Molecular Epidemiology of *Mycobacterium abscessus complex* in Cystic Fibrosis and Non-Cystic Fibrosis patients in New South Wales

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Statement of Originality

This is to certify that to the best of my knowledge; the content of this thesis is my own work.

This thesis has not been submitted for any degree or other purposes.

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Andrea Bustamante

Abstract

To improve management of infectious caused by *Mycobacterium abscessus* and to translate genomic analysis of *M. abscessus* into practice of the New South Wales Mycobacterium Reference Laboratory. Members of the *Mycobacterium abscessus complex* are emerging pathogens of increasing importance, causing both respiratory and soft tissue infections. It includes three clinically relevant subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletti*, and *M. abscessus* subsp. *massiliense,* with their own drug susceptibility profiles. The aim of this study was to examine the subspecies and antibiotic susceptibilities combining phenotypic and genotypic information. Clinical isolates were collected between 2015 to 2017 at the statewide New South Wales Mycobacterium Reference Laboratory, Australia. Drug susceptibility testing was performed for four main drugs (clarithromycin, amikacin, ciprofloxacin, linezolid) by broth micro dilution. Analysis of mutations associated with drug resistance was done by analyzing whole genome sequencing data. Furthermore, the analysis was extended to determine genetic diversity between subspecies and consecutives samples in the same patients.

We investigated 159 isolates and *Mycobacterium abscessus* subsp *abscessus* accounted for the majority of the *M. abscessus* isolates (n=124,77.4%). We isolated the first *M. abscessus* subsp *bolletii*. Both cohort of cystic fibrosis and non-cystic fibrosis patients were predominantly isolated from Female. Our findings confirmed that *Mycobacterium abscessus* subsp *abscessus* (87.3%) were the dominant subspecies in CF patients. Genotypic markers for Clarithromycin and Amikacin had strong association to the phenotypic susceptibility, with PPV value of 91.3% and 98.7%. There was still unexplainable resistance, related to Linezolid, Amikacin and Ciprofloxacin that can possibly involve other genes that have not been analyzed or other cell mechanism are involved to produce resistance.

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Glossary

%	Percent
°C	degree Celsius
23S rRNA	23S Ribosomal ribonucleic acid
3'	Three prime
5'	Five prime
AFB	Acid fast bacilli
ATTC	American Type Culture collection
BAL	Bronchoalveolar Lavage
BHQ	Black Hole Quencher
Bp	Base pair
BSC	Biosafety cabinet
CF	Cystic Fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
CIP	Ciprofloxacin
CLA	Clarithromycin
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
Cy5	Cyanine5
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EDTA	Ethylenediaminetetraacetic acid
erm(41)	erythromycin ribosomal methylase gene
Fam	Fluorescein amidites
gr	gram
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
hrs	Hours
ITS	Internal Transcribed Spacer
IWGMT	International working group of Mycobacterial Taxonomy
KOH	Potassium Hydroxide
LJ	Lowenstein Jensen

LZD	Linezolid
M	Molar
Mab	*Mycobacterium abscessus*
MabA	*Mycobacterium abscessus* subspecies *abscessus*
MabB	*Mycobacterium abscessus* subspecies *bolletii*
Mabc	*Mycobacterium abscessus* complex
MabM	*Mycobacterium abscessus* subspecies *massiliense*
MAC	*Mycobacterium avium* complex
Maldi-TOF	Matrix Assisted Laser Desorption
MB	Middlebrook
MGIT	Mycobacteria growth indicator tube
MIC	Minimum inhibitory Concentration
min	Minute
mL	Milliliter
MRL	Mycobacteria Reference Laboratory
MXF	Moxifloxacin
NACL	Sodium Chloride
NPAAC	National Pathology Accreditation Advisory Council
NSW	New South Wales
NTM	Non-tuberculosis Mycobacteria
OADC	Oleic Albumin Dextrose Catalase
PBS	Phenol buffer saline
PC3	Physical Contaminant Level III
PCR	Polymerase Chain Reaction
pH	potential of hydrogen
PTC	peptidyltransferase centre
QC	Quality control
QLD	Queensland
QRDR	Quinolone resistance-determining region
R.O	Reverse osmosis
rDNA	Ribosomal Deoxyribonucleic acid
RGM	Rapid Growing Mycobacteria
rpm	revolutions per minute
rpoB	Ribonucleic Acid Polymerase Beta Subunit
rRNA	Ribosomal ribonucleic acid

RT-PCR	Real time- Polymerase Chain Reaction
SNP	Single Nucleotide Polymorphism
SXT	Trimethoprim/sulfamethoxazole
TE	Tris Ethylenediaminetetraacetic acid
Tris-HCL	Tris-hydrochloride
US	United States
USA	Unites States of America
w/v	weight to volume
WGS	Whole Genome Sequencing
ZN	Ziehl-Neelsen
µL	Microliter
µM	Micromole

CHAPTER 1

1 Introduction

Increasing numbers of patients with Cystic Fibrosis (CF) are becoming infected with the multidrug-resistant *Mycobacterium abscessus* bacteria, which cause progressive lung damage and remain very challenging to treat. How this organism is acquired is not fully understood, but there are growing concerns about person-to-person transmission and prevention of drug resistance development. *M. abscessus* complex is a Rapidly Growing Mycobacterium (RGM) that includes three clinically relevant subspecies, *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense* (1).

The conventional methods of identifying *M. abscessus* include high-performance liquid chromatography (HPLC), biochemical tests and real-time PCR (2), however these methods cannot reliably discriminate subspecies within the *M. abscessus* complex. Further amplification and sequencing of house-keeping genes (e.g, *hsp65, rpoB, secA1, soda*) and Internal Transcribed Spacer (ITS) region (3) have been recommended, however, they are costly and time consuming. Identifying the correct subspecies can aid the treatment of *M. abscessus* complex infections (4). The current recommended antimicrobial regimen for chronic and persistent infections includes macrolides (azithromycin and/or clarithromycin) and a parental agent (amikacin, cefoxitin or imipenem) (5) (6). Unfortunately, the treatment does not necessarily lead to the elimination of infection in the lungs (2), due to resistant nature of *M. abscessus* complex (4) (7).

Thus, antimycobacterial testing of clinically significant isolates is recommended. Susceptibility testing can be achieved by broth micro dilution, which includes antibiotics used to test against RGM (CLSI M24) and PCR based techniques to detect the presence of the ribosomal methylase gene *erm*(41), which can confer inducible resistance to macrolides (8).

Currently, in New South Wales, as in other states of Australia, it is not mandatory for diagnostic laboratories to send all cultures of *M. abscessus* complex for identification to the state Mycobacteria Reference Laboratory (MRL). The prevalence of infection with Nontuberculous Mycobacterium have been underestimated and many isolates are not considered to be clinically significant for causing disease (9). Therefore, in NSW there is

no epidemiological surveillance data available that could assist in examining temporal trends of infection, drug resistance or potential outbreaks associated with these mycobacteria. This is of concern for immunocompromised patients, especially those affected by CF, who are highly susceptible to these opportunistic organisms. Infections with *M. abscessus* complex has been recognized as the main contributor to decreasing of the lung function in CF patients (1).

Whole genome sequencing (WGS) as a laboratory technique presents new opportunities to examine molecular epidemiology of *M. abscessus* complex with much higher resolution. The use of WGS can aid with identifying *M. abscessus* complex to species level. Recent evidence also suggests that WGS can identify clusters of epidemiologically linked cases, define markers of antibiotic resistance to macrolides, and examine molecular markers associated with infections in CF patients (10) (11) (12) (13). This information has the potential of improve clinical management of the infection and laboratory surveillance for Public Health and decrease the risk of any ongoing transmission of infection amongst CF patients.

This study aimed to examine the genotype and phenotype of clinical isolates of *M. abscessus* complex circulating in NSW in a three-year period, from 2015 to 2017. The general aim was to improve management of infections caused by *M. abscessus* complex and to translate its genomic analysis into practice at the NSW MRL.

Specific Aims:

- To investigate the molecular epidemiology of *M. abscessus* complex in NSW using WGS high-resolution approaches.
- To compare molecular markers of drug resistance to clarithromycin, amikacin and ciprofloxacin in *M. abscessus* complex with phenotypic susceptibility testing results.
- To explore associations between subspecies and subtypes of *M. abscessus* complex and chronic infection and colonization in patients with and without CF.

We hypothesized that: (i) Whole genome sequencing can improve the resolution of laboratory surveillance of *M. abscessus* complex; (ii) there are significant differences between genotypes of *M. abscessus* associated with infection in CF patients and infection and colonisation in other human hosts; (iii) genomic changes can be reliable predictors of *in vitro* phenotypic resistance in *M. abscessus* complex.

CHAPTER 2

2 Literature review

2.1 Evolution of *Mycobacterium abscessus* taxonomy

Mycobacterium abscessus complex (Mabc) is a representative of non-tuberculous mycobacteria (NTM) and Rapid Grower Mycobacteria (RGM) which produce rough to smooth colony (Figure 2-1) variants that are non-photochromogenic on a special media (7, 14). Mabc cells are ubiquitous in the environment and have found in soil and water (14) and are one of the few RGM to cause infections in human (2, 14, 15). It is an opportunistic pathogen responsible for causing infections in the skin, soft-tissue, solid organs and bones of humans. These infections often present as chronic and protracted diseases, especially chronic pulmonary disease (2, 14, 15).

