UNCOVERING GENETIC DETERMINANTS OF ANTIBIOTIC RESISTANCE IN *MYCOBACTERIUM ABSCESSUS*

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UNCOVERING GENETIC DETERMINANTS OF ANTIBIOTIC RESISTANCE IN *MYCOBACTERIUM ABSCESSUS*

By

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ABSTRACT

UNCOVERING GENETIC DETERMINANTS OF ANTIBIOTIC RESISTANCE IN *Mycobacterium abscessus*

Sharmilla Devi Jayasingam

Mycobacterium abscessus (Mab) is an emerging human pathogen notorious for its resistance to anti-mycobacterial drugs. Rapid and accurate determination of resistance is needed to deter the further emergence of resistance. One advancement in the molecular detection of antibiotic resistance is the *in silico* prediction of resistance-associated genes with the use of whole genome sequencing (WGS). This study aims to determine the antibiotic resistance pattern in Mab subspecies and to identify gene mutations associated with the resistance. Fifty-one Mab strains isolated from Malaysian patients were examined with Etest strips to determine their susceptibility to five antibiotics, namely, amikacin, clarithromycin, ciprofloxacin, imipenem, and linezolid. PCR-sequencing was used to amplify previously reported resistance-associated genes, while WGS data was imported into three online antibiotic resistance gene (ARG) databases to search for more resistance-associated genes. The putative genes predicted were then analyzed by multiple sequence alignment (MSA) of genes from susceptible and resistant phenotypes to confirm genotypic-phenotypic correlation and identify possible novel resistance-associated

mutations. The overall resistance rates of the Mab isolates were 0%, 6%, 22%, 33% and 39% to amikacin, clarithromycin, linezolid, imipenem and ciprofloxacin, respectively. Both amikacin and clarithromycin susceptibilities were in accordance with the mutations observed in the *rrs*, *erm*(41) and *rrl* genes. Mutations in the 23S rRNA previously reported to be associated with linezolid resistance were not found in any of the strains examined and imipenem resistance was not correlated with mutations in the Bla_{Mab} gene that encodes a carbapenemase in Mab. Although none of the ciprofloxacin resistant strains had the mutations in *gyr*A and *gyr*B reported by other researchers, the present study showed a strong correlation between ciprofloxacin resistance and mutations in *efr*A and *qep*A2, two genes associated with drug efflux pumps, that have not been reported in Mab. Although WGS facilitated the prediction of resistance genes and mechanisms, an increased knowledge on new resistance-associated genes or mechanisms in Mab is needed to further consolidate the genotypic-phenotypic correlation in this study.

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" If I have seen further, it is by standing on the shoulders of giants "

-Isaac Newton

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-Sharmilla

APPROVAL SHEET

This dissertation/thesis entitled "**UNCOVERING GENETIC DETERMINANTS OF ANTIBIOTIC RESISTANCE IN *Mycobacterium abscessus*"** was prepared by SHARMILLA DEVI JAYASINGAM and submitted as partial fulfillment of the requirements for the degree of Master of Medical Sciences at Universiti Tunku Abdul Rahman.

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Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that Sharmilla Devi Jayasingam (ID No: 16UMM06814) has completed this dissertation entitled "UNCOVERING GENETIC DETERMINANTS OF ANTIBIOTIC RESISTANCE IN *Mycobacterium abscessus*" under the supervision of Prof. Dr. Ngeow Yun Fong (Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences, and A. Prof. Dr. Thaw Zin (Co-Supervisor) from the Department of Pre-Clinical Sciences, Faculty of Medicine and Health Sciences.

I understand that the University will upload a softcopy of my dissertation in pdf format into UTAR Institutional Repository, which may be made accessible to UTAR community and the public.

Yours truly,

_____
(Sharmilla Devi Jayasingam)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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SHARMILLA DEVI JAYASINGAM

Date _____

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LIST OF ABBREVIATIONS

aa	Amino acid
AFB	Acid fast bacilli
Amk	Amikacin
AMR	Antimicrobial resistance
ARG	Antibiotic resistant genes
ARO	Antibiotic resistance ontology
AST	Antibiotic susceptibility testing
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSC	Biological safety cabinet
CF	Cystic fibrosis
Cip	Ciprofloxacin
Cla	Clarithromycin
CNS	Central nervous system
CO_2	Carbon dioxide
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
DW	Distilled water

ESBL	extended spectrum beta-lactamase
Erm	Erythromycin ribosomal methylase
FQ	Fluoroquinolone
gyr	gyrase
HGT	Horizontal gene transfer
HIV	Human immunodeficiency virus
I	Intermediate
IMP	Imipenemase
Imp	Imipenem
Lzd	Linezolid
Mab	*Mycobacterium abscessus* complex
MBL	Metallo- beta- lactamase
MIC	Minimum inhibitory concentration
NDM	New Delhi metallo- beta-lactamase
NGS	Next Generation Sequencing
NTM	Non-tuberculous mycobacteria
NC	Negative control
nt	Nucleotide
PBP	Penicillin binding protein
PC	Positive control
PCR	Polymerase chain reaction
R	Resistant

RC	Reagent control
RFLP	Restriction fragment length polymorphism
RGM	Rapid growing mycobacteria
rRNA	ribosomal RNA
RNA	ribonucleic acid
rpm	rotations per minute
S	Susceptible
SGM	Slowly growing mycobacteria
SNP	Single nucleotide polymorphism
SSTI	Skin and soft tissue infections
TBE	Tris Borate EDTA
TSB	Tryptic soy broth
VIM	Verona imipenemase
WGS	Whole Genome Sequencing
ZN	Ziehl–Neelsen

CHAPTER 1

INTRODUCTION

Mycobacterium abscessus (Mab) is an emerging human pathogen associated with both superficial and deep infections in immunocompromised individuals. It is responsible for about 65-80% of lung diseases caused by the rapidly growing mycobacteria (RGM) (Jeon *et al*, 2009) and is notorious for its resistance to the standard anti-tuberculous drugs and multiple antibiotics, including those commonly used for the treatment of RGM infections. Macrolides (clarithromycin and azithromycin), aminoglycosides, fluoroquinolones, imipenem, tigecycline, cefoxitin and linezolid are among the antibiotics recommended for susceptibility testing by the Clinical and Laboratory Standards Institute (CLSI, 2011).

Owing to the limited options for treatment and the poor clinical response seen in patients with Mab infections, there comes a critical need to determine the resistance profile of Mab isolates in a fast and accurate manner. Conventional methods (disk diffusion/ microbroth dilution) are time-consuming and labour-intensive. Thus, a quick and reliable molecular method of detection is necessary in most diagnostic laboratories. Common molecular methods include restriction fragment length polymorphism (RFLP), polymerase chain reaction (PCR) and

hybridization-based macro- and micro-arrays (Woodford and Sunsfjord, 2005). These molecular methods not only provide rapid results, but also require less hands-on time, and are becoming more affordable even in less affluent countries. Most importantly, rapid detection enables early appropriate treatment of these patients.

On the other hand, molecular methods are not without limitations. A bacterium with a positive genotypic result for an antibiotic resistance could still be phenotypically susceptible to the antibiotic if the resistance gene was not expressed. Besides, diagnostic molecular tests can only identify known mechanisms of resistance. Genuinely novel mechanisms will be missed.

One alternative to molecular identification of resistance is the *in silico* prediction of resistance-associated genes using whole genome sequencing (WGS) analysis. With the decreasing cost of sequencing technology, WGS data can now be obtained for many bacterial isolates for timely clinical applications (Metzker, 2010). The availability of WGS data has assisted in the genetic determinants of drug resistance in many established antibiotics and is helpful in terms of in-depth studies on bacteria (Ng and Kirkness, 2010). However, its usefulness is still unproven for recently introduced drugs and in less studied species, such as the Mab.

Hypothesis:

The spectrum and level of antibiotic resistance in *Mycobacterium abscessus* complex is determined by the genotype of Mab.

Aim:

To describe the spectrum and level of antibiotic resistance in clinical isolates of Mab subspecies from Malaysia, and to define the genetic basis of the phenotypic resistance observed.

Objectives:

I. To determine the spectrum and level of antibiotic resistance in Mab subspecies isolated in Malaysia
II. To identify gene mutations associated with phenotypic antibiotic resistance in these isolates
III. To correlate genotypic and phenotypic resistance in these isolates

CHAPTER 2

LITERATURE REVIEW

2.1 *Mycobacterium abscessus*: The origin and taxonomy

The mycobacteria are divided into two main groups: *Mycobacterium tuberculosis* complex and the non-tuberculous mycobacteria (NTM) which includes all types of mycobacterial species that do not cause tuberculosis (Lee *et al.*, 2015). The NTM group is then further divided into two categories: slowly growing mycobacteria (SGM) which take about one to two weeks to produce visible growth in a culture medium and the rapidly growing mycobacteria (RGM) which can form colonies within three days of incubation (Shallom *et al.*, 2013).

Mycobacterium abscessus is a RGM in the NTM group. It was first isolated from a knee abscess in 1953 (now known as ATCC 19977) (Brown-Elliott and Wallace, 2002). This bacterium was originally grouped together with *M. chelonae* since they shared almost identical biochemical features and differed by only four base pairs (bp) in their 16S rRNA sequence (Kusunoki and Ezaki, 1992). Hence, it was known as *M. chelonae* subspecies *abscessus* until it was reclassified as an individual species in 1992.

With support from comparative genomic studies (Leao *et al.*, 2011; Teng *et al.*, 2013; Davidson *et al.*, 2014; Sassi and Drancourt, 2014), the taxonomy of *M. abscessus* underwent further changes with the inclusion of *M. bolletii* and *M. massiliense* into a *M. abscessus* complex (Mab) comprising three subspecies named *M. abscessus* subspecies *abscessus*, *M. abscessus* subspecies *massiliense* and *M. abscessus* subspecies *bolletii*. In this dissertation, these subspecies will henceforth be referred to as *M. abscessus*, *M. massiliense* and *M. bolletii* while the *M. abscessus* species complex will be abbreviated to Mab.

The Mab is an environmental bacterium found in abundance in soil and water (Lee *et al.*, 2015). The cells are non-motile, acid-fast and about 1.0 to 2.5 µm in length and 0.5 µm in width. They form white or greyish colonies with either a smooth or rough texture and are non-photochromogenic (Kusunoki and Ezaki, 1992).

2.2 Pathogenesis

Mab has emerged as an important human pathogen over the last two decades. It is responsible for a wide variety of diseases, ranging from skin and soft tissue infections to pulmonary and central nervous system infections. It causes tuberculosis-like infections, especially in immunocompromised individuals (Luo *et*

al., 2016). In patients with underlying lung disease such as tuberculosis, bronchiectasis and cystic fibrosis (CF), Mab is the most prevalent cause which could eventually lead to acute lung failure or chronic disease with progressive decline in lung function (Brown-elliott, Nash and Wallace, 2012; Soroka *et al.*, 2014).

Skin and soft tissue infections (SSTI) on damaged skin lesions are also often caused by Mab. The infections span from deep tissue infections to localized skin infections (Lee *et al.*, 2015). SSTI can be caused by either direct contact with contaminated water or material, such as through traumatic injury, surgical wound or environmental exposure, or by secondary involvement of skin and soft tissues during disseminated disease (Kothavade *et al.*, 2013).

In rare cases, Mab can cause central nervous system (CNS) infection, whereby meningitis and cerebral abcesses are among the common manifestations reported. One study showed that while HIV-seropositive patients had mostly *M. avium* complex infections, most HIV-seronegative patients were infected with Mab (Lee *et al.*, 2012). Most of the patients with Mab infections had either undergone neurosurgery, had intracranial cathethers or had otologic diseases, suggesting that infection was spread via contaminated surgical instruments. A recent study utilizing WGS analysis (Bryant *et al.*, 2016) however, demonstrated that certain strains of

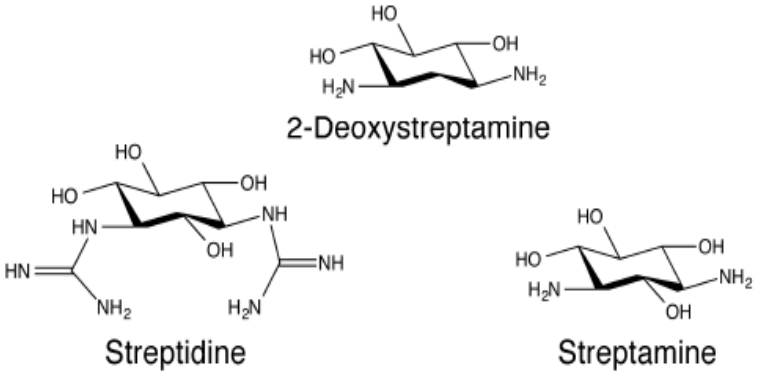
Mab can be transmissible between patients and that these strains are more virulent and resistant than those acquired from the environment.

2.3 Antibiotics and mechanisms of action

2.3.1 Amikacin

Amikacin is an aminoglycoside originally isolated from *Streptomyces* sp. All aminoglycosides have an aminocyclitol nucleus which is either streptamine, 2-deoxystreptamine or streptidine, linked to amino sugars (Veyssier and Bryskier, 2005) (Figure 2.3.1). They inhibit bacterial protein synthesis by binding to the bacterial 30S ribosomal subunit, thus changing the conformation of the A site and reducing the proofreading capabilities of the ribosome (Brown-elliott, Nash and Wallace, 2012).

A



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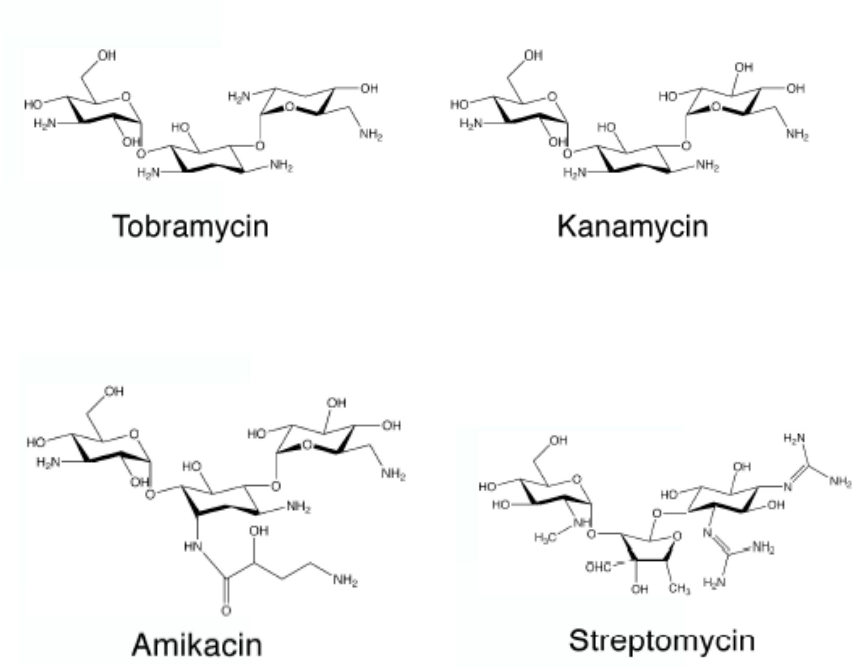


Figure 2.3.1: Basic chemical structures of aminocyclitols (A) and some of the representative aminoglycosides (B) (Ferro *et al*., 2016).

2.3.2 Clarithromycin

Clarithromycin is a semi-synthetic, second generation macrolide derived from erythromycin (Figure 2.3.2A) (Stout and Floto, 2012). It inhibits protein synthesis by binding reversibly to domain V of the 23S ribosomal RNA, (Figure 2.3.2B) thus preventing peptidyl transferase activity and interfering with the translocation of aminoacyl transfer-RNA to prevent peptide chain elongation during translation (Oh *et al.*, 2014).

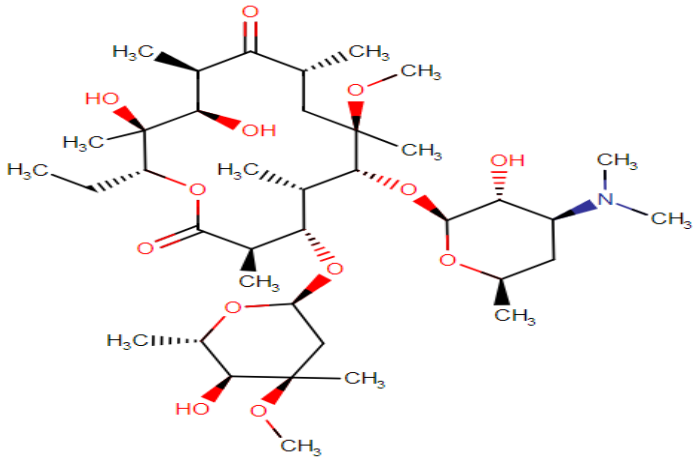


Figure 2.3.2A: Chemical structure of clarithromycin

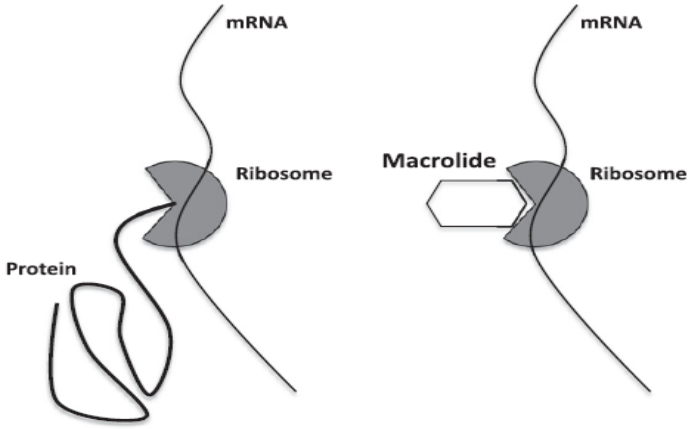


Figure 2.3.2B: Mechanism of macrolide action. The macrolide binds to the 23S rRNA to block bacterial protein synthesis **(**Stout and Floto, 2012**).**

2.3.3 Ciprofloxacin

Ciprofloxacin is the most potent bactericidal, second generation fluoroquinolone, that works against a wide variety of bacteria. Structurally, it is a quinolone antibiotic with a fluoro substitution (Figure 2.3.3). It inhibits the enzyme DNA gyrase to prevent replication of bacterial DNA during bacterial growth and reproduction (Brown-elliott, Nash and Wallace, 2012). The DNA gyrase has two A and two B subunits: The A subunit 'cuts' the DNA while the B subunit causes negative supercoiling, followed by resealing by the A subunit. Ciprofloxacin binds to the A subunit to restrict the nicking and resealing actions.

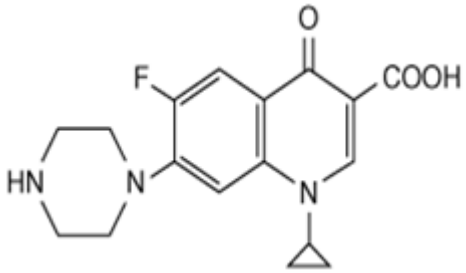


Figure 2.3.3: Chemical structure of ciprofloxacin

2.3.4 Imipenem

Imipenem is a beta-lactam antibiotic in the group of carbapenems (Figure 2.3.4). It has a broad spectrum of activity against aerobic and anaerobic Gram-positive and Gram-negative bacteria and is stable in the presence of many beta-lactamases.

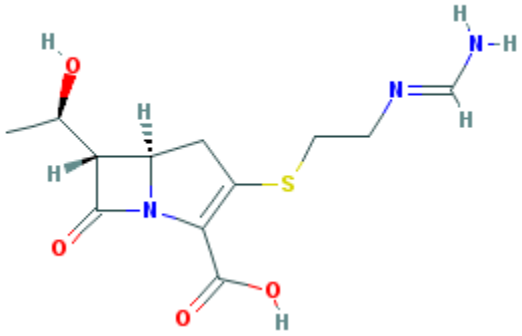


Figure 2.3.4: Chemical structure of imipenem

Imipenem inhibits bacterial cell wall synthesis by binding to, and inactivating penicillin binding proteins (PBPs) located on the inner membrane of the cell wall. PBPs are essential for assembling and reshaping bacterial cell wall during cell growth and division (Wang *et al.*, 2014). Inactivation of PBPs weakens the cell wall, leading to cell lysis.

2.3.5 Linezolid

Linezolid is a synthetic antibiotic, the first of the oxazolidinone class, discovered in the 1990s and approved for commercial use in 2000 (Li and Corey, 2013). The oxazolidinones are heterocyclic molecules with a nitrogen and oxygen in a five membered ring bridged with a carbonyl group. Linezolid is a member of the 3-aryl-2-oxazolidinones (Figure 2.3.5A) with excellent *in vitro* activity against most Gram- positive bacteria and atypical organisms like mycobacteria and *Nocardia*, including antibiotic resistant isolates.

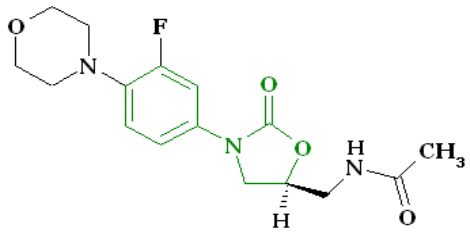


Figure 2.3.5A: Chemical structure of linezolid

Linezolid exerts its action by inhibiting the initiation of bacterial protein synthesis. Linezolid binds to the P-site of the bacterial 23S ribosomal RNA of the 50S subunit. This prevents the formation of the functional 70S initiation complex which is important in the bacterial translation process, hence, halting protein synthesis before it begins (Figure 2.3.5B). Owing to its unique mechanism of action, linezolid is effective against strains resistant to other antimicrobials, as cross-resistance between linezolid and other classes of antibiotics is highly unlikely.

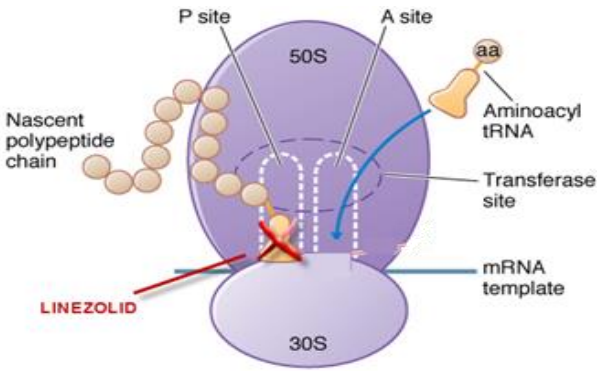


Figure 2.3.5B: Linezolid mechanism of action (Munita and Arias, 2016).

2.4 Mechanism of antibiotics resistance in Mab

Mab is now known as one of the most antibiotic resistant bacterial species. The emergence of resistance in this bacterium has been recognized as a public health threat affecting humans worldwide. The bacterium's waxy, impermeable cell wall and drug export systems are responsible for its innate resistance to disinfectants and a wide range of antimicrobials including the standard anti-tuberculous drugs, such as isoniazid, rifampicin, ethambuthol and pyrazinamide (Nessar *et al.*, 2012). With acquired resistance to many commonly used antibiotics, therapeutic options for treatment have been severely limited.

2.4.1 Amikacin

Amikacin resistance is rare in Mab (Nessar *et al.*, 2011). Resistance to 2-deoxystreptamine aminoglycosides has been linked to a single A to G mutation at nucleotide 1408 (*E. coli* numbering) of the *rrs* gene and this mutation has been reported to be accountable for a high level of resistance (MIC >1024mg/L) to amikacin, gentamicin and kanamycin (Prammananan *et al.*, 1998; Nessar *et al.*, 2011; Maurer *et al.*, 2015). Nessar *et al.* (2011) identified three other substitutions in the *rrs* gene (T1406A, C1409T and G1491T) that confer high level amikacin

resistance in Mab as in other bacteria such as *M. smegmatis*, (Shcherbakov *et al.*, 2010) *E. coli* (Shcherbakov *et al.*, 2010) and *M. tuberculosis* (Salvatore *et al.*, 2016). In Mab showing lower levels of amikacin resistance, mechanisms other than *rrs* mutations have been suspected (Li *et al.*, 2017).

2.4.2 Clarithromycin

Clarithromycin was the drug of choice for Mab infections until inducible resistance to macrolides was reported. Four main mechanisms of clarithromycin resistance have been described (Leclercq, 2002):

I. inducible methylase enzymes which modify ribosomal targets to reduce drug binding
II. drug efflux due to an active pump mechanism
III. macrolide hydrolysis by esterases in the *Enterobacteriaceae*
IV. chromosomal mutation that modifies the 50S ribosomal protein

In Mab, resistance to clarithromycin is often related to the inducible erythromycin ribosomal methylase enzyme gene, *erm*(41) and chromosomal mutations in the ribosomal proteins (Chew *et al.*, 2017). The main mechanism is the acquired constitutive resistance conferred by the mutations in nucleotides 2058

and 2059 (A2058/2059G) of the *rrl* gene which codes for the peptidyl transferase domain of the bacterial 23S rRNA, thereby preventing drug attachment (Maurer *et al.*, 2012). This mutation is often associated with very high level of clarithromycin resistance (MIC > 256mg/L).

The second mechanism is expressed by the inducible erythromycin ribosomal methylase gene, *erm*(41) gene. This gene, upon exposure to clarithromycin expresses RNA methylase, which mono- or di-methylates an adenine in the peptidyl transferase region of the 23S rRNA, reducing the binding of clarithromycin to the ribosome (Brown-elliott, Nash and Wallace, 2012).

The functioning of *erm*(41) gene varies according to the Mab subspecies. The e*rm*(41) gene in most *M. massiliense* strains is dysfunctional due to a 2bp deletion at nucleotides 64 and 65 and another 274 bp deletion from nucleotides 159 to 432 (*M. abscessus* numbering) (Bastian *et al.*, 2011). Hence, *M. massiliense* is often susceptible to clarithromycin, provided no mutations occur in the *rrl* gene. In *M. abscessus* and *M. bolletii*, the *erm*(41) is intact and functional, and clarithromycin resistance is inducible in the absence of *rrl* mutations. In both subspecies, the nucleotide at position 28 in the e*rm*(41) gene plays an important role in the inducible resistance (Rubio *et al.*, 2015). A T28 sequevar is associated with inducible resistance while a T28C substitution inactivates the e*rm(*41),

resulting in susceptibility in the C28 sequevar, if there is no mutation in the *rrl* gene. In T28 variants, the MIC for clarithromycin changes from susceptible to resistant, within 7 to 14 days of incubation.

2.4.3 Ciprofloxacin

Fluoroquinolones (FQ) are also active against the RGM group, including Mab. However, its increasing usage has caused the emergence of FQ-resistant bacteria (Kim *et al.*, 2016). Since there is a lack of evidence supporting the existence of topoisomerase IV in mycobacteria, it was hypothesized that DNA gyrase is the only target for FQ in mycobacteria (Brown-elliott *et al.*, 2012). Ciprofloxacin interacts with the DNA gyrase at the conserved regions known as quinolone resistance determining regions (QRDR) (de Moura *et al.*, 2012).

A Brazilian research in 2012 (Monego *et al.*, 2012) demonstrated that 88.6% of their ciprofloxacin-resistant strains had the substitution Ala-92 to Val-92, (*M. abscessus* numbering) in the QRDR of *gyr*A, suggesting that this mutation had a role in FQ-resistance. Meanwhile, a Korean study in 2014 only discovered five QRDR mutants from their 149 ciprofloxacin resistant strains. The mutations were in *gyr*A *(*Ala-92-Val in one strain, Asp-96-Asn in three strains) and in *gyr*B *(*Arg-

492-Cys in one strain). These five strains mutant showed MIC values of more than 16 mg/L, suggesting that mutations in *gyr*A and *gyr*B are more likely to occur in highly resistant strains (Lee *et al.*, 2014).

On the other hand, de Moura *et al.*, (2012) demonstrated that the peptide sequences of both *gyr*A and *gyr*B QRDR differ according to RGM species and may not be according to strains. For example, species that showed Ser-92 in *gyr*A were *M. chelonae* and *M. fourtuitum* while species that showed Ala-92 were *M. abscessus, M. bolettii* and *M. smegmatis.* These researchers also showed that all the RGM they tested had residues Arg-482 and Asn-499 in *gyr*B, while more susceptible species like *E. coli* had Lys-482 and Ser-499. Hence, Arg-482 and Asn-499 were seemingly associated with lower susceptibility to FQ, as in the RGM.

Since then, there have been numerous studies supporting the existence of a second factor which causes ciprofloxacin resistance in Mab, but the exact mechanism remains to be elucidated (Esfahani *et al.*, 2016; Kim *et al.*, 2016; Kim *et al.*, 2018).

2.4.4 Imipenem

Imipenem is one of the stronger, more stable carbapenems. However, resistance towards imipenem has emerged and is steadily increasing, especially in Mab (Lavollay *et al.*, 2013; Lefebvre *et al.*, 2017; Le Run *et al.*, 2018).

The main mechanisms of resistance in imipenem involve porins, efflux pumps, extended spectrum beta-lactamases (ESBLs) and carbapenemases, of which, the most prevalent mechanism is the production of beta-lactamases. Metallo-beta-lactamases (MBLs) are able to hydrolyze many beta-lactam antibiotics, including carbapenems. The common MBLs observed in clinical isolates are Imipenemase (IMP), Verona imipenemase (VIM) and New Delhi metallo- beta-lactamase (NDM) (Jiang *et al.*, 2018).

Analysis of the Mab genome (Soroka *et al.*, 2014) revealed an Ambler class A, beta-lactamase that is 48% homologous to BlaC, which is responsible for imipenem resistance in *M. tuberculosis* (Hoagland *et al.*, 2016). This gene was thereafter named $Bla_{Mab.}$ Soroka and her team, (2013) demonstrated that Bla_{Mab} could efficiently hydrolyze imipenem, rendering the bacteria resistant. Since then,

many other studies have proved support for the role of Bla_{Mab} in imipenem resistance (Dubee *et al.*, 2014; Lefebvre *et al.*, 2017; Le Run *et al.*, 2018).

