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EVALUATION OF THE *rpoB* GENE SEQUENCE IN THE IDENTIFICATION OF
MYCOBACTERIAL SPECIES

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DEDICATION

This work is dedicated to my family for all the support they rendered me during the entire course of my studies.

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LIST OF ABBREVIATIONS

“	:	Seconds
‘	:	Minutes
µg	:	Micrograms
µl	:	Microlitres
°C	:	Degrees Celsius
16S rRNA	:	ribosomal RNA small sub unit
AIDS	:	Acquired Immunodeficiency Syndrome

ATCC	:	American Type Culture Collection
BCCM	:	Belgian Coordinated Collection of Microorganisms
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
DGD	:	Directorate – General for Development Cooperation
DNA	:	Deoxyribonucleic Acid
g	:	Grams
HIV	:	Human Immunodeficiency Virus
ITM	:	Institute of Tropical Medicine
MAC	:	<i>Mycobacterium avium</i> complex
ml	:	Millilitres
mM	:	Millimolar
M. spp	:	Mycobacterial species
MTBC	:	<i>Mycobacterium tuberculosis</i> complex
NCBI	:	National Centre for Biotechnology Information
NCTC	:	National Collection of Type Cultures
No.	:	Number
NTM	:	Non-tuberculous mycobacteria
PCR	:	Polymerase Chain Reaction
RNA	:	Ribonucleic Acid
<i>rpoB</i>	:	RNA polymerase beta sub unit
S. no.	:	Serial number
TB	:	Tuberculosis
UP	:	University of Pretoria
UV	:	Ultraviolet
WHO	:	World Health Organisation

ABSTRACT

In this study, the use of the *rpoB* gene to identify mycobacterial species was evaluated. The *rpoB* gene encodes the highly conserved beta subunit of RNA polymerase. It is known to be more discriminatory in bacterial species identification compared to the widely used 16S rRNA gene sequence. This attribute can be exploited for correct identification of mycobacterial strains identified according to the 16S rRNA but requiring further identification to (sub-) species level.

A gene fragment of approximately 760 bp was targeted using primer pair My9 – MycoR. The study involved evaluating the primer set on 30 reference mycobacterial strains from the public culture collection (BCCM/ITM). The tests were performed using an optimised in-house PCR system followed by gel electrophoresis and sequencing of the amplicons. BLAST analysis was performed to identify the species. Twenty-five (83%) of the strains were in agreement with the sequence data available from GenBank (NCBI). For three species namely *Mycobacterium sphagni*, *M. komossense* and *M. parafortuitum*, the sequence information was not available on GenBank (NCBI). Test attributes including repeatability and reproducibility were performed on two strains (*M. ulcerans* and *M. abscessus*) and results were consistent with tested strains.

The *rpoB* was further evaluated on 71 mycobacterial strains from BCCM/ITM, which required further identification. According to the test results, thirty one of the strains (43%) were identified to the respective species. Using the *rpoB* gene sequence, it was possible to distinguish *M. marinum* (ITM M000140/NCTC 2275) from *M. ulcerans* and *M. montmartrense* (NCBI strain 028/ITM 981197) from *M. peregrinum* and *M. septicum* which 16S rRNA is not able to do.

The *rpoB* gene sequence has an added value in mycobacterial species identification. However, some *rpoB* gene sequences are not available on Genbank. It is therefore important to establish a complete *rpoB* gene sequence data bank for mycobacterial species especially the non-tuberculous mycobacteria (NTM) to improve their identification and to aid in disease diagnosis.

Keywords: Mycobacteria; *rpoB*; 16S rRNA, PCR; Electrophoresis, Sequencing; Identification

CHAPTER ONE: INTRODUCTION

The genus *Mycobacterium* consists of organisms which are opportunistic or obligatory pathogens for humans and animals, as well as saprophytic species found in nature. Some of the notorious human pathogens are *Mycobacterium tuberculosis* and *M. leprae*. Others are ubiquitous mycobacteria, collectively known as non-tuberculous mycobacteria (NTM) (Tortoli 2009a). Infections due to mycobacteria have increased since the 1980s, affecting both immuno-competent and immuno-compromised persons (Lee, H., Park, H. J., Cho, S. N., Bai, G. H., & Kim, S. J. 2000). About one third of the world's population is infected with the tuberculosis (TB) bacillus. TB affects mainly the lungs, but may also involve other parts of the body. The classic symptoms of active TB are chronic cough with blood-staining sputum, fever, night sweats and weight loss. People with a compromised immune system - especially those living with human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) - are at greater risk of developing active TB disease (WHO 2016).

Infections due to NTM occur in the presence of predisposing factors, such as restrictive or obstructive respiratory disease in the elderly and in immune-compromised individuals. Infection may involve the lungs or other body tissues, such as joints, bone marrow or the skin. Members of the *Mycobacterium avium* complex, such as *M. avium* and *M. intracellulare* are implicated most commonly in disease causation among the NTM (Tortoli 2009b).

Mycobacteria are acid-fast bacilli which are straight or slightly curved rods between 0.2 and 0.6 μm wide by 1.0 and 10 μm long. Presently, over 160 species of mycobacteria are recognized (<http://www.bacterio.net/mycobacterium.html> 2016). The genus is divided into rapid and slow growing bacteria, depending on the time they take to form colonies on selective media. Rapid growers typically take less than seven days whereas slow growers take more than seven days to form visible colonies.

Species in the genus *Mycobacterium* are grouped into complexes, depending on their disease involvement and phenotypic-genotypic relationship (Saviola and Bishai 2006). Examples of complexes among the slow growers include: *M. tuberculosis* complex (MTBC) with members causing tuberculosis in humans and animals such as *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. caprae*, *M. microti*; members of the *M. avium* complex (MAC) cause a

disseminated infection but with no lung involvement and they include *M. avium*, *M. paratuberculosis*, *M. intracellulare*, *M. colombiense*, *M. avium silvaticum*. Complexes among the rapid growers include *M. chelonae* complex with species such as *M. abscessus*, *M. chelonae*, *M. bolletii*; *M. fortuitum* complex, with members such as *M. fortuitum*, *M. peregrinum*, *M. porcinum*, *M. septicum*, *M. houstonense*, *M. mucogenicum*, *M. senegalense*, *M. cosmeticum*; and *M. parafortuitum* complex, with members such as *M. parafortuitum*, *M. diernhoferi*, *M. austroafricanum*, *M. hodleri* and *M. neoaurum* (McMurray 1996).

For a long time, identification of mycobacteria species has been dependent on morphological and biochemical characteristics. With the introduction of molecular techniques, 16S rRNA gene sequencing has been most widely used for characterization and has contributed to the establishment of more than 45 novel NTM species and to the description of emerging rapidly growing mycobacterial (RGM) infections. Experience however, indicated that the 16S rRNA molecular tool underestimates the diversity of this group and does not distinguish between all recognized mycobacterial taxa, especially species which are closely related, such as *M. peregrinum* and *M. septicum*, or to differentiate among species belonging to complexes, for example, *M. chelonae*-complex or MAC (Adékambi et al. 2006).

Identification of mycobacteria to the species level is important for clinical decision making, patient management and epidemiological analysis. However, due to inaccuracies in diagnosis, treatment regimens may sometimes be instituted for *M. tuberculosis* which would otherwise not be necessary (Joao et al. 2014).

To respond to this shortcoming, *rpoB* gene sequencing, which encodes the highly conserved beta sub unit of the bacterial RNA polymerase, has been developed. It has been reported to correctly identify bacterial strains to the (sub-) species level (Mollet, Drancourt & Raoult 1997). The *rpoB* gene sequence can offer rapid mycobacterial species identification, especially in the diagnosis of NTM.

It is nevertheless important to evaluate the *rpoB* assay prior to its implementation in the laboratory setting, to determine its added value in the identification of NTM strains belonging to the public culture collection of mycobacteria-BCCM/ITM (<http://bccm.belspo.be/about-us/bccm-itm>, 2016).

1.1 Aims

- To optimize the *rpoB* PCR system by comparing different commercial polymerase kits and amplification settings on a selection of mycobacterial species.
- To characterise the preferred *rpoB* PCR system and sequencing on a panel of well documented reference strains: BCCM/ITM - Belgian Coordinated Collection of Microorganisms, ATCC – American Type Culture Collection and NCTC – National Collection of Type Cultures and;
- To compare the added value of *rpoB* sequencing over 16S rRNA gene sequencing by applying the characterized method on mycobacterial strains identified by sequencing of the 16S rRNA gene and reference strains from the public culture collection (BCCM/ITM).

CHAPTER TWO: MATERIALS AND METHODS

The study was performed in three main steps: optimization of the PCR kits, characterization of the test method and evaluation of the assay for routine practice.

2.1 Optimization of 3 commercial PCR kits and 1 standard in-house PCR kit mix

Primers My9 and MycoR were used that target the *rpoB* gene. These primers amplify an approximately 760 bp region containing significant variation among mycobacterial species (Table 1).

Table 1: Primers used for amplification of the rpoB region based on Mtb H37Rv sequence (Accession number AL123456.3)

Primer	Forward or Reverse	Sequence (5' – 3')	Location (bp)	Source
My9	F	ATCGGCGCCGAGGTCCGCGA	2356 – 2376	(Andre et al. 2015)
MycoR	R	AGCGGCTGCTGGGTGATCATC	3135 – 3152	(Adekambi, Colson & Drancourt 2003)

The mycobacterial strains used for the optimization are listed in Table 2, with their ITM number and species identification according to the 16S rRNA gene.

Table 2: Mycobacterial strains used in PCR kit optimisation

	<i>Strain number (ITM)</i>	<i>Species name</i>
1.	960298	<i>M. tuberculosis complex</i>
2.	152446	<i>M. chelonae complex</i>
3.	9484	<i>M. simiae</i>
4.	152167	<i>M. intracellulare</i>

Three (3) commercial PCR kits (Qiagen, Promega and Thermo) and the standard in-house PCR mix were compared under their respective PCR conditions. Details of the reaction mixes for the four kits (i.e. In-house, Qiagen, Promega and Thermo) are given in Annex 1. All kits were run along a temperature gradient (56 – 70°C) to determine the one that gave the most specific reaction.

Gel electrophoresis was performed with a 2% agarose gel: 2g agarose mixed with 100ml 0.5x TAE buffer (40mM Tris, 20mM Acetic Acid, 1mM EDTA). Two µl of amplicons were mixed with loading buffer and dye (2µl) and run through the gel at 100V for about 25 minutes. A molecular marker/ladder (100+bp) was used to estimate the size of the bands. The gel was then soaked in ethidium bromide (0.5µg/ml) for between 30 - 45 minutes and then viewed under UV light on a trans-illuminator.