Figure 2-1: Growth appearance of Mycobacterium abscessus ATCC 19977 on Middlebrook 7H10 agar plate

One of the first reported cases of *Mycobacterium abscessus* (*Mab*) association with human disease was a knee abscess described by Moore and Frerichs in 1953 (16) where the organism was defined as an abundant rapid growing acid-fast bacterium. It was initially classified under the Mycobacteriacae family as it did not resemble any other typical acid-fast bacilli (AFB). It was classified as a novel species *Mycobacterium abscessus (16)* according to capability to produce deep subcutaneous abscesses. In 1972 an International Working Group of Mycobacterial Taxonomy (IWGMT) was formed to work out a systematic species identification for Mycobacteria and the Group proposed *M. abscessus*

to be classified as a subspecies of *Mycobacterium chelonae* subsp. *abscessus* under the *Mycobacterium chelonae* group (17). Then in 1992 Kusunoki and Ezaki (18) suggested that *M. abscessus* should become an independent species from *Mycobacterium chelonae* as they concluded that the difference between the two subspecies were their reannealing DNA values and systematic differences in the ordinary biochemical tests (18). However, the 16S rDNA sequencing analysis displayed only 4bp difference between *M. chelonae* and *M. abscessus*. Therefore, significant limitations of the identification solely based on 16S rRNA gene Polymerase Chain Reaction (PCR) and the sequencing analysis of this PCR product were recognized (19).

Over the following years there were confusion and inconsistency in naming *M. abscessus* as a distinct species and it commonly became known as *M. chelonae/abscessus* group, which consisted of three species - *M. chelonae*, *M. abscessus* and *M. immunogenum* (20, 21). These species were then separated by their biochemical properties. Specifically, *M. abscessus* could be distinguished from *M. chelonae* and *M. immunogenum* by its growth at 5% salt tolerance and the utilization of citrate (21). However, these biochemical tests were time consuming. Also, 16S rRNA PCR and gene sequencing analysis were producing only suggestive results. It was not until the PCR assay targeting another house-keeping gene *rpoB* with amplicon sequencing was developed and evaluated for RGM that the subspecies of *M. chelonae/abscessus* group was re-evaluated.

In 1999 Kim et al (19) conducted a *rpoB* PCR study with 44 Mycobacteria reference strains and 107 clinical isolates. Their findings have proven that the *rpoB* gene was able to distinguish Mycobacteria sub-species even further. For example, a pathogenic Mycobacteria species *Mycobacterium kansasii* was able to be distinguished from a nonpathogenic species *Mycobacterium gastri* (19). However, the section of *rpoB* gene selected as the target for the PCR and sequencing had its limitations.

Adékambi et al (22) suggested to use a complete *rpoB* gene to determine a better target when evaluating 20 reference strain of RGM with 9 other mycobacteria clinical strain of specific interest. The final evaluation determined an alternative partial *rpoB* (723-bp) target for the RGM sequence evaluation that was better. As it could clearly distinguish *Mycobacterium mucogenicum* and *M. abscessus* from *M. chelonae.*

Results from Adékambi (22) based on *rpoB* gene PCR and sequencing analysis have indicated that isolates with greater than 3% difference in the sequence could be considered a novel species, and the difference of less than 2% should be identified as the same species even though the 16S rRNA gene sequence comparison of the isolates was 100% (21, 22). Based on these observation, two novel species were identified by Adékambi et al from human sputum in 2004 as *M. massiliense* and in 2006 *M. bolletii* (21, 23) The isolate had similar biochemical properties to *M. chelonae-abscessus* group but the *rpoB* gene had a 96% to 97 % similarity to *M. abscessus*. However, the susceptibility properties of the novel species *M. massiliense* and *M. bolletii* were different in its susceptibility or resistance to clarithromycin (23).

In recent years there have been many studies suggesting that *M. abscessus* comprises of three subspecies: *Mycobacterium* a*bscessus* subspecies *abscessus* (MabA) *Mycobacterium abscessus* subspecies *massiliense* (MabM) and *Mycobacterium abscessus* subspecies *bolletii* (MabB) (10, 15, 23, 24, 25, 26). All three form the *Mycobacterium abscessus complex* (MabC). The 16S rRNA gene sequence for *M. abscessus* and *M. bolletii* are identical and *M.massiliense* share 99% sequence identity (1). Also, the three sub-species share a high genetic nucleotide identity of 99.1% (10) by Whole Genome Sequencing (WGS).

Despite multiple demonstrations of three species of *M. abscessus*, the classification remains debatable. For example, Leao et al (27) suggested that the *M. massiliense* and *M. bolletii* be combined and called *M. abscessus* subsp. *bolletii* comb.nov. However, in 2013 Heydari *et al* (28) and Bryant *et al* (29) confirmed through WGS and phylogenetic tree analysis that the three species can be classified as *M. abscessus* sensu stricto, *M. bolletii* and *M. massiliense.*

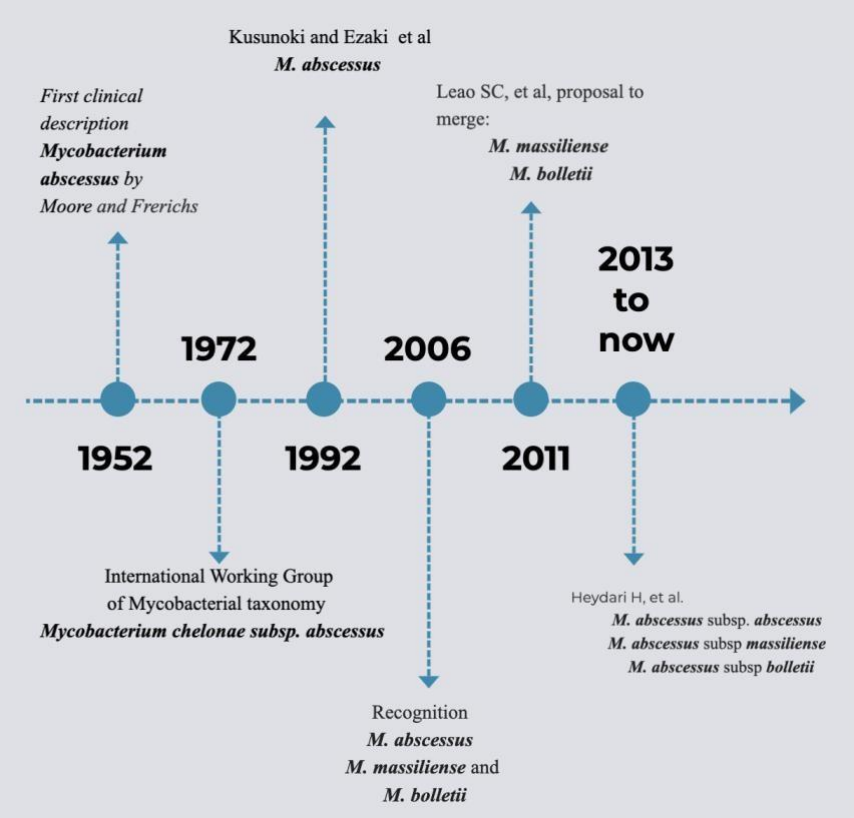

Figure 2-2 Timeline for M. abscessus complex subspeciation

2.2 Global epidemiology of *M. abscessus*

There has been an increased awareness of *M. abscessus* complex globally due to its emerging pathogenicity and drug resistant patterns (4, 10, 15, 26). *M. abscessus* complex has been associated to cause significant invasive disease in immunocompromised patients and patients with chronic pulmonary disease (10, 26). *M. abscessus* has become a significant pathogen for individuals affected by Cystic Fibrosis (CF) and is the main contributor to decreasing lung function in CF patients (30).

The infection caused by *M. abscessus* have been reported world-wide. Studies from the Unites States (US) and Korea have shown that *M. abscessus* complex is the second most widespread NTM species in pulmonary infectious disease (31, 32, 33). *M. abscessus* complex was the common NTM causing infection associated to CF patients in France (34). Of the 1,582 patients involved in the CF multicenter study 215 NTM were recovered, the most common NTM was *M. abscessus* (n=40) compared to 17 *Mycobacterium avium*

complex (MAC). Other countries like Taiwan have also shown an increase in prevalence of NTM infection (35), from 2.7 to 10.2 cases per 100,000 population.

In Australia national data about infection associated to NTM is lacking as it is considered not to be public health concern and hence not notifiable, with the exception of Queensland (QLD), where this pathogen is a reportable organism under the *Queensland Public Health Act (2005)* (36). It was reported in QLD that *M. abscessus* isolates increased from 0.065/ 100,000 in 1999 to 1/100,000 in 2005 (9). In New South Wales (NSW) it is not mandatory for external laboratories to send cultures of NTM for identification to the Mycobacteria Reference Laboratory (MRL). Nevertheless, from 971 cultures of NTM that were received by the NSW MRL in 2016, *M. abscessus* complex was identified as the third most common after *M. avium* and *M. intracellulare* (unpublished data). These were from various sources such as sputum, bronchial lavage and washing, tissue and skin abscesses.