2.4.5 Linezolid

Linezolid was introduced to the clinical world in 2000 because of its unique activity against multiple antibiotic resistant, Gram-positive bacteria. Up to 2014, bacterial resistance to linezolid has remained low (Mendes *et al.*, 2014) but since then, reports on resistance to this antibiotic have steadily increased.

Resistance to linezolid is often associated with mutations in the 23S rRNA, of which G2061, C2452, A2503, U2504, G2505, A2062, G2447T, A2453, C2499, U2500 and G2576U (*E. coli* numbering) are the most commonly reported (Papadimitriou-olivgeris, 2014). The mutation G2447T in particular, has been found in mutated *M. smegmatis* (Sander *et al.*, 2002) with high linezolid resistance.

Although the ribosomal proteins, L3 and L4 are located further from the antibiotic target, mutations in these regions seem to contribute to oxazolidinone resistance as well (Long and Vester, 2012). For example, mutations in *rplC* that

encodes protein L3 were reported to be involved in the acquisition of resistance to oxazolidinone (Kim *et al.*, 2017). Furthermore, the mutation T460C in the *rplC* of *M. tuberculosis* was also known to cause linezolid resistance (Beckert *et al.*, 2012).

Despite the numerous reports on the *in vitro* activity of linezolid in Mab, (Tang *et al.*, 2015, Luo *et al.*, 2016; Mougari *et al.*, 2016; Li *et al.*, 2017; Jeong *et al.*, 2018) there have been no thorough studies on the genetics of resistance in this species complex.

2.5 Antibiotic Susceptibility Testing (AST) methods for Mab

The proper management of Mab infections requires initiating effective therapy as soon as possible (Jayasingam *et al*., 2017). The availability of antibiotic susceptibility patterns of the bacteria is the key for a swift and accurate treatment (Cirillo *et al*., 2017). Furthermore, given the evidence regarding different antimicrobial susceptibility patterns in different subspecies, (Novosad *et al.*, 2016) local antibiotic susceptibility data is essential to guide antibiotic therapy. The two main methods for the AST of Mab are the conventional, culture-based testing and the molecular detection of resistance-associated genetic elements (Cirillo *et al*., 2017).

The conventional microbroth dilution method is the gold standard for the AST of NTM species (Brown-elliott *et al.*, 2012). This test system can be learned and standardized easily in laboratories. In addition, there is now the automated versions, such as the Vitek and Sensititre systems, to reduce labour cost and technical errors. One main disadvantage of this method is its rigidness in the choice of antibiotics to test (Kadlec *et al.*, 2015). Changing the test antimicrobial agents for different samples is not possible. Moreover, Mab colonies tend to form clumps in the broth, making interpretation of turbidity difficult. Knowledge and experience are needed to differentiate Mab culture from contamination in the broth (McLain *et al.*, 2016). Another disadvantage is that the MIC determined by broth microdilution does not represent the absolute value of the MIC for a strain. For example, if the MIC is 32µg/ml, the actual value would fall between the lowest concentration that inhibits bacterial growth (32 µg/ml) and the next lowest concentration (16 µg/ml) (Brown-elliott *et al.*, 2012).

Compared to microbroth dilution, the Epsilometer, or Etest method is more convenient as it provides the ease of agar disk diffusion with the application of a strip impregnated with an exponential gradient of antimicrobial, to yield an MIC (Brown-elliott *et al.*, 2012). Once the Etest strip is applied on an isolate-inoculated agar plate, the antimicrobial diffuses out, resulting in a stable concentration gradient in the medium (Reller *et al*., 2009). The MIC value is determined after incubation,

at the point of intersection of the organism growth and the MIC range printed on the Etest strip. The Etest MIC generally correlates well with the MIC obtained by broth dilution method. However, for some organism-antimicrobial combinations, there may be some variations. For example, a study by Papp *et al*., 2018 to detect antibiotic-resistant *Neisseria gonorrhoeae* showed that the Etest method showed good correlation for azithromycin and ceftriaxone, but not for cefixime.

Nevertheless, regardless of the type of phenotypic testing, conventional methods still require a lot of time and labour. It is not possible to get the AST result of an isolate within a day, and this can delay treatment. Another limitation is the inflexibility of antibiotic selections available in standard commercial panels. The quality of the media and antibiotics used and the experience and technical skills of the staff performing the test can strongly affect the reliability of the phenotypic test result (Cirillo, *et al*., 2017). Thus, the molecular testing of antibiotic susceptibility is a welcomed change in most laboratories. DNA hybridization and the polymerase chain reaction (PCR) are among the commonly utilized molecular methods (Anjum *et al*., 2018).

Molecular methods detect resistance-associated genes in a bacterium. PCR for example, amplifies a target DNA sequence in a rapid and exponential level, to a point that will be detected with the aid of gel electrophoresis and UV light

illumination (Anjum *et al*., 2018). The advantages of molecular methods are rapidity (only about four to five hours to detect resistance) and simplicity, thus incurring less technical errors (Cheng *et al*., 2014). These advantages enable early and appropriate treatment of infections.

However, molecular methods are not without limitations. The presence of a resistance gene does not necessarily equate to treatment failure, because resistance is also dependent on the mode and expression level of these genes (Cirillo *et al*., 2017). One major drawback is the conflicting results obtained by molecular and phenotypic testing. The discrepancies could be due to the detection of silent mutations in molecular assays that are not expressed in phenotypic tests. On the other hand, previously unreported or novel resistance mechanisms are not detected in standard molecular assays. Owing to the large diversity of possible mechanisms involved in antimicrobial resistance, it is challenging to transform all these mechanisms into sequence-based detection algorithms, especially when new resistance mechanisms are continuously being discovered (Rupp *et al.*, 2017). This limitation posed a huge problem to researchers until whole genome sequencing (WGS) was introduced.

2.6 Whole genome sequencing (WGS)

Whole genome sequencing (WGS) is the cornerstone in the evolution of antibiotic susceptibility testing methods. It is a process where the complete DNA sequence of an organism's genome can be determined. WGS data are produced by sophisticated sequencing platforms like Illumina and Ion Torrent that generate huge amounts of sequence data compared to the traditional Sanger sequencing (Anjum *et al*., 2018).

The rising awareness of Mab as an emerging pathogen reinforces the importance of understanding Mab at both subspecies and genomic levels. WGS has an edge over conventional molecular methods in that it is able to cover a broad spectrum of genetic determinants and to subtype specific genetic variants at the same time (Zankari *et al.*, 2012; Gupta *et al.*, 2014). It enables high resolution analysis of genetic variants, ranging from single nucleotide polymorphisms (SNP) to large-scale deletions (Davidson *et al.*, 2014). Furthermore, it allows new target sequences to be rapidly added to the analysis database, allowing back-screening or re-analysis on previously analyzed isolates (Anjum *et al*., 2018). WGS could also help to shed light on the discrepancies between phenotypic and genotypic results (Drobniewski *et al.*, 2015). Thus, WGS is fast replacing the phenotypic methods of AST.

Overall, WGS is a powerful alternative for rapid access to universal AST patterns and may overcome limitations of current phenotypic and genotypic methods. WGS can also help to personalize antibiotic therapy for each patient in the near future

2.7 Antibiotic Resistance Gene (ARG) databases

In order to extract crucial information to detect genetic determinants of antimicrobial resistance (AMR) from WGS data, an exhaustive, well-curated bioinformatics library containing relevant DNA or protein sequences is essential (Drobniewski *et al.*, 2015). There are many of these bioinformatics tools freely available to researchers worldwide. These tools or antibiotic resistance gene (ARG) databases are either available as a web service, downloaded stand-alone program or as command-line tools (Anjum *et al*., 2018). Among the most popular ARG databases are the ResFinder, CARD and ARG-ANNOT.

The ResFinder gives information on antibiotic resistance genes from sequenced or partially-sequenced bacterial isolates (Xavier *et al.*, 2016). One major

advantage of ResFinder is that it accepts both preassembled genome and raw, next generation sequencing (NGS) data from different sequencing platforms, such as Illumina, Ion Torrent and SOLiD as its query sequence (Zankari *et al.*, 2012). The uploaded raw WGS data is assembled by Velvet before analysis (Thomsen *et al.*, 2016). However, ResFinder only detects acquired genes and chromosomal mutations. Intrinsic resistance genes like protein pumps and multidrug transporters are not included (Zankari *et al.*, 2012). Hence, it is not a suitable alternative for phenotypic susceptibility testing in health centers.

The Comprehensive Antibiotic Resistance Database or CARD is a free web service which provides knowledge on AMR genes, their proteins and mutations involved in the AMR (Jia *et al.*, 2017). CARD is an all-inclusive ARG library as it owns an advanced antibiotic resistance ontology (ARO) platform which includes the classification of AR genes, functional ontology information, SNPs for resistance genes, gene ontology and infectious disease ontology among some (Xavier *et al.*, 2016). An additional plus point is its user-friendly and illustrative graphical interface.

ARG-ANNOT stands for Antibiotic Resistance Gene-Annotation. This database uses a local BLAST algorithm with the aid of the BioEdit software (Gupta *et al.*, 2014). It is unique as it allows sequence analysis without the use of internet.

It does not automatically detect mutations, but provides all the sequences that match the query, so that users can manually search for possible mutations. In addition, ARG-ANNOT allows users to customize or modify the database according to their requirement.

The application of WGS for the detection of bacterial AMR is now expanding, with most studies reporting good concordance. One such study was by Gordon *et al*., 2014 who compared the WGS and phenotype data in 501 *S. aureus* isolates, for 12 types of antibiotics. Through a blind validation, they demonstrated sensitivity and specificity values of 97% and 99% respectively.

McDermott and his team in 2016 utilized WGS to identify known AMR determinants in 640 non-typhoidal *Salmonella* and correlated the results with susceptibility phenotypes to 14 antibiotics, to evaluate the accuracy of WGS in AMR surveillance. Overall, resistance phenotypes and genotypes correlated in 99% of the cases. Concordance was almost 100% in all groups of antibiotics except for aminoglycosides and beta-lactams (McDermott *et al.*, 2016).

Gupta *et al*., (2017) conducted a study to predict genes and mutations potentially associated with antibiotic resistance in the *M. ulcerans* strain, AGY99. WGS via ARG-ANNOT predicted 14 putative ARG from various antibiotic

classes. Mutations in *kat*G (R431G) and *pnc*A (T47A, V125I) genes, conferring resistance to isoniazid and pyrazinamide respectively, were also detected. However, no mutations were predicted in *rpo*B, *gyr*A, *gyr*B, *rps*L, *rrs*, *emb*, *eth*A and 23S rRNA genes. The researchers suggested that isoniazid and pyrazinamide are probably not effective for this strain, in contrast to rifampin, streptomycin, azithromycin, clarithromycin and fluoroquinolones.

Till date, there has been only one study on the application of WGS for the prediction of Mab antibiotic resistance determinants (Lipworth *et al.*, 2018). This study used a predictive algorithm, based on all known resistance determining mutations published, to test 209 clinical isolates with paired phenotype/genotype data. The results demonstrated 76.2% sensitivity for clarithromycin, 0% for ciprofloxacin and 5% for amikacin. These values suggested room for improvement in the application of WGS for the detection of antibiotic resistance genes, especially in Mab.

A major disadvantage of ARG databases is that they are all based on known, published resistance-determinants data. A regular curating of the database is necessary, to include the updates whenever new genes are published. Furthermore, since the genetic determinants for AMR vary according to bacterial species, the

sensitivity of these databases could be low and of less value in less studied bacterial species.

Overall, an ARG database is a valuable platform locally and globally for the surveillance of AMR, as it permits unprecedented resolution of gene variants, a feature that is not offered by phenotypic and other genotypic methods.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and reference strain used

The 60 Mab isolates studied were collected from sputum and bronchoalveolar lavage fluids of patients presenting with clinical signs of lower respiratory tract infections, from 2012 to 2014. *M. abscessus* ATCC 19977 was chosen as the reference strain for the determination of minimum inhibitory concentrations (MIC) while *S. aureus*, ATCC 29213 was used as the control strain.

3.1.1 Preparation of stock cultures

Archived *M. abscessus* clinical isolates were subcultured onto blood agar to test their viability and purity. Contaminated and non-viable cultures were excluded from the study. Uncontaminated cultures were further propagated to prepare stock cultures in tryptic soy broth (TSB) (BD, USA) with 15% glycerol and stored in 2.0 ml Eppendorf tubes at -20 ^{o}C until required for use.

3.1.2 Preparation of mycobacterial slides

A drop of sterile saline water was placed on a glass slide. A single colony from a pure culture was smeared in the saline, in a circular manner. The smear was left to dry in the biosafety cabinet (BSC II) (Esco, Singapore). Once dry, the slide was heat-fixed before staining.

3.1.3 Ziehl-Neelsen staining of mycobacterial culture

Ziehl–Neelsen (ZN) staining was performed using the instructions from the manufacturer (BD, USA). Slides were placed neatly on the staining rack before flooding with carbol fuschin reagent for 4 minutes. They were then washed gently with running water, decolorized with acid-alcohol for a few seconds, washed gently again with running water and then counterstained with methylene blue for 30 seconds before a last gentle wash under running water. Slides were left to air-dry prior to reading under the microscope (Olympus, Japan).

3.1.4 DNA extraction from mycobacteria: The boiling method

The boiling method is a fast and convenient way to extract mycobacterial DNA. Firstly, the mini heating dry bath incubator (Major Science, US) was switched on and set to 100° C for 15 minutes, to allow some time for the plate to heat up. A cell suspension was made by mixing a few colonies from a pure culture in 200 µl of nuclease-free water (Norgen, Canada). The suspension was then boiled on the heating plate at 100° C for 15 minutes. After centrifuging (Eppendorf, Germany) for 10 minutes at 1500 rpm, the supernatant was carefully transferred into a new 1.5 ml microcentrifuge tube.

3.2 Polymerase chain reaction (PCR)

PCR assays were set up to identify *M. abscessus* subspecies and gene mutations previously reported to be associated with resistance to the five classes of antibiotics under study. All PCRs were performed using the Veriti 96-Well Thermal Cycler (Thermo Fischer Scientific, USA).

Primers and PCR parameters were obtained from literature wherever applicable, otherwise they were designed using Primer Blast, NCBI and other tools as described in Section 3.7. The annealing temperature (Ta) was optimized by

performing a gradient PCR with the annealing temperature ranging 3° C above and below the calculated annealing temperature. The optimal annealing temperature was chosen from the temperatures that gave the brightest band, with no non-specific products amplified, in the agarose gel.

3.3 Agarose gel electrophoresis

Gel electrophoresis was carried out to estimate the size of the DNA amplicons obtained using the PowerPac electrophoresis (Bio-Rad, USA).

3.3.1 Preparation of agarose gel

Agarose powder, 0.3g (Hydragene, USA) was measured and transferred into a conical flask. 1X TBE, (1st Base, Singapore) 15ml was then poured into the flask. The flask was microwaved for approximately 20 seconds before it was removed and swirled. It was microwaved again for another 10 seconds until the agarose powder was completely dissolved and the liquid came to a boil. The solution was left to cool for 1 minute before 2.5 µl of SYBR Safe DNA gel stain (Invitrogen, USA) was added. The solution with the gel stain was poured gently into the gel tray with the well combs in place to avoid causing bubbles. The gel was

then left to cool and solidify completely for 30 minutes. The combs were removed carefully before placing the gel into the electrophoresis chamber.

3.3.2 Loading samples into the gel

The gel chamber was filled with 1X TBE buffer (Appendix A) until it covered the gel surface. The first lane of the gel was filled with 5 µl of 100 bp PCR sizer (Norgen, Canada), while the rest were filled with 5 µl of PCR amplicons. The gel was run at 80 V, for 40 minutes. After electrophoresis, the DNA bands were viewed under a UV-transilluminator with digital camera-based gel documentation system (INTAS, Germany). The fragment size was assessed by comparing with the DNA ladder.

3.4 Purification of PCR amplicons for Sanger sequencing

All amplicons obtained were purified following the instructions in the QIAquick PCR purification kit (Qiagen, Germany). Buffer PB, 100 µl was added to 20 µl of the PCR reaction. A QIAquick column was placed in the 2 ml collection tube provided. The sample was then applied to the QIAquick column and was centrifuged at 13 000 rpm for 60 seconds to bind the DNA. The flow-through was then discarded before placing the QIAquick column back in the same tube.

To wash, 750 µl of Buffer PE was added to the QIAquick column and was centrifuged again at 13 000 rpm for 60 seconds. The flow-through was discarded and the QIAquick column was placed back in the same tube. In the same 2 ml collection tube, the QIAquick column was centrifuged again at the same rpm for 1 minute to remove any residual wash buffer.

Once washed, each QIAquick column was placed in a clean 1.5 ml microcentrifuge tube. Buffer EB, 50 µl (10 mM Tris·Cl, pH 8.5) was added to the center of the QIAquick membrane prior to centrifuging the column for 1 minute. This step was to elute the DNA.

To increase the DNA concentration, 30 µl of elution buffer was added to the center of the QIAquick membrane. The column was then left to stand for 1 minute before centrifuging for another few minutes. The eluted DNA was stored in Eppendorf 1.5 ml microcentrifuge tube at -20° C until further analysis.

All DNA samples were evaluated for purity using the Thermo Scientific NanoDrop Spectrophotometer before they were out-sourced to 1st BASE Malaysia for Sanger sequencing with the same primers used for PCR.

3.5 Identifying *M. abscessus* subspecies

M. abscessus subspecies were identified by the DNA sequence analysis of *hsp65* and *erm*(41) genes which were amplified using primers that were described by Telenti *et al.*, 1993 and Kim *et al.*, 2010 respectively. The primers were ordered from 1st BASE, Malaysia. Details of the primers and thermal profiles used are elaborated in Table 3.5. Each PCR mixture consisted of 6 µl of ddH_2O, 12.5 µl of Promega Master mix, 2 µl each of forward and reverse primer and 2.5 µl of DNA template amounting to a total volume of 25µl.

*Hsp*65 gene sequences were analyzed using NCBI BLASTN and *hsp*65BLAST (http://hsp65blast.phsa.ca/) while *erm*(41) gene sequences were aligned and analyzed using MEGA6 software, an integrated tool which conducts automatic and manual sequence alignments (Tamura *et al*, 2013). The *erm*(41) gene in *M. massiliense* is typically characterized by a 2 bp deletion at nucleotides 64-65 and another 274bp deletion of nucleotides 159-432 (*M. abscessus* numbering), which causes this subspecies to be 276 bp shorter compared to the other two subspecies (Maurer *et al.*, 2012).

For each gene, a dendogram was constructed using MEGA6 software with bootstrap values of 1,000 replicates to show the phylogenetic relationships among

samples and reference sequences. Sequence alignment was performed with MultAlin (Corpet, 1998) for a better presentation.

Table 3.5: PCR profiles used for *M. abscessus* subspecies identification

Gene	Primers (5' – 3')	PCR profile	Product size	References
*hsp*65	Tb11: ACCAACGATGGTGTGTCCAT Tb12: CTTGTCGAACCGCATACCCT	Denaturation 94 °C 1 min Annealing 60 °C 1 min Extension 72 °C 1 min (45 cycles) Final extension 72 °C 10 min	439 bp	Telenti *et al.*, 1993
erm(41)	*erm*F: TGGTATCCGCTCACTGATGA *erm*R: GCGGTGGATGTAGGAAAG	Initial denaturation 95 °C 5 min Denaturation 94 °C 30 sec Annealing 55 °C 30 sec Extension 72 °C 60 sec (40 cycles) Final extension 72 °C 10 min	450 bp/ 174 bp	Maurer *et al.*, 2012

3.6 Antibiotic susceptibility testing with Etest

Only 51 clinical strains from a total of 60 were viable and of pure colony. These strains were examined with Etest strips (bio Merieux, France) using Mueller Hinton agar with 5% sheep blood (Thermo Scientific, USA) as per Biomerieux instructions. The five antibiotics tested were amikacin, ciprofloxacin, clarithromycin, imipenem and linezolid. Inocula (obtained by touching at least six different colonies from the pure culture) from a suspension prepared in broth to 1 McFarland standard, were plated on the blood agar. Etest strips were placed on the air-dried inoculated plates which were then incubated in an ambient air incubator at 30 ^{o}C. All the plates were incubated within 15 minutes to prevent pre-diffusion of the antibiotics in room temperature.

The MICs were read after 72 hours of incubation, except for clarithromycin which were read on the 3rd, 7th and 14th day of incubation, for the detection of inducible resistance. Etest was repeated for the reference strain, ATCC 19977 from the colony which grew on the plate with clarithromycin strip after 14 days of incubation to detect any inducible resistance. The MICs were interpreted according to the Clinical and Laboratory Standards Institutes (CLSI, 2011) breakpoints. Table 3.6 below shows the guideline from the CLSI which was used to interpret the results. The Etest for the five antibiotics was also repeated for all fifty-one strains to evaluate its reproducibility.

Table 3.6: The MIC breakpoints for amikacin, ciprofloxacin, clarithromycin, imipenem and linezolid (CLSI, 2011)

Antibiotic	MIC (µg/ml) by category		
	Susceptible	**Intermediate**	**Resistant**
Amikacin	≤ 16	32	≥ 64
Clarithromycin	≤ 2	4	≥ 8
Ciprofloxacin	≤ 1	2	≥ 4
Imipenem	≤ 4	8-16	≥ 32
Linezolid	≤ 8	16	≥ 32

3.7 PCR amplification and sequencing of known resistance-associated genes

PCR primers and protocols were used to amplify genes known to be associated with resistance to amikacin, clarithromycin and ciprofloxacin. The primers for imipenem and linezolid resistance were designed for the IMP-1 gene and 23S rRNA respectively, using Primer-BLAST from NCBI. This programme was also used to check primer properties and self and self-3' complementarity, while Oligo Analyzer 1.0 was used to examine primer-primer compatibility. The full list of target genes and mutations associated with resistance is given in Table 3.7.1. Meanwhile, Table 3.7.2 shows the PCR amplification parameters for these

genes while the composition of the master mix is detailed in Table 3.7.3. Each PCR assay included a reagent control, ATCC 19977 as reference strain and distilled water in place of DNA template as negative control. The PCR products were viewed with gel electrophoresis and purified as previously described in Sections 3.3 and 3.4. The purified products were sent to 1st Base Malaysia for sequencing with the same primers used for PCR.

Table 3.7.1: List of target genes associated with resistance and the mutations involved

Antibiotic	Target gene	Resistance-associated mutations	References
Amikacin	*rrs*	A1408G, T1406A, C1409T, G1491T	Nessar *et al*.,2012
Clarithromycin	*rrl* *erm*(41)	A2058/2059G/C Deletion of 276 bp, C28T	Rubio *et* al., 2015 Maurer *et al.*, 2012
Ciprofloxacin	*gyr*A *gyr*B	Ala-92-Val, Asp-96-Asn Arg-492-Cys,	Monego *et al.*, 2012, Lee *et al.*, 2014
Imipenem	IMP-1	Presence of gene	Saderi *et al*., 2010
Linezolid	23S rRNA	G2061T, G2447T, G2576T	Papadimitriou-Olivgeri *et al.*, 2013

Table 3.7.2: Profiles of PCR amplification of resistance-associated genes for selected antibiotics

Drug	Target gene	Primers (5'- 3')	Thermal profile	Product size (bp)	Reference
Amikacin	*rrs*	Forward: ATGACGTCAAGTCATCATGCC Reverse: AGGTGATCCAGCCGCACCTTC	94°C -10 min 94°C -30 sec 55°C -30 sec 72°C -60 sec 72°C -5 min 35 cycles	344	Nessar *et al*, 2011
Clarithromycin	*rrl*	Forward: CCTGCACGAATGGCGTAACG Reverse: CACCAGAGGTTCGTCCGTC	94°C -10 min 94°C -30 sec 55°C -30 sec 72°C -60 sec 72°C -5 min 35 cycles	728	Maurer *et al*, 2012
	erm(41)	Forward: GACCGGGGCCTTCTTCGTGAT Reverse: GACTTCCCCGCACCGATTCC		673/397	Rubio *et al*, 2015

Linezolid	23S rRNA	Forward: CGGCGAAATTGCACTACGAG Reverse: GGCGGATAGAGACCGAACTG	94°C - 3 min 94°C -45 sec 55°C -30 sec 72°C- 60 sec 72°C -5 min 30 cycles	613	Self
Ciprofloxacin	*gyr*A *gyr*B	Forward: GTCCGCGATGGCCTCAA Reverse: TGAGCCGAAGTTGCCCTG Forward: GACCCGTCGAAATCGGAACT Reverse: TGTCGAACTCGTCGTGGATG	98°C-2 min 98°C-10 sec 57°C-5 sec 72°C-45sec 30 cycles 72°C- 2mins 95°C- 5min	225 211	Self Self
Imipenem	IMP-1	Forward: TTTCACGAGGACCATGTGGG Reverse: CGCTGGAAAGTGGGACATCT	94°C-30 sec 55°C-30sec 72°C-60sec 40 cycles 72°C-10 min	523	Self

Table 3.7.3: Composition of PCR master mix

Components	Volume (µl)
Sterile, nuclease free H_2O	6
Promega master mix	12.5
Forward primer (10 µM)	2
Reverse primer (10 µM)	2
DNA template	2.5
Final volume	25

3.8 Whole genome sequence-based analysis

Twenty-one out of the 51 strains were previously sent for WGS (with the Illumina HiSeq2500 platform) for a separate project. The WGS data of these 21 Mab strains were retrieved and used to search for resistance determinants in amikacin, clarithromycin, ciprofloxacin, imipenem and linezolid using ARG-ANNOT, CARD and ResFinder ARG platforms. The search results were used to correlate with the results of the conventional PCR assays described above, and to detect resistance genes not identified by the conventional PCR-sequencing assays.

3.8.1 *In silico* sequence analysis using Antibiotic Resistance Gene-Annotation (ARG-ANNOT)

The 21 assembled WGS (Fasta format) data were uploaded into the ARG-ANNOT database v7.0.5 to search for antibiotic resistance genes and point mutations in target genes. The e value was set to 0.001.

The long list of hits was filtered to include only acquired resistance genes specific to amikacin, clarithromycin, ciprofloxacin, imipenem and linezolid. Similarly, the point mutation database available in Bioedit was utilized to search for chromosomal mutations associated with resistance to these five antibiotics. The filtered list was then sorted to include only the top 20 hits with the lowest e value, highest percentage of identity and highest bit score (significance of match). The local hits were then compared with NCBI BLAST to validate the results.

3.8.2 *In silico* sequence analysis using Comprehensive Antibiotic Resistance Database (CARD)

The 21 assembled WGS data (fasta format) were uploaded into the online CARD database which provides curated reference sequences and SNPs in annotated genomes, plasmids, and whole-genome shotgun assemblies. DEFAULT-DNA sequence was the data type selected while the criteria was set as DISCOVERY-Perfect, Strict and Loose hits. Nucleotide sequences were then searched against the CARD via the Resistance Gene Identifier (RGI) tool. The RGI result in Tab-delimited Summary format was exported to Microsoft Excel to be viewed.

As described above, the top 20 hits were identified for the five antibiotics.

3.8.3 *In silico* sequence analysis using ResFinder

The 21 assembled WGS data were uploaded into the Browser in the ResFinder tool and blasted to search for chromosomal mutations and horizontally-acquired resistance-associated genes. In the ResFinder home web, the option 'Acquired Antimicrobial Resistance' genes was selected. The antimicrobial classes

of aminoglycoside, beta-lactam, fluoroquinolone, MLS (macrolide/ lincosamide/ streptogramin) and oxazolidinone were selected from the drop down list. The threshold for both percentage of ID and minimum length was set at 30%. Type of read was set as Assembled Genome/Contigs prior to uploading the sequence file. Query was then submitted. These steps were repeated for the option 'Chromosomal point mutation'.

3.9 Analysis with Multiple sequence alignment (MSA)

The top 20 resistance genes in each database were identified. These gene sequences (in FASTA format) from all phenotypes were multiply aligned with MEGA version 6 (Tamura *et al*, 2013) using MUSCLE under the default settings. From these genes, those showing synonymous mutations were excluded; those showing non-synonymous mutations but have low consistency with resistance expression were also excluded. Two putative genes (*efr*A and *qep*A2) were chosen for PCR-sequencing to verify the resistant phenotype in the Mab strains without WGS data as described in the section below.

3.10 Confirmation of the *efr*A and *qep*A2 homologues

The two putative genes shown to have the highest association with ciprofloxacin resistance by WGS analysis were chosen for PCR amplification to verify the mutations detected in the MSA analysis. PCR primers were designed as described in Section 3.7 and PCR was performed as described in Sections 3.2 until 3.4. Table 3.10 below illustrates the primers used, product sizes and the thermal profile of the two genes.

Table 3.10: Thermal profiles for the PCR amplification of *efr*A and *qep*A2 homologues found in Mab

Gene	Primers	Thermal profile	Size (bp)
*efr*A	F: TGAACTTGATACCCCGCCT R: ATCTCCCGATAGGTCCCGC	94°C-3 min 94°C-30 sec 58°C-30 sec 72°C-1 min (35 cycles) 72°C- 10 min	565
*qep*A2	F:CTGGGGTGCAACACTTTTCG R:GTCGAAACCGAGAACGGAC T	94°C-3 min 94°C-30 sec 58°C-30 sec 72°C-1 min (35 cycles) 72°C- 10 min	652

3.11 Summary of methodology

The following flow chart (Figure 3.11) illustrates an overview of the methodology used in this study.