2.2 Characterisation of the *rpoB* gene assay

Characterisation was done to ensure that the correct test is performed for its intended purpose. In order to characterise the *rpoB* gene assay, the PCR kit selected in 2.1 was used to perform the following test attributes;

2.2.1 Test for sensitivity of the *rpoB* assay

PCR and sequencing were done on 30 reference mycobacterial strains from the BCCM/ITM, ATCC and NCTC. The list of mycobacterial strains used is provided in Annex 3.

2.2.2 Test for specificity of the *rpoB* assay

The same PCR was applied to 20 non-mycobacterial strains of closely related genera (*Corynebacterium*, *Rhodococcus*, *Nocardia*; Annex 4) to test its specificity.

2.2.3 Test for repeatability and reproducibility

To test for repeatability (intra-run variability) and reproducibility (inter-run variability), two strains were used; *M. ulcerans* - ITM C05142 / ATCC 19423 and *M. abscessus* – ITM M001007 / ATCC 23006.

For repeatability, PCR and sequencing were done in triplicate for the two strains in the same run by the same operator. For reproducibility, PCR was done in four different runs by 2 operators. PCR products were sequenced (for more details see 2.4).

2.3 Evaluation of the *rpoB* assay using strains from the public culture collection

The selected test method was applied to a selection of 71 mycobacterial strains from the public culture collection (BCCM/ITM) which required further identification. The list of the strains used, together with their corresponding ITM numbers and species identification according to 16S rRNA sequencing are given in Annex 7.

2.4 Sequencing, sequence data analysis and phylogenetic trees

All sequencing of amplicons was done by the Sanger automated sequencing method which was outsourced to BaseClear in the Netherlands. The sequences were provided as ABI files which were read by SnapGene viewer version 3.2.1.0 (GSL Biotech LLC).

According to the sequence results, the forward primer My9 yielded some unspecific products leading to background noise in the ABI file output. The reverse primer MycoR on the other hand yielded very specific products with an average score of 704 bp with no background noise. MycoR was therefore used as the query sequence in the analysis and identification process.

Nucleotide sequences were compared to similar sequences in the National Centre for Biotechnology Information (NCBI) gene bank using the Basic Local Alignment Search Tool (Blastn) programme optimised for Megablast which searches for highly similar sequences.

All sequences from the tested mycobacterial strains (30 reference strains and the 71 strains from ITM/BCCM) were aligned using the CLC sequence viewer (version 7.7.1.). According to the sequence alignment, the region of hypervariability for the *rpoB* gene is between 260 – 310 bp (Figure 1).

		250		280		300		320
<i>M. chimaera</i> (130005)	TCGGGCAGGT	TCACCGCCCA	-----	-----	-ATCGGGTGA	GCCCTC----	-----	-GATGTTCCA 284
<i>M. intracellulare</i> (M001702)	TCGGGCAGGT	TCACCGCCCA	-----	-----	-ATCGGGTGA	GCCCTC----	-----	-GATGTTCCA 284
<i>M. avium</i> (M9016)	TCGGGCAGGT	TGACCGCCCA	-----	-----	-CTCGGGATT	GCCGTC----	-----	-GATGTTCCA 284
<i>M. paratuberculosis</i> (C02666)	TCGGGCAGGT	TGACCGCCCA	-----	-----	-CTCGGGATT	GCCGTC----	-----	-GATGTTCCA 284
<i>M. scrofulaceum</i> (C04988)	TCGGGCAGGT	TGGCGGCCCA	-----	-----	-GGCGGGCAC	GCCCTC----	---GCAACAT	TGATGTTCCA 293
<i>M. simiae-like</i> (M003260)	TCGGGCAGGT	TGACCGCCCA	-----	-----	-GTCCGGGCGT	GCCCTC----	-----	-GATGTTCCA 284
070247(<i>M. celatum</i>)	TCCGGCAACT	TGGAGGCCCA	-----	-----	-GTCCGGGCTT	GCCCTC----	-----	-GACCTTCCA 284
<i>M. terrae</i> (M001718)	TCGGGCAGCT	TCTCCGCCCA	-----	-----	-GTCCGGGCAC	GCCGGC----	---AGCAACAT	CGATGTTCCA 293
2015-00294(<i>M. kumatonense</i>)	TCGGGAAGCT	GATCGGCCCA	-----	-----	-GTCCGGGAAC	GCCGCCCGG	CCGTCCTTAT	CGATGGTCCA 299
<i>M. nonchromogenicum</i> (M001682)	TCGGGCAGCT	TGTCCGCCCA	-----	-----	-GTCCGGGCAC	GCCGGC----	---GCCACAT	CGATGTTCCA 293
M005766(<i>M. hiberniae</i>)	TCGGGCAGCT	GATCCGCCCA	-----	-----	-ATCCGGGCGT	ACCGGCC----	---GCCACAT	CGATGTTCCA 293
M001827(<i>M. angelicum</i>)	TCGGGCAGGT	TGACCGCCCA	-----	-----	-CTCGGGAGC	ACCCTC----	-----	-GATGTTCCA 284
M000996(<i>M. parafortuitum</i>)	TCGGGCAGCT	TGGACGCCCA	-----	-----	-GTCCGGGTAC	ACCGGC----	---GGCGACGT	CGATGTTCCA 293
<i>H. smegmatis</i> (M010691)	TCGGGCAGCT	TGGACGCCCA	-----	-----	-GTCCGGGCAC	GCCCGC----	---GGCGACGT	CGATGTTCCA 293
<i>M. gilvum-like</i> (M006995)	TCGGGCAGCT	TGGCTGCCCA	CGCGGGAACC	TCGGTCGAGC	CGTCCGCTCC	ATCACT----	---GGCAAGCC	GGATATTCCA 314
<i>M. neoaurum</i> (M002379)	TCGGGCAGGT	TGGCGGCCCA	T-----	-----	--TCGGGTGA	ACCC-----	-----T	CGATGTTCCA 284
<i>M. fortuitum-like</i> (M00995)	TCGGGCAGCT	TGACCGCCCA	T-----	-----	--TCCGGAGT	GCCCGC----	---AGCCACAT	CGATGTTCCA 293
<i>M. rhodesiae</i> (M003300)	TGCGGCAGGT	TGGCAGCCCA	C-----	-----	--TCGGGCGT	CCCGCC----	---GACCACAT	CGATGTTCCA 293
<i>M. aurum</i> (M006993)	TCGGGCAGGT	TGCGCGCCCA	CTCCGGGGT-	-----	-----	---GCT-----	---TGCACGT	CGATGTTCCA 290
<i>M. moniliformis</i> (981197)	TCCGGCAAGT	TGACGCCCA	AGCAGGAAGT	-----	--TCACGGGC	ACCATC----	---GGCACGT	CGATGTTCCA 302
<i>M. chitiae</i> (M002386)	TCGGGCAACC	CCTCGGCCCA	C-----	-----	--TCCGGTAC	GCCCGC----	---GGCGACGT	CGATGTTCCA 293
<i>M. thermoresistibile</i> (M007001)	TCGGGCAGCT	TGGACGCCAG	-----	-----	---GTCCCTT	GTCCGC----	---GACGAGGT	TGATGTCCCA 290
C01828(<i>M. komossense</i>)	TCAGGGAGCT	TGACGCCCA	C-----	-----	--TCGGGCAC	ACCACT----	---GGCAACCT	CGATGTTCCA 293
M002321(<i>M. kansasii</i>)	TCGGGCAGGT	TGGCGGCCCA	-----	-----	-GTCCGGTGT	ACCCGC----	-----	-GATCTGCCA 284
<i>M. abscessus</i> (M001045)	GCAGGCAGAT	TGGCGGCCCA	C-----	-----	--TCGGGCTC	ACCCTC----	-----	-GATGTTCCA 284
<i>M. chelonae</i> (M002656)	TCGGGCAGGT	TCTCGGCCCA	C-----	-----	--TCGGGGTT	GCCCTC----	-----	-GATGTTCCA 284
<i>M. triale</i> (M001179)	TCGGGCAGCT	TGGTCGCCCA	-----	-----	-GTCCGGGGT	GCCCGC----	---GGCGACGT	CGATGTTCCA 293
<i>M. Marinum</i> (M000140)	TCGGGCAGCT	TGCGCGGCCCA	C-----	-----	--TCCGGCAC	TCCGTT----	---GGCGACGT	CGATGTTCCA 293
<i>M. ulcerans</i> (C05142)	TCGGGCAGCT	TGCGCGGCCCA	C-----	-----	--TCCGGCAC	TCCGTT----	---GGCGACGT	CGATGTTCCA 293
<i>M. tuberculosis</i> (H3715)	TCGGGCAGCC	TGGCGGCCCA	-----	-----	-GTCCGGGAAC	CCCTT-----	---GGCGGCGT	CGACCTTCCA 293
<i>hly-rpoB</i> ref (H37Rv)	TCGGGCAGCC	TGGCGGCCCA	-----	-----	-GTCCGGGAAC	CCCTT-----	---GGCGGCGT	CGACCTTCCA 293

Figure 1: Sequence alignment showing region of hypervariability of the *rpoB* gene.

After the sequence alignment, phylogenetic trees were drawn which infer relationship among species. The trees were constructed using the Neighbor Joining method, Kimura 80 was used to measure the nucleotide distance and Bootstrap analysis was performed.

CHAPTER THREE: RESULTS

3.1 PCR kit optimization

A detailed overview of the results obtained for the optimization step is available in Annex 2. According to the results of the gel electrophoresis, all the kits gave amplification at high annealing temperatures from 68°C – 70°C (Figure 2). The Thermo kit produced amplicons that showed long smears with no clear bands (Image a), the Promega kit produced bands of the expected size but with a lot of background noise (Image b), Qiagen was specific but did

not produce bands for all strains tested (Image c), while the in-house PCR produced the expected bands for all the strains but with some background noise especially at lower annealing temperatures (Image d).



Image a: Thermo kit with samples A, B, C, D, molecular marker (M), + and - controls



Image b: Promega kit with samples A, B, C, D, + and - controls

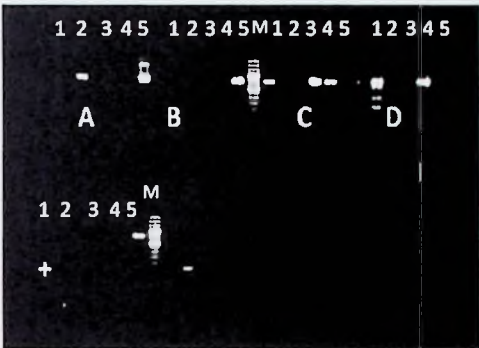


Image c: Qiagen kit with samples A, B, C, D, + and - controls

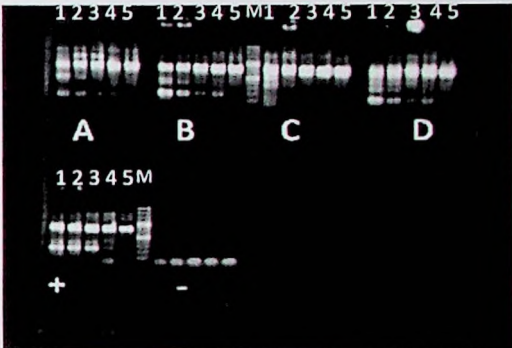


Image d: In-house kit with samples A, B, C, D, + and - controls

Figure 2: Images of the 4 PCR kits run along a temperature gradient from 56°C to 70°C between lanes 1 – 6 (thermo) and lanes 1 to 5 (Promega, Qiagen and In-house) that represent an increase of 2.8°C and 3.5°C per lane respectively.