2.3 Human disease associated with *M. abscessus*

NTM are abundant in the environment and can naturally colonise lungs making it difficult to differentiate between true infection and colonization or contamination (37, 38). Therefore, not all NTM isolated or cultured from clinical samples should be considered significant as its detection could be due to contamination or colonization. Hence, a clinically significant NTM sample is dependent on the type of NTM species isolated, type of clinical samples, clinical presentation, and epidemiological data (38, 39, 40). The recommended microbiological, clinical and radiographic guidelines for diagnosis of NTM associated to pulmonary disease suggest strict criteria for isolates to be considered clinically significant. Specifically, there needs to be two or more positive culture results from separate expectorated sputum's, one bronchial washing or lavage and lung biopsies showing AFB or granulomatous inflammation that also culture positive, NTM isolated from sterile sites e.g., CSF or blood and any isolated from seriously immunosuppressed patient (2, 38, 40).

In the case of pulmonary infection associated with the *M. abscessus* complex as the pathogen, two or more positive cultures of the same subspecies should be isolated before

establishing laboratory diagnosis or considering treatment (40). However, most of these guidelines lack detailed evidence for diagnosis and treatment for pulmonary disease associated with CF, immunocompromised or suppressed patients. Amongst the CF patient *M. abscessus* have been associated with progressive inflammatory lung damage and considered as a contraindication for lung transplant surgery (41).

M. abscessus complex has been recognized as a causative agent in skin and soft tissue infections following trauma that involves skin penetration. Such penetrating injuries include tattooing, acupuncture, intramuscular injection and reconstructive surgery (42, 43). Reports have suggested that the infections could be caused by contaminated water sources, medical instruments, material, poor infection control or the intrinsic nature of the pathogen, as the cell wall of the pathogen can be resistant to most disinfectants (4) (43) (42). Other infections that have been describe involving this pathogen include catheter infection, eye infection, otitis media and disseminated disease as a secondary infection (44).

2.4 Cystic Fibrosis Disease and its association to NTM colonisation and infection

Cystic Fibrosis (CF) is an autosomal recessive genetic condition that mainly affects digestive and lung system due to a malfunction in the exocrine system (97). This can cause impaired breathing due to the thickening and stickiness of mucus that can trap bacteria. Also damages in digestive function of the pancreas. In Australia over 3,600 people are affected (97), and according to the Woolcock Institute of Medical Research (98) (50) it affects many young Australians and can be life-limiting.

The diagnosis in Australia is usually routine screen testing at birth known as a heel prick test and confirmed with further testing. An inherited disease where one copy of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene mutations comes from both parent (99) The *CFTR* gene encodes the cell-surface chloride ion channel and this is important for the transport of chloride and bicarbonate to apical epithelial cell (45). The mutation of the gene can cause disruption in the ion channel and can lead to excess mucus

production. The thick and viscous mucus can cause blockage to many organs making it prone for bacterial colonisation and infection (46).

The thick secretion in areas like the lung has the potential to retain bacteria and promote bacterial infection and colonization that can lead to chronic pulmonary infection (46) (47). One of the common CF pathogens are NTM, with *Mycobacterium avium* complex (MAC) and *M. abscessus* complex the most frequently observed (47). The later becoming the most prevalent mycobacteria causing chronic pulmonary infection in studies from USA, Europe, Taiwan and Australia (10). The difficulty in treating infection related to *M. abscessus* in CF is that there is no specific guideline to follow for treatment and diagnosis due to the lack of evidence on clinical efficacy for the treatment (48)

2.5 Challenges of laboratory identification

Currently, there are no consistent reliable results in identifying all Mycobacteria species or sub-species from all positive cultures in liquid or solid media. The laboratory methods for identification of NTM can include biochemical testing, High-performance liquid chromatography (HPLC) and PCR. However, these are dated methods as they are less discriminatory in identifying species specific Mycobacterium and are considered costly.

A novel identification method known as Matrix Assisted Laser Desorption ionization-time of flight (MALDI-TOF) is an efficient and rapid method of NTM identification, however, it still requires further evaluation and optimisation (38, 40). As, MALD-TOF can often have less than 75% accuracy level in identifying positive mycobacteria culture (43).

The current gold standard for identification of NTM are molecular methods. There are a combination of methods such as DNA line probe assay, Real time-PCR targeting specific house-keeping genes, and 16S rRNA gene sequencing analysis (2, 26, 38). Furthermore, amplification and sequencing of several genes in combination (e.g., *hsp65*, *rpoB*, *secA1*, *soda*) and Internal Transcribed Spacer (ITS) region have been recommended (26) for speciation of NTM like *M. abscessus* complex for sub-speciation. Identifying the correct

sub-species can guide the antimicrobial treatment of *M. abscessus* infection (4, 38) and provide important clinical and epidemiological information (40). However, these methods are slowly becoming superseded as novel methods such as whole genome sequencing are becoming readily available and can provide extra data of antibiotic resistance, virulence and molecular epidemiology (10) (49).

Whole genome sequencing (WGS) has recently emerged as an important tool in the identification and characterization of Mycobacteria. WGS allows the study of the entire genome of a bacterium and the comparison of it to others. It is a widely used technique in research, clinical diagnostic, and public health laboratories. It is most likely to be the gold standard for bacteria identification in the future as it has better resolution, and rapidly becoming more affordable. In recent studies the use of WGS had aid the speciation of *M. abscessus* complex, identification of drug resistance markers, linkage of cases with and without epidemiological data and possible molecular markers associated with infections in CF patients (10, 11, 49). The capacity of WGS to support the investigation of transmission events has become also relevant as the transmissions of *M. abscessus* between patients within CF clinics have been recognized (50) (51) (10) (52) (53).

2.6 Challenges of antibiotic susceptibility testing for *M. abscessus* infections

M. abscessus complex is considered to be the most resistant known pathogenic NTM, with daunting innate antibiotic resistance mechanisms that defeat most antibiotics (5). Currently there is no established therapeutic regimen for *M. abscessus* complex associated with pulmonary disease with proven effectiveness (2). However, the recommendation for patients that are diagnosed with CF and have an NTM isolated from numerous respiratory samples rely on the use of macrolide antibiotics (2, 37). If a patient still recovers NTM from sputum after monotherapy, the treatment shouldn't be repeated (2, 37). However, this is not recommended for *M. abscessus* complex pulmonary infection (24). A standard therapy for chronic and persistent infection with *M. abscessus* is a macrolide (azithromycin or clarithromycin) and one or more parental agents (e.g., cefoxitin, imipenem and/or amikacin) (2, 26, 37, 54). Unfortunately, the treatment does not necessarily lead to the

elimination of infection in the lungs (2) due to *M. abscessus* complex resistant nature (4, 15).

The laboratory susceptibility testing for *M. abscessus* has not been fully validated. The standard drugs used to test in-vitro susceptibility for NTM include agents belonging to macrolides, aminoglycosides, quinolones, tetracyclines, oxazolidinones, beta-lactamase and sulphonamides (2, 44, 55, 56). Unfortunately, a resistance of majority standard NTM drugs to macrolides, linezolid, quinolones, tetracyclines and aminoglycosides is becoming widespread in NTM species (56) (44) and these drugs are important to use for the treatment. The cure rate for infection caused by NTM like *M. avium* complex (MAC) is 50-70% and *M. abscessus* is only 30-50% from a retrospective study of NTM patients in the United States between 2001 and 2008 (100). *M. abscessus* complex species is one of the main antibiotic-resistant NTM in-vitro (4, 56) with the exception of macrolides (clarithromycin and azithromycin) and aminoglycoside (amikacin, tobramycin and kanamycin) and they tend to be susceptible to these two agents (4, 56). Unlike pulmonary disease infection related to skin and soft tissue caused by *M. abscessus* complex are found to be active against the implicated isolate in-vitro (2).

Regimens for treatment for clinically significant isolates should be primarily based on in vitro drug susceptibility testing (DST) results. However, there are relevant discrepancies between drug susceptibilities measured in the vitro and the activity of the drug in vivo, resulting in therapy failure (57). The unreliable correlation between in vitro findings and clinical outcomes is probably multifactorial in nature. The values obtained for DST are the result of complex interactions between intrinsic resistance, inducible resistance, and mutational resistance. Intrinsic resistance to antimicrobial drugs could be due to the interference with the uptake of the drug, its biotransformation inside the cell, or the decrease in affinity of the drug target (57). Porins are important in nutrient acquisition but their frequency is significant lower in the cell wall of *M.abscessus* making it highly impermeable. In addition, several active drug efflux pumps have been identified as capable of transporting antimicrobial drugs, including the MmpS-MmpL which up regulation was reported in association with cross resistance to clofazimine and bedaquiline (58).

Drug resistance by acquired mutations occurs when a strain emerges from a population that was initially drug sensitive (59). This could happen during a prolonged and/or suboptimal

drug exposure. The acquired resistance due to a single-point mutations are significant for *M. abscessus* complex that only have a single copy of genes encoding common target proteins, such as *rrs* and *rrl* genes, therefore increasing the risk of acquiring mutations with single drug-treatments.

In 2011 the Clinical and Laboratory Standards Institute (CLSI) recommended to test RGM such as *M. abscessus* complex for susceptibility to the following antimicrobial drugs: amikacin, cefoxitin, doxycycline, linezolid, moxifloxacin, imipenem, ciprofloxacin, clarithromycin, trimethoprim-sulfamethoxazole and tobramycin (55). The latter should not use to treat infections related to *M. abscessus* as tobramycin is predominantly used to treat infection related to *M. chelonae* as there is insufficient data related to *M. abscessus* (55) (85).