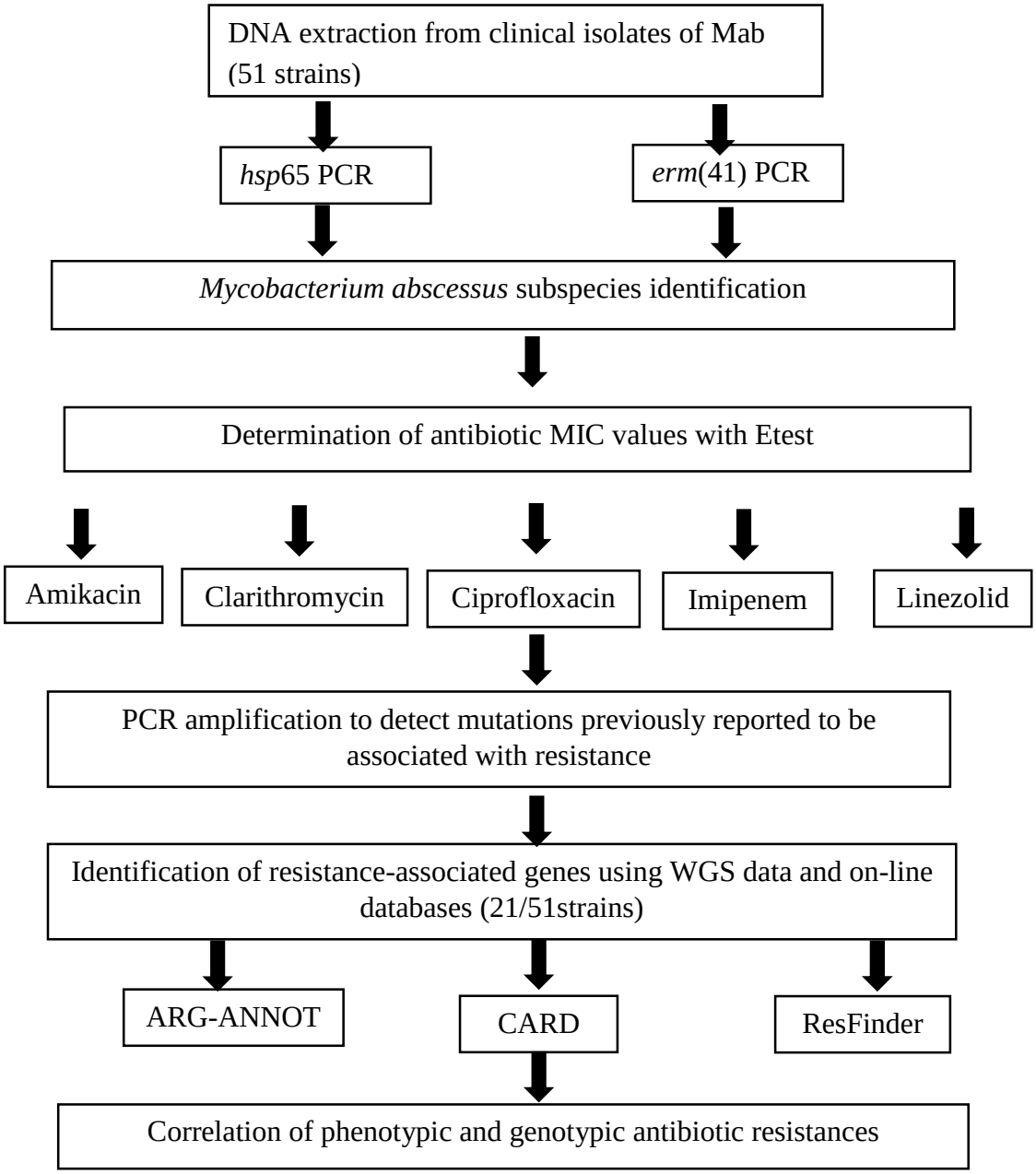


Figure 3.11: An overview of the methodology used

CHAPTER 4

RESULTS

4.1 *M. abscessus* growth and colony morphology

Of the 60 isolates retrieved from stock cultures, only 51 were viable and pure. These colonies were visible on blood agar after 3 days of incubation at 30˚C. They were all non-pigmented with either a smooth or rough texture (Figure 4.1.1) and were acid-fast on ZN staining (Figure 4.1.2). Samples of Mab growth on blood agar are shown in Appendix B while the complete list of colony morphology for the 51 strains is listed in Appendix C.

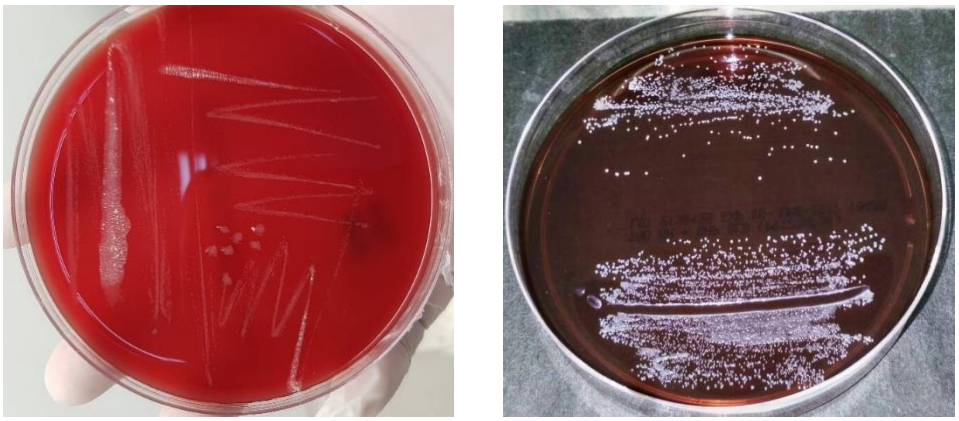


Figure 4.1.1: M. *abscessus* colonies on blood agar

Left: M61 showing dry, clumpy colonies
Right: M24 showing white, smooth colonies with elevated centers

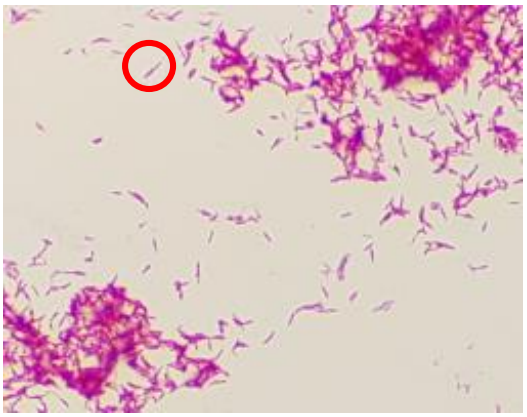


Figure 4.1.2: AFB morphology under 100X oil immersion

The acid fast bacilli (AFB) observed had rod shape and were pink in colour

4.2 Subspecies identification of the *M. abscessus* complex

Based on the *hsp*65 and *erm*(41) analysis (Figure 4.2.1 to 4.2.6), the 51 strains of Mab were identified as 12 strains of *M. abscessus*, 38 of *M. massiliense* and only one *M. bolletii*. The *M. massiliense* subspecies is characterized by a truncated *erm*(41) gene which is 276 bp shorter than the gene in the other two subspecies. This feature was seen in 37 strains classified as *M. massiliense* but not in M139 which had a full length *erm*(41). The *erm*(41)-based dendrogram showed this strain in the same group as the *M. abscessus* but the *hsp*65-based dendrogram

identified it with the *M. massiliens*e cluster. Its classification as *M. massiliense* was also supported by other investigations (Tan *et al*., 2013).

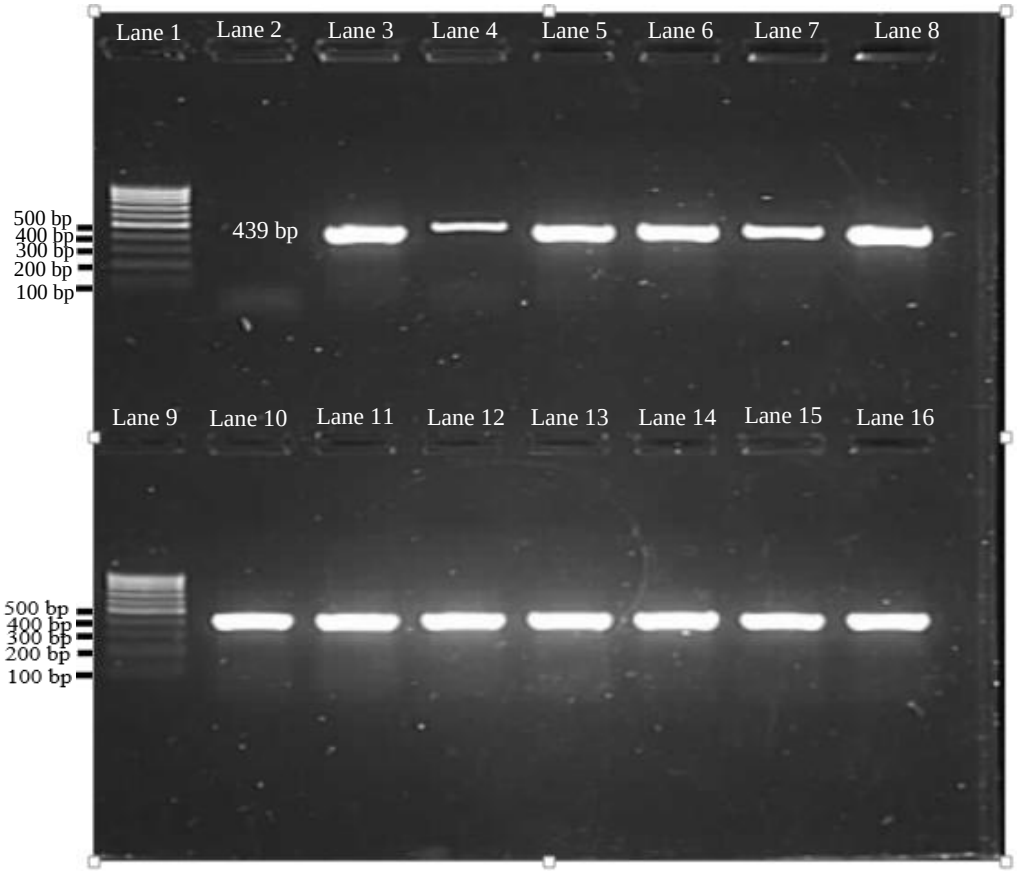


Figure 4.2.1: The image of *hsp*65 gene gel electrophoresis

From lane 1 to lane 16 are 100 bp ladder, RC, M02, M04, M18, M24, M27, M57, 100 bp ladder M61, M93, M94, M115, M117, M1119, and ATCC 19977 respectively. The product size is 439 bp.

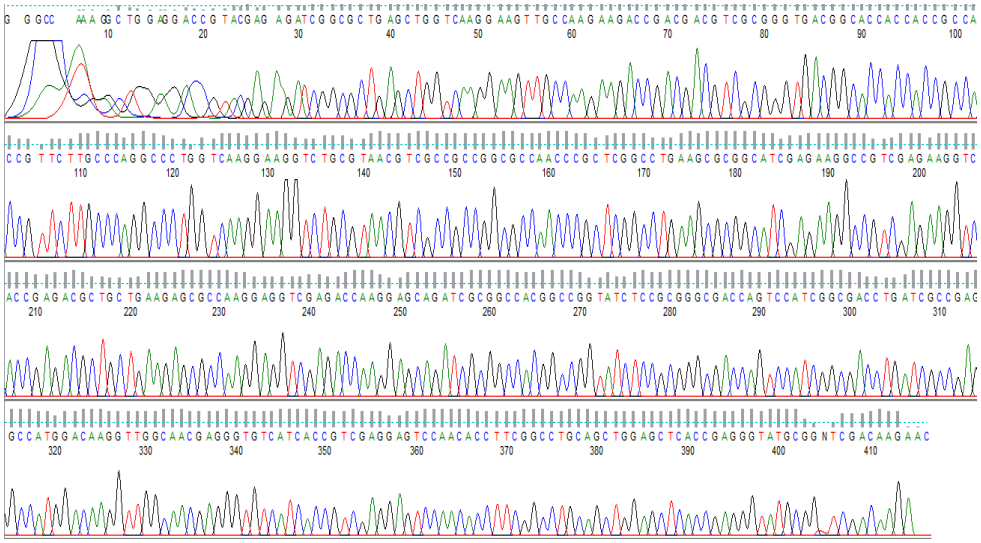


Figure 4.2.2: *hsp*65 chromatograph of M245, a *M. abscessus* subspecies. The color-coded peaks represent DNA bases: A (green), C (blue), G (black) and T (red). The numbers above the corresponding peaks indicate the nucleotide positions in the sequence.

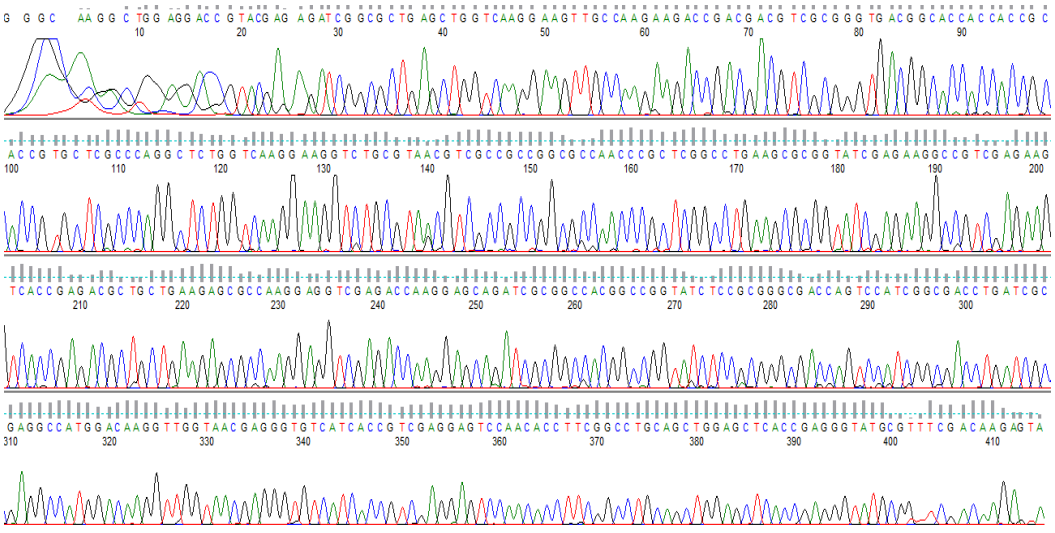


Figure 4.2.3: *hsp*65 chromatograph of M04, a *M. massiliense* subspecies. The color-coded peaks represent DNA bases: A (green), C (blue), G (black) and T (red). The numbers above the corresponding peaks indicate the nucleotide positions in the sequence.

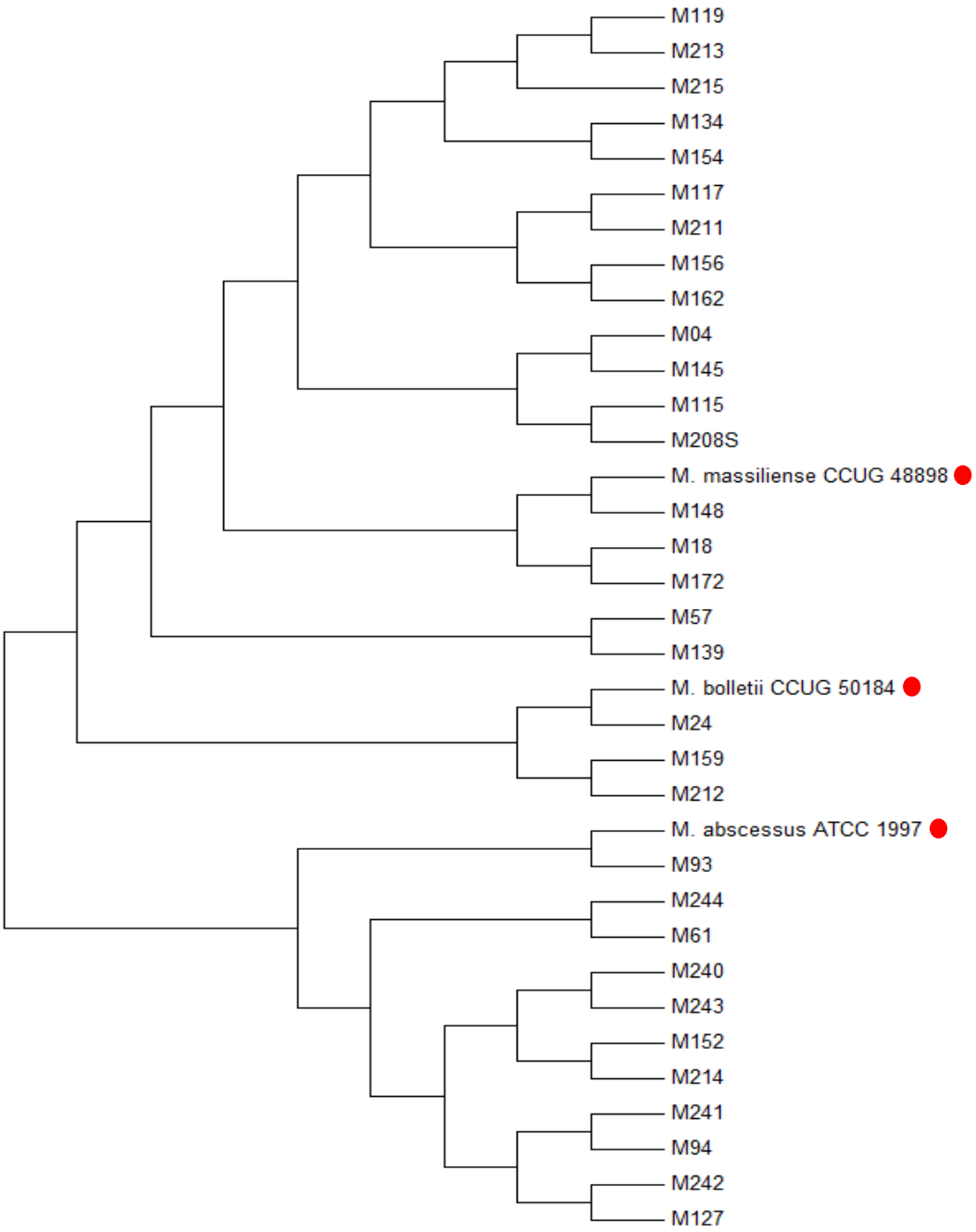


Figure 4.2.4: The *hsp65* dendogram showing 3 clusters representing *M. abscessus, M. bolletii* and *M. massiliense*.

Red dots ● indicate reference strains for each subspecies. M139 was classified under *M. massiliense*

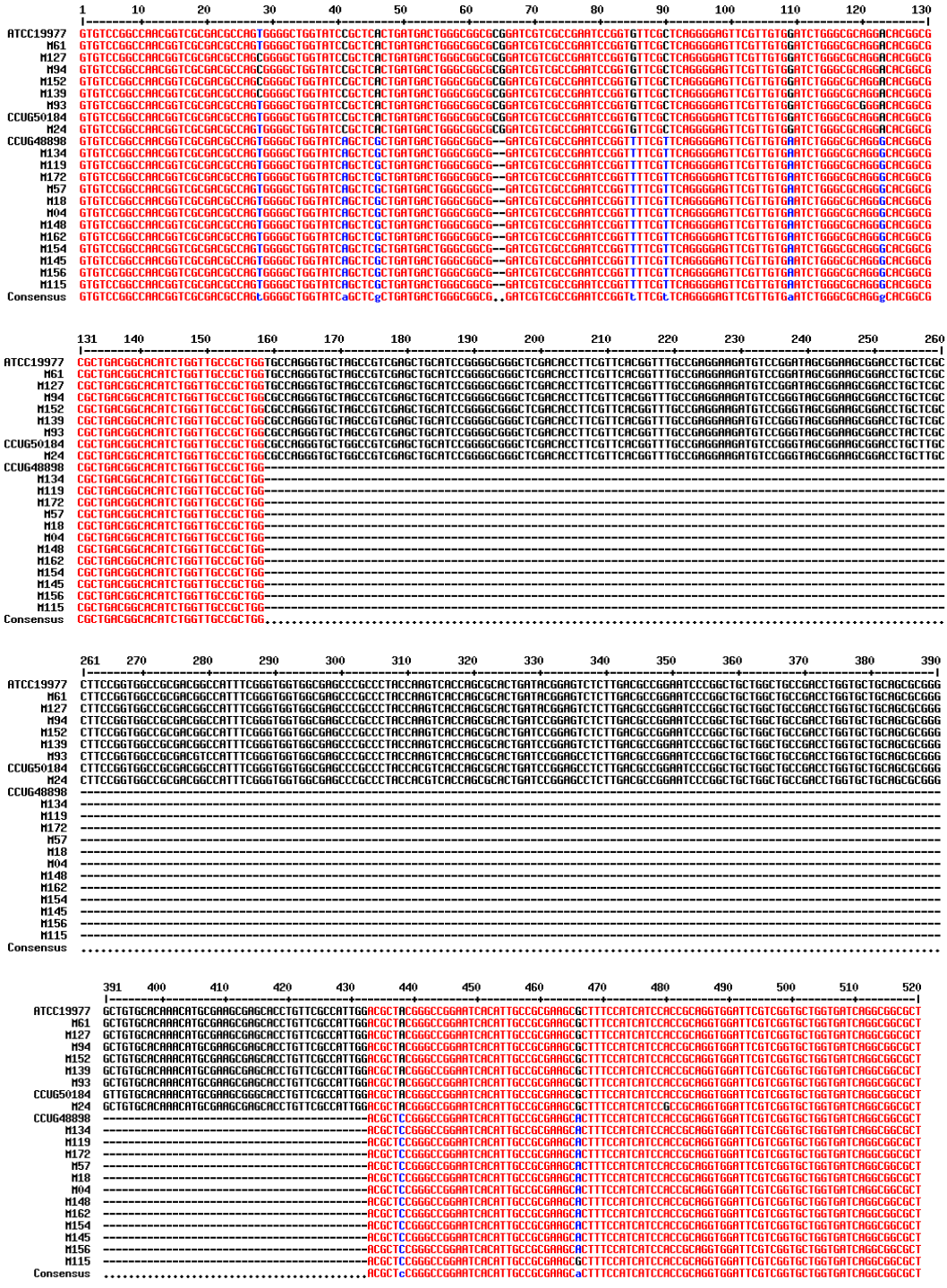


Figure 4.2.5: The MSA of *erm*(41).

There is a 2bp deletion at nucleotide position 64-65 and another 274 bp deletion from nucleotides 159-432 in *M. massiliense* strains, except in M139.

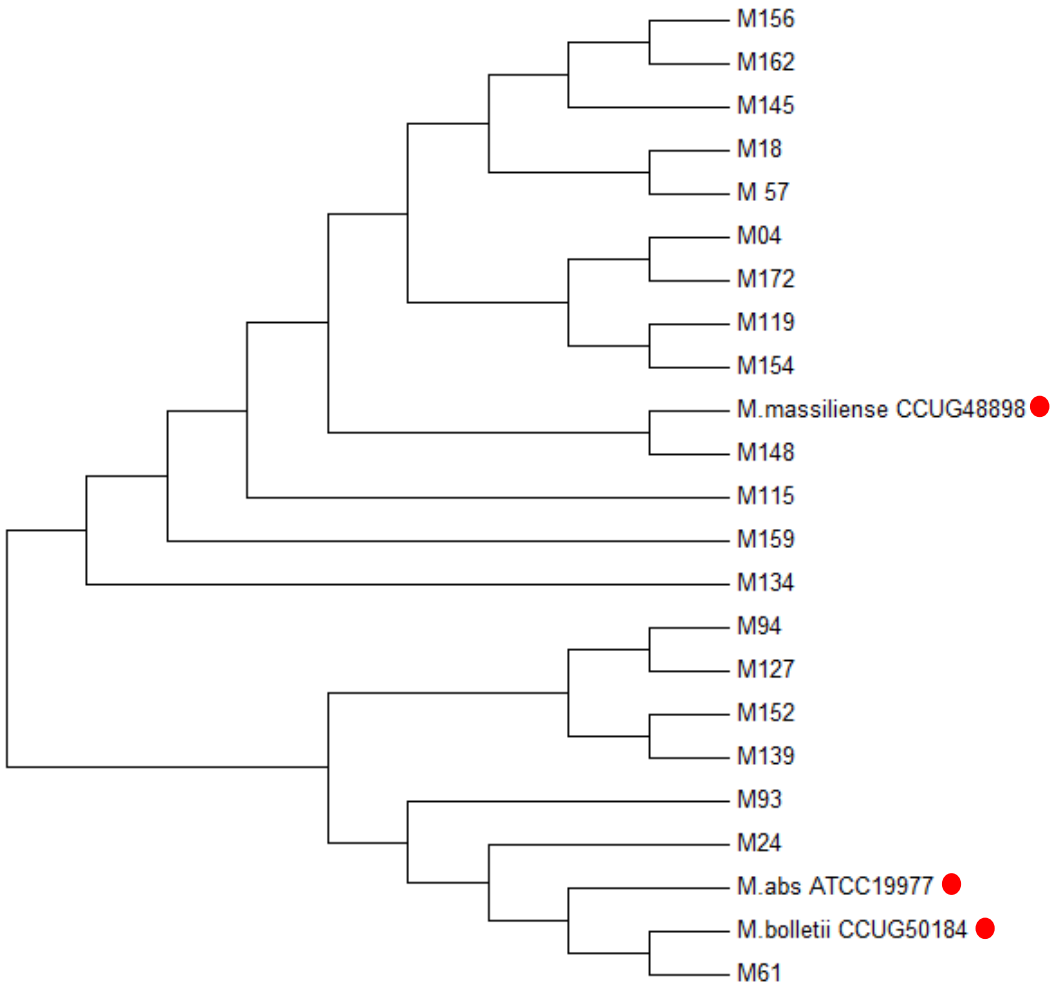


Figure 4.2.6: The dendogram of the *erm*(41) gene distinguished the M. *massiliense* strains from the *M. abscessus* and *M. bolletii*.

Red dots ● indicate reference strains for each subspecies. M139 was classified under *M. abscessus.*

4.3 Antibiotic susceptibility towards amikacin, clarithromycin, ciprofloxacin, imipenem and linezolid.

The antibiotic susceptibilities of the strains examined are summarized in Table 4.3 and Figures 4.3A and 4.3B. The quality control and Etest picture for some strains are shown in Appendix D while the complete list of MIC for all 51 strains is attached in Appendix E. Amikacin seems to be the most effective antibiotic *in vitro*, with no strain showing full resistance. Clarithromycin, likewise, showed good antimicrobial activity on the Mab. In contrast, imipenem resistance was seen in 23.7% to 66.7% among the subspecies. Similarly, ciprofloxacin resistance was high among *M. massiliense* (36.8%) and *M. abscessus* (33.3%). M24, the only *M. bolletii*, was also resistant to ciprofloxacin. This finding agrees with the observation of de Moura *et al.,* (2012) that all *M. bolletii* exhibit resistance towards fluoroquinolones. Linezolid resistance was 13.2% in *M. massiliense* and 16.7% in *M. abscessus*.

Overall, 25% of *M. abscessus* and 55.3% of *M. massiliense* strains showed *in vitro* susceptibility to all five of the antibiotics. None were resistant to all five antibiotics, two (one *M. bolletii* and one *M. massiliense)* were resistant to four antibiotics and only four were resistant to three antibiotics. The only *M. bolletii* in the collection was resistant to all antibiotics, except for amikacin. On the other

hand, no antibiotic showed consistent activity on all strains. Amikacin and clarithromycin had the best activity for the Mab.

Table 4.3: Summary of resistance rates in *M. abscessus* complex

Bacterium (n)	**Cip**			**Imp**			**Amk**			**Cla**			**Lzd**		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
***M abscessus* (12)**	7	1	4	4	0	8	12	0	0	11	1	0	9	1	2
% Resistance			33.3			66.7			0			0			16.7
***M massiliense* (38)**	14	10	14	26	3	9	31	7	0	36	0	2	31	2	5
% Resistance			36.8			23.7			0			5.3			13.2
***M bolletii* (1)**	0	0	1	0	0	1	1	0	0	0	0	1	0	0	1
Total	21	11	19	30	3	18	44	7	0	47	1	3	40	3	8
% Resistance			37.3			35.3			0			5.9			15.7

S:susceptible, I:intermediate, R:resistant

Cip: ciprofloxacin, Imp: imipenem, Amk: amikacin, Cla: clarithromycin, Lzd: linezolid

SIR breakpoints (µg/ml): ciprofloxacin (1,2,4); imipenem (4,8,16); amikacin (16,32,64); clarithromycin (2,4,8); linezolid (8,16,32) (CLSI, 2011)

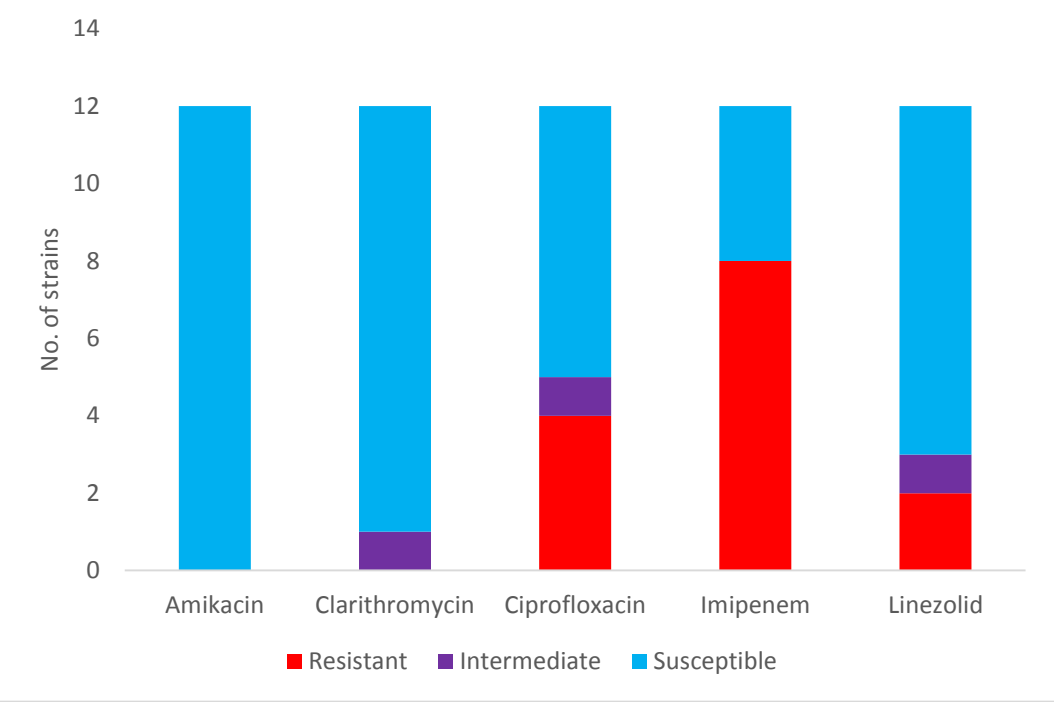


Figure 4.3A: Resistance pattern of *M. abscessus*

Amikacin has the best antimicrobial activity, with no resistance recorded, followed by clarithromycin and linezolid. Imipenem has the poorest antimicrobial activity with more than half of the strains being resistant.