The Qiagen and in-house kits were then subjected to the same annealing temperature of 72°C under their respective PCR conditions. The kits produced clear specific bands, but the in-house PCR kit produced more consistent amplification for all the isolates, including the positive control (Figure 3). Therefore, and to be in line with the other in-house PCRs implemented in the unit, the in-house kit was selected for *rpoB* gene based identification.

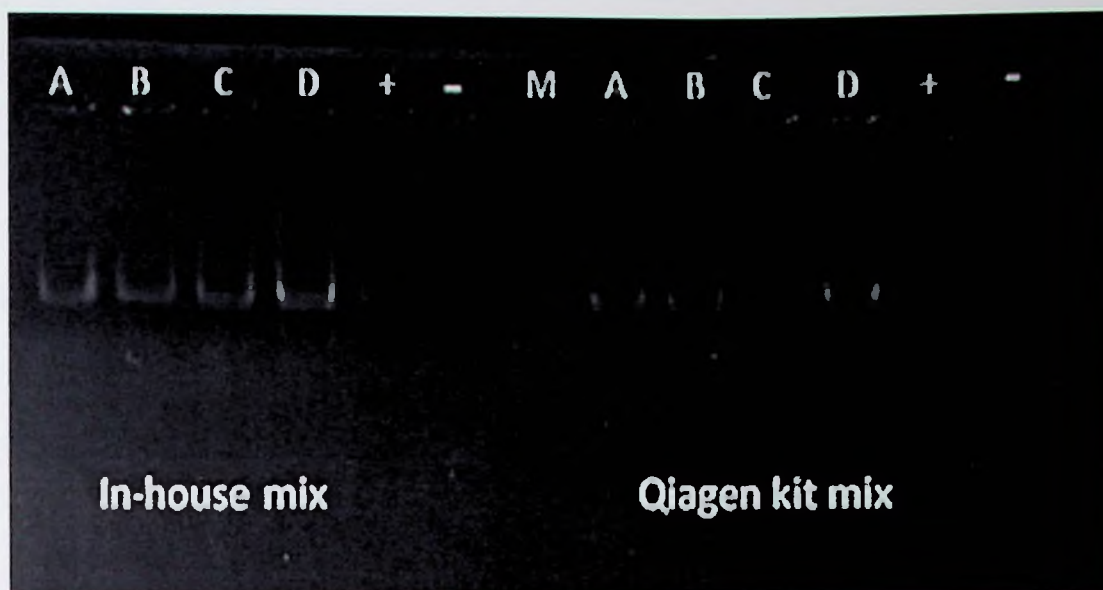


Figure 3: Qiagen and In-house compared at the same annealing temperature (72°C). Samples are marked A, B, C and D; + and – controls and molecular marker (M).

3.2 Characterisation of the *rpoB* assay using reference mycobacterial strains

3.2.1 Sensitivity of the *rpoB* assay

PCR and gel electrophoresis was performed on 30 strains using the optimised in-house PCR system. Most of the isolates were amplified producing bands of the expected size except for isolate marked 11 (M001179; *M. triviale*) which produced a weak band (Figure 4).



Figure 4: Test for sensitivity of the *rpoB* assay using the in-house PCR assay on 30 reference mycobacterial strains marked 1 – 30 (Annex 3) and molecular marker (M).

Sequencing of amplicons and Blastn analysis results are given in Annex 5. According to the sequence results, 25 of the 30 strains (83%) corresponded to identification provided on GenBank. Sequences for 4 strains belonging to 3 species namely *M. sphagni* (ATCC 33026 and ATCC 33027), *M. komossense* (ATCC 33013) and *M. paraafortuitum* (ATCC 19686) were not available in this public repository. Efforts to get similar sequences from in-house data banks from colleagues in other institutes did not yield results. One other strain namely *M. triviale* (ATCC 23292) had more than 3 mismatches with the subject sequence from NCBI (n=5) and therefore could not be conclusively identified.

3.2.2 Specificity of the *rpoB* assay

Ten (50%) out of the 20 non-mycobacteria test strains were amplified as shown in Figure 5. This implied that primers My9-MycoR were not specific for mycobacteria and amplified bacteria belonging to closely related genera such as *Corynebacterium*, *Nocardia* and *Rhodococcus*.

However, sequencing of the products revealed that the amplification was non-specific. The ABI file was unreadable; hence no identification could be carried out.

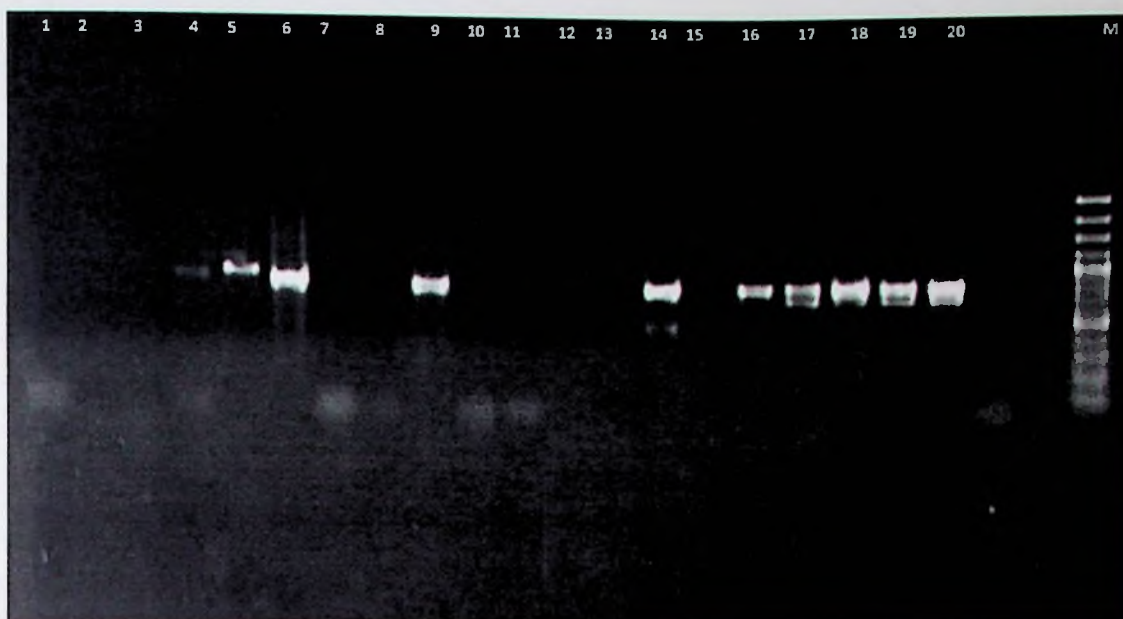


Figure 5: Twenty non-mycobacterial strains (Annex 4) used to test the specificity of the *rpoB* assay, M is the molecular marker.

3.2.3 Repeatability and reproducibility of the *rpoB* PCR assay

Bands of the expected size were produced in all the replicates from the repeatability (Figure 6) and reproducibility tests (Figure 7).

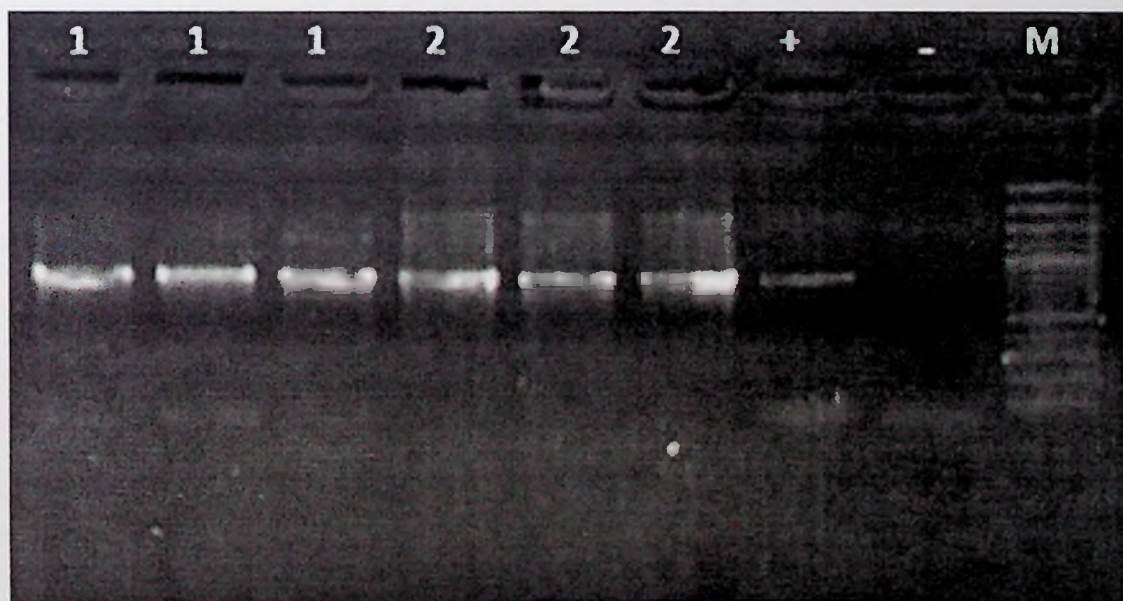


Figure 6: Test for repeatability of the *rpoB* assay using 2 samples marked 1 (*M. ulcerans*) and 2 (*M. abscessus*), a positive and negative control. M is the molecular marker (100+ bp).