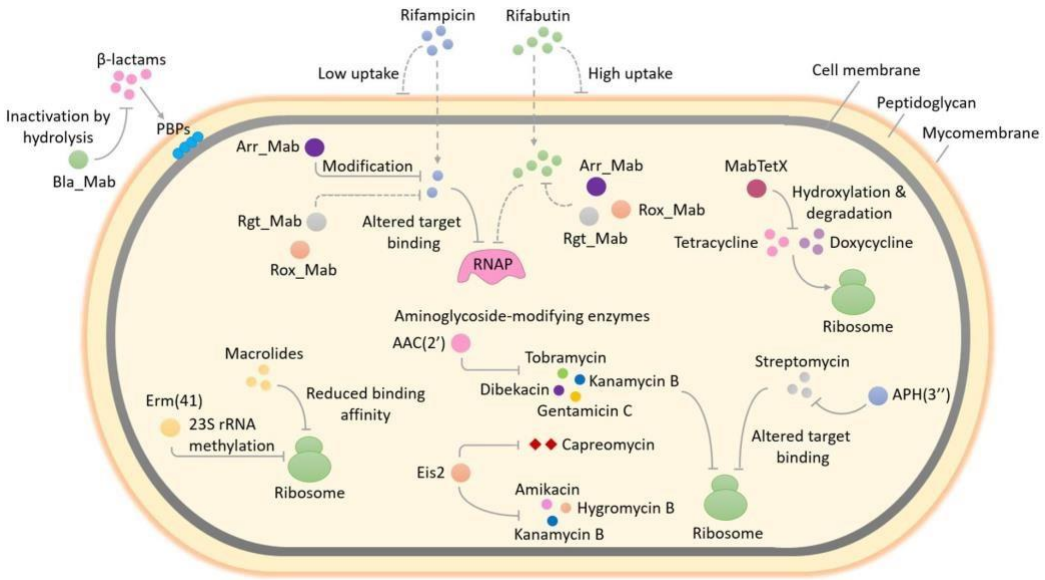

Figure 2-3. *Representation of enzyme mediated antibiotic resistance in M. abscessus complex. Reprinted from Luthra S, et al, 2018. The Role of Antibiotic-Target-Modifying and Antibiotic-Modifying Enzymes in Mycobacterium abscessus Drug Resistance.*

Front Microbiol. doi: 10.3389/fmicb.2018.02179.

2.6.1 Macrolides

Macrolides antibiotics includes erythromycin and second generation like clarithromycin and azithromycin. They are mostly used to treat skin, genital, and respiratory infections that are caused by gram positive pathogens and fastidious to gram negative pathogens (60). Macrolides target the nascent polypeptide exit tunnel within the 50S ribosomal subunit, which contains the newly synthesized polypeptide chain as it emerges from the ribosome. After the macrolide binding, the elongation of polypeptide chain extends for a few amino acids before the peptidyl-tRNA dissociates form the ribosome thereby disrupting protein synthesis (61) (Figure 2.5).

Clinically relevant resistance to macrolides could be the result of efflux of the antibiotic, drug inactivation, or through target-site modification by methylation or mutation that prevents the binding of the antibiotic to the ribosomal subunit. Although, acquired resistance to macrolides occurs at lower rates through point mutations at positions 2270 or 2271 (*Escherichia coli* (*E. coli*) numbering 2058/2059) of the 23S rRNA *rrl* gene (62), a study by Lipworth and colleagues in 2018 (63) and Realegeno (64) showed how this should be the first gene to evaluate as part of a decision algorithm for predicting drug resistance (see figure 2- 4).

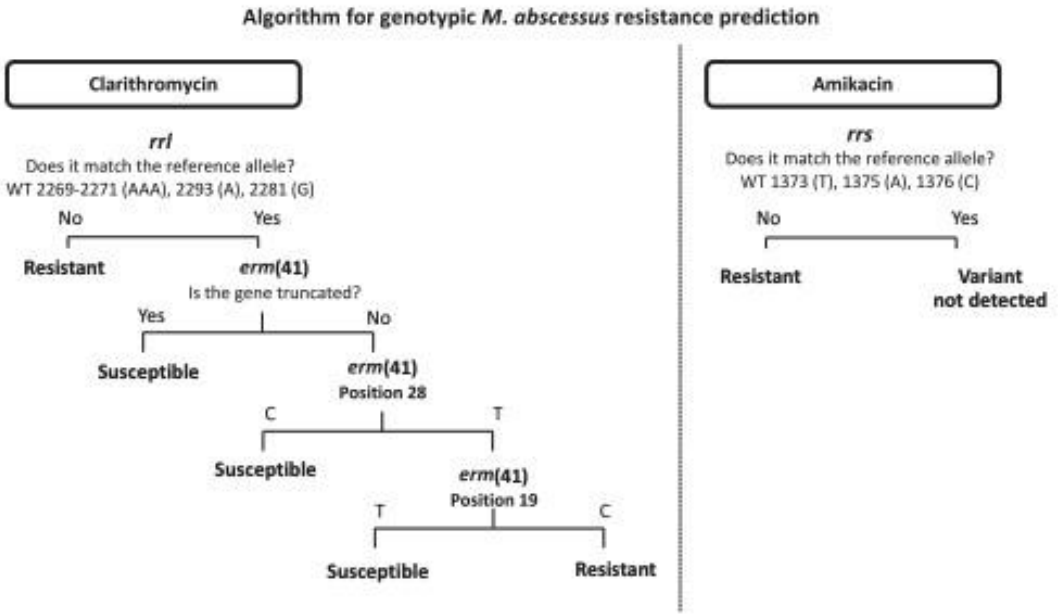

Figure 2-4. *The Algorithm used to analyse Clarithromycin and Amikacin genotype data. Reprinted from Realegeno S, et al, 2021. Clinical Whole Genome Sequencing for Clarithromycin and Amikacin Resistance Prediction and Subspecies Identification of Mycobacterium abscessus The Journal of Molecular Diagnostics. doi:10.10.16/j.moldx.2021.07.023.*

In *M. abscessus* complex the erythromycin ribosome methylase (Erm) enzyme has been well studied and associated with inducible macrolide resistance (65). The enzyme methylates A2058 nucleotide of 23S rRNA and reduces the macrolide affinity for ribosome exit tunnel (66). Noteworthily, the *erm*(41) gene (MAB_2297 as per annotation in reference strain *M. abscessus* ATCC 19977) is only functional in *M. abscessus* and *M. bolletii*; subspecies of *M. massiliense* harbor deletions in the *erm*(41) gene which cause the Erm(41) enzyme to be inactive and the subspecies susceptible to macrolides (67). Inducible resistance to macrolide in the subspecies of *M. abscessus* and *M. bolletii* can occur by a T/C polymorphism at position 19 and 28 of the *erm*(41) gene (see figure 2- 4).

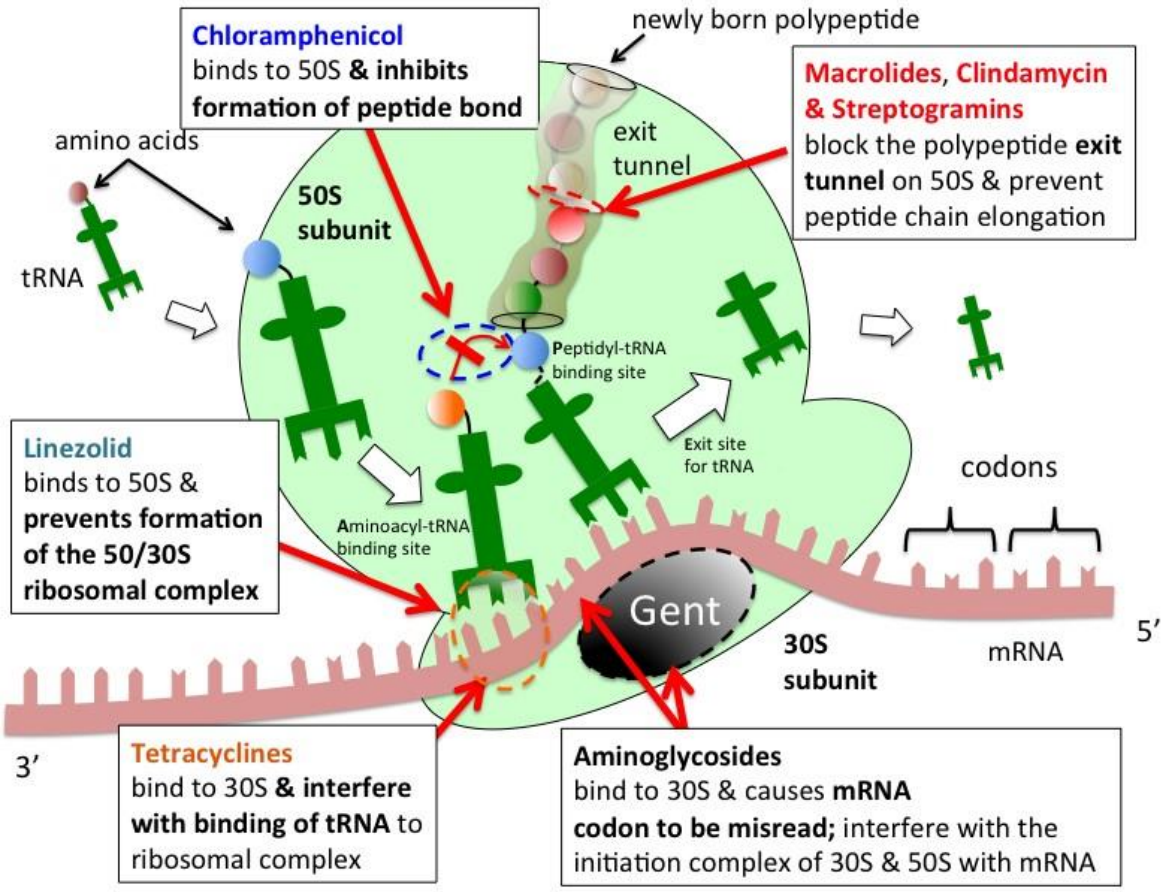

Figure 2-5 Sites of action of antibiotics that interfere with bacterial protein synthesis.Image reprinted from https://tmedweb.tulane.edu/pharmwiki/doku.php/ribosomal_antibiotics

Furthermore, the impermeability of its cell membrane and the induction of efflux activity is linked to clinical resistance to macrolides and other drugs (68). Studies using efflux pump inhibitors and measurement of RNA gene expression have shown a significant decrease in MIC of clarithromycin for non-susceptible isolates (69) (68).