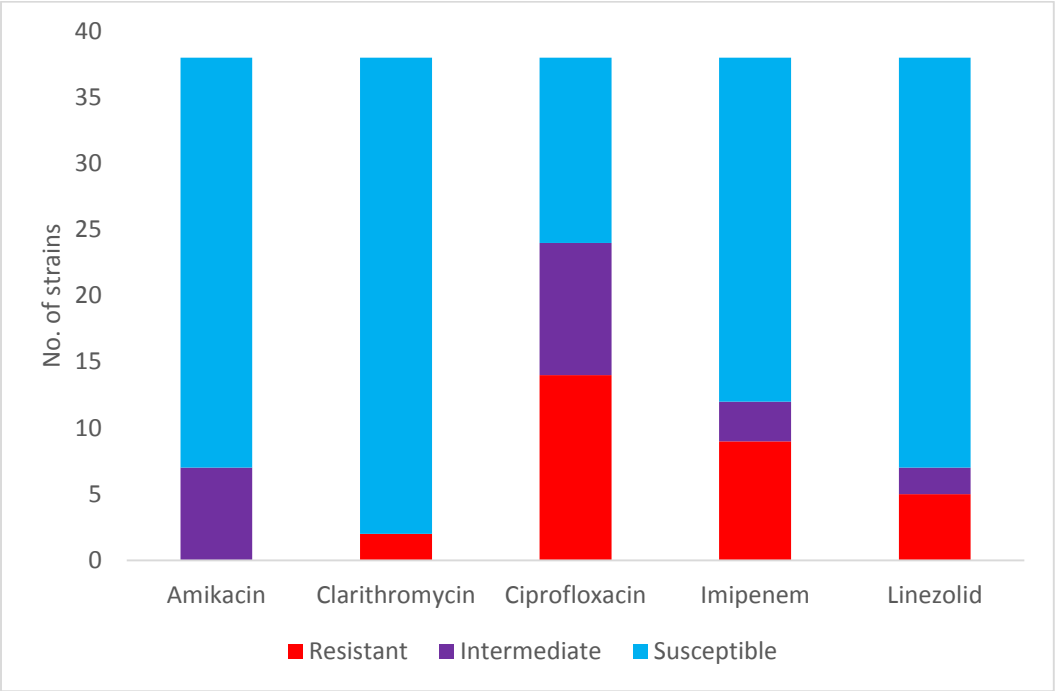


Figure 4.3B: Resistance pattern of *M. massiliense*

Clarithromycin has the best antimicrobial activity, followed by amikacin and linezolid. Ciprofloxacin has the poorest antimicrobial activity with many strains being resistant or have intermediate MIC value.

4.3.1 Detection of inducible clarithromycin resistance in T28 variants of *M. abscessus* strains

M. abscessus ATCC 19977 and two other T28 sequevars were tested for inducible resistance to clarithromycin by extending the duration of incubation in the Etest to 14 days. Surprisingly, the MIC of all three strains (0.125-2 µg/ml) did not increase with prolonged incubation. When the Etest was repeated with clarithromycin pre-incubated ATCC 19977, a slight increase in MIC from 0.125 to 0.75 µg/ml was observed on day 14, which was far below the resistance breakpoint of 8 µg/ml (Figure 4.3.1).

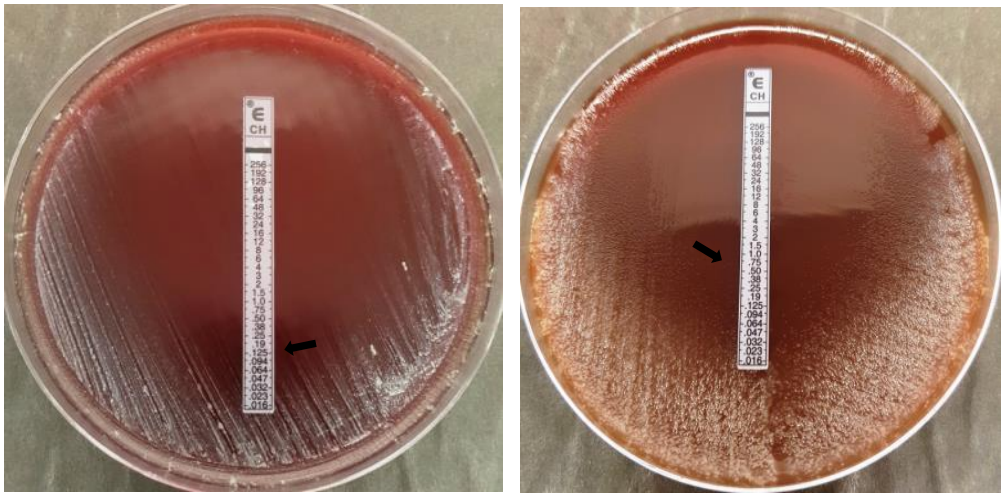


Figure 4.3.1: Comparison of Cla MIC values for ATCC 19977 on day 14 of incubation

The picture on the left shows the MIC (0.125 µg/ml) obtained without pre-incubation in clarithromycin; the picture on the right shows the MIC (0.75 g µ/ml) obtained with pre-incubation.

4.4 PCR-sequencing for known resistance-associated genes

4.4.1 Amikacin

All 51 strains tested did not show phenotypic resistance to amikacin. This susceptibility is 100% correlated with the absence of mutations in the *rrs* gene, including the mutations at nucleotides 1406, 1408, 1409 and 1491 (*E. coli* numbering) that have been reported to be associated with amikacin resistance in Mab. Figure 4.4.1 shows the multiple sequence alignment of some of the strains.

Figure 4.4.1: The *rrs* gene sequence analysis of selected strains. The mutations T1406A, A1408G, C1409T and G1491T in amikacin susceptible strains (*E. coli* numbering) are absent. Alignment was performed with MultAlin (Corpet, 1998).

4.4.2 Clarithromycin

Only three (two *M. massiliense* and one *M. bolletii*) of the 51 strains tested showed phenotypic clarithromycin resistance. With the exception of one strain (M139), all clarithromycin-susceptible *M. massiliense* (n=35) showed the typical 276 bp deletion of the *erm*(41) gene (Figure 4.2.5). The T to C substitution at nucleotide 28 in M139 explains its susceptibility although M139 has the full length *erm*(41) typical of clarithromycin resistance.

Figure 4.4.2A below shows the gel image for *erm*(41) and *rrl* PCR products. The size of the *erm*(41) gene in M24 (*M. bolletii*) is 450 bp while in M134 (*M. massiliense*) is 174 bp. The *rrl* gene is 728 bp in size.

None of the 12 *M. abscessus* strains tested was phenotypically clarithromycin resistant. Ten of these 12 were C28 sequevars usually associated with clarithromycin susceptibility. The two *M. abscessus* strains which were T28 sequevars did not show the inducible resistance (within 14 days of incubation) described as a characteristic of T28 sequevars. In the only highly clarithromycin-resistant *M. massiliense*, M134 (MIC>256µg/ml), the presence of the A2058G (*E. coli* numbering) mutation in the *rrl* gene (Figure 4.4.2B) explained the high level

resistance in the presence of a truncated *erm*(41) gene, which is associated with clarithromycin susceptibility.

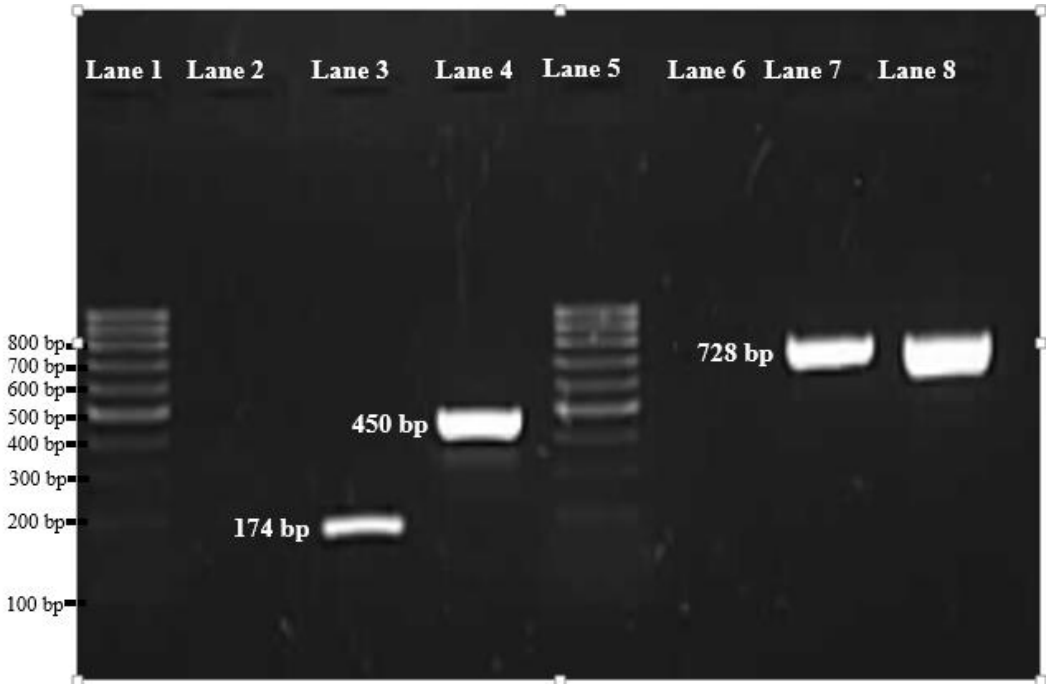


Figure 4.4.2A: Gel image of *erm*(41) and *rrl* PCR products

Lanes 1 to 8 are 100 bp ladder, RC, M134, M24 with amplified *erm(*41); 100 bp ladder, RC, M134 and M24 with amplified *rrl* respectively. There is a clear difference in size between the *erm*(41) in M24 (*M. bolletii*) and in M134 (*M. massiliense*)

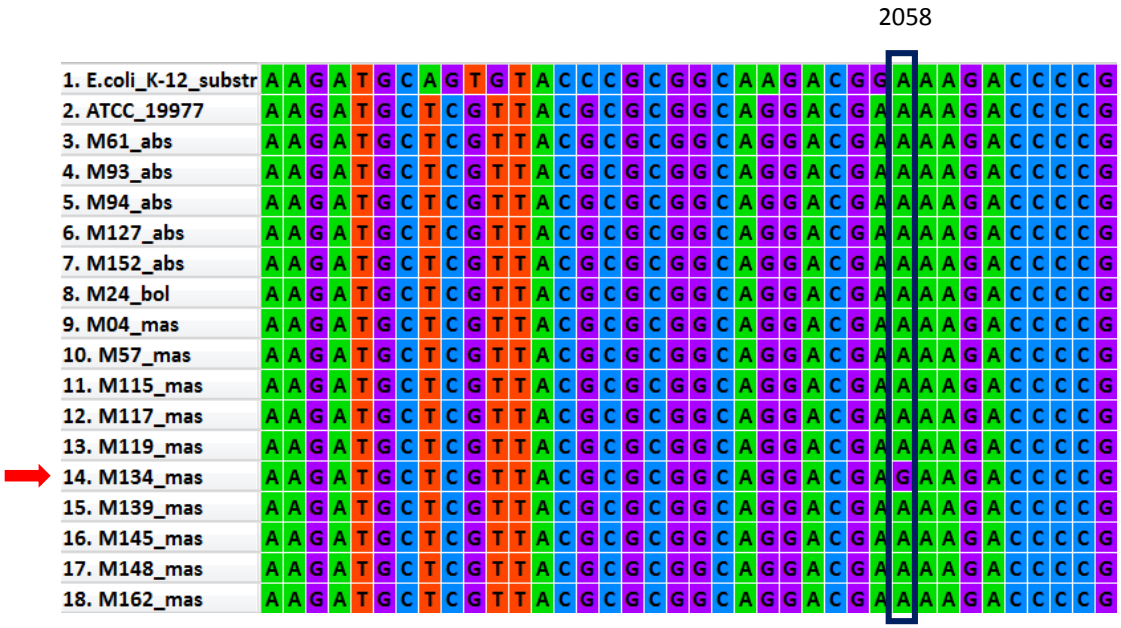


Figure 4.4.2B: Nucleotide sequence of the *rrl* gene.

The A2058G mutation in M134 causes high resistance to clarithromycin.

4.4.3 Ciprofloxacin

The *gyr*A and *gyr*B genes were successfully amplified in all 19 ciprofloxacin-resistant strains (Figure 4.4.3A) but no previously described resistance-associated mutations, Ala-92-Val, Asp-96-Asn in *gyr*A and Arg-482-Cys in *gyr*B (*M. abscessus* numbering), were seen in the resistant strains (Figures 4.4.3B and 4.4.3C). Furthermore, the amino acid sequences were also almost identical in both the resistant and susceptible strains, suggesting mechanisms other

than mutations in *gyr*A and *gyr*B to be responsible for the ciprofloxacin resistance exhibited by the Mab in this study.

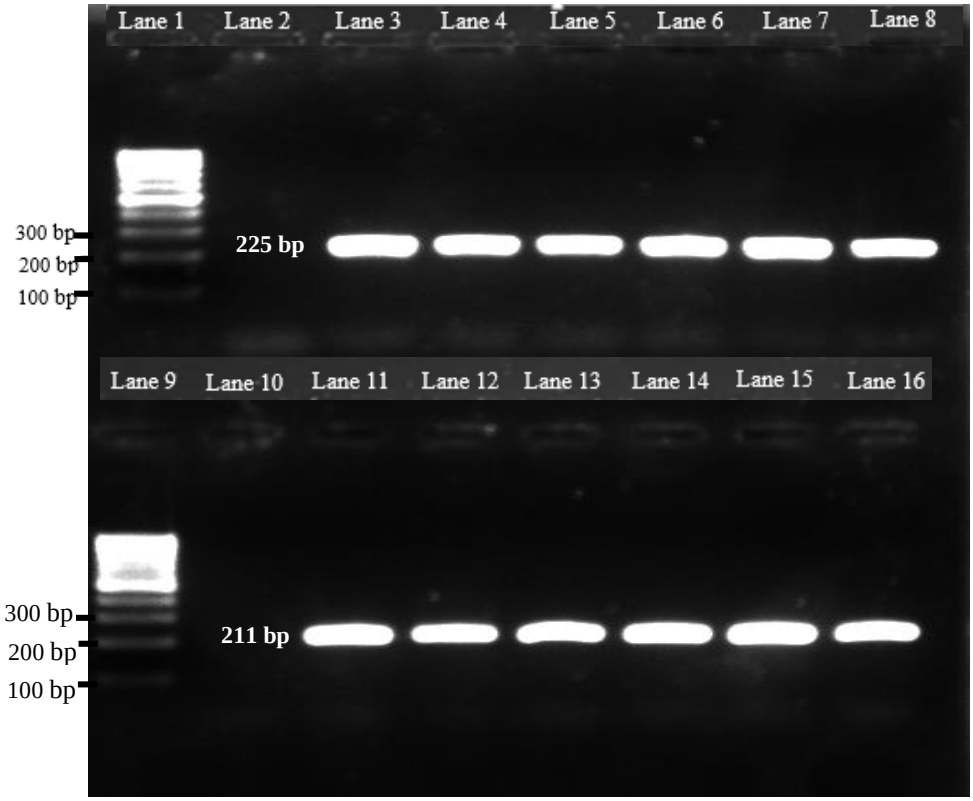


Figure 4.4.3A: Gel image of *gyrA* and *gyrB* PCR products

Lanes 1 to 8 (*gyr*A):100 bp ladder, RC, M240, M241, M242, M243, M244, M245. The product size is 225 bp

Lanes 9 to 16 (*gyr*B): 100 bp ladder, RC, M240, M241, M242, M243, M244, M245. The product size is 211 bp

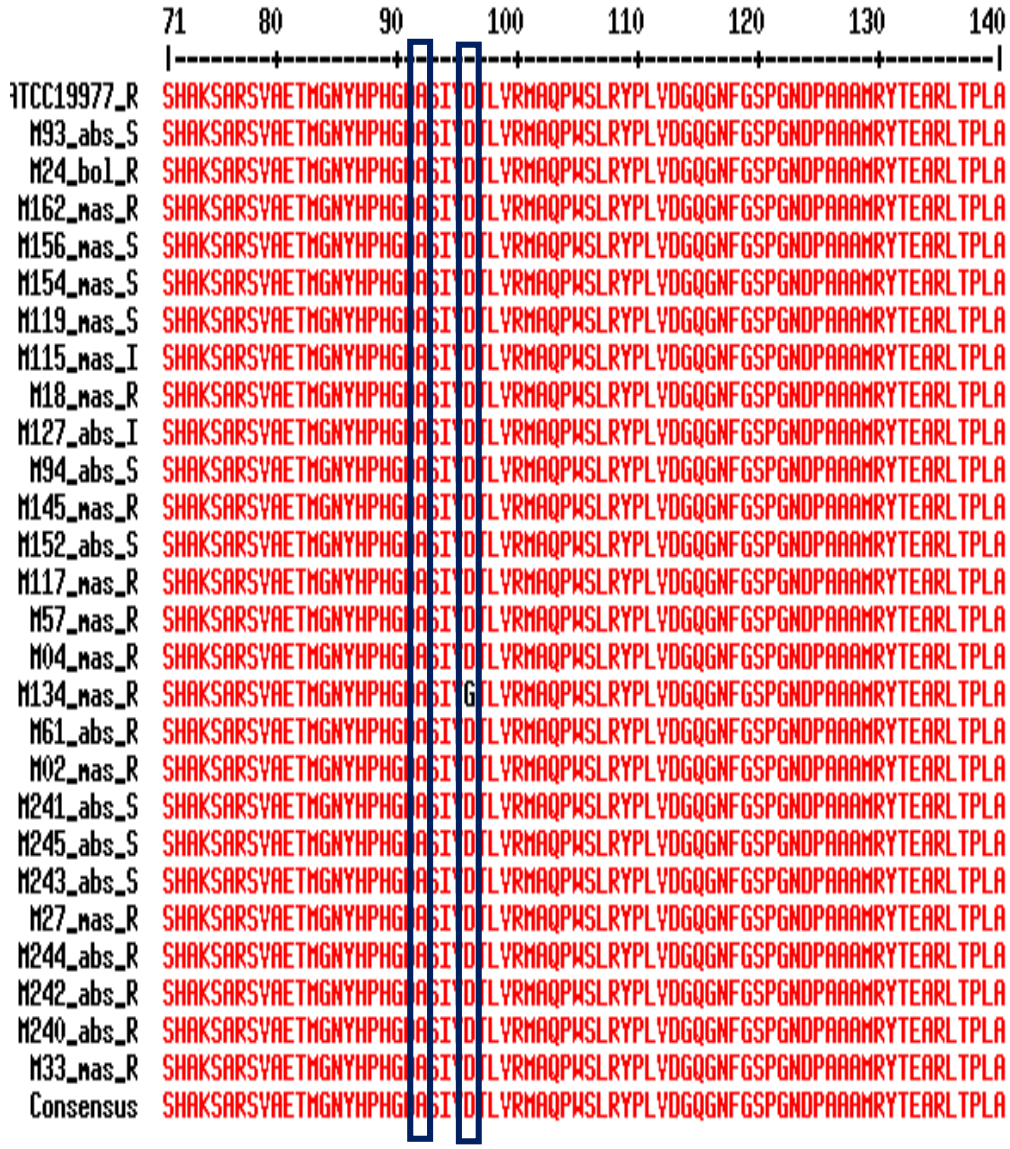


Figure 4.4.3B: The amino acid alignment of *gyr*A gene.

*Gyr*A QRDR extends from amino acid 86 to 115 (*M. abscessus* numbering). With one exception (M134), amino acid at positions 92 and 96 are the same regardless of the susceptibility phenotype.

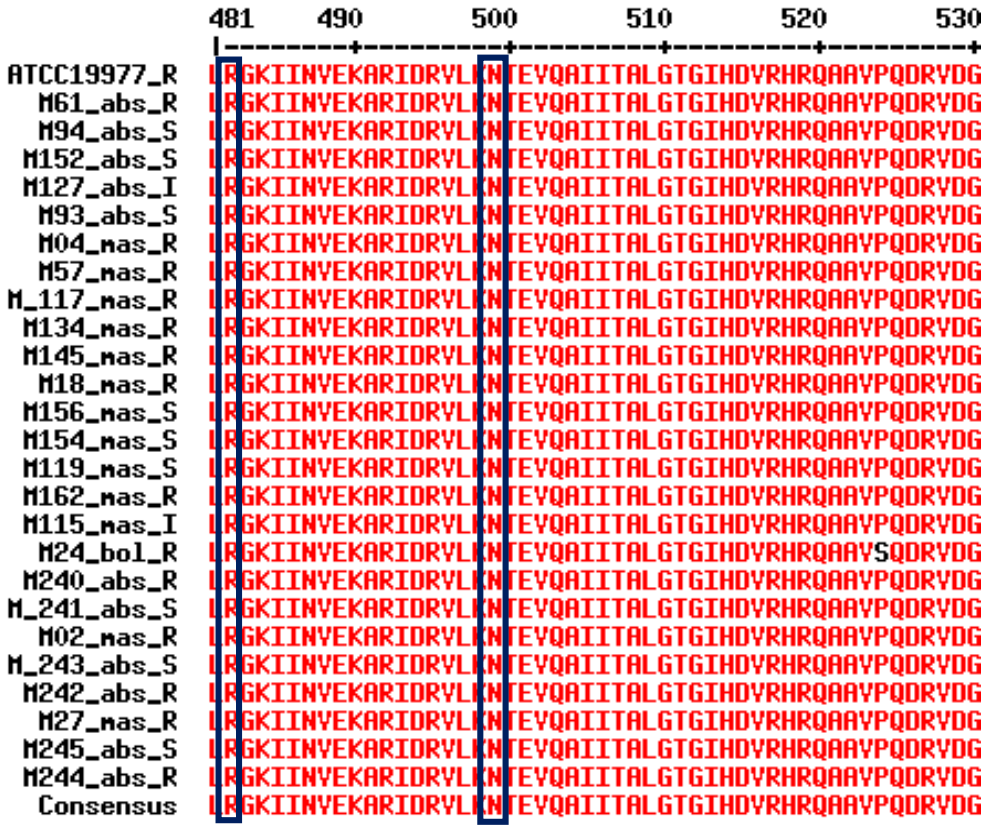


Figure 4.4.3C: The amino acid alignment of *gyr*B gene

The *gyr*B QRDR extends from amino acid residues 472 to 499, in the numbering system used for *M. abscessus.* Amino acids at position 482 and 499 are the same regardless of the susceptibility phenotype.

4.4.4 Imipenem

The MBL gene (IMP-1), a plasmid-encoded carbapenemase reported in *P. aeruginosa*, was amplified in all 18 imipenem-resistant strains (MIC>32 µg/ml) as well as all 30 susceptible strains (MIC< 2 µg/ml) (Figure 4.4.4A). BLAST search revealed this homologue to belong to the putative metallo-beta-lactamase superfamily while Interpro analysis predicted the protein domain as metallo-beta-lactamase (Figure 4.4.4B).

The gene sequence was, however, only 38 % similar to the IMP-1 in *P. aeruginosa (*Appendix F). It is not clear why this IMP-1 homologue is silent in the Mab strains examined in this study but the results suggest that IMP-1 is not likely to be responsible for imipenem resistance in Mab.

Figure 4.4.4A: Metallo- beta-lactamase (IMP-1) gel image

Lanes 1 to 15 are 100 bp ladder, RC, M57, M 145, M 161, ATCC 19977, M61, M24, ladder, M93, M94, M119, M120, M148 and M152 respectively. The product size is 523 bp

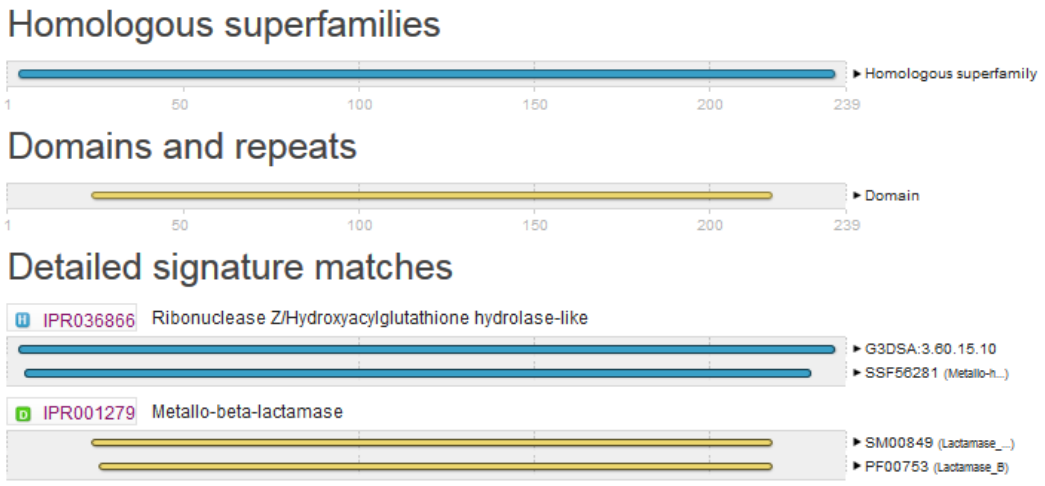


Figure 4.4.4B: Interpro analysis of IMP-1 homologue in Mab. The protein domain predicted was metallo- beta- lactamase

4.4.5 Linezolid

Figure 4.4.5A below is the gel image of the 23S rRNA. The mutations G2061T, G2447T, G2576T reported to be associated with resistance were not seen in the eight linezolid resistant strains. The gene sequence was almost identical in the resistant and susceptible phenotypes (Figure 4.4.5B). Unlike many other bacterial species, linezolid resistance in Mab might not be due to ribosomal mutations.

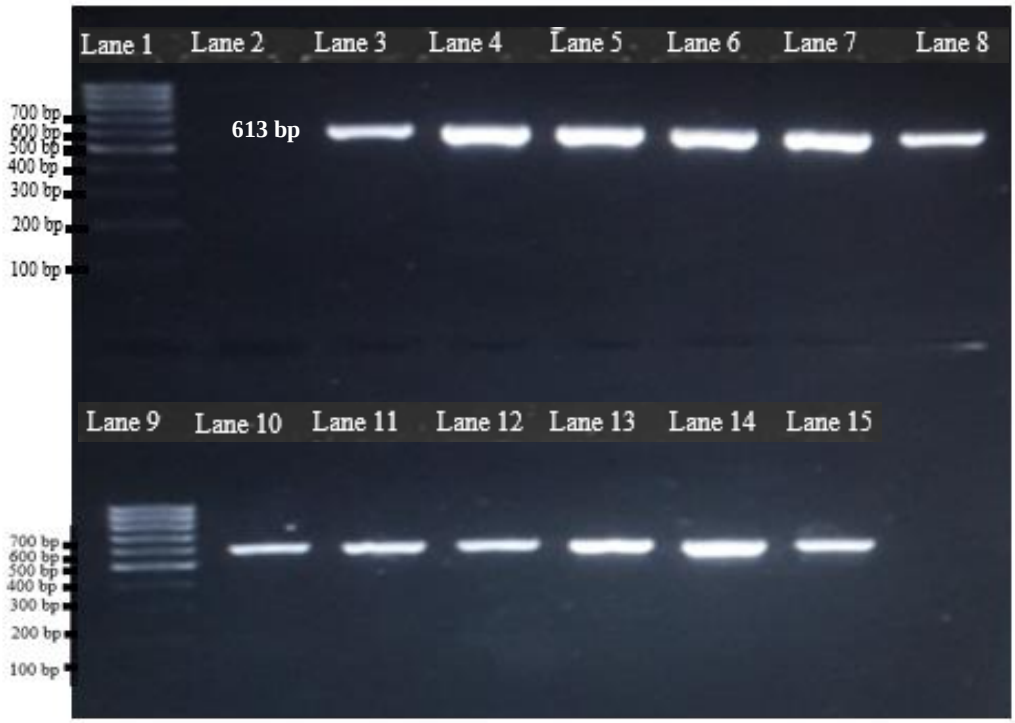
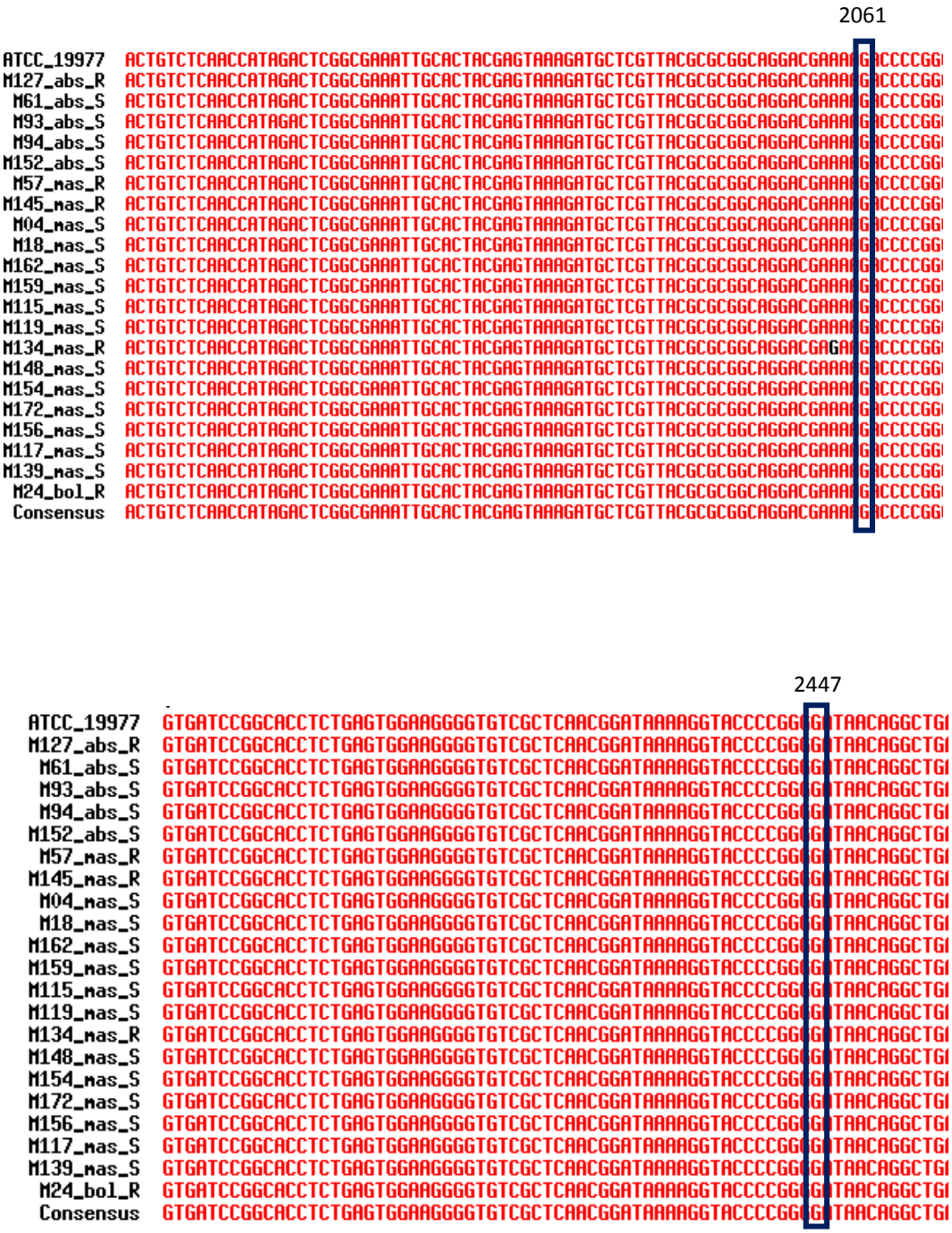


Figure 4.4.5A: 23S rRNA gel image

Lanes 1 to 15 are 100 bp ladder, RC, M61, M93, M94, M127, M240, M241, ladder, M04, M18, M27, M33, M57 and M120 respectively. The product size is 613 bp.