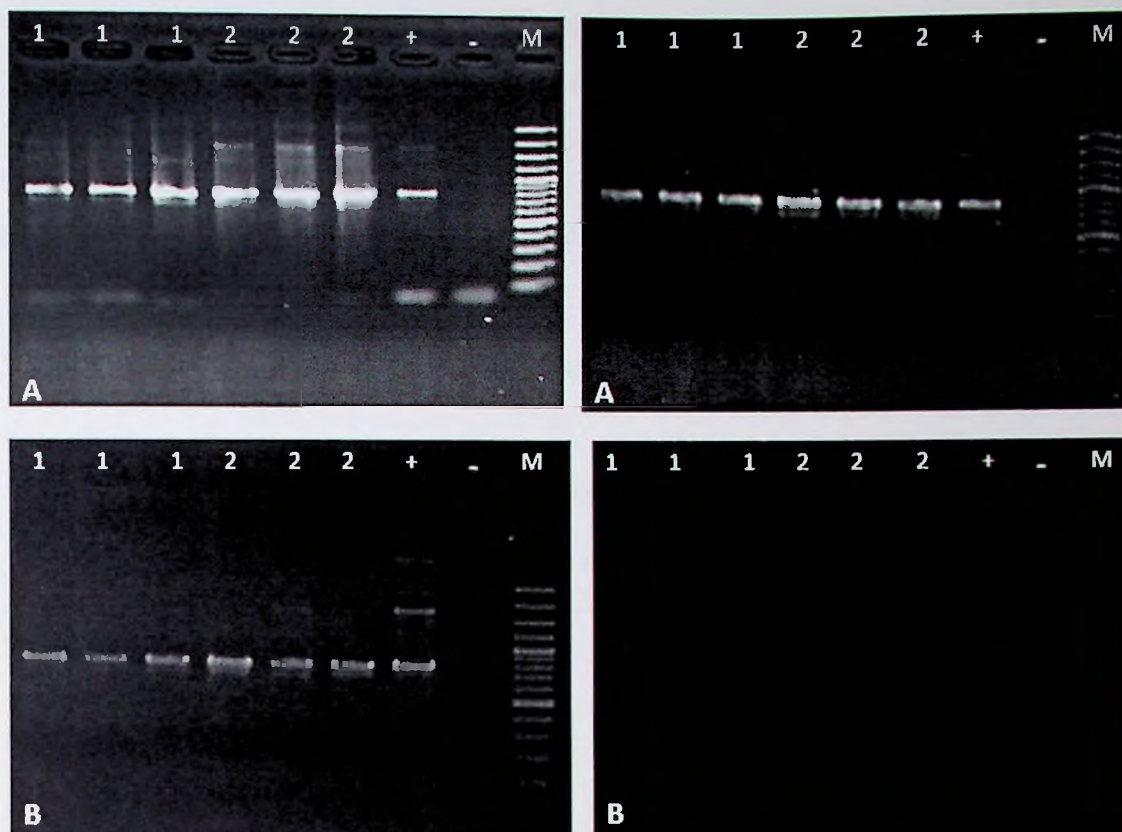


Figure 7: Test for reproducibility of the *rpoB* assay by 2 operators A and B. Two samples marked 1 (*M. ulcerans*) and 2 (*M. abscessus*), a positive and negative control and molecular marker (M).

PCR products from the 2 strains (*M. ulcerans* - ITM C05142/ATCC 19423 and *M. abscessus* - ITM M001007/ATCC 23006) used to test for repeatability and reproducibility were sequenced. A summary of the sequence results for repeatability and reproducibility is given in Annex 6. All replicates yielded a valid (correct length and good quality) and correct (correct species after blasting) sequence.

3.3 Evaluation of the *rpoB* assay using public culture collection (BCCM/ITM)

Most of the strains were amplified producing clear DNA bands except 3 strains marked 57 (M005090; *M. avium complex*), 63(040538; *M. celatum*) and 70 (M005189; *M.sp*) which produced double bands. These strains were re-run on gel electrophoresis and the DNA band corresponding to the expected band size was cut out of the gel and sequenced together with the rest of the tested strains (Annex 8).

Results from sequencing and Blastn analysis are shown in Annex 9. Thirty one (43%) of the 71 strains were identified to species level. Seventeen of these strains exhibited 100% identity with the sequence available in the database, whereas 14 had three or less mismatches with the subject sequences (Table 3). Strain (130545; *M. intracellulare*) could not be clearly distinguished from *M. chimaera* by the *rpoB* sequence. Further phenotypic and/or genotypic analysis may be required to confirm its identity.

The rest of the 40 strains could only be identified to the genus as mycobacteria but could not be assigned to their respective species identities.

Table 3: Mycobacterial strains from ITM that were identified by the *rpoB* gene assay

S. no	Species identity	No. of strains with 100% identity	No. of strains with 3 or less mismatches (n ≤ 3)
1	<i>M. avium</i>	3	
2	<i>M. avium sub-spp. avium</i>	1	
3	<i>M. intracellulare</i>	4	
4	<i>M. montmartrense</i>	1	
5	<i>M. chelonae</i>	3	
6	<i>M. abscessus</i>	2	
7	<i>M. peregrinum</i>	1	
8	<i>M. scrofulaceum</i>	1	
9	<i>M. chimaera</i>	1	
10	<i>M. avium</i>		1
11	<i>M. abscessus</i>		1
12	<i>M. chelonae</i>		4
13	<i>M. intracellulare</i>		1
14	<i>M. hiberniae</i>		1
15	<i>M. heidelbergense</i>		1
16	<i>M. chimaera</i>		1
17	<i>M. sinense</i>		1
18	<i>M. simiae</i>		1
19	<i>M. chimaera/M. intracellulare</i>		1
20	<i>M. chimaera</i>		1
TOTAL		17	14

3.4 Sequence alignment and phylogenetic trees

Species belonging to the *M. avium* complex were presented on a phylogenetic tree (Figure 8) and they showed the distinctive species groupings. Another tree for the *M. chelonae* complex was likewise drawn and similar groupings emerged (Figure 9).

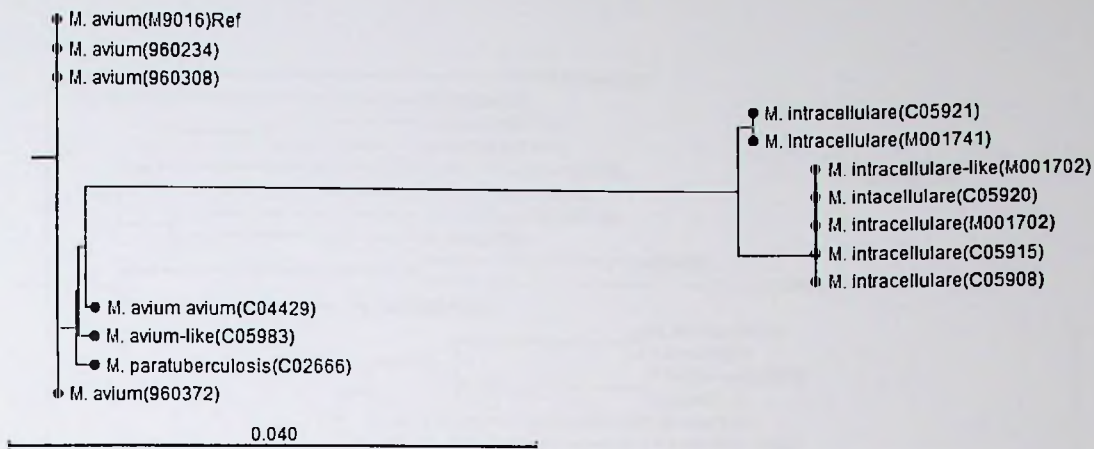


Figure 8: Neighbour-joining phylogenetic tree based on the *rpoB* sequence for species of *M. avium* complex.

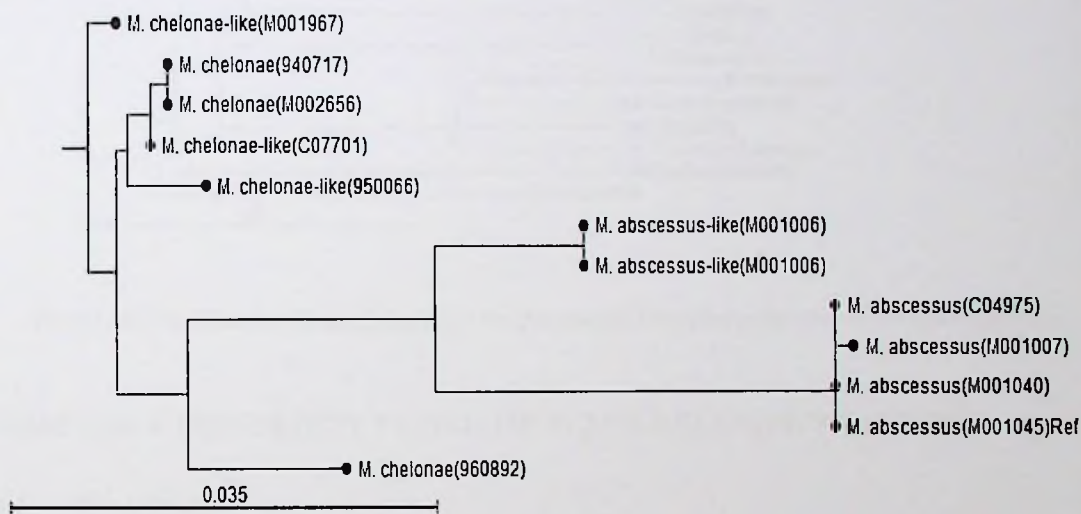


Figure 9: Neighbour-joining phylogenetic tree based on the *rpoB* sequence for species belonging to *M. chelonae* complex.

A phylogenetic tree was drawn for all strains identified by the *rpoB* gene sequence and their phylogenetic relationships were inferred. This tree grouped slow growers from the rapid growers. Strains that could not be identified using the *rpoB* gene such as *M. angelicum* (M001827), *M. kansasii* (M002321), *M. celatum* (070247), *M. hiberniae* (M005766), *M. kumatonense* (2015-00294) and *M. parafortuitum* (M000996) grouped correctly as either slow or rapid growers according to the *rpoB* gene phylogenetic tree (Figure 10).

For reference purposes, trees for all strains tested by the *rpoB* gene and the *16S rRNA* gene sequences are provided in Annex 10.