2.6.2 Amikacin

Aminoglycosides are potent bactericidal drugs that prevent protein synthesis by attaching themselves to the 30S ribosomal subunit effecting bacterial translation process and leading

to bacteria death (4, 56, 70). They have been used since 1944 treating infection related to gram negative rods (39) and have been a key component in treating infections related to *Mycobacterium spp.* The most common aminoglycoside used for mycobacteria treatment include streptomycin, kanamycin, tobramycin, and amikacin (71). The latter is the main parenteral agent used in the treatment of infections caused by *M. abscessus* complex specially in chronic pulmonary conditions (54). Amikacin administration is recommended to be done in two phases; the intensive phase with 3 to 12 weeks of intravenous amikacin and daily macrolide, follow up by the continuation phase which include a daily macrolide and inhaled amikacin (24, 40).

Although, naturally *M. abscessus* complex is susceptible to amikacin (6), resistance to it have been reported due to long use of the drug or previous exposure of it. Mutation in the 16S rRNA gene has been associated to cause resistance to amikacin and as *M. abscessus* complex has only one copy of this gene, alterations to critical positions may be responsible for the high-level drug resistance observed (72) (71).

In *M. abscessus* complex a single point mutation in the *rrs* gene (MAB_5051, encoding 16S rRNA) at position 1374 (1408, *E. coli* numbering) from Adenine to Guanine, has been reported to lead to high level resistance to amikacin, kanamycin and gentamicin (MICs > 1024 mg/mL) (72) (71).

2.6.3 Fluroquinolones

Fluroquinolones are a fetching option for therapy due to their excellent oral bioavailability and good performance. Although *M. abscessus* complex is not intrinsically resistant to fluoroquinolones, only a few point mutations are needed in DNA gyrase subunits A and B to confer fluoroquinolone resistance (MICs > 8 mg/ML) (73). Thus, monotherapy is not recommended. In addition, it has been shown that it has potential antagonism with macrolides for *M. abscessus* subsp. *abscessus* (74) (75).

Fluroquinolones mode of action is to inhibit topoisomerase IV and DNA gyrase, the latter enzyme causes the DNA supercoiling which is essential for gene replication and

transcription in bacteria (76). This enzyme introduces negative supercoils into DNA by generating a DNA double-stranded break, passes a separate segment of double-stranded DNA through the break, and then reseals the DNA molecule (76). There are reported mutations in the DNA gyrase Quinolone Resistance Determining Region (QRDR) that are responsible for resistance to fluoroquinolones in *Mycobacterium tuberculosis*. However, in *M. abscessus* complex no mutations are found in this region associated with resistance (77), suggesting that there could be other mechanisms involved in causing fluoroquinolones resistance.

2.6.4 Linezolid

Linezolid is a synthetic drug from the family oxazolidinones that inhibit protein synthesis in bacteria. It is a bacteriostatic drug that inhibits the binding of the 23S rRNA to the 50S subunit by overlapping with the binding sites of antibiotics (78).

Although it is active against both aerobic and anaerobic Gram-positive bacteria and some Gram-negative anaerobes, it is often use as a last resort drug for infections (79). It has been demonstrated that linezolid *in vitro* activity against most NTM is a modal MIC of 16 μg/mL (80). The exception being M. *abscessus* complex with 32 μg/mL.

Resistance to linezolid has been reported in NTM globally (78), with rates varying from 51% to 42%. Noteworthy, Tu et al. reported significant discrepancies in resistances rates due to the use of different methods (81). A well-known mechanism for linezolid resistance is a mutation in the ribosome structure near the peptidyl transferase center (PTC). The ribosomal protein L3, one of the components of the 50S ribosomal subunit and involved in the PTC, is encoded by the *rplC* gene. Mutations in this gene sequence have been demonstrated to increase the linezolid MIC (78) (82). In addition, mutations in the *rrl* gene sequence, which encoded for the 23S rRNA, are also quite frequently observed in clinical isolates (80).

2.7 Molecular epidemiology of *M. abscessus* complex in NSW

Molecular epidemiology focuses on the identification of genotype and phenotype components at the molecular level that have the potential to impact on the cause, distribution, and prevention of disease. WGS techniques have help to breach the gap of knowledge between genotype and phenotype as discussed in previous sections. Simultaneously, WGS allows the inference of the phylogenetic relationships and clusters. Single Nucleotide Polymorphism (SNP) based analysis are commonly used to create distance matrix between a set of strains, which allows the construction of a phylogenetic tree via diverse clustering algorithms. SNPs are detected by mapping sequence reads against a closely related reference genome and calling nucleotide differences.

Whole genome sequencing (WGS) as a laboratory technique presents new opportunities to examine molecular epidemiology of *M. abscessus* complex with much higher resolution. The use of WGS can aid with the speciation of *M. abscessus* complex. Recent evidence also suggests that WGS can identify clusters of epidemiologically linked cases, define markers of antibiotic resistance to macrolides, and examine molecular markers associated with infections in CF patients (10) (11) (12) (13).

Global studies have observed the distribution of dominant clades which accounted for most infections in patients with cystic fibrosis (10) (50) (53). The hypothesize that widespread recent transmission was most likely due to indirect person-to-person transmission through environmental contamination (eg, via fomites or aerosols) in health-care settings and other shared venues. This drive changes in international guidelines, suggesting that person-to person transmission might be an important mechanism for *M abscessus* acquisition in patients with cystic fibrosis.

Lipworth et al. (2021) (83) undertook an extensive study of more than 2200 isolates (906 patients in England) which recognised non-CF patients were also colonized with a clustered isolate. These results are in contrast with the current dogma that outbreak strains are primarily propagated in nosocomial cystic fibrosis environments (13). Phylogeny and geographical distribution linkage did not aid to explain clusters, with patients in clusters

not having identifiable epidemiological links. Furthermore, Commins et al (2023) recent publication on *M. abscessus* molecular clock rate supported the hypothesis of a slow clock rate could have a stronger association with the phylogenetic clusters than patient-to-patient transmission (12).

CHAPTER 3

3 Methods

3.1 Materials

3.1.1 Buffers and Solutions

Buffers and solutions used in this work were made by the NSW Pathology West-Media Laboratory following NATA accredited protocols. Commercial kits were provided by the Mycobacterium Reference Laboratory at ICPMR, NSW Health Pathology.

- Low TE Buffer

  20µL of 0.5M EDTA, ph 8 was added to 1mL of 1M Tris-HCL, pH8 and combined with 98.98 mL of UltraPure™ DNase/RNase-Free Distilled water to make 100mL of buffer. The buffer was sterilised by filtering the solution through 0.2µM filter unit and then aliquoted to 2ml lo-bind tube and stored in a fridge.

- 3M Sodium Acetate

  24.61 gr of Sodium Acetate (CH_3COONa) was added to 70 mL of nuclease-free water in a beaker. This solution was mixed with a magnetic stirrer until it completely dissolved. The solution was poured into a sterile volumetric flask and then additional nuclease free water was added until the solution totaled 100mL. This was sterilised by filtering through a 0.2µM filter unit, aliquoted to 2mL lo-bind tube and stored in room temperature.

- Zirconia Beads extraction vials

  Approximately 0.4 gr or 0.25mL of 0.1 mm diameter zirconia beads were aliquoted to 2mL screw top tubes. These were aliquoted as per Whole Genome sequence (WGS) extraction for each sample.

- 70% Ethanol

  Fresh 70% ethanol was prepared for each WGS run. Each sample required 1 ml of 70% ethanol (e.g. For 10 samples - 7 mL of 100 % ethanol combined with 3 mL nuclease free water).

• 80% Ethanol

Fresh 80% ethanol was prepared for each WGS run. Each sample required 1 ml of 80% ethanol (e.g. For 10 samples - 8 mL of 100 % ethanol combined with 2 mL nuclease free water).

• Phosphate Buffer Solution

1.53g NaCl, 1.16g $NaHPO_4$, 0.6g KH_2PO were combined. The buffer solution was made up to 200 mL with distilled H_2O. This was sterilized by autoclaving at 121℃ for 20 min.

• 3% Acid Alcohol

Inside a fume hood 3880 mL of absolute ethanol was added to 120 mL of HCL. No pH testing was required.

• 0.5% Methylene Blue

250 mL of 1% Methylene blue was added to 250 mL of distilled water. No pH testing was required.