2061


2061
ATCC_19977 ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M127_abs_R ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M61_abs_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M93_abs_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M94_abs_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M152_abs_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M57_mas_R ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M145_mas_R ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M04_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M18_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M162_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M159_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M115_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M119_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M134_mas_R ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAGAAGACCCCGG
M148_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M154_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M172_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M156_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M117_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M139_mas_S ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
M24_bol_R ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
Consensus ACTGTCTCAACCATAGACTCGGCGAAATTGCACTACGAGTAAAGATGCTCGTTACGCGCGGCAGGACGAAAAGACCCCGG
2447
ATCC_19977 GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M127_abs_R GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M61_abs_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M93_abs_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M94_abs_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M152_abs_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M57_mas_R GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M145_mas_R GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M04_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M18_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M162_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
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M119_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M134_mas_R GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M148_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M154_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M172_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M156_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M117_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M139_mas_S GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
M24_bol_R GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG
Consensus GTGATCCGGCACCTCTGAGTGGAAGGGGTGTCGCTCAACGGATAAAAGGTACCCCGGGGGTAACAGGCTG

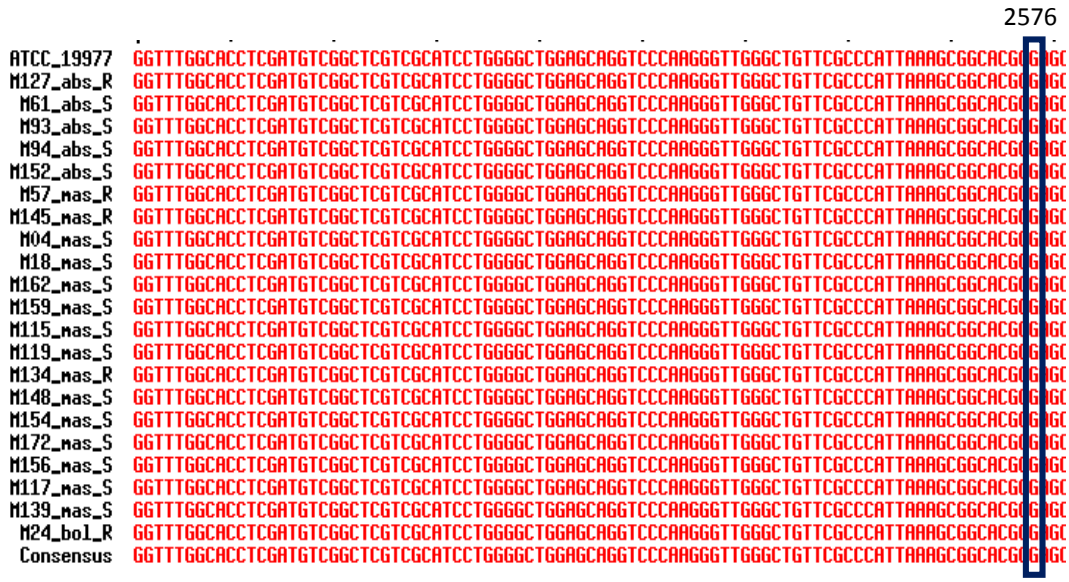


Figure 4.4.5B: The 23S rRNA sequence alignment.
Mutations G2061T, G2447T and G2576T were not seen in any of the strains (*E. coli* numbering)

4.5 *In silico* prediction of antibiotic resistant genes

Genes associated with resistance to the five antibiotics examined in this study were found in all three databases (Table 4.5). Appendix G lists the predicted genes by each database. CARD provided the most number of predicted genes with high specificity. ResFinder predicted the least number of genes since transporter genes and efflux pump genes like *mtr*A and *qep*A2 were not included in its database. The ARG-ANNOT database was highly sensitive, revealing many AR genes with more than 90% of sequence similarity for all classes of antibiotics

studied, except for oxazolidinone (linezolid). However, it was also highly non-specific, yielding hits with low query coverage (35% and lower). The genes detected by more than one database shared identical sequences e.g. the *qep*A2 gene sequence detected by both CARD and ARG-ANNOT.

Following MSA with gene sequences of susceptible and resistant phenotypes from the 21 strains with WGS data, the most likely resistance-associated genes were identified to be *rrl*, *erm*(41) (clarithromycin), *gyr*A, *efr*A, *qep*A2 (ciprofloxacin), KPC-16, PBP2 (imipenem) and *optr*A, *clb*B (linezolid).

Table 4.5. Comparison of top resistance-associated genes predicted by the three ARG databases

Antibiotic	ARG-ANNOT	CARD	ResFinder
Amikacin	*aph* (3")-*Ic*	*aph(3")-Ia*	*aac*(2')-*Ib*
	aac(3)-*VII*	*kdp*E	*rrs*
	aph(6)-*Ia*	*sme*S	
	aac(3)-*IIIb*	*bae*S	
	rrs	*cpx*A	
Clarithromycin	*erm*(41)	*erm*(41)	*erm(*41)
	*srm*B	*mtr*A	*rrl*
	*tlr*C	*efr*A	
	*ole*B	*ole*B	
	rrl	*rrl*	
Ciprofloxacin	*gyr*A	*gyr*A	*gyr*A
	*qep*A	*qep*A2	
	*par*C	*efr*A	
		*pat*A	
		*mfp*A	
Carbapenem	*far*-1	KPC-16	Nil
	*oxy*1-2	PBP2	
	ctx-M-139	*mec*B	
	*aqu*2	*spg*-1	
	pam-1	*nmc*-R	
Oxazolidinone	Nil	*clb*B	Nil
		*optr*A	

4.6 Significance of predicted resistance genes

4.6.1 Amikacin

A number of ribosomal and efflux-related genes were predicted but MSA could not be carried out to see whether they carried mutations that could be associated with resistance because there were no amikacin-resistant phenotypes among the strains examined. The similarity between these predicted genes in Mab and their counterparts in the ARG databases ranged from 29.3% to 70% (Appendix H). A number of aminoglycoside modifying enzymes such as *aac*(3)-*VII* and *aac*(2')-*Ib* were predicted as well. Possible reasons for their non-expression in the susceptible phenotypes will be discussed in chapter 5. The *eis*2 which was reported to be responsible for clinical resistance in Mab infections (Rominski *et al.*, 2017) was not predicted.

4.6.2 Clarithromycin

The *erm*(41) and *rrl* genes were predicted in all the three databases. The mutations in both genes showed good correlation with phenotypic resistance in the MSA and with the results of PCR-sequencing.

4.6.3 Imipenem

Many genes encoding beta-lactamase enzymes were identified in the CARD and ARG-ANNOT databases but, none showed consistent correlation with the resistant phenotype. Nevertheless, the MSA showed differences in gene sequence among the resistant and susceptible strains. For instance, in the KPC-16 homologue identified by BLAST search to be Bla_{Mab}, an Ambler class A beta-lactamase (Soroka *et al.*, 2017), it was noted that the three resistant *M. abscessus* strains had threonine at position 141 while two of the three susceptible strains had alanine instead (Figure 4.6.3A). Among the *M. massiliense*, five of the eight imipenem susceptible strains had amino acid (aa) differences in nine positions : Asp instead of Asn at aa 33, Ala instead of Thr at aa 63, Arg instead of His at aa 83, Arg instead of Ser at aa 85, Gln instead of Arg at aa 94, Ser instead of Pro at aa 115, Thr instead of Ser at aa 161, Gly instead of Asp at aa 252 and Thr instead of Ala at aa 280

(Figure 4.6.3B). While it is possible that these amino acid mutations could have rendered the Bla_{Mab} beta-lactamase non-functional, the small number of phenotypes examined here particularly in the *M. abscessus* makes it difficult to confirm the significance of these mutations.

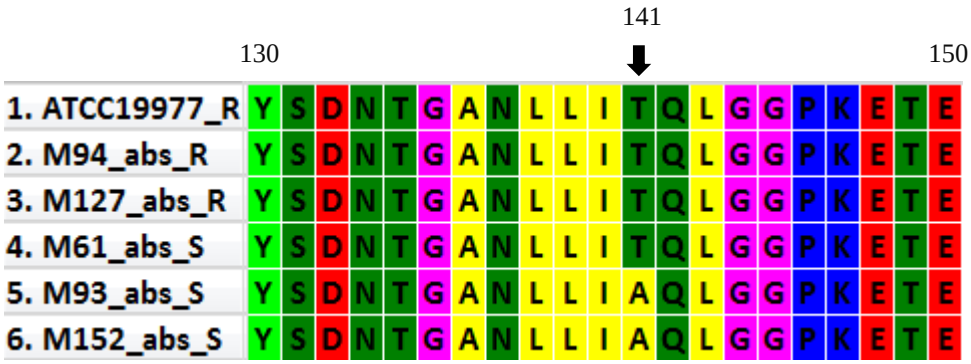
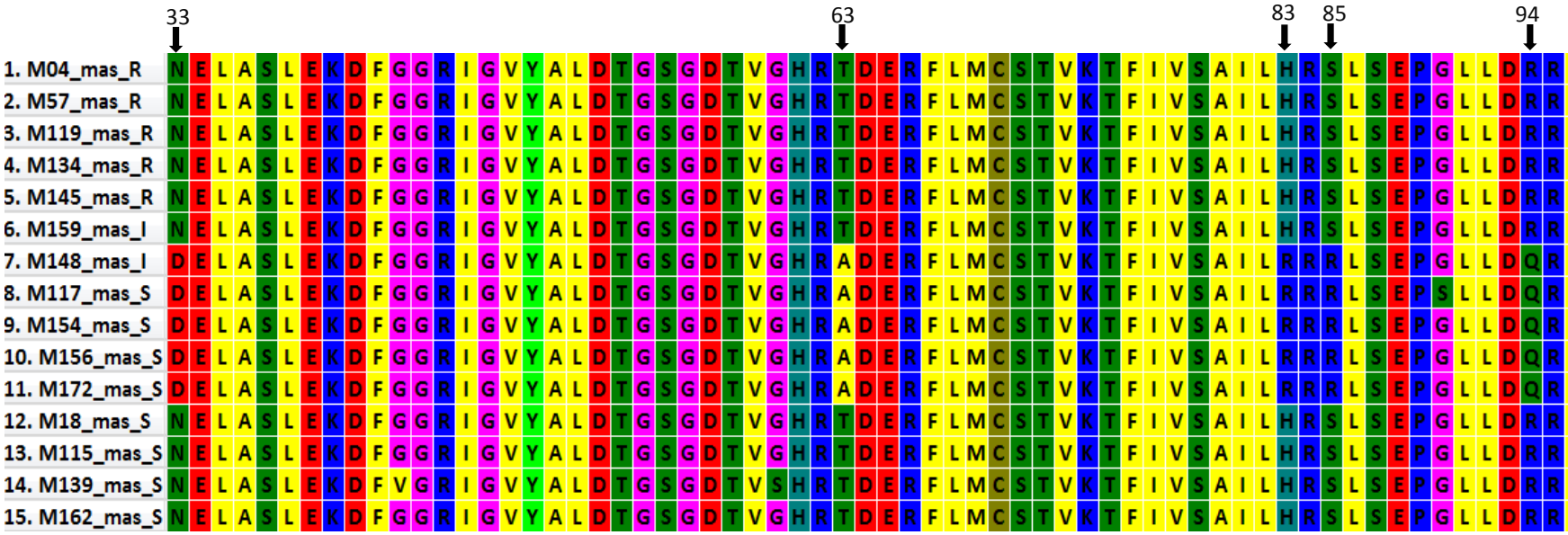
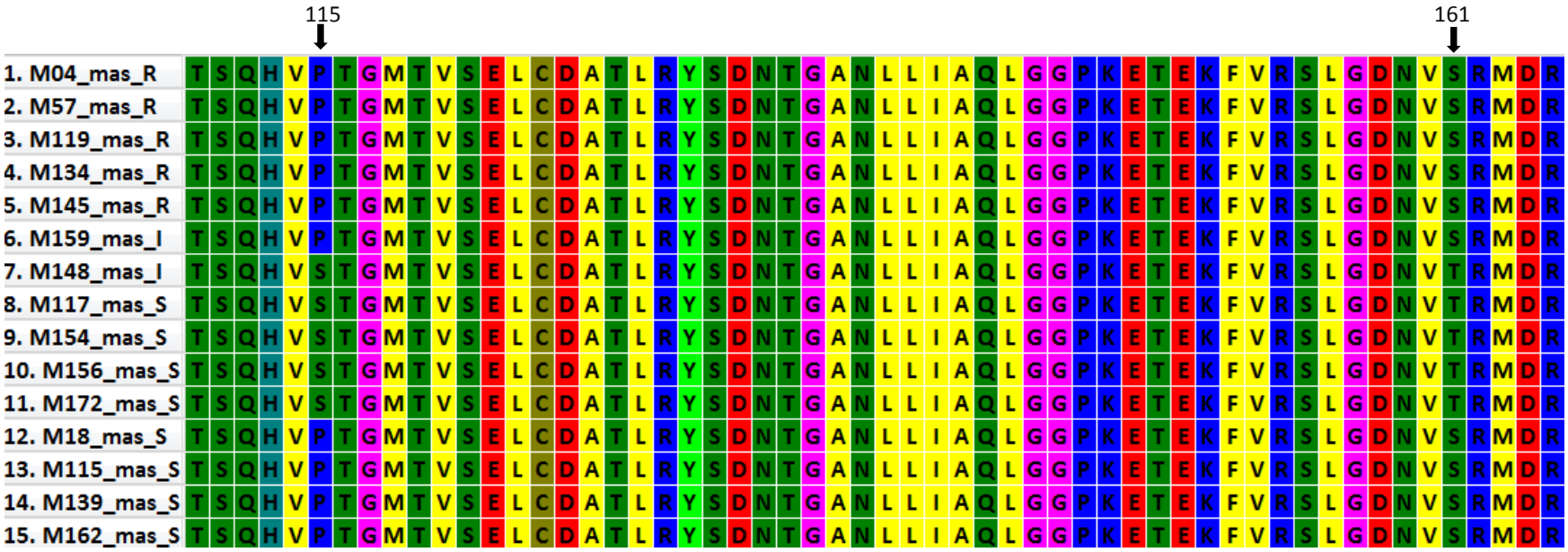


Figure 4.6.3A: MSA alignment of the Bla_{Mab} gene in *M. abscessus* strains

The susceptible strains had alanine instead of threonine at position 141, except for M61.


33
63
83
85
94
1. M04_mas_R NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
2. M57_mas_R NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
3. M119_mas_R NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
4. M134_mas_R NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
5. M145_mas_R NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
6. M159_mas_I NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
7. M148_mas_I DELASLEKDFGGRIGVYALDTGSGDTVGHRADERFLMCSTVKTFIVSAILRRRLSEPGLLDQR
8. M117_mas_S DELASLEKDFGGRIGVYALDTGSGDTVGHRADERFLMCSTVKTFIVSAILRRRLSEPSLLDQR
9. M154_mas_S DELASLEKDFGGRIGVYALDTGSGDTVGHRADERFLMCSTVKTFIVSAILRRRLSEPGLLDQR
10. M156_mas_S DELASLEKDFGGRIGVYALDTGSGDTVGHRADERFLMCSTVKTFIVSAILRRRLSEPGLLDQR
11. M172_mas_S DELASLEKDFGGRIGVYALDTGSGDTVGHRADERFLMCSTVKTFIVSAILRRRLSEPGLLDQR
12. M18_mas_S NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
13. M115_mas_S NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
14. M139_mas_S NELASLEKDFVGRIGVYALDTGSGDTVSHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR
15. M162_mas_S NELASLEKDFGGRIGVYALDTGSGDTVGHRTDERFLMCSTVKTFIVSAILHRSLSEPGLLDRR


115
161
1. M04_mas_R T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
2. M57_mas_R T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
3. M119_mas_R T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
4. M134_mas_R T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
5. M145_mas_R T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
6. M159_mas_I T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
7. M148_mas_I T S Q H V S T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V T R M D R
8. M117_mas_S T S Q H V S T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V T R M D R
9. M154_mas_S T S Q H V S T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V T R M D R
10. M156_mas_S T S Q H V S T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V T R M D R
11. M172_mas_S T S Q H V S T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V T R M D R
12. M18_mas_S T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
13. M115_mas_S T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
14. M139_mas_S T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R
15. M162_mas_S T S Q H V P T G M T V S E L C D A T L R Y S D N T G A N L L I A Q L G G P K E T E K F V R S L G D N V S R M D R

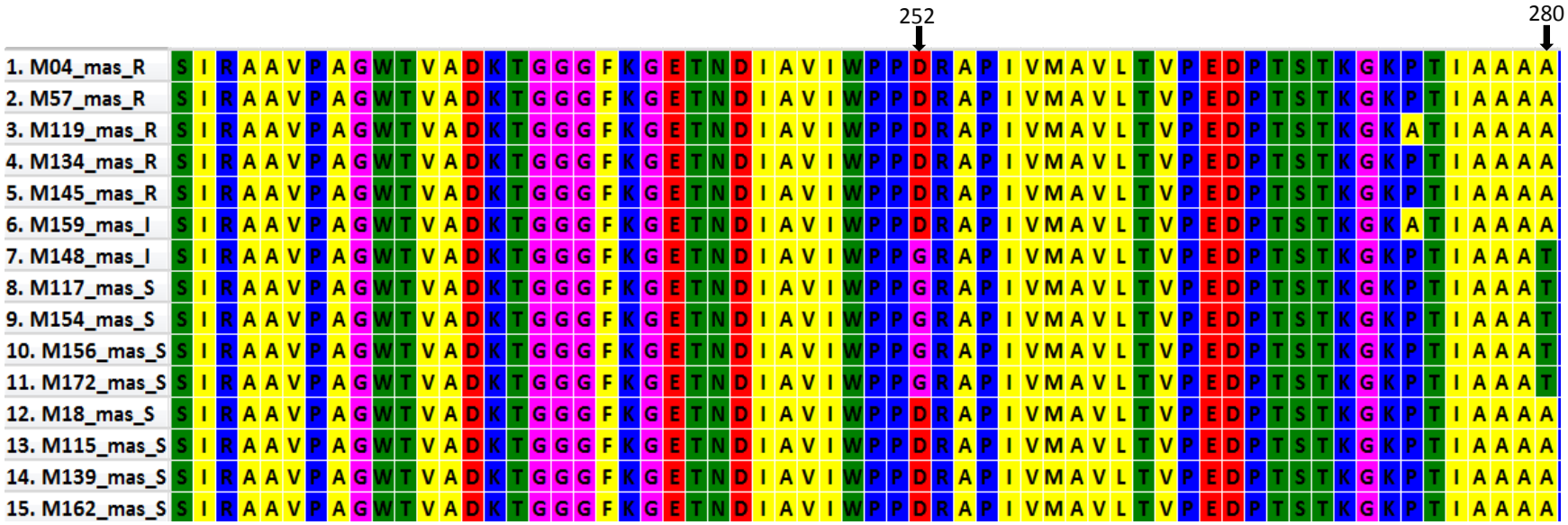


Figure 4.6.3B: Bla$_{Mab}$ alignment of *M. massiliense* strains.

The five imipenem-susceptible strains had amino acid differences at nine positions:Asp instead of Asn at aa 33, Ala instead of Thr at aa 63, Arg instead of His at aa 83, Arg instead of Ser at aa 85, Gln instead of Arg at aa 94, Ser instead of Pro at aa 115, Thr instead of Ser at aa 161, Gly instead of Asp at aa 252 and Thr instead of Ala at aa 280.

Another beta-lactamase protein with amino acid sequence differences in different phenotypes is the SPG-1 homologue, the carbapenemase reported in *Sphingomonas sp.* (Gudeta *et al.*, 2016). It was observed that all the resistant *M. massiliense* strains and M24 have alanine at position 2, (*M. abscessus* numbering) leucine at position 54 and valine at position 115 while the susceptible and intermediate strains (with the exception of M18, M139 and M162) have serine, valine and isoleucine respectively (Figure 4.6.3C). Here again, further investigations are required to assess the significance of these mutations.

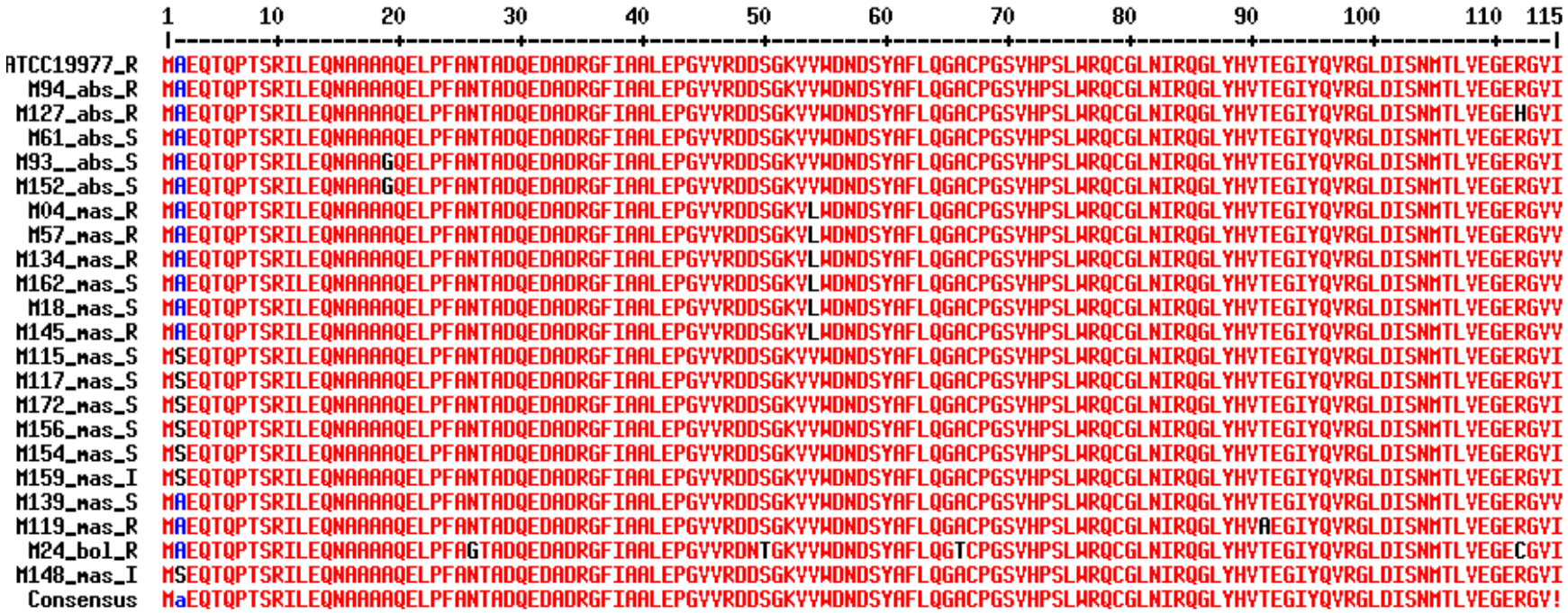


Figure 4.6.3C: MSA of the SPG-1 protein.

Resistant *M. massiliense* strains and M24 have alanine at position 2, (*M. abscessus numbering*) leucine at position 54 and valine at position 115 while the susceptible and intermediate strains have serine, valine and isoleucine respectively, except for M18, M 139 and M162. At position 19, the resistant *M. abscessus* strains have alanine while M93 and M152 which were susceptible to imipenem have glycine, except for M61

4.6.4 Linezolid

Linezolid is a relatively new antibiotic, compared to the other four antibiotics. Although various mutations have been associated with linezolid resistance (Long and Vester, 2012), they have not been adequately studied in Mab. In this study, the search through ARG databases did turn up several genes which could play a role in resistance, such as the *optr*A which encodes an ABC-transporter (Wang *et al.*, 2015) and *clb*B, a cfr gene that methylates the 23S ribosomal RNA (Hansen *et al.*, 2012; Atkinson *et al.*, 2013) , but the MSA of resistant and susceptible strains did not show a clear genotypic-phenotypic correlation.

4.6.5 Ciprofloxacin

Among the genes identified by the three databases, *gyr*A, *efr*A, *qep*A2, *pat*A and *mfpA* had the highest bitscore values under the FQ antibiotic class. *Pat*A, a part of an ABC transporter (Baylay *et al.*, 2015) and *mfp*A, a quinolone resistant (qnr) protein homologue that confers resistance to fluoroquinolones in *M. smegmatis* (Jacoby and Hooper, 2013), did not exhibit any correlation with the Mab phenotypes in this study.

Several studies have suggested that point mutations in *gyr*A confers resistance in Mab (Monego *et al.,* 2012; Lee *et al.,* 2014). However, MSA analysis showed that there was no correlation between these mutations (Ala-92-Val, Asp-96-Asn, *M. abscessus* numbering) and the phenotypic resistance observed in the Mab strains. Moreover, the amino acid sequence of all the strains in this study were almost identical, regardless of the phenotypic susceptibility, indicating lack of association between this gene and ciprofloxacin resistance.

Nonetheless, synonymous nucleotide differences between susceptible and resistant strains were seen at 15 locations in *M. abscessus* i.e. A231C, C378G, G1077T, T1119C, C1128T, C1290T, G1509C, C1545T, C1626T, A1860G, T1977C, T2013C, C2074T, T2151C and G/C2160T and at 18 locations in *M. massiliense* i.e G141C, T147C, A378C, C486T, A501G, C591T, A957G, T972C, T999C, A1017G, C1128T, C1251T, C1458T, T1485C, C1551T, A1695G, C2176T and G2373A (*M. abscessus* numbering). Further studies with larger sample sizes are needed to investigate whether synonymous mutations could contribute to a change in the antibiotic susceptibility of the Mab.

***efr*A**

The *efr*A gene codes for part of the efrAB efflux pump, an ABC multidrug efflux pump that confers resistance to macrolides, tetracyclines and fluoroquinolones (Jia *et al.*, 2017). The *efr*A in *M. abscessus* shares 40.8% homology with the *efr*A from *Enterococcus faecium* (Jia *et al.*, 2017). Sequence analysis of the *efr*A homologues in the Mab subspecies revealed some interesting patterns.

In *M. massiliense,* ciprofloxacin-resistant strains had leucine at position 429 (*M. abscessus* numbering) in the efrA protein, while the susceptible and intermediate strains had valine at this position. The nucleotide change was from GTG in susceptible strains to CTG in resistant strains. In contrast, all *M. abscessus* strains had valine at this position regardless of their susceptibility (Figure 4.6.5A). Hence, the Val-429-Leu mutation seems to be *M. massiliense* -specific.

At amino acid position 509, ATCC 19977 and M24 (*M. bolletii*) which were ciprofloxacin-resistant, had histidine (CAC), while the susceptible and intermediate *M. abscessus* strains had arginine (CGC). All *M. massiliense* strains had arginine at this position, except for M139 which had a histidine (Figure 4.6.5A). Here, the

Arg-509-His mutation in *efr*A appears to be associated with ciprofloxacin resistance only in *M. abscessus* and *M. bolletii*.

