reporting of false negative results. An IAC specific for *M. smegmatis* (MSMEG_0660) was designed and incorporated. This particular gene was chosen as from BLAST analysis no significant homology was present to any member of the MTC or NTM.

The multiplex real-time PCR assay was empirically demonstrated to be specific and LODs 3.95, 3.55 and 14.77 genome equivalents were determined for the *M. bovis/M. bovis* BCG/*M. caprae* the *M. bovis/M. caprae* and the *M. caprae* assays respectively with 95% probability.

To summarise **Chapter 4**, a method was developed to specifically detect and differentiate between the members of the MTC most commonly associated with

zoonotic TB in humans. This test could be used specifically when zoonotic TB is expected, or alternatively as a follow on test from the one described in Chapter 3 resulting in the unambiguous detection and identification of 5 out of 8 members of the MTC.

In **Chapter 5** a two stage, unidirectional, internally controlled multiplex real-time PCR based method, *Seek*TB, is described for the detection and accurate identification of each specific member of the MTC, including the two clades of *M. africanum*. This method takes approximately 1.5 - 3 hrs post DNA extraction depending on which, if any member of the MTC is present in a sample.

The first multiplex real-time PCR consisted of the MTC (*lepA*), *M. tuerbculosis*/*M. canettii* (*wbbl1*), *M. canettii* specific ($RD^{canettii1}$), *M. africanum* West African 1 (RD713) and IAC (MSMEG_0660) diagnostics assays. The second multiplex real-time PCR diagnostics assay consisted of the *M. bovis*/*M.caprae*/*M. bovis* BCG (*lpqT*), *M. bovis*/*M. caprae* (RD1), *M. caprae* (*lepA*), *M. africanum* West African 2 (RD701) and IAC (MSMEG_0660) assays. To specifically identify the remaining members of the MTC, namely *M. microti* and *M. pinnipedii*, the combined results from both multiplex assays must be taken into account, as is outlined in **Chapter 5**.

The specificity of the targets used were validated against an extensive panel of well characterised MTC isolates, NTM and other bacteria. Using multiplex 1 analytical LOD's of 9.04, 5.88, 0.4 and 5.08 genome equivalents respectively were determined for the MTC, *M. tuberculosis*/*M.canettii*/*M. africanum* West African 1 and *M. canettii* specific and the *M. africanum* West African 1 diagnostics assays respectively. Using multiplex 2 analytical LOD's of 5.66, 6.05 98.28 and 24.9 genome equivalents respectively were determined for the *M. bovis*/*M. bovis* BCG/*M. caprae* (*lpqT*), *M. bovis*/*M. caprae* (RD1), *M. caprae* and *M. africanum* West African 2 specific diagnostics assays respectively. In both multiplex assays, the IAC at a concentration of 100 cell equivalents was detected in all samples tested.

After optimisation and validation of the multiplex real-time PCR assays developed, a clinical liquid culture positive investigation was performed to demonstrate whether *Seek*TB could be used directly on clinical culture positive isolates. To perform this investigation, 125 MGIT positive clinical isolates were blindly tested using *Seek*TB and the Genotype MTBC (Hain Lifescience) and the results were 100 % concordant. The results were then compared to the TBc ID test (BD) which revealed discordance in 9 samples. Further nucleotide sequence analysis of the discordance revealed the results obtained using *Seek*TB and the Genotype MTBC were correct, outlining the superior performance of the molecular methods over the TBcID test.

In this blind study evaluation all culture positive MTC isolates were determined to be *M. tuberculosis*. However, only a relatively small biobank of samples from one urban region in Lusaka, Zambia were tested. Evaluation of a much larger biobank is required to give a clearer picture as to the prevalence of members of the MTC other than *M. tuberculosis* present in this region.

Furthermore, clinical culture positive isolates from other regions in Zambia including more rural areas and other geographical regions, where members of the MTC other than *M. tuberculosis* are thought to cause human TB, are necessary to ascertain the global prevalence of members of the MTC.

## 6.2 Current application of *Seek*TB

*Seek*TB is currently validated for use on pure culture or clinical liquid culture positive isolates. *Seek*TB has the potential to be a useful methodology for epidemiological studies and could also be a useful methodology to ascertain if the transmission of TB is human to human or zoonotic. In low incidence countries it has become routine for reference laboratories to type all TB isolates. In such settings *Seek*TB could easily be adapted for the rapid typing of liquid culture positive isolates. Also, in high incidence settings, *Seek*TB could be used in retrospective

studies for typing of liquid culture positive isolates. Our blind study evaluation of liquid culture positive isolates from Zambia indicates that *M. tuberculosis* is the predominant cause of human TB in this region, however further studies are required to confirm this observation. For example, a larger panel of MTC isolates from this region need to be analysed using *Seek*TB.

Our blind study did show the importance of developing and utilising more robust methods in the diagnosis of TB. Our results suggest that approximately 7 % of the patients in this cohort were misdiagnosed. According to the data generated, if the results of the TBc ID test are implemented, 4 patients (3.2 % of cohort) may be receiving treatment for TB who in fact do not have TB, whereas an additional 4 patients (3.2 %) may not be receiving the appropriate treatment they require.