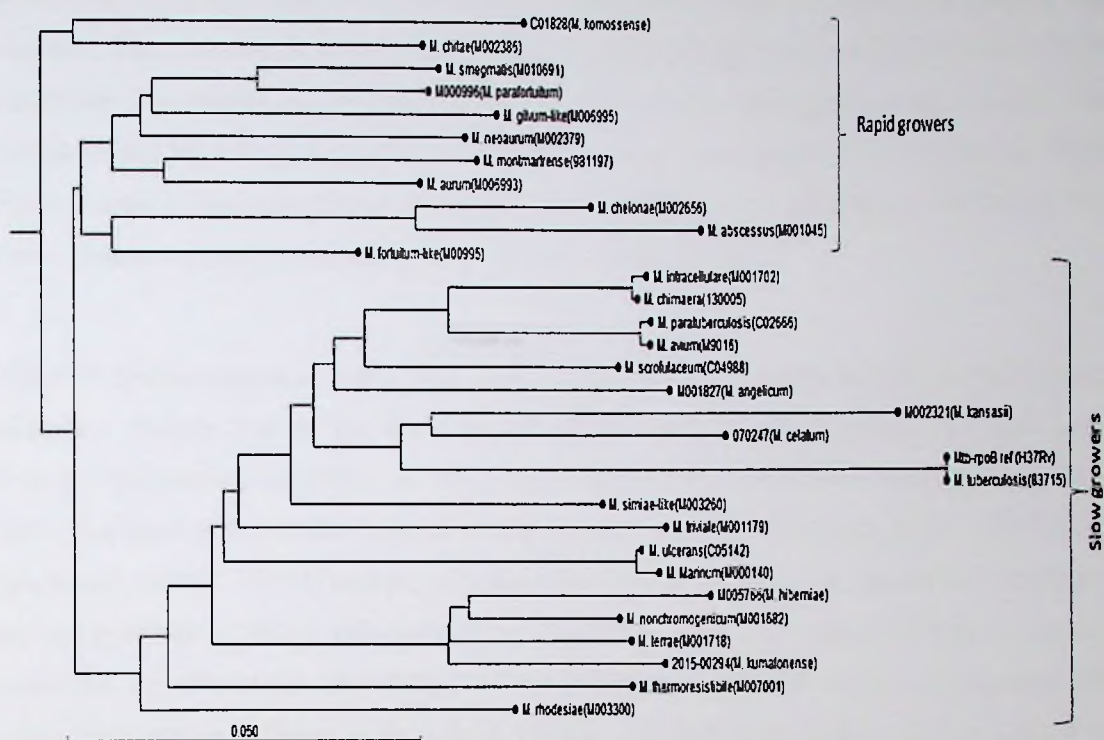


Figure 10: Neighbour-joining phylogenetic tree for species identified according to the *rpoB* sequence.

CHAPTER 4: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Discussion

Identification of mycobacterial species to the correct (sub) species level is key in ensuring correct therapeutic interventions, patient management and in epidemiological studies. The 16S rRNA sequence analysis is widely used in mycobacterial species identification. However, it does not distinguish some closely related species, hence the need for alternative tests to achieve a correct identification. The *rpoB* gene sequence is very discriminative for bacterial species identification (Drancourt, Raoult 2002) particularly in the diagnosis of mycobacterial infections (Kim et al. 1999). It offers a useful tool to distinguish mycobacterial strains that require further identification in addition to the 16S rRNA gene sequence. In this study, we evaluated the use of the *rpoB* gene in mycobacterial species identification.

Using primers My9-MycoR, commercial kits including Qiagen, Promega, Thermo and an in-house PCR kit were compared to each other to select one which was the most specific and sensitive. The in-house PCR mix yielded the most specific products at the optimised PCR conditions. The primer set anneals specifically at relatively high temperatures (72⁰C). The reverse primer MycoR gave more consistent results as compared to the forward primer My9. There is need to improve the specificity of the forward primer in subsequent studies as this allows sequence comparison during the process of identification.

This PCR system was used to test 30 mycobacterial reference strains from the public culture collection. Twenty five of the thirty strains (83%) were identified using the *rpoB* gene sequence information available on the NCBI website with similarities ranging from 99 – 100%. For three of the strains under study (*M. sphagni* (ATCC 33026 and ATCC 33027), *M. komossense* (ATCC 33013) and *M. parafortuitum* (ATCC 19686), the sequence information was not available in NCBI. Efforts to obtain these sequences from other databases were not successful. Another strain *M. triviale* (ATCC 23292/ITM C07736) had 5 mismatches and hence could not be assigned to its species identity. Additional phenotypic and/or genotypic analysis may be required to determine the true identification of this reference strain, and to check whether it is entirely pure after long-term storage and use in the laboratory.

Further evaluation of the test on selected mycobacterial strains from the BCCM/ITM collection, allowed for correct species identification in 43% of the isolates tested. Species of the MAC such as *M. avium*, *M. intracellulare* and *M. chelonae* complex such as *M. chelonae*, *M. abscessus* were clearly identified. These species are important medically as they are isolated frequently from clinical specimens and are implicated in disease causation (van Ingen et al. 2009). Genomic data for NTM which are occasionally encountered in clinical examinations, yet with a much lower or no clinical impact, such as *M. komossense*, *M. sphagni* and *M. parafortuitum* are not yet available in the NCBI, and hence cannot be readily identified by the BLAST programme.

The *rpoB* gene sequence showed its added value over 16S rRNA gene sequencing by distinguishing *M. marinum* (ITM M000140/NCTC 2275) from *M. ulcerans* and *M.*

montmartrense (NCBI strain 028/ITM981197) from *M. peregrinum* and *M. septicum* which was not possible with the latter assay.

Test attributes - including repeatability and reproducibility - were consistent for the 2 strains tested; *M. ulcerans* - ITM C05142/ATCC 19423 and *M. abscessus* – ITM M001007/ATCC 23006.

The My9-MycoR primer pair is not specific for mycobacteria. Half of the tested strains belonging to the genera *Rhodococcus*, *Corynebacteria* and *Norcadia* were amplified, although subsequent sequence analysis did not yield identification to the species level. This implies that the test as such should not be used as a first-step detection/identification of mycobacteria, but rather as a follow-up assay on cultures already identified as being mycobacteria.

The *rpoB* phylogenetic trees drawn from the tested strains were consistent with the 16S rRNA tree. The slow growers were grouped distinctly from the rapid growers. From the tree, the newly named *M. montmartrense* was clearly grouped among the rapid growers. Some strains such as *M. angelicum* (M001827), *M. kansasii* (M002321), *M. celatum* (070247), *M. hiberniae* (M005766), *M. kumatonense* (2015-00294) and *M. paraafortuitum* (M000996) that could not be identified by the *rpoB* gene sequence emerged in their expected corresponding groupings (Figure 9). This implies that their sequences are accurate. However, they are not available on the NCBI GenBank, hence could not be identified. The names of these strains were obtained based the 16S rRNA gene identification.

The *rpoB* assay was not able to identify forty (56%) of the 71 strains to species level. They were only identified as mycobacterium species (*M. spp*); such as (M010254, 132007, 070247, M002055, 961284, 941342). These strains were scattered among the slow and rapid growers (Annex 11). Again, further phenotypic and/or genotypic testing may be required to determine their species identification. As they are scattered over the 16S- and *rpoB*-based phylogenetic trees, they do not represent a single (possibly new) species.

4.2 Conclusions

The *rpoB* gene sequence has an added value in mycobacterial species identification over 16S rRNA as it was possible to distinguish *M. marinum* (ITM M000140/NCTC 2275) from *M. ulcerans* and *M. montmartrense* (NCBI strain 028/ITM 981197) from *M. peregrinum* and *M. septicum* which was not possible with the latter assay.

However most of the mycobacterial *rpoB* gene sequences are not available on the NCBI website. It is therefore not possible to identify all the mycobacterial species using the GenBank based on *rpoB* sequences. The GenBank is limited only to the genomic data that has been deposited.

4.3 Recommendations

Given the usefulness of the *rpoB* gene sequence in mycobacterial species identification, and the fact that many NTM are increasingly encountered in clinical examinations, it is important to have a full data bank based on the *rpoB* gene sequence as this will improve disease diagnosis and epidemiological research.

It is recommended that sequence data for the strains tested in this study which is not yet on the GenBank be deposited to aid in the identification process. Public collections, such as BCCM/ITM, could play an important role in expanding the *rpoB* sequence repository by submitting their sequences for well documented strains.

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Annex 1: PCR conditions for the 4 kits

In-house kit mix

	Reaction using Taq DNA polymerase (Promega)	50 µl reaction (1 tube reaction) - µl
1.	dH ₂ O	18
2.	Buffer (10x)	25
3.	Q-buffer (20%)	10
3.	dNTPs (10mM)	4
4.	Primer 1 (25µM)	0.5
5.	Primer 2 (25µM)	0.5
6.	Polymerase Taq (5units/µl)	0.2
7.	Template DNA (2 µl sample)	

Qiagen kit mix

	Reaction using HotstarTaq DNA polymerase	50 µl reaction (1 tube reaction) - µl
1.	dH ₂ O	28
2.	Buffer (10x)	5
3.	Q-buffer (20%)	10
3.	dNTPs (10mM)	4
4.	Primer 1 (25 µM)	0.5
5.	Primer 2 (25 µM)	0.5
6.	Polymerase Taq (5units/µl)	0.25
7.	Template DNA (2µl sample)	

Promega kit mix

	Reaction using Colourless GoTaq polymerase	50 µl reaction (1 tube reaction) - µl
1.	dH ₂ O	29.2
2.	Buffer (10x)	10
3.	MgCl ₂ solution (25mM)	3
4.	dNTPs (10mM)	4
5.	Primer 1 (25 µM)	0.8
6.	Primer 2 (25 µM)	0.8
7.	Polymerase Taq (5units/µl)	0.25
8.	Template DNA (2 µl in each tube)	

Thermo kit mix

	Reaction using Phire Hot Start II DNA polymerase	50 µl reaction (1 tube reaction) – µl
1.	dH ₂ O	31
2.	Phire green buffer (5x)	10
3.	dNTPs (10mM)	4
4.	Primer 1 (5µM)	1
5.	Primer 2 (5µM)	1
7.	Taq polymerase (5units/µl)	1
	DNA template (2µl in each tube)	2

PCR conditions for optimizing the kits along a temperature gradient

PCR Kit	Initial temp.*	Hot start **	Denaturation	Annealing	Extension	Final extension
In-house	92°C	95.5°C for 5'	95.5°C for 45"	56-70°C for 45"	72°C for 45"	72°C for 10'
Promega	92°C	95.5°C for 2'	95.5°C for 45"	56-70°C for 45"	72°C for 45"	72°C for 10'
Qiagen	92°C	95.5°C for 15'	95.5°C for 45"	56-70°C for 45"	72°C for 45"	72°C for 10'
Thermo	92°C	98°C for 30"	98°C for 30"	56-70°C for 45"	72°C for 45"	72°C for 10'

The stages of denaturation, annealing and extension are repeated 45 times

The annealing is done along a temperature gradient as follows;

56.0°C, 56.6°C, 58.0°C, 59.9°C, 62.0°C, 64.0°C, 66.1°C, 68.0°C, 69.4°C and 70.0°C

*template DNA is added: **DNA template denaturation and enzyme activation

Kits compared at the most appropriate annealing temperature

PCR Kit	Initial temp.*	Hot start **	Denaturation	Annealing	Extension	Final extension
In-house	92°C	95.5°C for 5'	95.5°C for 45"	72°C for 45"	72°C for 45"	72°C for 10'
Promega	92°C	95.5°C for 2'	95.5°C for 45"	72°C for 45"	72°C for 45"	72°C for 10'
Qiagen	92°C	95.5°C for 15'	95.5°C for 45"	72°C for 45"	72°C for 45"	72°C for 10'
Thermo	92°C	98°C for 30"	98°C for 30"	72°C for 45"	72°C for 45"	72°C for 10'