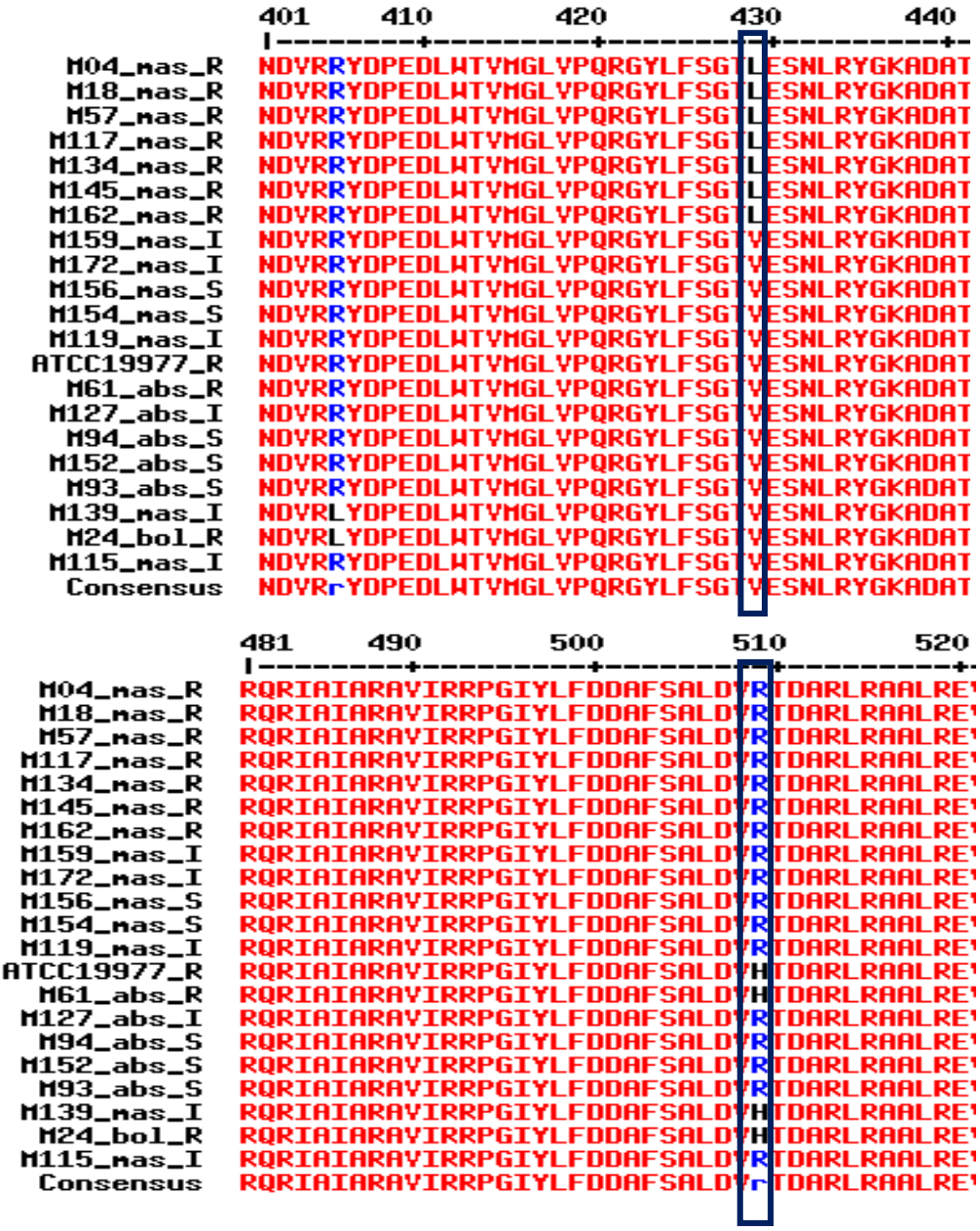


Figure 4.6.5A: Amino acid sequences of the *efr*A gene in the 21 strains with WGS data.

Resistant *M. massiliense* strains showed leucine at position 429 while the susceptible and intermediate strains had valine at this position. All *M. abscessus* strains and M24 had valine regardless of their susceptibility. At position 509, resistant *M. abscessus* and *M. bolettii* strains had histidine while the susceptible and intermediate strains had arginine. All *M. massiliense* strains possess arginine regardless of their susceptibility. M139, an ambiguos *M. massiliense* strain with features of both *M. abscessus* and *M. massiliense* had histidine instead of arginine at this position.

To further evaluate this hypothetical protein, a domain search was carried out with InterPro (Finn *et al.,* 2017), a resource for classifying proteins into families and predicting domains, repeats and functional sites, for the query hit in ATCC 19977 and the CARD protein sequence hit. Based on the search result, it is highly likely that the *efr*A sequence of the ATCC 19977 is indeed the gene for the EfrA pump. The EfrA in ATCC 19977 shares the exact protein domain, biological process and molecular functions with the EfrA in *E. faecium*. Furthermore, the general domain composition is very similar in both the protein sets. They share the exact detailed signature matches. Both are in the ABC transporter type 1, transmembrane domain superfamily (Figure 4.6.5B).

Furthermore, a BLAST search of the ATCC 19977 *efr*A homologue also showed its similarity to the *efr*A in *E. faecium* in their involvement in transmembrane transport and ATP binding function (Table 4.6.5A).

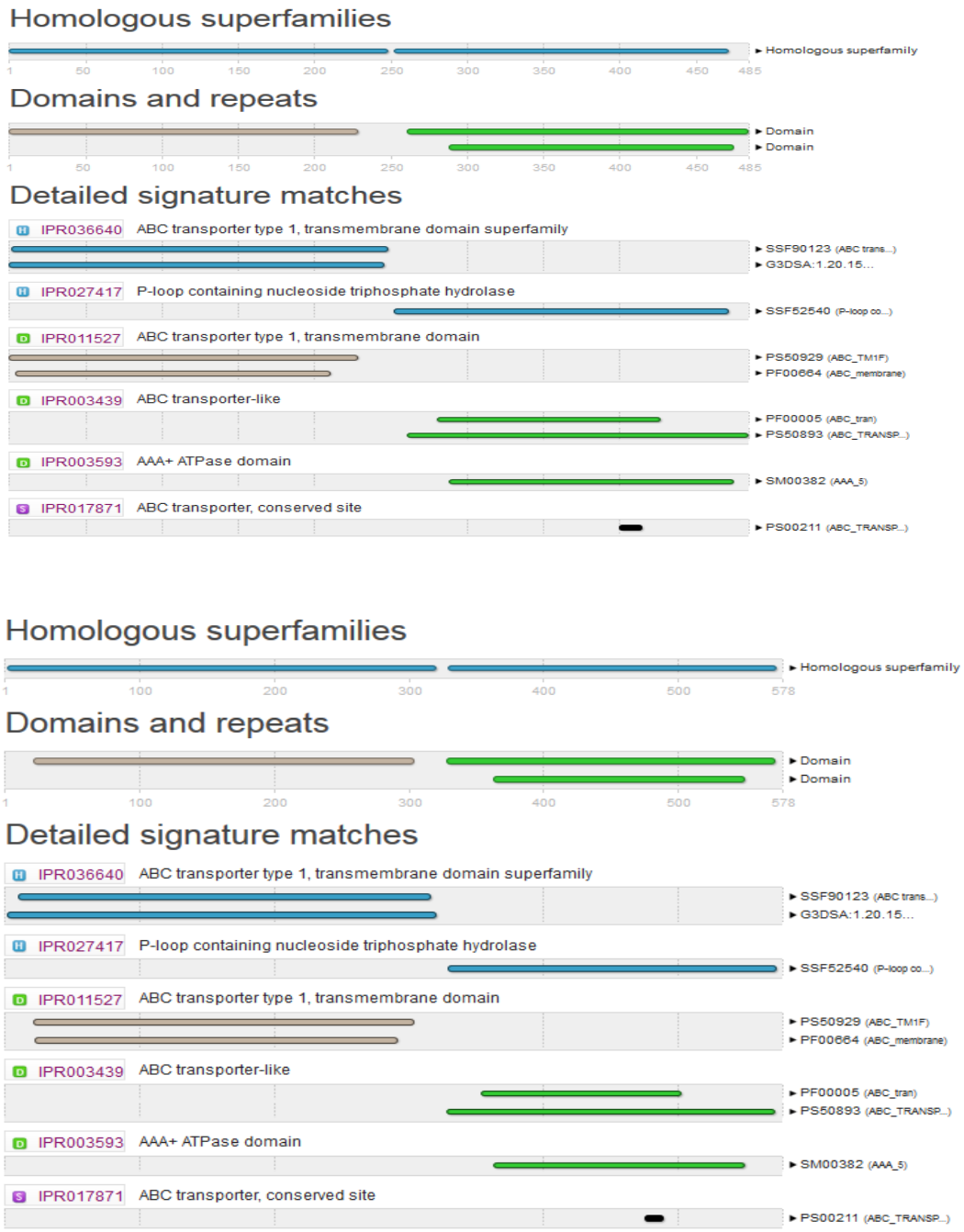


Figure 4.6.5B: Analysis of *efr*A gene with Interpro.

Both *Enterococcus faecium* (top) and ATCC 19977 (bottom) are probable ABC transporters, sharing the same protein family domains.

Table 4.6.5A: Comparison between the EfrA protein in *E. faecium* and the putative EfrA of ATCC 19977

Protein	**EfrA from *E. faecium***	**EfrA protein of ATCC 19977**
Size	485 amino acids	578 amino acids
Biological process	transmembrane transport	transmembrane transport
Molecular function	ATP binding ATPase avtivity	ATP binding ATPase avtivity
Cellular component	integral component of membrane	integral component of membrane

***Qep*A2**

QepA2 is a plasmid-mediated quinolone resistance pump found in an *Escherichia coli* isolate from France which confers high resistance to hydrophilic fluoroquinolones such as norfloxacin, ciprofloxacin, and enrofloxacin (Cattoir *et al.*, 2008). The *qep*A2 homologue found in this study shares 40.9% sequence identity with the *qep*A2 gene in *E. coli* (Jia *et al.*, 2017).

In all the ciprofloxacin-resistant *M. massilense* strains, the *qep*A2 homologue showed amino acid change from isoleucine to valine at position 155,

whereas the resistant *M. abscessus* strains showed amino acid changes at Asn-199-Asp and Val- 209- Ile (*M. abscessus* numbering) (Figure 4.6.5C).

As with the *efr*A gene, InterPro was used to compare the *qep*A2 gene of *E. coli* with the homologue found in *M. abscessus* complex (Figure 4.6.5D). Similarly, the *qep*A2 genes of both *E. coli* and ATCC 19977 share the same protein domain and signatures and the two proteins are similar in their biological process and cellular components (Table 4.6.5B).

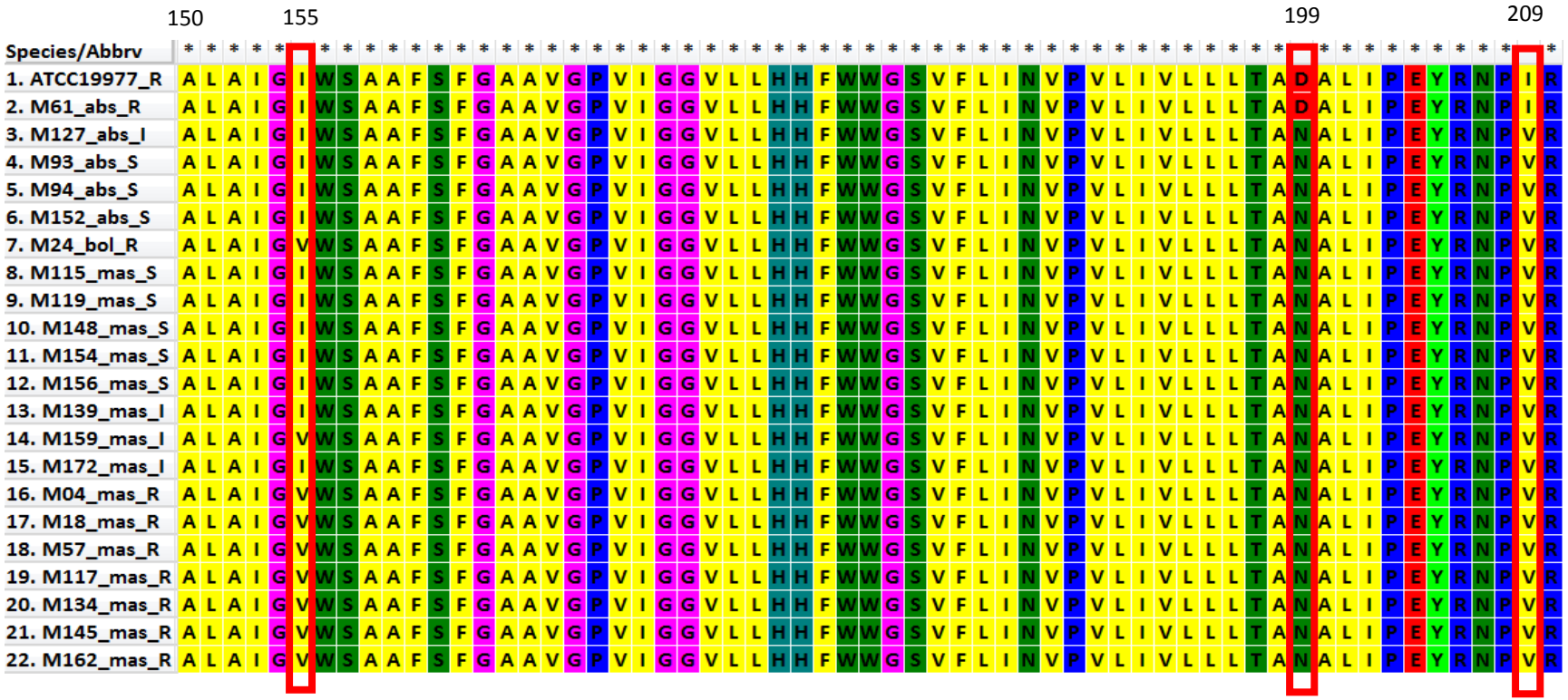


Figure 4.6.5C: Amino acid sequences of *qep*A2 in the 21 strains with WGS data.

Resistant *M. massiliense* strains showed valine at position 155 while the susceptible and intermediate strains had isoleucine at this position. All *M. abscessus* strains had isoleucine regardless of their susceptibility. At positions 199 and 209, the resistant *M. abscessus* strains had mutations Asn-199-Asp and Val-209-Ile respectively. The amino acids for the *M. massiliense* remained the same at these position

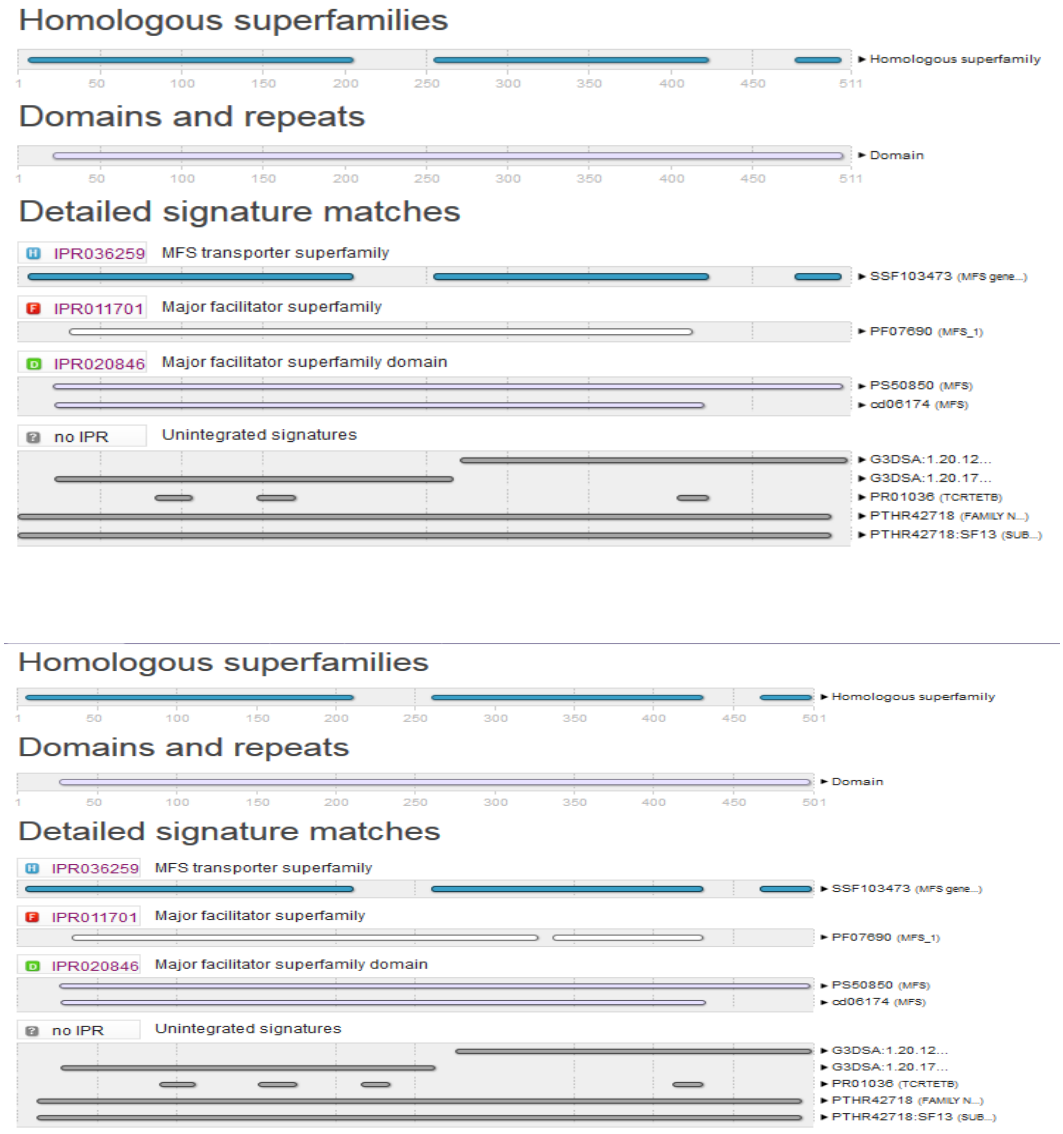


Figure 4.6.5D: Analysis of *qep*A2 gene with Interpro. Both *E. coli* (top) and *M. abscessus* (bottom) share the same protein domains and signatures

Table 4.6.5B: Comparison between the QepA2 protein in *E. coli* and the putative QepA2 in ATCC 19977

Protein	**QepA2 from *E. coli***	**QepA2 of ATCC 19977**
Size	511 amino acids	501 amino acids
Biological process	transmembrane transport	transmembrane transport
Cellular component	integral component of membrane	integral component of membrane

Genotypic-phenotypic correlation of *efrA* and *qepA2*

To further analyze the significance of the amino acid changes in *efr*A and *qep*A2, PCR-sequencing was carried out on Mab strains without WGS data. In *M. massiliense,* the results showed excellent correlation between the presence or absence of SNPs and the corresponding phenotype for *qepA*2 but not for *efr*A. In *M. abscessus,* however, the correlation was poor (Table 4.6.5C). This poor correlation could be due to the small number of *M. abscessus* strains examined, or the existence of other mechanisms of ciprofloxacin resistance in the *M. abscessus.*

Table 4.6.5C Comparison between *efr*A and *qep*A2 genes

Strain	Type	Susceptibility	*efrA*	*qepA2*
M02	*M. massiliense*	Resistant	Yes	Yes
M27	*M. massiliense*	Resistant	No	Yes
M33	*M. massiliense*	Resistant	No	Yes
M120	*M. massiliense*	Susceptible	Yes	Yes
M149	*M. massiliense*	Intermediate	Yes	Yes
M202	*M. massiliense*	Resistant	No	Yes
M240	*M. abscessus*	Resistant	No	No
M241	*M. abscessus*	Susceptible	No	No
M242	*M. abscessus*	Resistant	No	No
M243	*M. abscessus*	Susceptible	No	No
M244	*M. abscessus*	Resistant	No	No
M245	*M. abscessus*	Susceptible	Yes	Yes

The ‘Yes” and “No” indicate whether the genotype of the gene screened matches the phenotype observed. As seen**,** the genotype-phenotype correlation is better for *qep*A2. This indicates that *qep*A2 is a better candidate for future studies in ciprofloxacin resistance in Mab.

CHAPTER 5

DISCUSSION

5.1 Subspecies distribution of the *M. abscessus* complex (Mab)

For this study, Mab isolates from a routine diagnostic laboratory were collected over a period of three years. From this collection, it appears that *M. massiliense* is more common than *M. abscessus*, while *M. bolletii* is rare among clinical isolates from Malaysian patients with respiratory disease. However, this subspecies distribution may not be representative of the distribution in the rest of the country.

Previous studies have shown that the prevalence of Mab subspecies varies by geographical location. For example, *M. abscessus* predominates in Korea, (Lee *et al.*, 2014), Australia (Chua *et al.*, 2015) and China (Luo *et al.*, 2016), while *M. massiliense* is more common in Singapore (Chew *et al.*, 2017). *M. bolletii* is uncommon in most countries, except Brazil, where there was an outbreak of post-surgical *M. bolletii* infection (Baruque Villar *et al.*, 2015).

The small number of isolates examined in this study reflects the lack of facilities for the identification of Mab, particularly the subspecies, in routine diagnostic laboratories in the country.

5.2 Antibiotic susceptibility of Mab

Antibiotic susceptibility testing is important to guide therapy and to monitor patient's response to therapy. Since antibiotic susceptibility varies in different geographic locations for most bacteria, it is essential to have local antibiotic susceptibility data for empirical therapy.

The antibiotic susceptibility results for Mab in this study showed that amikacin and clarithromycin have a good antimicrobial activity overall, with 0% resistance to amikacin and only 5.9% resistance to clarithromycin. This finding is consistent with the common use of these two drugs for Mab infections world-wide (Tang *et al.*, 2014; Novosad *et al*., 2016).

The least effective antibiotics appear to be ciprofloxacin and imipenem which showed overall high resistances at 37.2% and 35.3% respectively. This high prevalence of resistance has also been reported by other researchers (Lee et al.,

2013; Chua et al., 2015; Luo *et al*., 2016). Similarly, the relatively low resistance to linezolid (15.7%) has been previously reported (Broda *et al.*, 2013).

As there is evidence of varying antimicrobial susceptibility patterns in different Mab subspecies (Koh et al., 2011; Novosad *et al.*, 2016), it is equally important to determine antibiotic susceptibilities at the subspecies level. From the results obtained, imipenem appears to be the least effective antibiotic for *M. abscessus* (66.7% resistance) and ciprofloxacin the least effective for *M. massiliense* (36.8% resistance). These results support the need for subspecies identification prior to antibiotic treatment.

5.3 Antibiotic resistance determinants

The usual mechanisms of antibiotic resistance include mutations in antibiotic targets, production of antibiotic inactivating enzymes, presence of protective proteins, and efflux systems. These resistance elements can be acquired through evolution or horizontal gene transfer (HGT) of genetic elements. With the availability of WGS, it is now possible to identify with speed, antibiotic resistance genes in clinical isolates. To facilitate the prediction of an antibiotic resistance genotype, online databases have been developed that enable the prediction and description of antibiotic resistance genes within whole or partial genomes as well

as provide useful information on the antibiotics and their targets. This information, gathered from literature and gene banks, is updated regularly to capture newly discovered resistance elements and curated in a user-friendly format that enables analysis and query of antibiotic resistances. The databases currently available differ mainly in their scope and frequency of updating.

In this study, PCR-sequencing and three ARG databases were used to identify resistance-associated genes in the Mab examined. The results are discussed below.

5.3.1 Amikacin

Amikacin has been the parenteral antibiotic against Mab infection for many years (Ferro *et al.*, 2016). It is currently the preferred treatment (Prammananan *et al.*, 1998; Maurer *et al.*, 2015) usually given in combination with a macrolide and cefoxitin or imipenem (Ferro *et al.*, 2016). However, there has been an increasing trend of amikacin resistance worldwide. The *rrs* mutations A1408G, T1406A, C1409T and G1491T (*E. coli* numbering) have all been strongly associated with amikacin resistance, but they were not found in any of the fifty-one strains

examined by PCR-sequencing. Nor were they found in the *rrs* predicted in the ARG databases. This corresponds well with the susceptible phenotype of the strains.

A number of genes encoding aminoglycoside modifying enzymes (phosphoryltransferases and acetyltransferases) were also identified with the ARG databases. The aminoglycoside modifying enzymes are usually associated with aminoglycoside resistance but it has also been hypothesised that they are likely to play a role in normal cellular metabolism (Perry *et al.*, 2014). This possible native function could provide an explanation for their existence in the amikacin susceptible strains in this study.

5.3.2 Clarithromycin

Clarithromycin is an important antimicrobial for the treatment of Mab infections. One drawback with this therapy is the occurrence of inducible resistance in apparently clarithromycin-susceptible isolates. This inducible resistance is not detected in routine antibiotic susceptibility tests and hence, will not be reported to referring physicians. It is deduced when resistance emerges after the initiation of therapy and is believed to be linked to the presence of an intact functional *erm*(41) gene in the Mab. In most *M. massiliense* strains, the *erm*(41) is truncated and

inactive. It is generally assumed that this subspecies is not affected by inducible resistance and thus, susceptible phenotypes can be safely treated with clarithromycin. In agreement with the observations made by others (Lee *et al.*, 2014; Li *et al.*, 2017), the results in this study showed all but two *M. massiliense* strains to be clarithromycin susceptible and all but one strain to have a truncated *erm*(41) gene. The exceptions are one susceptible strain (M139) with a complete *erm*(41) but with a T28C substitution, one resistant strain (M134) with a truncated *erm*(41) but a mutation in the *rrl* gene and one strain (M218) that is resistant without an obvious mutation. These exceptions illustrate the insufficiency in the use of the *erm*(41) alone for the subspecies identification of Mab and the prediction of clarithromycin susceptibility in *M. massiliense.*

In *M. abscessus*, on the other hand, most strains have a complete *erm*(41) with some showing a T → C polymorphism at nucleotide 28 of the gene. T28 variants show inducible clarithromycin resistance (Maurer *et al*., 2014; Mougari *et al*., 2016; Jeong *et al*., 2017) while C28 variants are susceptible to the antibiotic. The two *M. abscessus* T28 variants in this study did not show significant inducible resistance when tested with the standard methodology. Other researchers (Brown *et al*., 2015; Chua *et al*., 2015; Li *et al.*, 2017) have also found that the link between *erm*(41) and inducible clarithromycin resistance is not always observed. Hence, the method of demonstrating this phenomenon and its underlying mechanism(s) need to be further studied (Maurer *et al.*, 2014; Kim *et al.*, 2016).

5.3.3 Ciprofloxacin

Ciprofloxacin is active against the mycobacteria, especially the RGM (de Moura *et al.*, 2012). However, the wide usage of fluoroquinolones for the treatment of infections in the respiratory, gastrointestinal, urinary and genital tracts (Malik *et al.*, 2012) has led to the emergence of resistance in this class of antibiotics, including ciprofloxacin. Many investigations have been carried out to determine the cause for ciprofloxacin resistance in the Mab but till date, no consistent mechanism of resistance has been shown.

In 2012, Monego and his team (Monego *et al.*, 2012) noted that more than 80% of the ciprofloxacin resistant *M. massiliense* strains in their study had a Ala-92-Val mutation in the *gyr*A gene (*M. abscessus* numbering). In this study however, this mutation was not seen in any of the ciprofloxacin resistant strains. Moreover, the strains in this collection were mostly highly resistant (MIC>32μg/ml) compared to those from Monego's study (MIC 4-16 μg/ml) suggesting that there might be other mechanisms conferring high level resistance to ciprofloxacin.

Similarly, a Brazilian team in 2012 discovered that the *M. massiliense* strains in their collection did not possess the Ala-92-Val mutation in *gyr*A despite being resistant to all generations of quinolones. Instead, their strains showed the

amino acids Arg-482 and Asn-499 in *gyrB*, that have been linked to intrinsic low level resistance to quinolones in mycobacteria (de Moura *et al.*, 2012). This further strengthens the hypothesis that an unknown mechanism is likely to be responsible for high level ciprofloxacin resistance in Mab.

In Korea, Lee *et al. (*2014) identified five mutants with gyrase mutations Ala-92-Val, Asp-96-Asn in *gyr*A and Arg-482-Cys in *gyr*B. As the ciprofloxacin MIC values of these mutants were all >16μg/ml, the researchers suggested that the mutations observed were likely to be responsible for high level ciprofloxacin resistance.

In a separate Korean study, Kim *et al.* (2016) could not detect any *gyr*A and *gyr*B mutations in their resistant clinical isolates, although a synonymous mutation at Arg-516 (CGC to CGA) of *gyr*B was identified in half of their strains. They noticed that the resistance rate to ciprofloxacin was higher in CGC sequavars than the CGA sequavars. In this study however, the resistant strains had CGA, and not CGC at Arg-516.

Hernández *et al.*, 2011 had reasoned that apart from target genes, a combination of other mechanisms such as efflux pumps, target-protecting proteins, and even quinolone-modifying enzymes could contribute to high level quinolone

resistance. The discovery of the *efr*A and *qep*A2 pumps in this study complements their reasoning, as discussed further in Section 5.4 below.

5.3.3 Imipenem

Most beta-lactam antibiotics are ineffective against mycobacteria, owing to beta-lactamase production and low permeability of the mycomembrane (Soroka *et al*.,2014). However, the exact mechanism underlying this insensitivity is still unclear. Genetic analysis to date, has revealed the presence of an Ambler class A beta-lactamase gene within the Mab (Nessar *et al.*, 2012) but not any of the metallo-beta-lactamases that have been reported in bacteria like *Enterobacteriaceae, Pseudomonas aeruginosa,* and *Acinetobacter bumannii.* (Palzkill, 2013). One of these enzymes, the IMP-1, was reported to cause carbapenem resistance in *P. aeruginosa* (Saderi *et al.*, 2010; Ghamgosha *et al*, 2015), The gene homologue for this enzyme was detected by PCR-sequencing in all the Mab in this study, regardless of their susceptibility but it was not predicted in any of the three ARG databases. These results suggest that MBL genes may not play a role in imipenem resistance in the Mab.