3.1.2 HPLC Reagents

• Reagent B

Concentrated hydrochloric acid (32% w/v) was diluted 1:1 with water and mixed in the chemical fume hood while wearing gloves (safety glass, face shield and rubber apron may be used if preferred). The reagent was poured into 500 mL deionised water and stored in a Teflon bottle.

• Reagent C

10g of Potassium bicarbonate was dissolved in 250 mL deionised water. 250 mL Methanol was added to the solution, mixed by swirling and stored in a Teflon bottle.

- Reagent E

100 ml of Reagent B was poured in 100 ml of Methanol mixed by swirling and stored in a Teflon bottle.

- Reagent M

50 gr Potassium hydroxide was dissolved in 70mL deionised water using a magnetic rod and placing it on a magnetic plate stirrer. Deionised water was added to make up the volume to 100mL and stored in a Teflon bottle.

- Reagent N

50mL of chloroform was added to 62.5 mg of 4bromomethyl-6-7 dimethoxycoumarin and 7.5 mg of 18-crown-6 ether and stored in a Teflon tube.

- Chloroform and Internal Standard

50 µL each of High and Low standard was added to 14 µL of Chloroform and stored in a Teflon tube.

3.1.3 Broth, agar, and media

The following broth, agar and media were made by the NSW Health Pathology-ICPMR Media laboratory as per NATA accredited protocols.

• OADC

20 gr Bovine serum albumin fraction V, 8 gr glucose, 3.4 gr NaCL, 200 µL Oleic acid, 0.016 gr Catalase was added to 400 mL of sterile Reverse Osmosis (R.O.) water and stirred with a magnetic stirrer until dissolved. The solution was sterilized by filtration and stored in freezer.

- Middlebrook 7H10 plates (MB)

  76 gr MB 7H10 and 20 mL glycerol was dissolved in 3600 ml of R.O. water and autoclaved at 121°C for 15 minutes. 20 mL Cyclohexamide and 400 mL of OADC was added to the previous autoclaved solution at pH 6.6. Solution was then poured into plates by automatic plate pourer.

- Lowenistein Jensen slope (LJ)

  600 mL of mineral salts and 40 mL of malachite green was added to 1000 mL of egg emulsion and mixed well at pH 7.4. 10 mL of solution was dispensed in 30 mL yellow top bottles and placed in 85°C oven for 2 hours on a tray at a 45-degree angle.

3.1.4 Commercial kits and Chemicals

- BBL™ MGIT™ (Becton Dickinson REF-245122)

  Approximately 5.9 gr Modified Middlebrook 7H9 broth base and 1.25 gr Casein peptone per L of purified water.

- Kinyoun's Carbol Fuchsin (Australian Biostain P/L)

  8% Phenol, 10% Ethanol and 4% Basic Fuchsin Certified

- 1% Aqueous Methylene Blue (POCD Healthcare)

  1% w/v certified Methylene blue powder with D.I. water.

- Potassium Hydroxide Pellets (Ajax Finechem Pty Ltd 405-500G)

  Potassium hydroxide solid

- Chloroform (Sigma Aldrich- Merck 270636)

  HPLC grade > 99.8%

- 37 % Hydrocholic Acid (Sigma Aldrich CAT-320331)

- Methanol (VWR Life Science CAT-97064-856),

  HPLC grade > 99.8%

- Potassium Hydrogen carbonate (BDH Laboratory Supplies 10206)

- Instagene™ Matrix (Bio-Rad CAT-732-6030)

  20 mL of 6% Instagene matrix and a magnetic stirrer.

- Immomix™ PCR reagent Kit (Bioline BIO-25020)

  10 x 1.25 mL Immomix [IMMOLASE Polymerase (quantity not defined), 1.5 mM MgCl2, 1 mM dNTPs, 16 mM NH4SO4, 62.5 mM Tris-HCl, 0.01% Tween 20, Stabilizer] and 1.2 mL 50 mM $MgCl_2$ Solution

- SensiFAST™ Probe No-Rox Mix (Bioline BIO-86020)

  20 x 1 mL 2x SensiFAST Probe No-ROX mix (contents not supplied)

- EDTA 0.5M, pH 8 (Millipore Sigma-Aldrich CAT-632415)

  Ethylenediaminetetra-acetic Acid in distilled water adjusted to pH 8.

- AMPure Xp magnetic beads (Beckman Coulter Inc A63880)

  Magnetic particles suspended in water, Tris buffer, salt, sodium azide and polyethylene glycol

- RNase A, DNase and protease-free (Thermo Fisher CAT-EN0531)

  RNase enzyme at 10 mg/mL

- Sensititre™ Cation adjusted Mueller-Hinton broth with TES (Thermo Scientific 3462)

  11 mL of Mueller-Hinton broth and TES biological buffer in an aqueous solution containing magnesium and calcium ions.

• Sensititre RAPMYCOI microtiter plate (Thermo Scientific TREK diagnostics RAPMYCOI)

Table 3-1Antibiotics agents included and dilution range.

Antibiotic	Abbreviation	Concentration (µg/ml)
Trimethoprim/sulfamethoxazole	SXT	0.25/4.75 - 8/152
Ciprofloxacin	CIP	0.12 - 4
Moxifloxacin	MXF	0.25 - 8
Cefoxitin	FOX	4 - 128
Amikacin	AMI	1 - 64
Doxycycline	DOX	0.12 - 16
Tigecycline	TGC	0.015 - 4
Clarithromycin	CLA	0.03 - 16
Linezolid	LZD	1 - 32
Imipenem	IMI	2 - 64
Cefepime	FEP	1 - 32
Amoxicillin/clavulanic acid 2:1 ratio	AUG2	2/1 - 64/32
Ceftriaxone	AXO	4 - 64
Minocycline	MIN	1 - 8

Tobramycin	TOB	1 - 16

Plate Code: **RAPMYCO**

	1	2	3	4	5	6	7	8	9	10	11	12
A	SXT	SXT	SXT	SXT	SXT	SXT	LZD	LZD	LZD	LZD	LZD	LZD
	0.25/4.75	0.5/9.5	1/19	2/38	4/76	8/152	1	2	4	8	16	32
B	CIP	CIP	CIP	CIP	CIP	CIP	IMI	IMI	IMI	IMI	IMI	IMI
	0.12	0.25	0.5	1	2	4	2	4	8	16	32	64
C	MXF	MXF	MXF	MXF	MXF	MXF	FEP	FEP	FEP	FEP	FEP	FEP
	0.25	0.5	1	2	4	8	1	2	4	8	16	32
D	FOX	FOX	FOX	FOX	FOX	FOX	AUG2	AUG2	AUG2	AUG2	AUG2	AUG2
	4	8	16	32	64	128	2/1	4/2	8/4	16/8	32/16	64/32
E	AMI	AMI	AMI	AMI	AMI	AMI	AMI	AXO	AXO	AXO	AXO	AXO
	1	2	4	8	16	32	64	4	8	16	32	64
F	DOX	DOX	DOX	DOX	DOX	DOX	DOX	DOX	MIN	MIN	MIN	MIN
	0.12	0.25	0.5	1	2	4	8	16	1	2	4	8
G	TGC	TGC	TGC	TGC	TGC	TGC	TGC	TGC	TGC	TOB	TOB	TOB
	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	1	2	4
H	CLA	CLA	CLA	CLA	CLA	CLA	CLA	CLA	CLA	TOB	TOB	POS
	0.06	0.12	0.25	0.5	1	2	4	8	16	8	16	

ANTIMICROBICS

SXT	Trimethoprim / sulfamethoxazole
CIP	Ciprofloxacin
MXF	Moxifloxacin
FOX	Cefoxitin
AMI	Amikacin
DOX	Doxycycline
TGC	Tigecycline
CLA	Clarithromycin
LZD	Linezolid
IMI	Imipenem
FEP	Cefepime
AUG2	Amoxicillin / clavulanic acid 2:1 ratio
AXO	Ceftriaxone
MIN	Minocycline
TOB	Tobramycin
POS	Positive Control

Figure 3-1 Sensititre RAPMYCOI microtiter plate layout

3.1.5 Primers and probes

Primers were provided by NSW Mycobacterium Reference Laboratory. All primers and probes were HPLC or RPC pure.

Table 3-2 Primers for species identification

OLIGO NAME	SEQUENCE ITS (5'-3') & LABEL
FORWARD PRIMER	**AAGGAGCACCATTTCCCAG**
REVERSE PRIMER	**TCGACGTTTTGCCGACTAC**
M.ABSCESSUS PROBE	**CY5-YCACTACAGATGCCTACTTTA-BHQ2**
M.CHELONAE PROBE	**6FAM-TTTCTGTAGTGGTTACTCGC-BHQ1**

3.2 Methods

3.2.1 Biosafety Practices

The NSW MRL comply with the Australian/New Zealand Standard 2243.3 Safety in Laboratories- Microbiological aspects and contaminant facilities, and with the National Pathology Accreditation Advisory Council (NPAAC) requirements.

Clinical and Culture specimen from the NSW MRL have been all processed in a Class I or Class II Biosafety cabinet which are located in an accredited Physical Contaminant Level III (PC3) laboratory. When processing the specimen protective laboratory clothing must be worn, this includes wrap around or solid front gown, protective gloves, safety glasses and protective respiratory mask.

3.2.2 Bacteria strains

Clinical isolates of *Mycobacterium abscessus* complex characterized in this study were collected from isolates referred to and identified by the New South Wales (NSW) Mycobacterium Reference Laboratory (MRL) between January 2015 to December 2017.