*Seek*TB is the first description of a multiplex real-time PCR diagnostics assay which can accurately and unambiguously differentiate all members of the MTC. If it is used in a clinical setting it will allow for accurate epidemiology studies to be performed. Currently, literature suggests that TB caused by members of the MTC other than *M. tuberculosis* is underrepresented. *Seek*TB may also be useful geographical regions which report a prevalence of members of the MTC other than *M. tuberculosis*. For example, in a recent study in the Republic of Djibouti 11.2 % of human TB cases were caused by *M. canettii* (7) whereas in certain regions of Africa up to 50 % of human TB caused by *M. africanum* has been reported (6). In addition, it has been suggested that bovine TB in developing countries may be responsible for up to 15 % of TB cases (5). *Seek*TB could be a useful tool to evaluate if this perceived prevalence of specific members of the MTC causing TB is in fact true. In instances where zoonotic transmission of TB occurs, it would be possible to intervene more promptly allowing for the implementation of control measures. When appropriate control measures are implemented, subsequent transmission of TB to other patients is disrupted which is important in the global fight to eliminate TB as a public health problem. The method presented in this study may also provide clinically relevant improvements in the context of patient treatment and care. When using *Seek*TB it is possible to detect the presence of members of the MTC with intrinsic resistances to

PZA (*M. bovis M. bovis* BCG and *M. canettii*). By specifically identifying these members of the MTC, the clinician can administer the more appropriate 9 month anti-TB regimen. This may help in preventing further drug resistances owing to inadequate anti-TB treatment, which may also reduce the likelihood of patients relapsing.

## 6.3 Future work

While *Seek*TB, a unidirectional two stage internally controlled multiplex real-time PCR method described in this thesis offers advantages over other tests described in the literature and tests which are also commercially available, further improvements can be made to *Seek*TB in the near future. In its current format, *Seek*TB could be used to evaluate larger biobanks of liquid culture positive isolates to determine if the perceived prevalence of members of the MTC other than *M. tuberculosis* causing TB in humans is in fact the true scenario.

There is also a need to evaluate the method developed directly on clinical samples (eg. sputum) without the requirement for culture in liquid medium. To determine the optimal method for recovery of bacterial DNA which is also suitable for use with *Seek*TB, a variety of purification methods for isolating mycobacterial genomic DNA from sputum need to be evaluated.

Subsequently, testing of *Seek*TB on these samples could be performed to optimise and validate the performance characteristics of *Seek*TB when used directly on sputum. This step may involve further optimisation of the assays developed to ensure the specificity and sensitivity are acceptable for use in this setting. If *Seek*TB can be used directly on clinical samples it will offer the further significant advantages for monitoring recent outbreaks, which could be beneficial for implementing control measures more rapidly. It will also potentially allow for the more rapid initiation of the optimal therapeutic regimen for patients.

While there are a number of advantages relating to *Seek*TB, there are also limiting factors associated with this test in its present format. Firstly, the current cost associated with reagents and real-time PCR machines to perform the tests are limiting factors with regard to its widespread use. Also as *Seek*TB is presently not a fully integrated, ie. sample in - result out, there is a requirement for personnel highly skilled in molecular techniques to perform the test. Furthermore, in its current format, a skilled person is also required to interpret the results output generated when the test is carried out. Finally, there is also a requirement for a constant power supply when using real-time PCR, which may be problematic in low resource setting.

In the future, transfer of *Seek*TB to an alternative *in-vitro* amplification technology with greater multiplexing capabilities (ie. multiparametric) could be advantageous as only one test would be required to be performed to detect all the diagnostics targets in a sample, thereby reducing the time and costs associated with the test.

Multiparametric PCR technologies are rapidly becoming the methods of choice for infectious disease and will represent a significant presence in the *in-vitro* diagnostics market place in the coming years. Some examples of these technologies include; xTAG® Technology (Luminex, Austin Texas) which reports a multiplexing capacity of 100 analytes in approx 3 hours; the GeneSpress® Platform (Genewave, Paris France) with a multiplexing capability of up to 50 analytes in approximately 2 hours and finally Biocartis' fully integrated molecular diagnostics platform currently in development (1-3).

In the context of TB diagnostics, these technologies represent an exciting opportunity which may aid in the global fight to control TB. Ideally, in the future, *Seek*TB will be further developed on a multiparametric *in-vitro* amplification technology such as those listed above. A diagnostics assay which can accurately identify the causative agent of TB infection, along with incorporation of diagnostics targets specific for common first and second line drug resistances and possibly some NTM associated with human infection would be particularly useful.

A fully integrated sample in - answer out diagnostics technology with the ability to guide the most appropriate therapeutic approach for a patient, which can also give a result on the day the patient presents at a hospital, could represent a significant improvement in the global fight to control TB. The transfer of *Seek*TB to a fully integrated platform would reduce the requirement for skilled personnel to perform the diagnostics tests. Furthermore, a platform with greater levels of multiplexing will result in improved patient management, reduced patient morbidity and mortality, and reduced TB associated healthcare costs.

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