5.3.4 Linezolid

Linezolid being relatively new compared to the other four antibiotics, has shown good activity overall, though reports on its resistance are now emerging (Tang et al., 2015). There is still a lack of studies investigating the genetic mechanisms involved in Mab resistance to linezolid. For many bacteria, the mechanism of resistance is often associated with mutations in the 23S rRNA region. Among the mycobacteria, the 23S rRNA mutations G2061T and G2576T were found in resistant *M. tuberculosis* whereas G2447T was found in resistant *M. smegmatis* (*E. coli* numbering). However, none of these mutations were found in the Mab in this study. In fact, the nucleotide sequence of the 23S rRNA in both resistant and susceptible phenotypes was almost 100% identical, indicating a possible non-23S ribosomal mechanism of resistance.

5.4 Prediction of resistance genes with ARG databases

Each of the resistance detection methods used in this study has its own strengths and weaknesses. Phenotypic testing shows expressed resistance that is useful for making therapeutic decisions but in mycobacteria, this is technically challenging as there are insufficient guidelines for the testing protocol and the

interpretation of antibiotic susceptibility or resistance, especially for the RGM. With the Etest, the MIC reading is often made difficult by the slow growth (3 days) of the Mab and the irregular edge of the zone of growth inhibition. PCR-sequencing allows a more rapid determination of resistance by demonstrating the presence of resistance-associated genes but it can only be used to detect known genes with sequence information for the designing of PCR primers. In contrast, WGS data can potentially enable the identification of all resistance-related loci and all types of mutations including silent mutations that do not alter the phenotype of the bacterium tested. Free online ARG databases using WGS data have enabled the *in silico* detection of resistance-associated genes. Although their usefulness has been demonstrated for common pathogens like *E. coli, S. aureus* and *P. aeruginosa* (Koser *et al.*, 2014), they have been much less developed for less studied bacterial species, including the Mab.

The analysis of the Mab resistome is challenging owing to the number, complexity, and distribution of the resistance loci to be examined. The reliability and accuracy of WGS-based resistance detection relies heavily on adequate DNA extraction, bioinformatics expertise, the accuracy and inclusiveness of the reference library of resistance mutations used and flexible data management procedures to accommodate new resistance markers as they are discovered and validated. Hence, accuracy may be low where there is poor quality sequence data and incomplete

knowledge regarding resistance-causing mutations which may vary in different geographic origins.

In this study, the databases ARG-ANNOT, CARD and ResFinder were chosen because they are popular among researchers and are frequently updated. Table 5.4 below describes some of the differences among these ARG databases. One important factor that determines the popularity of the databases is the size of the database. An incomplete and small database is less useful as many, especially newly discovered resistance genes may be missed out. The response time is another important deciding factor. In the early part of this study, the ResFinder had a long responding time of two to three days for each query. After its update in March 2018, the response time became much shorter.

Table 5.4: Differences among ARG-ANNOT, CARD and ResFinder

Database	**ARG-ANNOT**	**CARD**	**ResFinder**
***Last update**	May 2018	October 2018	September 2018
Coverage	All AR genes	All AR genes	All AR genes
Special feature	allows user to analyze sequences without web interface	highly descriptive, with function-based classification of AR genes	accepts NGS raw reads, including *de novo* assembled contigs
Analysis duration	35 seconds	56 seconds	120 seconds
RG criteria	adjustable e value	bit-score, e value, percentage of identity	adjustable percentage of identity and length coverage.

*last updated as in October 2018

In this study, CARD performed better compared to the other two databases. ResFinder is limited in its scope with a relatively small AR gene spectrum, while ARG-ANNOT lacks regular updates. The three databases differed in their sensitivity and specificity for the detection of resistance determinants in Mab but enabled the identification of more resistance genes than the traditional PCR-

sequencing which was limited to only one or two previously described genes for each antibiotic because of time and cost constraints.

Nonetheless, the results from the database predictions did not correlate well with the phenotype or the PCR-sequencing detections. For instance, the IMP-1 and *gyr*B genes were amplified in all Mab strains but were not identified in any of the databases. Among the resistance genes predicted by the databases, the Bla_{Mab} gene (KPC-16 homologue) was not correlated with imipenem phenotypic resistance, while the PBP2 protein sequence was almost identical for both phenotypic imipenem resistant and susceptible strains. Neither were the *pat*A, *mfp*A and *gyr*A that confer resistance to fluoroquinolones, the *optr*A and *clb*B associated with linezolid resistance and all the genes associated with aminoglycoside resistance. The reason for this poor correlation could be that the homologues of resistance genes described in other bacterial species are not involved in resistance in Mab. For most antibiotics there are multiple resistance mechanisms. The three databases used may not be suitable for the prediction of resistance genes in Mab because there is relatively little data on resistance mechanisms in this species and much less in the subspecies.

Of all the genes predicted, only KPC-16 (Bla_{Mab}), *erm*(41) and *rrl* have been identified as resistance-associated genes in Mab. As the mutations in the

erm(41) and *rrl* in Mab have been well studied, with few exceptions, the predicted mutations in these two genes correlated well with the expected phenotypes. As for the Bla_{Mab} this gene has been described only in *M. abscessus* ATCC 19977. To date, there are no reports of its role in *M. massiliense* or *M. bolletii*. Thus, the evaluation of this gene is greatly limited by the small number of *M. abscessus* in this study.

The most exciting contribution from the ARG databases in this study is the discovery of *efr*A and *qep*A2 homologues in the Mab strains examined. These genes suggest a previously unreported mechanism of fluoroquinolone resistance in Mab. The SNPs in these genes correlated well with the phenotypes in different subspecies. Their role in ciprofloxacin resistance in Mab awaits confirmation.

The ARG database prediction might have been compromised by the importation of draft genome data. The WGS data available in this study were all from draft genomes. As described in many papers (Ricker *et al.,* 2012; Hahn *et al.,* 2014), working with draft genomes is a challenge, since there may be many gaps within the genome sequence, and the resistance genes involved could be in those gaps, thus giving a false negative detection.

Another disadvantage is that the names used for the genes predicted varied among the databases. For example, the *qep*A2 gene detected by CARD is the same as the *qep*A gene detected by ARG-ANNOT. Similarly, the *aph (*3*'')-Ic* detected by ARG-ANNOT is the same as the *aph(*3*'')-Ia* gene detected by CARD. It would be helpful if online ARG databases could reach a consensus on the naming of genes.

CHAPTER 6

CONCLUSION

The findings in this study highlight a considerable problem with antibiotic resistance among locally prevalent Mab strains. Although the overall resistance rates to five antibiotics recommended for use in Mab infections ranged from 0% (amikacin) to 37% (ciprofloxacin), subspecies analysis showed a 66.7% resistance to imipenem among *M. abscessus* and a high level of resistance to both imipenem and ciprofloxacin in many strains.

The genotypic-phenotypic correlation for these strains revealed limitations in the conventional culture-dependent susceptibility testing of resistance as well as the more recently introduced molecular detection with PCR-sequencing and *in silico* detection using the importation of WGS data into ARG databases. There was inconsistency in the detection of resistance and overall poor genotypic-phenotypic correlation. Although synonymous and non-synonymous SNPs were found that could be related to resistance to some antibiotics, further investigations with larger numbers of Mab and subspecies is required to determine their significance as resistance determinants. Generally, a large sample size is particularly important for studies on bacteria harbouring multiple resistance mechanisms.

The WGS-based ARG databases used in this study provided insufficient information for an ample understanding of resistomes in the Mab. This is largely because there is relatively scarce published data on antibiotic resistance determinants in Mab and its subspecies. The homologues of resistance genes described for other bacterial species may not be resistance genes in Mab. To be useful, ARG databases have to be comprehensive, well-curated and regularly updated. Because of database-specific limitations, it is important to use at least two ARG databases for *in silico* prediction to avoid missing out on significant genes.

Collectively, the results from this study demonstrate the need for both phenotypic and genotypic testing of antibiotic susceptibility. While the former informs on expressed resistance that the physician needs to initiate antibiotic therapy, the latter alerts the physician on the potential for resistance after starting therapy which may affect treatment response and recovery. With further improvements in technology, it may be possible, in the near future, to provide rapid access to reliable antibiotic resistance information to clinicians to enable personalized optimal antibiotic therapy.

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APPENDICES

APPENDIX A

Reagents preparation

I. 1X TBE solution

50 ml of 10X TBE into 450 ml of DW

II. Primer

90 µl of DW into 10 µl of stock primer

III. 15% glycerol with TSB

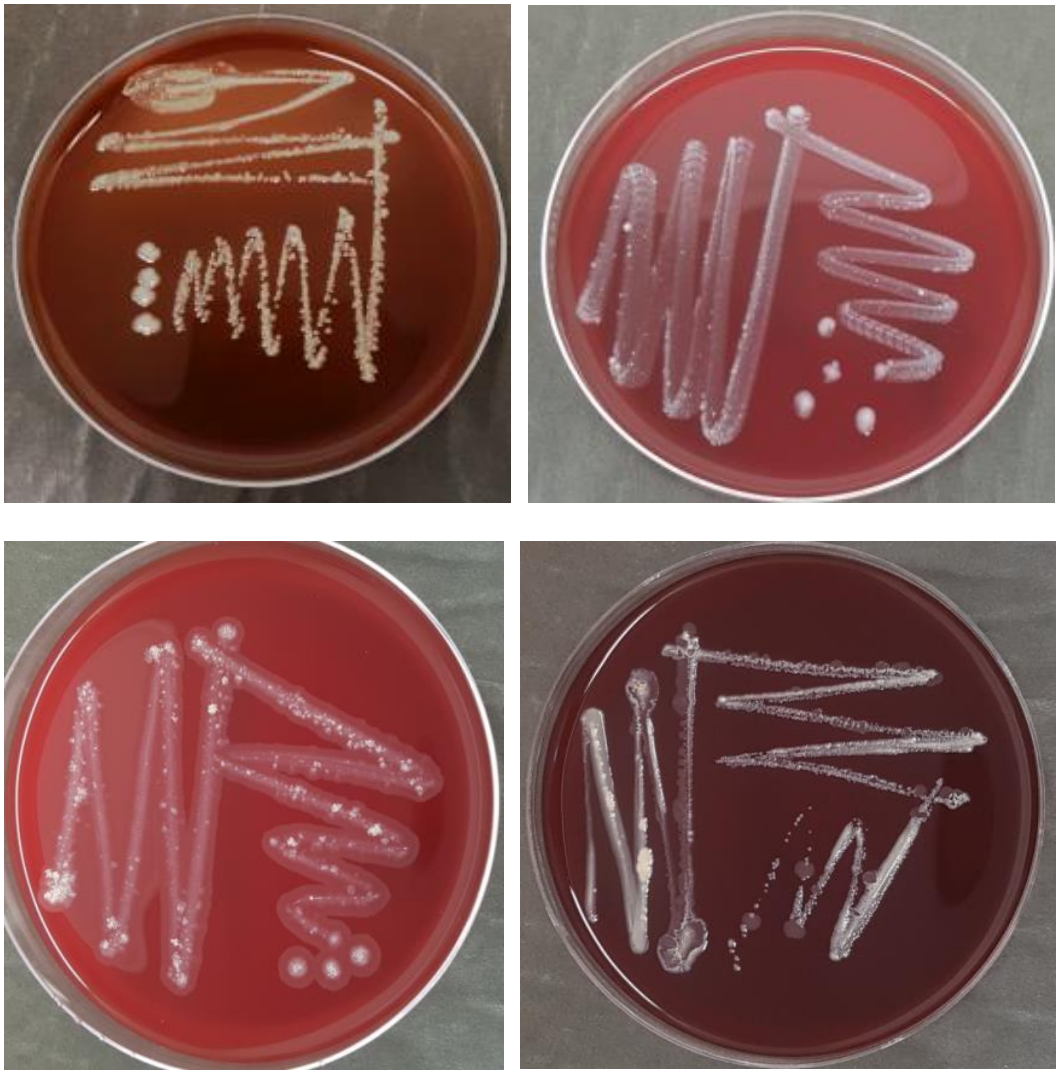
7.5 ml of 100% glycerol into 50 ml DW. Add in 1.5 g of TSB powder. Autoclave before use

IV. Nutrient agar

11.5 g of agar nutrient powder into 500 ml of DW. Autoclave before use

APPENDIX B

Samples of *M. abscessus* complex growth on blood agar



From top left to bottom right: ATCC 19977, M04, M93 and M94

From top left to bottom right: M120, M154, M161, M162 and M172

APPENDIX C

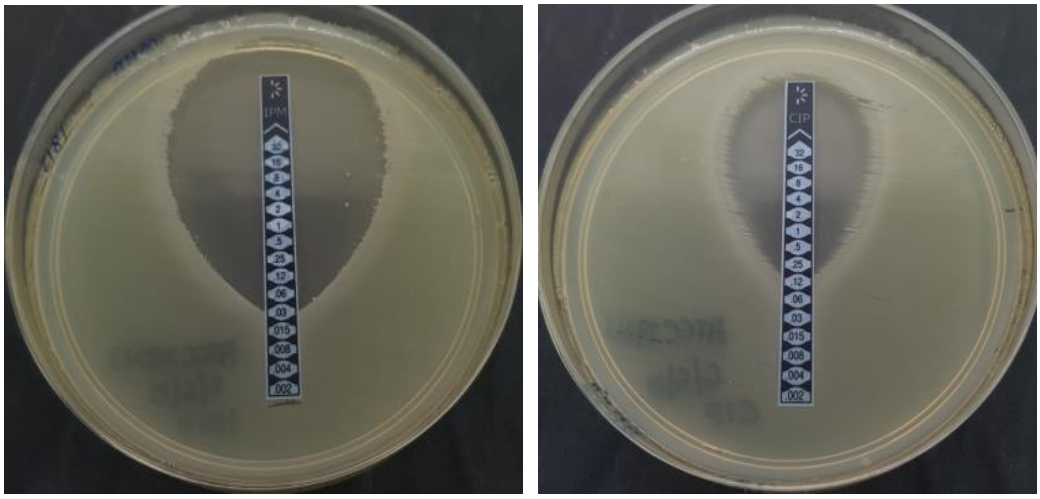
Colony morphology of *M. abscessus* subspecies

No	Strain	Morphology	Subspecies type
1	M 61	rough,clumpy	*M. abscessus*
2	M 93	rough, clumpy	*M. abscessus*
3	M 94	rough, clumpy	*M. abscessus*
4	M 127	rough, clumpy	*M. abscessus*
5	M 152	rough, clumpy	*M. abscessus*
6	M 214	smooth	*M. abscessus*
7	M 240	rough, clumpy	*M. abscessus*
8	M 241	smooth, mucoid	*M. abscessus*
9	M 242	smooth, mucoid	*M. abscessus*
10	M 243	smooth, mucoid	*M. abscessus*
11	M 244	smooth, mucoid	*M. abscessus*
12	M 245	rough, clumpy	*M. abscessus*
13	M 24	smooth, mucoid	*M. bolletii*
14	M 02	smooth,few clumps	*M. massiliense*
15	M 04	rough, clumpy	*M. massiliense*
16	M 18	smooth, mucoid	*M. massiliense*
17	M 27	smooth, round colonies	*M. massiliense*
18	M 33	Smooth, many clumps	*M. massiliense*
19	M 57	rough, clumpy	*M. massiliense*
20	M 97	Rough ,dry clumps	*M. massiliense*
21	M 115	smooth, mucoid	*M. massiliense*
22	M 117	Rough ,dry clumps	*M. massiliense*
23	M 119	smooth, mucoid	*M. massiliense*

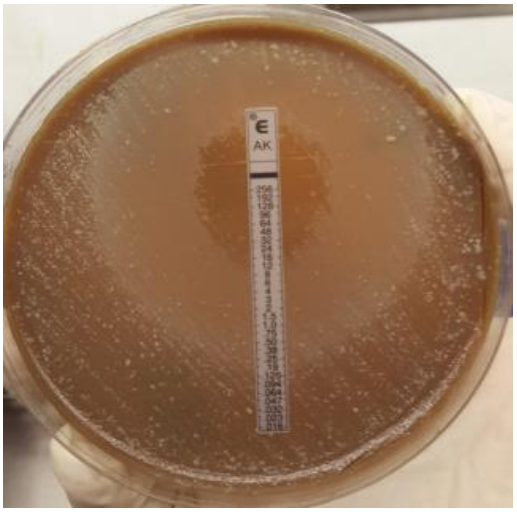
24	M 120	Smooth	*M. massiliense*
25	M 134	Rough ,dry clumps	*M. massiliense*
26	M139	Rough ,dry clumps	*M. massiliense*
27	M 145	smooth, mucoid	*M. massiliense*
28	M 148	Clump, dry	*M. massiliense*
29	M 149	smooth, mucoid	*M. massiliense*
30	M 154	Rough ,dry clumps	*M. massiliense*
31	M 156	smooth, mucoid	*M. massiliense*
32	M 159	smooth, mucoid	*M. massiliense*
33	M 161	smooth, mucoid	*M. massiliense*
34	M 162	smooth, mucoid	*M. massiliense*
35	M 172	Clump, dry	*M. massiliense*
36	M 202	Smooth, mucoid	*M. massiliense*
37	M 208	both smooth and rough	*M. massiliense*
38	M 211	smooth, mucoid	*M. massiliense*
39	M 212	Rough ,dry clumps	*M. massiliense*
40	M 213	smooth, mucoid	*M. massiliense*
41	M 215	smooth, mucoid	*M. massiliense*
42	M 216	Rough ,dry clumps	*M. massiliense*
43	M 217	Rough ,dry clumps	*M. massiliense*
44	M 218	Rough ,dry clumps	*M. massiliense*
45	M 219	smooth, mucoid	*M. massiliense*
46	M 220	Rough ,dry clumps	*M. massiliense*
47	M 221	smooth, mucoid	*M. massiliense*
48	M 222	smooth, mucoid	*M. massiliense*
49	M 223	smooth, mucoid	*M. massiliense*
50	M 224	Rough ,dry clumps	*M. massiliense*
51	M 225	Rough ,dry clumps	*M. massiliense*

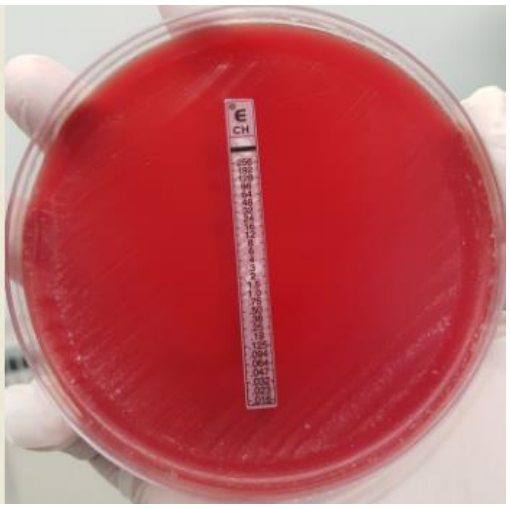
APPENDIX D

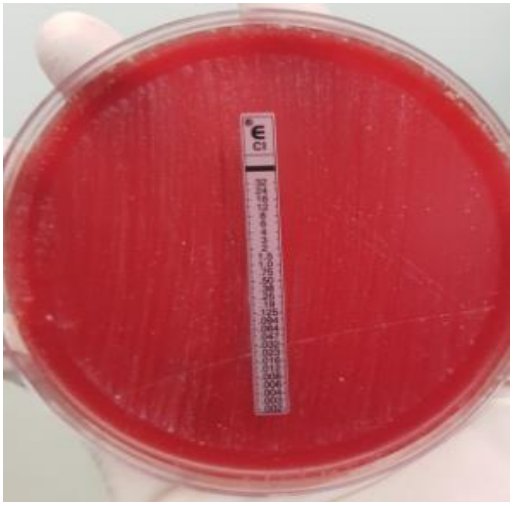
Etest pictures

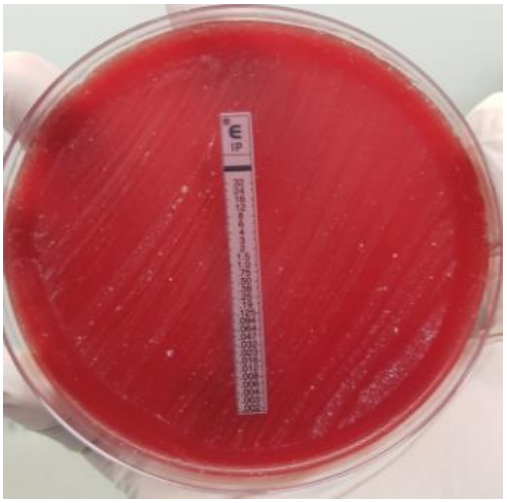


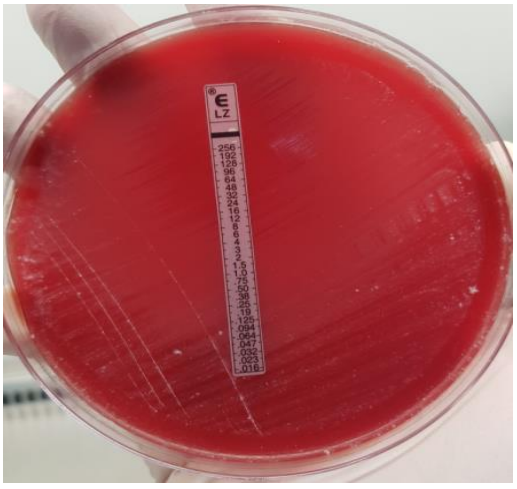
Quality control check for Etest with control strain ATCC 29213 for Imp and Cip



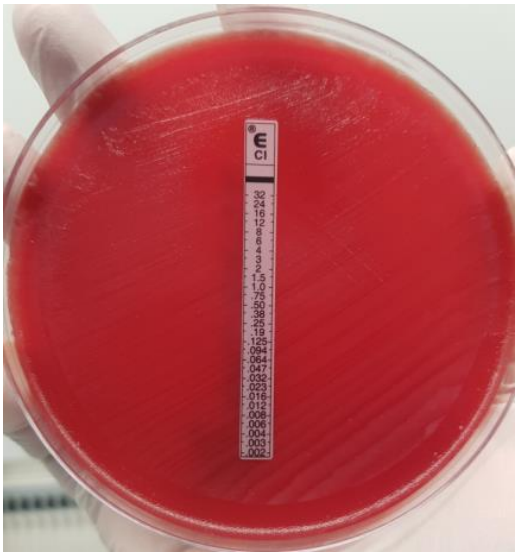




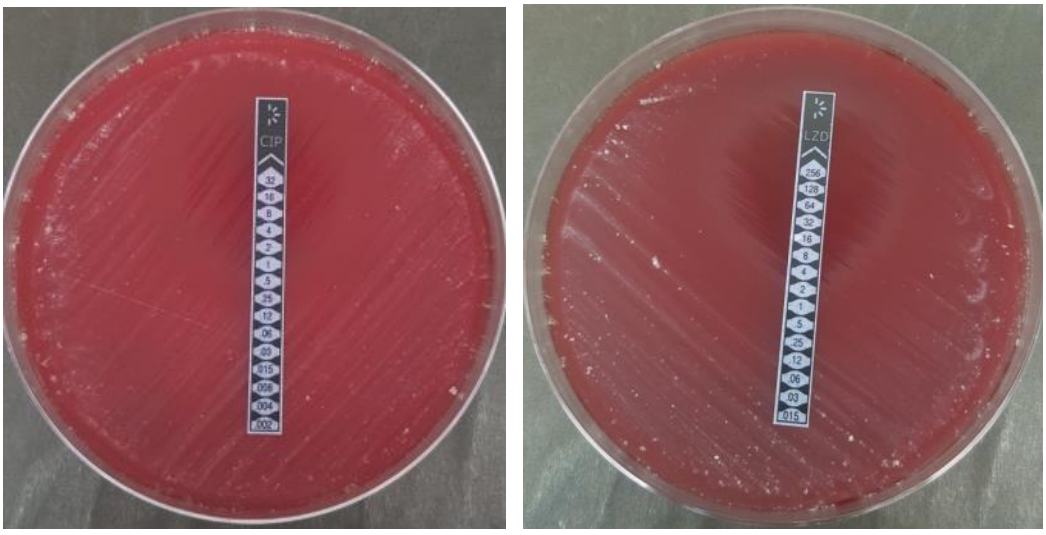




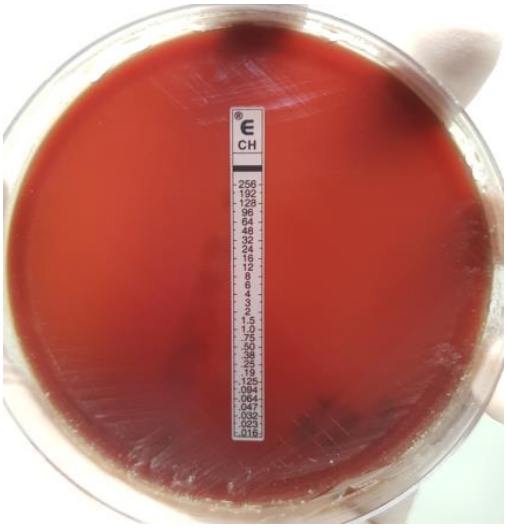
Etest for M04: Ak,Cla,Cip,Imp and Lzd. MIC is read at the point where there is complete inhibition of growth.