Specimens were collected from sputum, induced sputum, Bronchial Alevolar Lavage (BAL), tissue and aseptic body fluids, Cerebrospinal Fluid (CSF), blood and bone marrow and respiratory children's samples. The clinical specimens required to be received within 24 hours from collection and preferably within 1 to 2 hours, refrigeration at 2 to 8 degrees could be done reduce growth of contaminants. Except for whole blood which should not be refrigerated.

Reference and commercial strains were also used in this study. Control isolates *Mycobacterium abscessus* ATCC 19977 and *Mycobacterium peregrinum* ATCC 700686.

3.2.3 Culture conditions and storage of bacteria

Isolates were subcultured onto Middlebrook 7H10 (MB) plates. When a subculture from a liquid culture was required approximately 10-20 µL of sample was placed on a MB and then streaked by using the quadrant streaking technique. When a subculture from a solid culture was required, a sweep of 10 µL loopful of bacteria colonies were collected, placed on the MB plate and then streaked by using the quadrant streaking technique.

Subculture plates were incubated at 37°C for 4 weeks. After 4 weeks a 10 µL loopful sweep was taken from the MB plate and submerged in cryotubes with 10% glycerol broth and stored in a -80°C freezer for further investigation if needed.

3.2.4 Microscopic Smear

Smears are made to detect Acid Fast Bacilli (AFB) as all *Mycobacterium spp.* are AFB positive, due to high levels of mycolic acid located in the cell wall. However, *Corynebacteria* and *Nocordia* can also be partially AFB positive. The direct smears are prepared from tissue, decontaminated respiratory clinical specimen and concentrated body fluid specimen. Culture smears are performed on all positive Mycobacteria Growth Indicator Tube (MGIT) and solid media (LJ) to confirm the presence of AFB. The smears are stained using Ziehl-Neelsen (ZN) cold.

Slide Preparation of a Positive Culture

This method was always performed in a Biosafety cabinet (BSC) located at the NSW MRL PC3 laboratory. The albumin slides were labelled with an accession number and then a 200 µL aliquot of a positive MGIT were fixed on an albumin slide. If the sample was from solid media 200 µL of PBS was placed on the slide and 2-3 colonies from the media was added and mixed using a 1 µL disposable loop. The slides were then placed on a 70°C heating block for 2 hrs to be heat fixed.

Ziehl-Neelsen (ZN) Kinyoun cold stain Method

The heat-fixed slide was flooded with Kinyoun carbol fuchsin stain for 5 minutes, decolourized with 3% acid alcohol for 2 minutes followed by a counterstain of 0.5% methylene-blue for 1 minute, slide is also gently rinsed with water between each step.

Microscopic Method:

An optic microscope was used to assess if the slides were AFB positive. 10X objective was used for preliminary scan followed by 100X objective using oil immersion. Which was used to focus on acid fast bacilli. Pink to red bacilli is positive for Acid fact bacilli (Figure 3.2) and non-acid fast bacilli is blue.

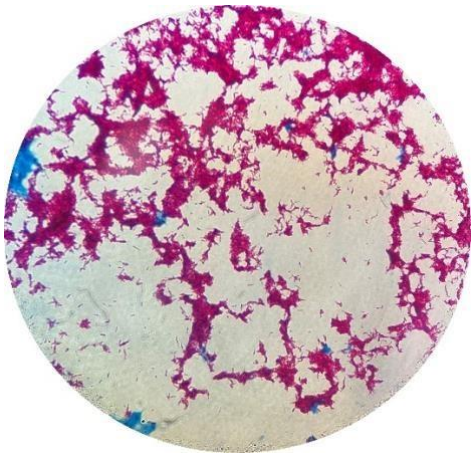

Figure 3-2 AFB positive of M. abscessus ATCC 19977 strain at 100X objective

3.2.5 Identification by HPLC

The isolates were initially identified by high performance liquid chromatography (HPLC).

10 µl loopful of colonies from solid media was added to a glass tube containing 1 mL of reagent M. If the sample was from a MGIT, 3ml of MGIT liquid culture was added to a yellow top centrifuge tube and centrifuged for 15 minutes at 5000 rpm, the supernatant was discarded and the pellet was transferred to the glass tube containing reagent M. The glass

tube was then vortexed for 30 seconds in the BSC II cabinet and then placed in a 90- 100°C water bath for 2 hours or autoclave at 121°C for 1 hour. The glass tubes were then cooled and the following was performed in a chemical fume cabinet. 2ml of chloroform was added to the tubes followed by 2ml of reagent B, this was slowly added to avoid excess bubbles.

The tubes were then vortexed for 1 minute, forming two layers. The bottom layer (containing mycolic acid and chloroform) was aspirated and transferred to the second glass tube. The glass tubes were placed into a 90°C heating block, metal needles were then lowered into tubes. The nitrogen gas supply was turned on to release 50kpa through the needles for 15 minutes to dry.

After drying 100 µL of reagent C was added to the tubes and dried for 10 minutes with the presence of Nitrogen and heat. Then 100 µL of fluorescent reagent N was added. Lids were placed on tubes and then transferred to the heating block for 20 minutes. After 20 minutes, 2ml of chloroform and reagent E was added to the tubes and vortexed for 60 seconds and two layers were formed again. The bottom layer was aspirated to the third glass tube and dried like previously with nitrogen for 15 minutes.

Once tubes were dried 80 µl of chloroform with internal standards was added and vortexed to mix. The mixture was then transferred to specific HPLC tubes for processing, reading and analysis on the High-Performance liquid chromatography Shimadzu system. A chromatographic mycolic acid pattern was produced from the samples. These patterns were visually evaluated and compared to a reference species of known *Mycobacterium abscessus* and *Mycobacterium chelonae* templates (Figure 3.3). If the patterns of the sample resembled the template of these reference species due to the mycolic acid profile being similar further work to speciate and confirm was completed.

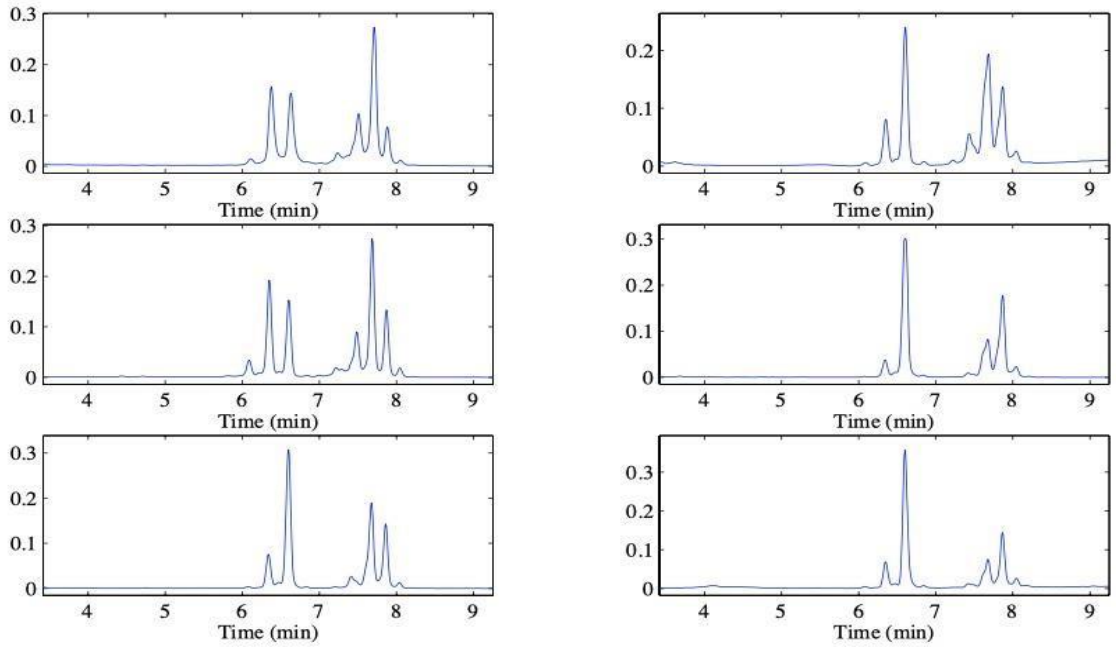

Figure 3-3 *Different HPLC profile of Mycobacterium abscessus and Mycobacterium chelonae. Reprinted from USA Centers for Disease Control and Prevention (CDC, February 1999) Mycolic Acid Pattern Standards for HPLC Identification of Mycobacteria.*

3.2.6 DNA Culture Extraction

The method was slightly adjusted according to manufacture insert of InstaGene™ Matrix BIO-RAD Catalog # 732-6030 Section 2 IFU 2.3 DNA preparation from bacteria.

The method was performed in a BSC II within the PC3 facility. The procedure was performed from positive culture specimen grown on a MGIT, LJ or MB. From a MB or LJ, 10 µL loopful of bacterial cell was scraped from the culture and placed in a labelled sterile screw-capped tube that contained 1 ml of sterile distilled water. From a culture MGIT broth 1ml of mixed liquid was collected and placed in a labelled sterile screwed-capped tube. The tubes were then put in a water bath sonicator for 20 minutes and tubes were then centrifuged for 2 minutes at 11,000rpm.

Supernatant was removed from the tube and 200 µL of mixed Instagene™ matrix was added to each tube and incubated at 56°c for 20 minutes. Tubes were then vortexed and placed in boiling water bath for 8 minutes and vortex again and centrifuged for 2 minutes at 11000 rpm. Once the samples were extracted the samples were stored in -20°C freezer until tested.