The stages of denaturation, annealing and extension are repeated 45 times

*template DNA is added: **DNA template denaturation and enzyme activation

Annex 3: List of reference mycobacterial strains used for the characterisation of the *rpoB* assay

S.no.	Isolate nr (CULT)	Biological Origin	Identification	Other culture collection number
1	M9016		<i>M. avium</i>	ATCC 25291
2	C05142	Human	<i>M. ulcerans</i>	ATCC 19423
3	083715	Human	<i>M. tuberculosis</i>	ATCC 27294; H37Rv; TMC 102
4	M003300	Unknown	<i>M. rhodesiae</i>	ATCC 27024; NCTC 10779
5	C04988	Unknown	<i>M. scrofulaceum</i>	CIPT 140220031
6	M010691	Unknown	<i>M. smegmatis</i>	ATCC 11759
7	C01830	Unknown	<i>M. sphagni</i>	ATCC 33026
8	C01831	Unknown	<i>M. sphagni</i>	ATCC 33027
9	M001686	Unknown	<i>M. terrae</i>	ATCC 25113
10	M001718	Unknown	<i>M. terrae</i>	ATCC 25146
11	M001179	Unknown	<i>M. triviale</i>	ATCC 23292
12	C07736	Unknown	<i>M. triviale</i>	ATCC 23292; CIPT 140330002
13	M007001	Environment	<i>M. thermoresistibile</i>	NCTC 10409; ATCC 19527;316; DSM 44167
14	M001702	Unknown	<i>M. intracellulare</i>	ATCC 25131
15	M001741	Unknown	<i>M. intracellulare</i>	ATCC 25171
16	C01828	Environment	<i>M. komossense</i>	ATCC 33013
17	M000140	Animal	<i>M. marinum</i>	NCTC 2275
18	M002379	Environment	<i>M. neoaurum</i>	ATCC 25795
19	M001682	Unknown	<i>M. nonchromogenicum</i>	ATCC 19530
20	M000996	Environment	<i>M. parafortuitum</i>	ATCC 19686
21	C02666	Animal	<i>M. paratuberculosis</i>	ATCC 19698
22	M001006	Human	<i>M. abscessus</i>	ATCC 23006
23	M001007	Human	<i>M. abscessus</i>	ATCC 23007
24	M001040	Unknown	<i>M. abscessus</i>	ATCC 23040
25	M001045	Human	<i>M. abscessus</i>	ATCC 23045
26	M006993	Environment	<i>M. aurum</i>	ATCC 10437; NCTC 10437
27	M002373	Environment	<i>M. aurum</i>	ATCC 23366
28	M002386	Environment (Soil)	<i>M. chitae</i>	ATCC 19628
29	M000995	Animal (Cow)	<i>M. fortuitum</i>	ATCC 19542
30	M006995	Human	<i>M. gilvum</i>	NCTC 10942

Annex 4: List of non-mycobacterial strains used to test specificity of the rpoB assay

Isolate nr	Biological Origin	Identification	Other culture collection number
M008189	Human	<i>Corynebacterium</i>	
M008190	Human	<i>Corynebacterium</i>	
C01817	Human	<i>Corynebacterium</i>	
M008273		<i>Nocardia amarea</i>	
M005352	Animal	<i>Nocardia asteroides</i>	
M008361		<i>Nocardia asteroides</i>	
C02135	Human	<i>Nocardia asteroides</i>	
M008277		<i>Nocardia carnea</i>	
M008297		<i>Nocardia caviae</i>	
M005417		<i>Nocardia farcinica</i>	ATCC 3318
M008283		<i>Nocardia otitidis-cavarium</i>	
M008299		<i>Nocardia paraguayensis</i>	
M008279		<i>Nocardia salmonicida</i>	
M008280		<i>Nocardia transvalensis</i>	
M008282		<i>Nocardia vaccinii</i>	
M003341		<i>Rhodococcus</i>	ATCC 271
M003343		<i>Rhodococcus</i>	ATCC 4001
M003344	Environment	<i>Rhodococcus</i>	ATCC 4004
M000997		<i>Rhodococcus</i>	ATCC 13808
M003346		<i>Rhodococcus</i>	ATCC 4276

Annex 5: Sequence results for 30 mycobacterial strains used to characterise the rpoB assay

P- number	Isolater number (CULT)	Biological Origin	Identification (SS rRNA)	Other culture collection number	Query length	Identities (Row data)	Identities (Corrected)	% identities	Identification by rpoB (Blastn)	M	Remarks
16-936	M001007	Human	<i>M. abscessus</i>	ATCC 23007	750	736/736		100	<i>M. abscessus</i>		Identified
16-937	M001040	Unknown	<i>M. abscessus</i>	ATCC 23040	761	738/738		100	<i>M. abscessus</i>		Identified
16-938	M001045	Human	<i>M. abscessus</i>	ATCC 23045	844	738/738		100	<i>M. abscessus</i>		Identified
16-939	M006993	Environment	<i>M. aurum</i>	ATCC 10337; NCTC 10437	770	644/644		100	<i>M. aurum</i>		Identified
16-940	M002373	Environment	<i>M. aurum</i>	ATCC 23366	769	643/644	643/643	100	<i>M. aurum</i>		Identified
16-941	M002386	Environment (Soil)	<i>M. chitae</i>	ATCC 19628	770	704/704		100	<i>M. chitae</i>		Identified
16-945	M001702	Unknown	<i>M. intracellulare</i>	ATCC 25131	762	738/738		100	<i>M. intracellulare</i>		Identified
16-946	M001741	Unknown	<i>M. intracellulare</i>	ATCC 25171	762	749/750	750/750	100	<i>M. intracellulare</i>		Identified
16-948	M000140	Animal	<i>M. marinum</i>	NCTC 2275	775	743/744	743/743	100	<i>M. marinum</i>		Identified
16-949	M002379	Environment	<i>M. neoaurum</i>	ATCC 25795	767	714/714		100	<i>M. neoaurum</i>		Identified
16-950	M001682	Unknown	<i>M. nonchromogenicum</i>	ATCC 19530	756	707/707		100	<i>M. nonchromogenicum</i>		Identified
16-952	C03666	Animal	<i>M. paratuberculosis</i>	ATCC 19698	765	738/738		100	<i>M. avium subsp. paratuberculosis</i>		Identified
16-953	M003300	Unknown	<i>M. rhodesiae</i>	ATCC 27024; NCTC 10729	772	704/706	705/705	100	<i>M. rhodesiae</i>		Identified
16-954	C04988	Unknown	<i>M. scrofulaceum</i>	CIP11402/20031	770	692/700	700/700	100	<i>M. scrofulaceum</i>		Identified
16-955	M010691	Unknown	<i>M. smegmatis</i>	ATCC 11759	770	745/747	747/747	100	<i>M. smegmatis</i>		Identified
16-959	M001718	Unknown	<i>M. terrae</i>	ATCC 25146	771	699/699		100	<i>M. terrae</i>		Identified
16-960	M001179	Unknown	<i>M. trivale</i>	ATCC 23292	778	680/683	681/681	100	<i>M. trivale</i>		Identified
16-962	83715	Human	<i>M. tuberculosis</i>	ATCC 27294; H37Rv; TMC 102	771	742/745	744/744	100	<i>M. tuberculosis</i>		Identified
16-963	C05142	Human	<i>M. ulcerans</i>	ATCC 19433	771	748/748		100	<i>M. ulcerans</i>		Identified
16-964	M9016		<i>M. avium</i>	ATCC 25291	759	733/736	735/735	100	<i>M. avium</i>		Identified
16-942	M000995	Animal (Cow)	<i>M. fortuitum</i>	ATCC 19542	768	749/754	750/752	99	<i>M. fortuitum</i> subsp. <i>fortuitum</i>	2	Mix <i>M. fortuitum</i> + <i>M. fortuitum</i> -like
16-943	M006995	Human	<i>M. gilvum</i>	NCTC 10042	791	770/773	770/771	99	<i>M. gilvum</i>	1	Mix <i>M. gilvum</i> + <i>M. gilvum</i> -like
16-944	M007001	Environment	<i>M. thermoresistibile</i>	NCTC 10409; ATCC 19327; 316; DSM 44167	770	693/698	693/696	99	<i>M. thermoresistibile</i>	3	M. thermoresistibile + M. thermoresistibile-like
16-935	M001006	Human	<i>M. abscessus</i>	ATCC 23006	763	733/735	733/735	99	<i>M. abscessus</i>	2	Mix <i>M. abscessus</i> + M. abscessus-like
16-958	M001686	Unknown	<i>M. terrae</i>	ATCC 25113	756	741/742	741/742	99	<i>M. terrae</i>	1	Mix <i>M. terrae</i> + <i>M. terrae</i> -like
16-947	C01828	Environment	<i>M. komossense</i>	ATCC 33013	773	668/746		90	<i>M. spp</i>	>3	No identification
16-951	M000996	Environment	<i>M. parafortuitum</i>	ATCC 19886	759	696/704		99	<i>M. spp</i>	>3	No identification
16-956	C01830	Unknown	<i>M. spoliophila</i>	ATCC 33026	766	694/747		93	<i>M. spp</i>	>3	No identification
16-957	C01831	Unknown	<i>M. spoliophila</i>	ATCC 33027	852	696/748		93	<i>M. spp</i>	>3	No identification
16-961	C07736	Unknown	<i>M. trivale</i>	ATCC 23292; CIP11403/30002	767	685/690		99	<i>M. spp</i>	>3	No identification

Annex 6: Sequence results for repeatability and reproducibility

Sequencing results for repeatability of the *rpoB* assay

P-number	Isolate/ Collection nr	Identification by 16S rRNA	Query length	Identities (Raw data)	Identities (Corrected)	% Identities	Identification by <i>rpoB</i> (Blastn)	Remarks
165392	C05142/ ATCC19423	<i>M. ulcerans</i>	768	750/750		100	<i>M. ulcerans</i>	No gaps
165393	C05142/ ATCC19423	<i>M. ulcerans</i>	768	751/753	753/753	100	<i>M. ulcerans</i>	No gaps
165394	C05142/ ATCC19423	<i>M. ulcerans</i>	771	751/752	751/751	100	<i>M. ulcerans</i>	No gaps
165395	M001007/ ATCC 23006	<i>M. abscessus</i>	762	748/751	748/748	100	<i>M. abscessus</i>	No gaps
165396	M001007/ ATCC 23006	<i>M. abscessus</i>	761	738/738		100	<i>M. abscessus</i>	No gaps
165397	M001007/ ATCC 23006	<i>M. abscessus</i>	760	738/738		100	<i>M. abscessus</i>	No gaps

Sequencing results for reproducibility of the *rpoB* assay performed by 2 operators; A and B