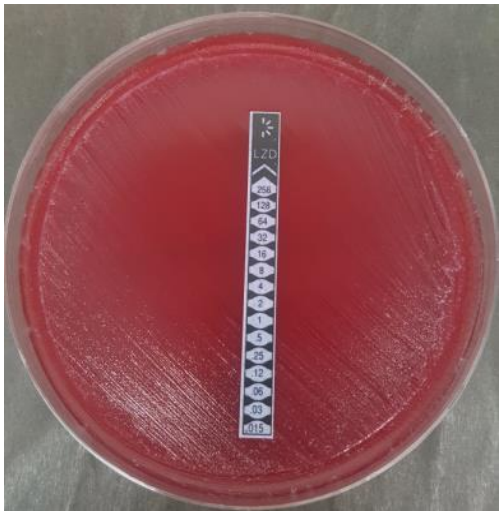
Etest for M18: Cip,Imp



Etest for M93: Cip, Lzd

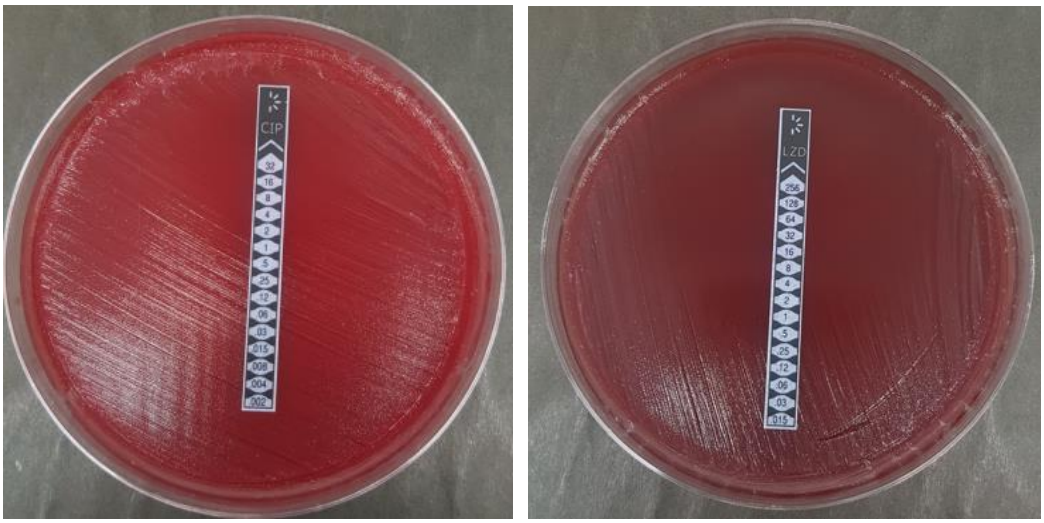


Etest for M94: Cla

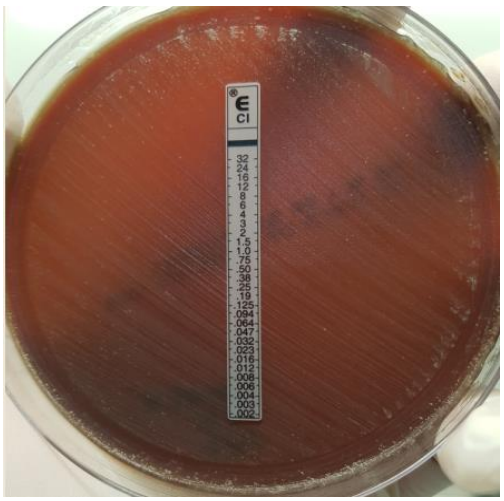


Etest for M97: Lzd

Etest for M162: Cla

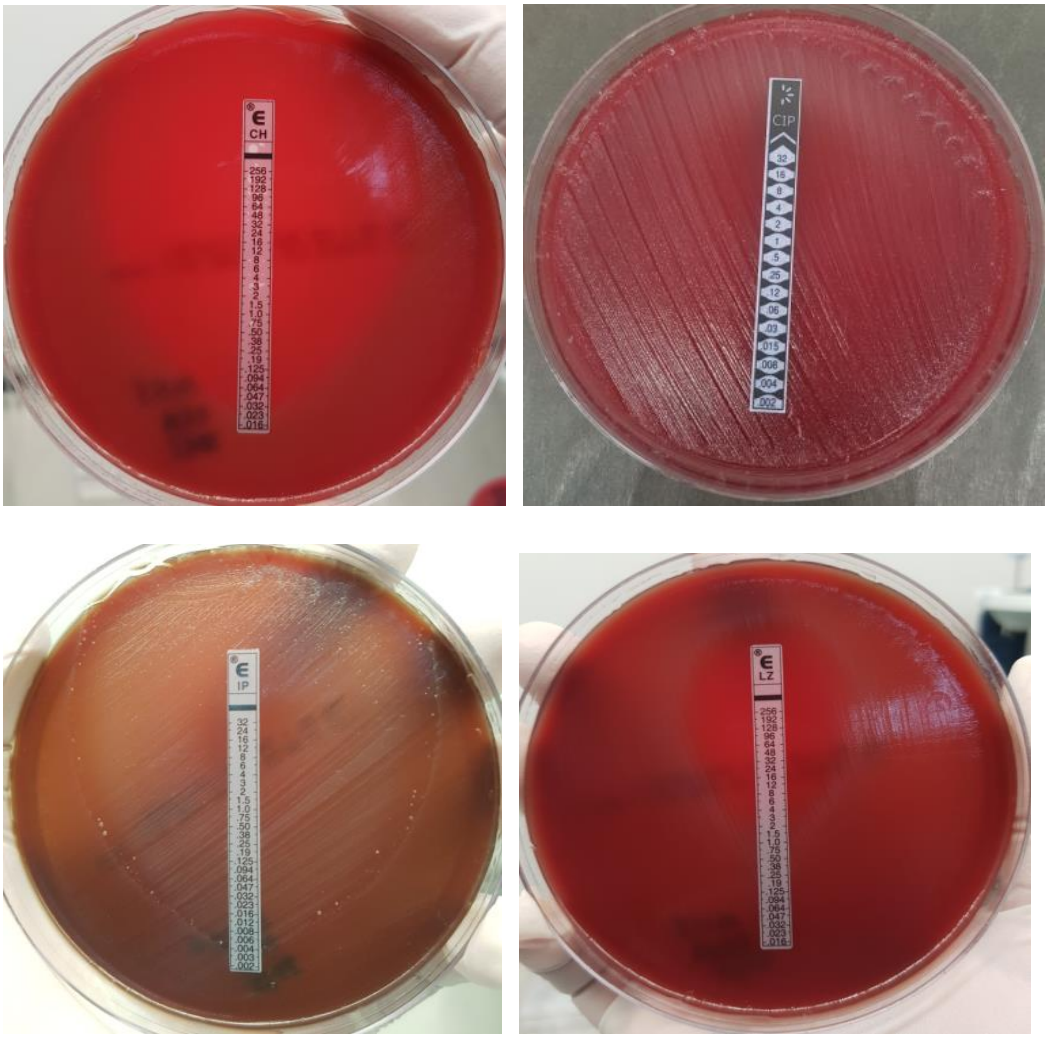


Etest for M120: Cip, Lzd

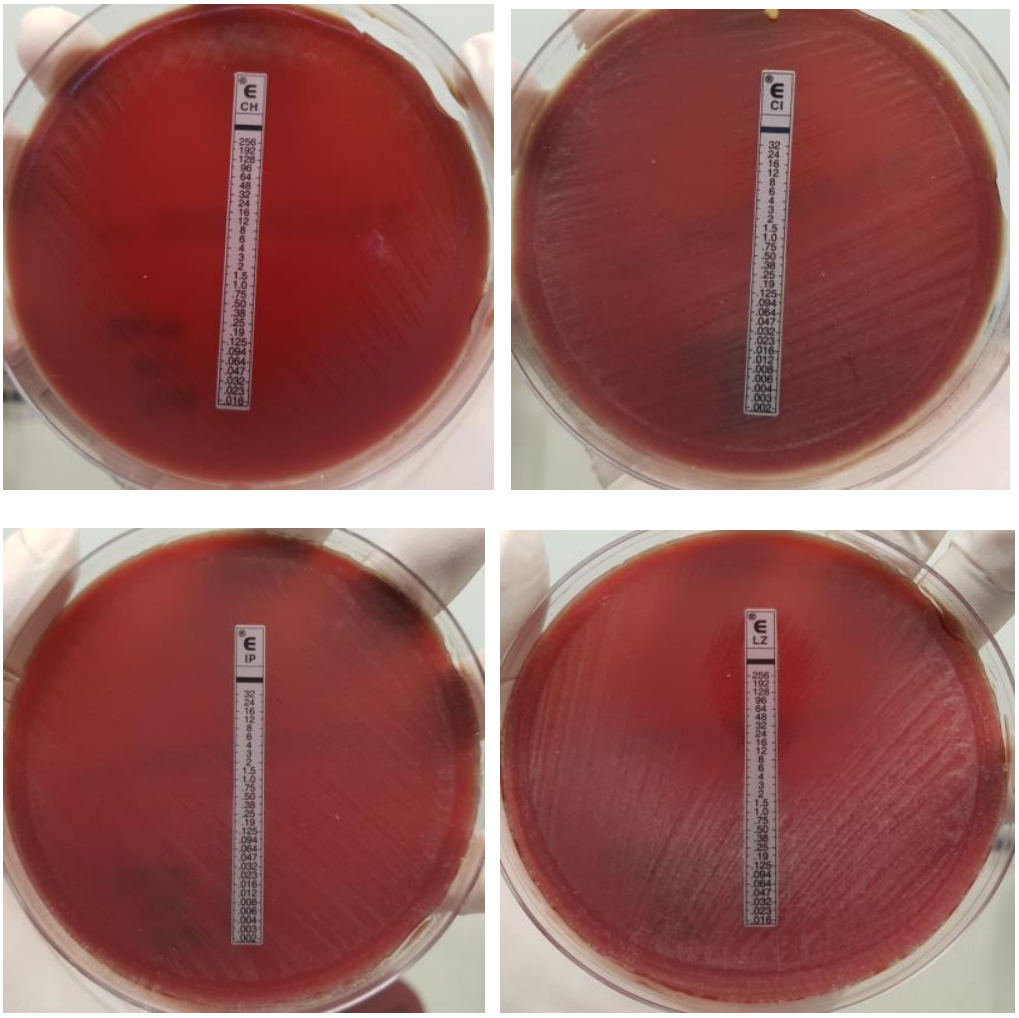


Etest for M240: Cip

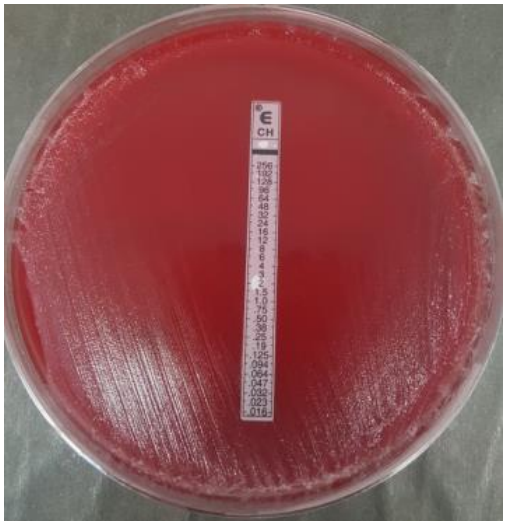
Etest for M241: Lzd

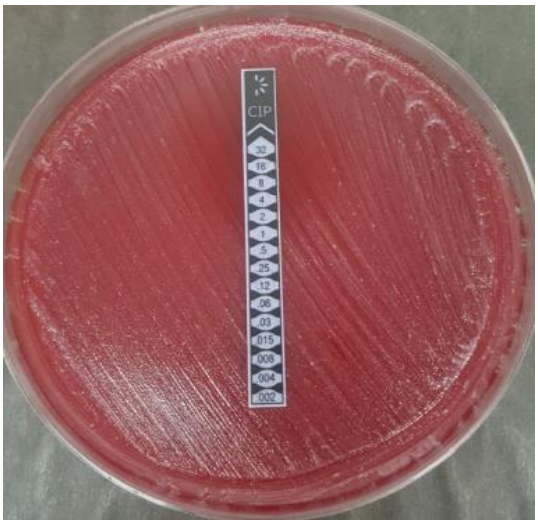


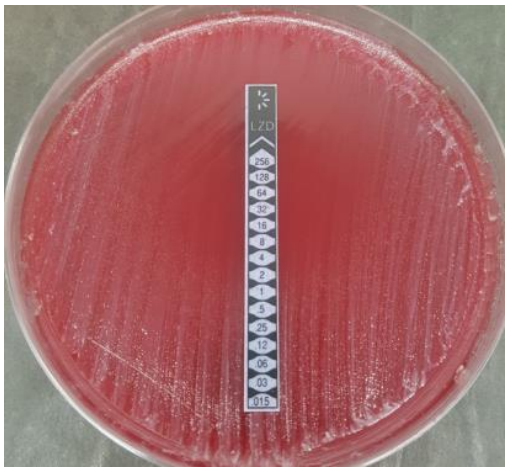
Etest for M115: Cla, Cip, Imp and Lzd



Etest for M148: Cla, Cip, Imp and Lzd







Etest for M245: Cip, Cla and Lzd

APPENDIX E

MIC of the five antibiotics for 51 clinical strains of the *M abscessus* complex

M. abscessus	Cip(1,2,4)	Imp(4,8,16)	Amk(16,32,64)	Cla(2,4,8)	Lzd (8,16,32)
*ATCC 19977	**8**	**>32**	4	0.125	**>256**
M240	**>32**	**>32**	8	0.125	**>256**
M241	0.75	**>32**	2	0.19	1.5
M242	**>32**	**>32**	4	2	8
M243	1	**>32**	2	2	12
M244	**>32**	**>32**	3	4	16
M245	1	**>32**	2	0.19	2
M61	**4**	1.5	12	2	0.25
M93	1	6	24	2	3
M94	0.5	**>32**	16	0.19	4
M127	2	**>32**	8	0.064	**>256**
M152	0.19	3	16	0.032	4
M214	0.75	6	24	0.5	4
	S=7/12(58.3)	S=4/12(33.3)	S=12/12(100)	S=11/12(91.7)	S=9/12(75)
	I=1/12(8.3)	I=0	I=0	I=1/12(8.3)	I=1/12(8.3)
	R=4/12(33.3)	R=8/12(66.7)	R=0	R=0	R=2/12(16.7)
M. bolletii					
M24	**>32**	**>32**	8	**>256**	**>256**
M. massiliense					
M02	**>32**	**>32**	32	0.125	4
M04	**>32**	**>32**	12	0.125	4
M18	**4**	3	12	0.19	3
M27	**8**	**>32**	4	0.032	16
M33	**>32**	2	32	0.25	0.75
M57	**>32**	**>32**	3	0.064	**>256**
M97	**>32**	6	4	0.38	2
M115	2	6	24	0.19	4
M117	**>32**	6	6	0.125	0.75
M119	1	**16**	8	0.19	4
M120	1	6	32	0.047	1
M134	**>32**	**>32**	4	**>256**	**>256**
M139	2	3	3	0.094	2
M145	**>32**	**>32**	8	0.125	**>256**
M148	0.75	8	6	0.094	2
M149	2	4	4	0.032	**>256**
M154	1	6	12	0.19	2
M156	1	4	12	0.25	6
M159	2	8	32	0.135	8
M161	**4**	**>32**	6	0.047	**>256**
M162	**>32**	2	24	0.032	3
M172	2	1.5	12	0.094	4
M202	**>32**	6	8	0.19	8
M208	0.75	6	24	0.125	4
M211	0.75	6	12	0.19	4
M212	2	3	32	0.125	8
M213	1.5	3	16	0.094	6
M215	0.38	6	32	0.125	6
M216	**>32**	**>32**	2	0.19	0.125
M217	0.75	2	12	0.094	2
M218	0.094	0.75	12	**12**	0.75
M219	2	12	24	0.125	2
M220	1.5	4	48	0.19	16
M221	2	2	8	0.125	1.5
M222	1.5	4	8	0.19	3
M223	2	6	16	0.5	0.75
M224	3	4	16	0.25	12
M225	0.047	1.5	2	0.25	0.25
	S=14/38(36.8)	S=26/38(68.4)	S=31/38(81.6)	S=36/38(94.7)	S=31/38(81.6)
	I=10/38(26.3)	I=3/38(7.9)	I=7/38(18.4)	I=0	I=2/38(5.3)
	R= 14 /38 (36.8)	R= 9/38 (23.7)	R= 0/38	R= 2/38 (5.3)	R=5/38(13.2)

APPENDIX F

Protein sequence and comparison between IMP-1 in *P. aeruginosa* and the homologue in Mab.

Amino acid sequence of IMP-1 in *P. aeruginosa* (GenBank: ABG67754)

MSKLSVFFIFLFCSIATAAESLPDLKIEKLDEGVYVHTSFEEVNGWGVVPKHGLV
VLVNAEAYLIDTPFT
AKDTEKLVTWFVERGYKIKGSISSHFHSDSTGGIEWLNSRSIPTYASELTNELLKK
DGKVQATNSFSGVN
YWLVKNKIEVFYPGPGHTPDNVVVWLPERKILFGGCFIKPYGLGNLGDANIEAW
PKSAKLLKSKYGKAKL
VVPSHSEVGDASLLKLTLEQAVKGLNESKKPSKPSN

Amino acid sequence of IMP-1 homologue in Mab (GenBank: CAM61202.1)

MSSPDLQQLAPSLFRLRIPGGRAHLLNCYLWLAPDGVTLIDTGWPDSAELIEQAL
HQLGRGRTDIVRIVL
THFHEDHVGAAAEIAAWSRAEVIAGEPDSPFITGERGGPVPVLTAGEQALHPGFT
EPPHGPVCRVDRAVK
DGEVLDFAGGAHVIAVPGHTPGSIALYLPAADAVLTGDAVAEFNGQVILGVFNS
DRQVAARSLSRLAATG
AEIGGFGHGEAILEKASARIATAIDAFGE

Putative metallo-beta-lactamase superfamily [Mycobacteroides abscessus ATCC 19977]

Sequence ID: CAM61202.1

▶ See 81 more title(s)

Range 1: 157 to 177 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
24.3 bits(51)	0.002	Compositional matrix adjust.	8/21(38%)	13/21(61%)	0/21(0%)

```
Query  155  PGHTPDNVVVWLPERKILFGG  175
            PGHTP ++ ++LP    +  G
Sbjct  157  PGHTPGSIALYLPAADAVLTG  177
```

Range 2: 39 to 79 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Method	Identities	Positives	Gaps
20.0 bits(40)	0.033	Compositional matrix adjust.	13/41(32%)	19/41(46%)	2/41(4%)

```
Query  64   LIDT--PFTAKDTEKLVTWFVERGYKIKGSISSHFHSDSTG  102
            LIDT  P +A+  E+ +         I   + +HFH D  G
Sbjct  39   LIDTGWPDSAELIEQALHQLGRGRTDIVRIVLTHFHEDHVG  79
```

Range 3: 27 to 53 GenPept Graphics ▼ Next Match ▲ Previous Match ▲ First Match

Score	Expect	Method	Identities	Positives	Gaps
13.1 bits(22)	5.7	Compositional matrix adjust.	12/43(28%)	17/43(39%)	16/43(37%)

```
Query  161  NVVVWLPERKILFGGCFIKPYGLGNLGDANIEAWPKSAKLLKS  203
            N  +WL             P G+  L D     WP SA+L++
Sbjct  27   NCYLWL------------APDGV-TLIDTG---WPDSAELIEQ  53
```

Overall percentage of identity: 38%

APPENDIX G
In silico prediction of resistant genes

Top 20 predicted resistance genes by ARG-ANNOT, for M61. Hits were organized from the highest to the lowest bitscore.

Query id	Database id	Alignment length	bit score
final_225_04790	(MLS)Erm41:EU590124:258-779:522	136	270
final_225_03492	(AGly)Aph3''Ic:DQ336355:603-1367:816	50	60
final_225_00412	(MLS)SrmB:X63451:558-2210:1653	35	54
final_225_00412	(MLS)TlrC:M57437:277-1923:1647	31	54
final_225_00412	(MLS)OleB:L36601:1421-3130:1710	36	48.1
final_225_00859	(Bla)FAR-1:AF024601:303-1196:894	72	48.1
final_225_00859	(Bla)OXY1-2:AJ871865:1-876:876	27	46.1
final_225_00859	(Bla)CTX-M-139:KC107824:1-876:876	26	44.1
final_225_03028	(MLS)OleC:L06249:1528-2505:978	30	44.1
final_225_04407	(AGly)Aac2-Ib:U41471:266-822:588	38	44.1
final_225_00051	(Bla)CTX-M-100:FR682582:1-876:876	21	42.1
final_225_00282	(MLS)TlrC:NC_016113:803268-384890:1623	21	42.1
final_225_00353	(AGly)Aph6-Ia:AY971801:1-924:924	21	42.1
final_225_00412	(MLS)CarA:M80346:411-2066:1656	25	42.1
final_225_00525	(Bla)AQU2:KF730243:1-1143:1143	21	42.1
final_225_00525	(AGly)Aac(3)-IIIb:L06160:984-1721:861	25	42.1
final_225_01175	(Flq)QepA:AB263754:7052-8587:1536	21	42.1
final_225_03714	(AGly)Aac3-VII:M22999:493-1359:867	21	42.1
final_225_04407	(AGly)Aac2-Ie:NC_011896:3039059-3039607:549	73	42.1
final_225_03423	(MLS)ErmR:M11276:333-1355:1023	20	40.1

AR genes by antibiotic class predicted by ARG-ANNOT for the 21 strains

The oxazolidinone antibiotic class was not predicted

Strains	**Antibiotic class**			
	Agy	**Beta-lactams**	**FQ**	**MLS**
ATCC 19977 abs	Aph3"Ic, Aac(3)-IIIb, Aph6-Ia,	FAR-1, OXY1-2, CTX-M, AQU2	*qep*A, *gyr*A, *par*C	erm(41),erm(38),SrmB, OleB,Car A,TlrC,OleC , *rrl*
M 61 abs	Aph3"Ic, Aac2-Ib, Aph6-Ia, Aac(3)-IIIb, Aac3-Vii,Aac2-Ie	FAR-1, OXY-1,CTX-M-139, CTX-M-100, AQU 2	*Qep* A *gyr*A, *par*C	*Er*m41, *Erm* 38,SrmB, TlrC M, TlrC NC, Ole B, Lmr A,Ole C, Car A, Erm O, Erm R,*rrl*
M 93 abs	Aph3"Ic, Aac(3)VII	FAR-1,AQU-2,CTX-M,OXY-1	*Qep*A *gyr*A, *par*C	TlrC, SrmB,CarA,OleC,OleB ,e*rm*41,*Erm* 38,ErmR,ErmO,r*rl*
M 94 abs	Aac(3)-IIIb, Aph3"Ic, Aac3-VII, rrs	AQU-2,AmpC1,CTX-M,FAR-1,	*Qep*A *gyr*A, *par*C	SrmB,TlrC,OleB, CarA, OleC, *Erm* 41,Erm R,*Erm* 38,*rrl*
M 127 abs	Aph3"Ic, Aac3-VII,Aac2-Ie, Aac2-Ib, rrs	FAR-1, OXY1-2, CTX-M-139, AQU 2, AmpC1, CTX-M-100	*Qep*A *gyr*A, *par*C	*Erm* 41, Tlr C M, TlrC NC, Srm B, Car A,Erm 38, Lmr A,Ole B, Ole C, Erm O,Erm R, rrl
M 152 abs	Aac3-VII, Aph3"Ic,	Far-1,OXY1-2,CTX-M-139,CTX-M-100, AQU2	*Qep* A *gyr*A, *par*C	*Erm*41, OleC, Car A, ,TlrC,SrmB,Erm O,Ole B,Erm R,ErmN, Lmr A,Erm38, *rrl*
M 24 bol	Aph3"Ic, Aac(3)-IIIb, Aac(2)-Ic, Aac(3)VII, rrs	FAR-1, CTX-M, AQU-2, HugA	*Qep*A *gyr*A, *par*C	*Erm*41, Tlr C,OleB,SrmB, OleC, Erm31,Erm R, , *rrl*
M 04 mas	Aac3-Xa, Aac2-Ic, Aph3-IIb,	CTX-M-100, AQU-2, GES-	*gyr*A, *par*C	Ole B,Srm B, Tlr C M, Tlr C NC, Car A, Ole

	Aac(3)-IIIb, Aph3"Ic,Aac2-Ic, Aac3-VII, rrs	23,FAR-1,Oxy1-2,CTX-M-139,CTX-M-75,Pam-1, Amp C1, LEN-37		C, Erm R, *Erm* 38, *Erm* 41, *rrl*
M 18 mas	Aph3"Ic,Aac2-Ic, Aac3-Xa, Aac(3)-IIIb, Aph3-IIb, Aac3-V11, rrs	FAR-1, CTX-M,Len-37, AmpC1,OXY1-2,GES-23,AQU-2,Pam-1	*gyr*A, *par*C	*Erm* 41, *Erm* 38,OleB, SrmB, Car A,TlrC, Ole C, ErmR, , *rrl*
M 57 mas	Aac2-Ic, Aac3-Xa, Aac(3)-IIIb, Aph3"Ic,Aph3-IIb,Aac2-Ie, Aac3-VII, rrs	Pam-1, FAR-1,OXY1-2, CTX-M-139, GES-23, CTX-M-100, Len-37, AQU2	*gyr*A, *par*C	Srm B, TlrC M, TlrC NC, Ole B, CarA, Ole C,Erm R, *Erm* 38,*Erm* 41, , *rrl*
M 115	Aac(3)-IIIb, Aac3-V11, Aph3-IIb, AadA8b, AadA24, AadA3, Aph3"Ic, Aac(2)-Ic, rrs	CTX-M-100,AQU-2, AIM-1,AmpC1,FAR-1,OXY1-2,CTX-M-139, LEN-37,Pam-1,	*gyr*A, *par*C	*Erm*38,CarA, OleC, TlrC, SrmB, ErmR, Ole B, *Erm* 41, *rrl*
M 117	Aph3-IIb, Aph3"Ic, Aac(3)-IIIb, Aac2-Ie,Aac3-VII, rrs	FAR-1, OXY1-2,CTX-M-139, CTX-M-130,Pam-1, AQU2,AmpC1,GES-23, LEN-37,CTX-M	*gyr*A, *par*C	Srm B, TlrC M, Tlr C NC, Ole B,Car A, Ole C, *rrl*
M 119	Aph3"Ic,Aac3-Xa, Aph3-IIb, Aac3-V11, Aac2-Ie,Aac2-Ic, rrs	FAR-1, OXY1-2, BKC-1, CTX-M-139, Pam-1,CTX-M-100,	*gyr*A, *par*C	*Erm*41, Srm B, TlrC, *Erm* 38,Erm R, Car A, Erm O, rrl

		AmpC1,AQU 2, LEN-37		
M 134	Aph3"Ic,Aac3-Xa, Aph3-IIb,Aac(3)-IIIb,Aac2-Ie, Aac3-VII,Aac2-Ic,	OXY1-2, Pam-1,GES-23,CTX-M-139, AQU2, CTX-M-100,AmpC1, FAR-1, LEN-37	*gyr*A, *par*C	*Erm*41, Ole B, Srm B, *erm* 38,TlrC M, Tlr C NC, Car A, Erm R, Ole C, rrl
M 139	Aac3-V11, Aac(3)-IIIb, rrs	AQU-2, FAR-1,Oxy1-2, CTX-M-139,Pam-1,PME-1, LEN-37, GES-23, CTX-M-100	*Qep*A *gyr*A, *par*C	*Erm3*8, Erm R, *Erm*41, OleC, Tlr C,Srm B,Ole B, Car A, *rrl*
M 145	Aph 3"Ic, Aac3-Xa, Aph3-IIb,Aac(3)-IIIb, Aac2-Ie, Aac3-VII,Aac2-Ic	OXY1-2, Pam-1, GES-23, CTX-M-139,Aqu-2, AmpC1, FAR-1, Lem-37,CTX-M-75	*gyr*A, *par*C	*Erm* 41,Srm B,Tlr C M,Tlr C NC, *Erm 38*, CarA, Ole B, Ole C, Erm R, *rrl*
M 148	Aph3"Ic, Aac3-V11, Aac(2)-Ic, Aac(3)-IIIb, rrs	AmpC1, LEN-37, Pam-1, AmpC2, FAR-1, OXY-1,CTX-M-100, CTX-M-139, AQU2	*gyrA, parC*	CarA, TlrC, OleB, ErmR, SrmB, *Erm* 41, , *rrl*
M 154	Aac(2)-Ic, Aac(3)-IIIb, Aac3-Xa, Aph3"Ic, Rmt F, Aac(3)-IIIb, Aac3-V11, rrs	FAR-1, OXY1-2, CTX-M-139, CTX-M-100,AQU-2, LEN-37, AmpC2,DHA-2, CTX-M, Pam-1	*gyr*A, *par*C	*Erm*41, Tlr C, *Erm38*, OleB, ErmR, SrmB,Car A,Ole C, *rrl*
M 156	Aph3"Ic, Aac3-V11, Aac(3)-IIIb, Aac3-Ib, Aac3-1,	AmpC1, CTXM-100, AmpC2, FAR-1, Pam-1, OXY1-2, CTX M-	*gyr*A, *par*C	Tlr C, Ole B, Erm R, *Erm* 38, CarA, Ole C, SrmB, *Erm* 41, *rrl*

	aadA24, Aac2-Ic	139, LEN-37, AQU-2		
M 159	Aph3”Ic, Aac2-Ic, Aac3-V11, Aac3-Xa, Aph3-IIb, rrs	AQU2, FAR-1, OXY1-2, BKC-1,CTX-M-139, Pam-1, CTX-M-100, LEN-37,AmpC1	*gyr*A, *par*C	TlrC, Srm B,CarA,OleC,OleB,Erm R,*Erm* 38,*Erm* 41, *rrl*
M 162	Aac3-Xa, Aac(3)-IIIb, Aph3-IIb, Aac2-Ie, Aac3-VII, Aac2-Ic, Aph3”Ic, rrs	FAR-1, OXY1-2,GES-23, CTX-M-139,AQU2, CTX-M-100,AmpC1,LEN-37	*Qep* A *gyr*A, *par*C	*Erm* 41,*Erm 38*,Tlr C NC,TlrC M, Ole B,Ole C, Srm B, Erm R, Car A, *rrl*
M 172	Aph3”Ic, Aac(3)-IIIb, Aac3-V11, Aac2-Ic, rrs	FAR-1, OXY1-2, CTX-M-139, Pam 1, AmpC1, CTX-M-100,AQU2, LEN-37,AmpC2, CTX-M-130	*gyr*A, *par*C	*Erm* 41, *Erm* 38, Srm B,OleB, Tlr C M, Tlr C NC, Car A,Ole C, Erm R, *rrl*

Top 20 resistance genes predicted by CARD, for M154.

Hits were organized from the highest to the lowest bitscore

ORF_ID	Bitscore	ARO	% id	SNPs	Other SNPs	Drug Class
M154_M00173612_	1462.2	gyrA	89.69	S95T	A85T:2412	nybomycin; fluoroquinolone antibiotic
M154_M00175994_	427.9	mtrA	95.11	n/a	n/a	macrolide antibiotic; penam
M154_M00175496_	332	efrA	40.76	n/a	n/a	rifamycin antibiotic; macrolide antibiotic; fluoroquinolone
M154_M00172253_	300.4	QepA2	40.91	n/a	n/a	fluoroquinolone antibiotic
M154_M00176398_	294.3	patA	38.92	n/a	n/a	fluoroquinolone antibiotic
M154_M00176497_	286.2	APH(3'')-Ia	59.29	n/a	n/a	aminoglycoside antibiotic
M154_M00172148_	250.8	KPC-16	48.16	n/a	n/a	monobactam; cephalosporin; penam; carbapenem
M154_M00176458_	235	patA	40.87	n/a	n/a	fluoroquinolone antibiotic
M154_M00174707_	228.8	mfpA	59.89	n/a	n/a	fluoroquinolone antibiotic
M154_M00172590_	228	oleB	36.11	n/a	n/a	macrolide antibiotic
M154_M00175671_	227.3	kdpE	50.67	n/a	n/a	aminoglycoside antibiotic
M154_M00172739_	224.2	PBP2	30	V316T	M400T:320	cephamycin; cephalosporin; penam; monobactam; carbapenem
M154_M00172000_	221.1	optrA	29.43	n/a	n/a	oxazolidinone antibiotic
M154_M00174346_	219.9	mtrA	49.55	n/a	n/a	macrolide antibiotic; penam
M154_M00172481_	191.8	clbB	37.39	n/a	n/a	lincosamide antibiotic; macrolide antibiotic; oxazolidinone
M154_M00174813_	176.4	arlR	40.18	n/a	n/a	acridine dye; fluoroquinolone antibiotic
M154_M00175170_	163.7	smeS	36.07	n/a	n/a	aminoglycoside antibiotic; cephalosporin; penam; cephamycin
M154_M00173434_	160.6	macB	39.09	n/a	n/a	macrolide antibiotic
M154_M00175239_	137.1	oleC	36.94	n/a	n/a	macrolide antibiotic
M154_M00176427_	135.6	soxR	47.73	n/a	n/a	triclosan; glycylcycline; cephalosporin; penam; fluoroquinolone

APPENDIX H

Summary of top genes predicted for each antibiotic class and the % of identity with the homologue in Mab

Antibiotic class	ARG-ANNOT		CARD		ResFinder	
Aminoglycoside	*aph (3'')-Ic*	70%	*aph(3'')-Ia*	58.5%	*aac(2')-Ib*	78.7%
	aac(3)-VII	35%	*kdp*E	32.8%	*rrs*	
	aph(6)-Ia	48%	*sme*S	36.1%		
	aac(3)-IIIb	39%	*bae*S	31.4%		
	rrs		*cpx*A	29.3%		
Macrolide	*erm*(41)	99.5%	*erm*(41)	99.3%	*erm(*41)	99.8%
	*srm*B	54%	*mtr*A	40.3%	*rrl*	
	*tlr*C	49%	*efr*A	41%		
	*ole*B	39%	*ole*B	36%		
	rrl		*rrl*			
Fluoroquinolone	*qep*A	42%	*qep*A2	41.3%	*gyr*A	
	*par*C		*efr*A	41%		
	gyrA		*pat*A	40.6%		
			*mfp*A	59.9%		
			*gyr*A			
Carbapenem	*far*-1	41%	KPC-16	47.8%	Nil	
	*oxy*1-2	57%	PBP2	30%		
	ctx-M-139	57%	*mec*B	25.9%		
	*aqu*2	50%	*spg*-1	30%		
	pam-1	46%	*nmc*-R	32.4%		
Oxazolidinone	Nil		*clb*B	37.4%	Nil	
			*optr*A	29.4%		