P-number	Isolate/ Collection nr	Identification by 16S rRNA	Query length	Identities (Raw)	Identities (Corrected)	% identities	Identification by <i>rpoB</i> (Blastn)	Remarks
				Operator A (1 st run)				
165398	C05142/ ATCC19423	<i>M. ulcerans</i>	771	746/746		100	<i>M. ulcerans</i>	No gaps
165399	C05142/ ATCC19423	<i>M. ulcerans</i>	771	752/753	752/752	100	<i>M. ulcerans</i>	No gaps
165400	C05142/ ATCC19423	<i>M. ulcerans</i>	768	752/754	752/752	100	<i>M. ulcerans</i>	No gaps
165401	M001007/ ATCC 23006	<i>M. abscessus</i>	747	744/746	744/744	100	<i>M. abscessus</i>	No gaps
165402	M001007/ ATCC 23006	<i>M. abscessus</i>	759	738/738		100	<i>M. abscessus</i>	No gaps
165403	M001007/ ATCC 23006	<i>M. abscessus</i>	762	738/738		100	<i>M. abscessus</i>	No gaps
				Operator A (2 nd run)				
165404	C05142/ ATCC19423	<i>M. ulcerans</i>	768	752/756	754/754	100	<i>M. ulcerans</i>	No gaps
165405	C05142/ ATCC19423	<i>M. ulcerans</i>	769	757/760	757/757	100	<i>M. ulcerans</i>	No gaps
165406	C05142/ ATCC19423	<i>M. ulcerans</i>	770	749/749		100	<i>M. ulcerans</i>	No gaps
165407	M001007/ ATCC 23006	<i>M. abscessus</i>	761	742/743	742/742	100	<i>M. abscessus</i>	No gaps
165408	M001007/ ATCC 23006	<i>M. abscessus</i>	753	748/751	748/748	100	<i>M. abscessus</i>	No gaps
165409	M001007/ ATCC 23006	<i>M. abscessus</i>	760	738/738		100	<i>M. abscessus</i>	No gaps
P-number	Isolate/ Collection nr	Identification by 16S rRNA	Query length	Identities (Raw)	Identities (Corrected)	% identities	Identification by <i>rpoB</i>	Remarks

				Operator B (1 st run)				
165410	C05142/ ATCC19423	<i>M. ulcerans</i>	772	748/748		100	<i>M. ulcerans</i>	No gaps
165411	C05142/ ATCC19423	<i>M. ulcerans</i>	769	747/747		100	<i>M. ulcerans</i>	No gaps
165412	C05142/ ATCC19423	<i>M. ulcerans</i>	771	751/752	751/751	100	<i>M. ulcerans</i>	No gaps
165413	M001007/ ATCC 23006	<i>M. abscessus</i>	763	738/738		100	<i>M. abscessus</i>	No gaps
165414	M001007/ ATCC 23006	<i>M. abscessus</i>	751	742/742		100	<i>M. abscessus</i>	No gaps
165415	M001007/ ATCC 23006	<i>M. abscessus</i>	762	738/738		100	<i>M. abscessus</i>	No gaps
				Operator B (2 nd run)				
165416	C05142/ ATCC19423	<i>M. ulcerans</i>	770	741/742	742/742	100	<i>M. ulcerans</i>	No gaps
165417	C05142/ ATCC19423	<i>M. ulcerans</i>	770	758/760	758/758	100	<i>M. ulcerans</i>	No gaps
165418	C05142/ ATCC19423	<i>M. ulcerans</i>	767	755/760	758/758	100	<i>M. ulcerans</i>	No gaps
165419	M001007/ ATCC 23006	<i>M. abscessus</i>	748	739/739		100	<i>M. abscessus</i>	No gaps
165420	M001007/ ATCC 23006	<i>M. abscessus</i>	751	739/739		100	<i>M. abscessus</i>	No gaps
165421	M001007/ ATCC 23006	<i>M. abscessus</i>	760	738/738		100	<i>M. abscessus</i>	No gaps

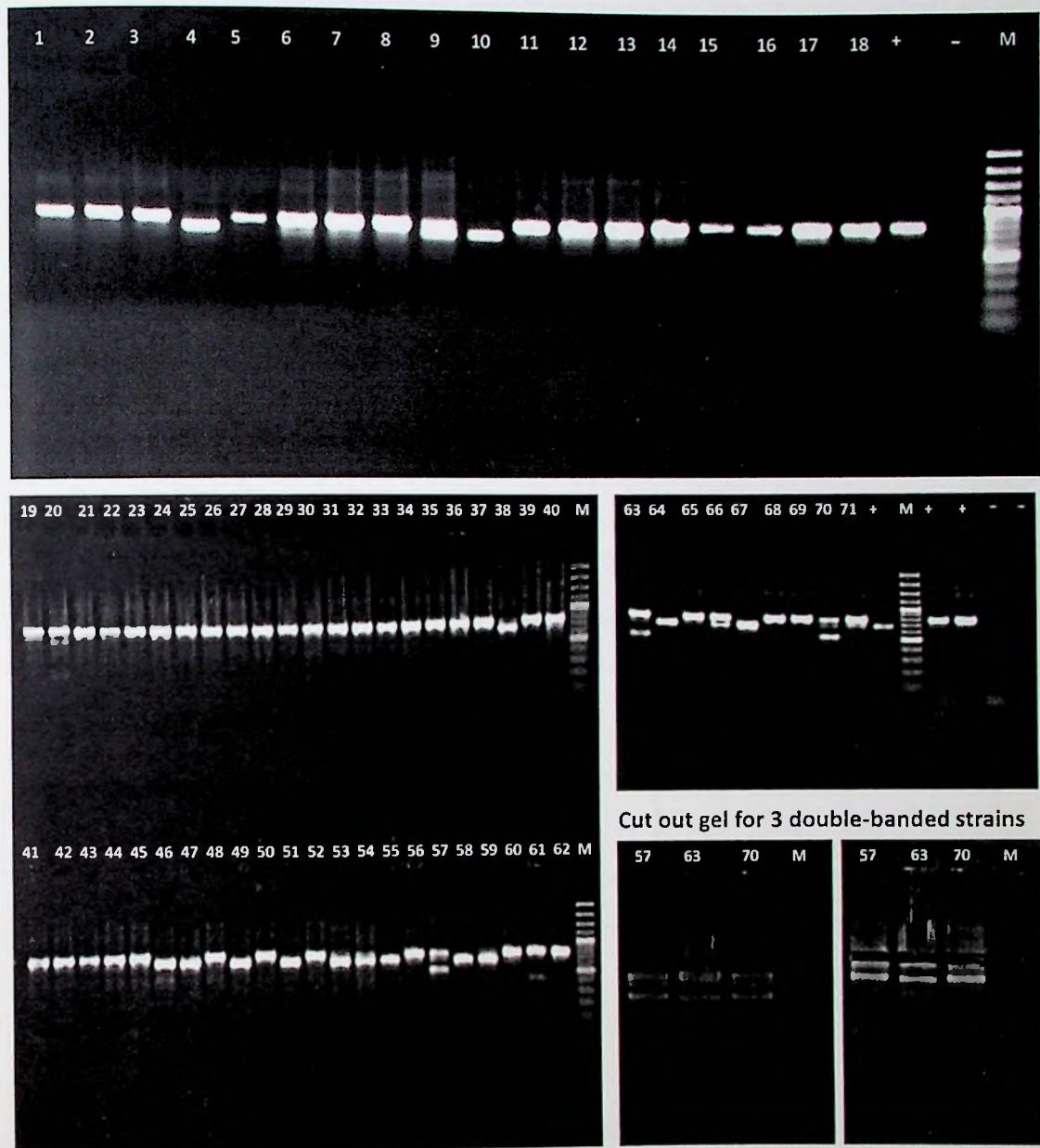
Annex 7: List of mycobacterial strains from ITM used for evaluation of the rpoB assay

S. No	Culture number	p-number	Species identity (16S rRNA)
1	072501	164116	<i>M. celatum</i>
2	2015-00294	164644	<i>M. kumatonense</i>
3	070247	155525	<i>M. celatum</i>
4	132007	155186	<i>M. interjectum</i>
5	960372	153513	<i>M. avium</i>
6	960308	153512	<i>M. avium</i>
7	960234	153511	<i>M. avium</i>
8	C05983	141272	<i>M. avium</i>
9	132008	150279	<i>M. lentiflavum</i>
10	M010254	150010	<i>M. avium</i>
11	C07971	141388	<i>M. chelonae</i>
12	C05921	142306	<i>M. intracellulare</i>
13	C04429	163735	<i>M. avium</i>
14	C01193	163735	<i>M. paraense</i>
15	981197	163872	<i>M. septicum</i>
16	M002656	152020	<i>M. chelonae</i>
17	070145	165301	<i>M. abscessus</i>
18	M001521	130515	<i>M. chelonae</i>
19	M001702	120454	<i>M. intracellulare</i>
20	M005766	122723	<i>M. lactis</i>
21	M001967	122725	<i>M. chelonae</i>
22	M001827	122775	<i>M. gastri</i>
23	M002321	122841	<i>M. kansasii</i>
24	M002055	122850	<i>M. diernhoferi</i>
25	970462	123062	<i>M. peregrinum</i>
26	960892	131516	<i>M. chelonae</i>
27	961284	131519	<i>M. chelonae</i>
28	940717	131975	<i>M. chelonae</i>
29	941342	132084	<i>M. chelonae</i>

30	981295	132188	<i>M. heidelbergense</i>
31	940332	132375	<i>M. chelonae</i>
32	940973	132376	<i>M. chelonae</i>
33	941332	132377	<i>M. chelonae</i>
34	C07701	132660	<i>M. chelonae</i>
35	950066	132661	<i>M. chelonae</i>
36	C00789	132675	<i>M. scrofulaceum</i>
37	961457	132730	<i>M. chelonae</i>
38	C01412	132741	<i>M. malmoense</i>
39	C04306	132974	<i>M. fortuitum</i> or <i>M. peregrinum</i>
40	C04988	133088	<i>M. scrofulaceum</i>
41	130005	140243	<i>M. chimaera</i>
42	130444	140245	<i>M. chimaera</i>
43	C05908	140354	<i>M. intracellulare</i>
44	C05920	140355	<i>M. intracellulare</i>
45	C05915	140357	<i>M. intracellulare</i>
46	M004528	140536	<i>M. intracellulare</i> -like
47	M004644	140538	<i>M. avium</i> complex-like
48	M004761	140540	<i>M. spp</i>
49	M004769	140541	<i>M. avium</i> complex-like
50	M005085	140544	<i>M. avium</i> -like
51	M005086	140545	<i>M. avium</i> -like
52	M004874	140546	<i>M. spp</i>
53	M005090	140548	<i>M. avium</i> complex
54	M005094	140554	<i>M. avium</i> complex
55	M005175	140555	-
56	M005177	140556	<i>M. spp</i>
57	M005189	140560	<i>M. avium</i> complex-like
58	M005191	140563	<i>M. avium</i> complex-like
59	M005229	140565	<i>M. avium</i> complex-like
60	130965	140572	<i>M. marseillense</i>
61	040353	140619	<i>M. paraterrae</i>

62	130545	140626	<i>M. intracellulare</i>
63	040538	140722	<i>M. celatum</i>
64	081344	140723	<i>M. angelicum</i>
65	C04975	142415	<i>M. abscessus</i>
66	M003260	143092	<i>M. Habana</i>
67	C04754	143104	<i>M. lufu</i>
68	M001006	151831	<i>M. abscessus</i>
69	M005791	132669	<i>M. species</i>
70	M005189	140561	<i>M. spp</i>
71	M1686	155523	<i>M. hinense/M. algericum</i>

Annex 8: Results of gel electrophoresis of the 71 strains used to evaluate the rpoB assay, M - molecular marker



Annex 9: Sequence results for 71 mycobacterial strains from ITM used for evaluation of the rpoB assay

Culture number	p-number	Position	Species (16S rRNA)	Query length	Identities (Raw)	Identities (Corrected)	% Identities	N	Identity (rpoB)	Remarks
960372	165378	TE8J20	<i>M. avium</i>	764	735/735		100		<i>M. avium</i>	Identified
960308	165379	TE8J19	<i>M. avium</i>	751	738/738		100		<i>M. avium</i>	Identified
960234	165380	TE8J18	<i>M. avium</i>	749	738/738		100		<i>M. avium</i>	Identified
C05921	165385	TE4O6	<i>M. intracellulare</i>	762	749/750	749/749	100		<i>M. intracellulare</i>	Identified
C04429	165386	TE3I4	<i>M. avium</i>	750	739/739		100		<i>M. avium</i> subsp. <i>avium</i>	Identified
981197	165388	TE12H24	<i>M. septicum</i> / <i>M. peregrinum</i>	780	723/723		100		<i>M. montmartrense</i>	Identified
M002656	165389	TE7 Q18	<i>M. chelonae</i>	751	738/739	739/739	100		<i>M. chelonae</i>	Identified
070145	165390	TE12P20	<i>M. abscessus</i>	759	750/751	750/750	100		<i>M. abscessus</i>	Identified
970462	166206		<i>M. peregrinum</i>	812	744/745	744/744	100		<i>M. peregrinum</i>	Identified
960892	166207		<i>M. chelonae</i>	759	693/694	693/693	100		<i>M. chelonae</i>	Identified
940717	166209		<i>M. chelonae</i>	747	737/738	737/737	100		<i>M. chelonae</i>	Identified
C04988	166221		<i>M. scrofulaceum</i>	769	702/702		100		<i>M. scrofulaceum</i>	Identified
130005	166222		<i>M. chimera</i>	761	736/736		100		<i>M. chimera</i>	Identified
C05908	166224		<i>M. intracellulare</i>	758	735/735		100		<i>M. intracellulare</i>	Identified

C05920	166225		<i>M. intracellulare</i>	758	736/736		100		<i>M. intracellulare</i>	Identified
C05915	166226		<i>M. intracellulare</i>	757	736/736		100		<i>M. intracellulare</i>	Identified
C04975	166246		<i>M. abscessus</i>	862	738/738		100		<i>M. abscessus</i>	Identified
C05983	165381	TE3E12	<i>M. avium</i>	761	740/744	740/742	99	2	<i>M. avium</i>	Mix <i>M. avium</i> + <i>M. avium</i> -like
M001521	165391	-	<i>M. chelonae</i>	711	690/695	691/694	99	3	<i>M. chelonae</i>	Mix <i>M. chelonae</i> + <i>M. chelonae</i> -like
M001702	166200		<i>M. intracellulare</i>	761	735/736		99	1	<i>M. intracellulare</i>	<i>M. Intracellulare</i> -like
M005766	166201		<i>M. lacticis</i>	768	700/702	700/701	99	1	<i>M. hiberniae</i>	<i>M. hiberniae</i> -like
M001967	166202		<i>M. chelonae</i>	749	690/694	690/693	99	3	<i>M. chelonae</i>	<i>M. chelonae</i> -like
981295	166211		<i>M. heidelbergense</i>	761	701/704		99	3	<i>M. heidelbergense</i>	<i>M. heidelbergense</i>
C07701	166215		<i>M. chelonae</i>	750	736/738	736/737	99	1	<i>M. chelonae</i>	<i>M. chelonae</i> -like
950066	166216		<i>M. chelonae</i>	760	694/696	694/695	99	1	<i>M. chelonae</i>	<i>M. chelonae</i> -like
130444	166223		<i>M. chimera</i>	761	736/737		99	1	<i>M. chimera</i>	<i>M. chimera</i> -like
M1686	166252		<i>M. hinense/M. algericum</i>	769	748/750	748/749	99	1	<i>M. sinense</i>	<i>M. sinense</i> -like
M003260	166247	L292	<i>M. habana</i>	750	735/737	735/736	99	1	<i>M. simiae</i>	<i>M. simiae</i> -like
130545	166243		<i>M. intracellulare</i>	760	741/744	741/742	99	1	<i>M. chimera/M. intracellulare</i>	<i>M. chimera</i> -like/ <i>M. intracellulare</i> -like
M001006	166249		<i>M. abscessus</i>	759	733/735		99	2	<i>M. abscessus</i>	<i>M. abscessus</i> -like
130965	166241		<i>M. marseillense</i>	765	731/739	731/732	99	1	<i>M. chimera</i>	<i>M. chimera</i> -like
072501	165374	TE12K07	<i>M. celatum</i>	918	711/732		97	>3	<i>M. spp</i>	No identification
2015-00294	165375	TE12N27	<i>M. kumatonense</i>	775	697/704		99	>3	<i>M. spp</i>	No identification
070247	165376	TE9D18	<i>M. celatum</i>	748	718/736		98	>3	<i>M. spp</i>	No identification

132007	165377	TE8Q27	<i>M. paraense</i>	774	672/700		96	>3	<i>M. spp</i>	No identification
132008	165382	TE3M22	<i>M. palustre</i>	769	680/703		97	>3	<i>M. spp</i>	No identification
M010254	165383	TE3M4	<i>M. avium</i>	914	6610/627		97	>3	<i>M. spp</i>	No identification
C07971	165384	TE4O15	<i>M. chelonae</i>	764	643/696		92	>3	<i>M. spp</i>	No identification
C01193	165387	TE12G12	<i>M. paraense</i>	769	684/699		98	>3	<i>M. spp</i>	No identification
M001827	166203	L596	<i>M. gastri</i>	762	682/695		98	>3	<i>M. spp (M. angelicum)</i>	No identification
M002321	166204		<i>M. kansasii</i>	748	676/678	676/676	100		<i>M. spp</i>	No identification
M002055	166205	L84	<i>M. diemhoferi</i>	749	723/733		99	>3	<i>M. spp</i>	No identification
961284	166208		<i>M. chelonae</i>	747	723/739		98	>3	<i>M. spp</i>	No identification
941342	166210		<i>M. chelonae</i>	759	721/737		98	>3	<i>M. spp</i>	No identification
940332	166212		<i>M. chelonae</i>	761	723/739		99	>3	<i>M. spp (M. chelonae)</i>	No identification
940973	166213		<i>M. chelonae</i>	748	723/739		98	>3	<i>M. spp (M. chelonae)</i>	No identification
941332	166214		<i>M. chelonae</i>							Bad sequence
C00789	166217	L2475	<i>M. scrofulaceum</i>	749	675/695		97	>3	<i>M. spp (M. europaeum)</i>	No identification
961457	166218		<i>M. chelonae</i>	764	722/738		98	>3	<i>M. spp (M. chelonae)</i>	No identification
C01412	166219	L3104	<i>M. malmoense</i>	764	664/697		95	>3	<i>M. spp (M. paraense)</i>	No identification
C04306	166220		<i>M. peregrinum</i>	754	667/674		99	>3	<i>M. spp (M. arceutense)</i>	No identification
M004528	166227	L278	<i>M. intracellulare-like</i>	794	596/642		93	>3	<i>M. spp (M. paraense)</i>	No identification

M004644	166228	L182	<i>M. avium complex-like</i>	765	617/632		98	>3	<i>M. spp (M. bochedurhonense)</i>	No identification
M004761	166229	L538	<i>M. spp</i>	749	677/740		91	>3	<i>M. spp (M. celatum)</i>	No identification
M004769	166230	L555	<i>M. avium complex-like</i>	763	613/631		97	>3	<i>M. spp (M. bochedurhonense)</i>	No identification
M005085	166231	L320	<i>M. avium-like</i>	770	643/703		91	>3	<i>M. spp</i>	No identification
M005086	166232	L221	<i>M. avium-like</i>	763	615/633		97	>3	<i>M. spp (M. avium complex)</i>	No identification
M004874	166233	L270	<i>M. spp</i>	751	679/741		92	>3	<i>M. spp (M. celatum)</i>	No identification
M005090	166234	L222	<i>M. avium complex</i>	763	614/627		98	>3	<i>M. spp (M. avium complex)</i>	No identification
M005094	166235	L225	<i>M. avium complex</i>	771	618/641		96	>3	<i>M. spp (M. avium complex)</i>	No identification
M005175	166236	L96	-	760	575/637		90	>3	<i>M. spp</i>	No identification
M005177	166237	L230	<i>M. spp</i>	750	683/738		93	>3	<i>M. spp (M. celatum)</i>	No identification
M005189	166238	L556	<i>M. avium complex-like</i>	757	712/748		95	>3	<i>M. spp (M. chimera)</i>	No identification
M005191	166239	L180	<i>M. avium complex-like</i>	775	614/629		98	>3	<i>M. spp (M. avium complex)</i>	No identification
M005229	166240	L194	<i>M. avium complex-like</i>	769	617/630		98	>3	<i>M. spp (M. avium complex)</i>	No identification
040353	166242		<i>M. paraterrae</i>	756	696/741		94	>3	<i>M. spp (M. sinense)</i>	No identification
040538	166244		<i>M. celatum</i>	768	700/759		92	>3	<i>M. spp (M. sinense)</i>	No identification
081344	166245		<i>M. angelicum</i>	1042	622/639		97	>3	<i>M. spp</i>	No identification
C04754	166248	L306	<i>M. lufu</i>	764	626/637		98	>3	<i>M. spp</i>	No identification
M005791	166250	L330	<i>M. spp</i>	767	693/755		92	>3	<i>M. spp</i>	No identification
M005189	166251	L513	<i>M. spp</i>	763	711/750		95	>3	<i>M. spp</i>	No identification

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Phylogenetic tree for 16S rRNA sequence of the strains used in test evaluation