3.2.7 Identification by Real Time PCR

Multiplex real-time PCR for confirmation of *Mycobacterium abscessus* species by targeting the internal transcribed spacer (ITS) region. This region is located between the 16S and 23S rRNA genes and can be differentiate between the species of *M. abscessus* and *M. chelonae*. Starting DNA material from extracted cultured DNA.

The instrument used for identification and detection changed during the time of this study.

Smart-cycler system

Each 25µL reaction contained 0.1µL 400nM of each primer, 0.1µL 100nM of each probe, 12.5µL of 1 x ImmoMix™ [components: IMMOLASE Polymerase (quantity not defined), 1.5 mM $MgCl_2$, 1 mM dNTPs, 16 mM NH_4SO_4, 62.5 mM Tris-HCl, 0.01% Tween 20, Stabilizer],2.1 µL of Qiagen H_2O and 10 µL of template DNA.

Thermo cycling was performed using the SmartCycler® system by Cepheid. The initial polymerase activation occurred during cycle 1 at 95°C for 590 seconds, cycles 2 to 40 there was denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 40 seconds and the instrument read fluorescence on each channel for each sample at the 60°C step. A positive result is signaled by an exponential increase in fluorescence (Figure 3-4) during the reaction at the individual channel (630nm-650nm), matching HPLC profile and clinical information.

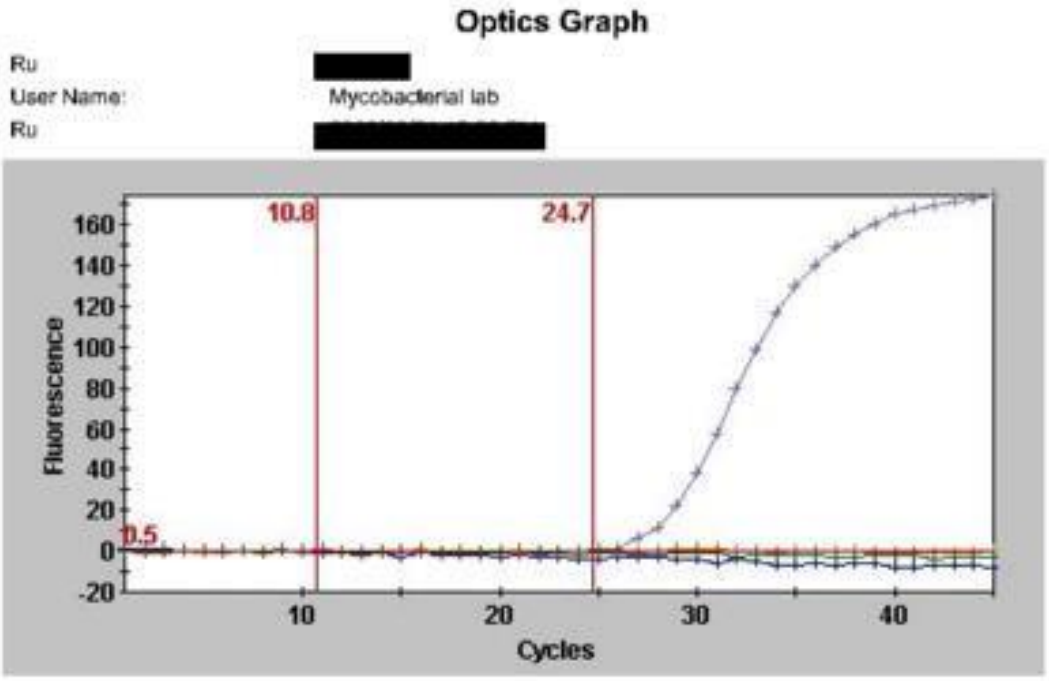

Figure 3-4 Exponential curve for a positive case using the SmartCycler

Roche Light Cycler® System

Each 25 µL reaction contained 0.1µL 400nM of each primer, 0.1µL 100nM of each probe, 12.5µL of 1 x SensiFAST™ Probe No-ROX MM (not supplied), 2.1 µL of Qiagen H2O and 10 µL of template DNA.

Thermo cycling was performed using the Roche LightCycler® 480 system. Initial polymerase activation occurred at cycle 1 at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 10 seconds and annealing and extension at 60°C for 40 seconds. A positive result for *M. abscessus* was signaled when the level of fluorescence of the CY5 channel (618nm-660nm) crossed the threshold as seen in Figure 3.5.

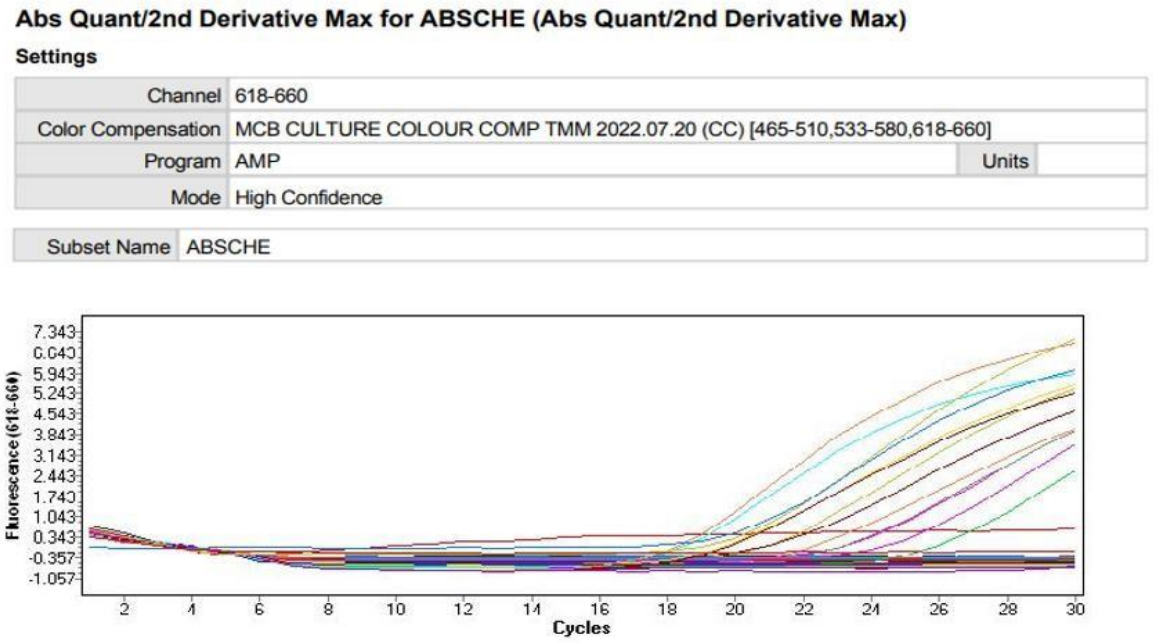

Figure 3-5*: Positive curve for M. abscessus at the 618-660 channel via Roche Lightcycler System*

During the time of gathering data and working on identification the Real Time PCR platform used for identification of *M. abscessus* was changed from the Smart-cycler (Cepheid) to the Light-cycler LC480 (Roche) system. The evaluation was performed and passed by the NSW MRL in 2017 and a total of 280 isolates were used.

3.2.8 Antibiotic Resistance

To determine the minimum inhibitory concentration (MIC) of strains for an antimicrobial drug a microbroth dilution method was used. The MIC is an in-vitro value that determines the lowest concentration of antimicrobial drug that prevents visible growth of bacterial strains. The method involves using a concentrated amount of bacteria in broth and equally distributing this medium to a micro-titre plate that contains dilution series of specific drugs.

The micro-dilution broth protocol used for *M. abscessus* was based on CLSI M24 (84) document and CLSI M62 (85) and MRL sensitivity procedure manual using the Sensititre Broth Microdilution for Rapidly Growing Mycobacteria (RGM) by TREK diagnostics Systems.

- **Quality Control**

Prior to routine use all new lot numbers of Sensititre RAPMYCOI microtiter plate and Sensititre™ Cation adjusted Mueller-Hinton broth with TES are QC tested with control strain *Mycobacterium peregrinum* ATCC 700686. Quality control is performed according to CLSI M62 1st edition, 2018 (84) and the recommended 72 hr MIC ranges (ug/ml) for the quality control organism are tabelised in M62 document (3rd edition, 2018) (85).

- **Inoculation Procedure**

The *M. abscessus* isolates tested were collected from a Middlebrook (MB) plate and emulsified in sterile water with beads. This was then adjusted to 0.5 McFarland turbidity using the Densicheck nephelometer. 50 µl of this suspension was added to tube of 10ml Mueller-Hinton broth with TES buffer and vortexed. This suspension was then poured gently into a sterile pipettor tray were 100 µl of this suspension was transferred to each well of the Sensititre plate using the Eppendorf multichannel pipettor. 1 µl of the sample from the positive growth well was placed onto MB plate and spread with a sterile spreader to create a lawn plate. This was for purity check and colony count. The sensititre plate was then covered with a clear adhesive seal and placed in a 35°c incubator aerobically for 72 hours.

- **Reading Procedure**

The sensititre plates (Figure 11) were read at 72 hours, parallel to the purity MB plate. The MB plate was checked for purity and colony count (100-1000 colonies), followed by reading of the growth control well. If the control well had growth and the purity plate had no other contaminate and were within the colony count the plate were interpreted. The minimal inhibitory concentration (MIC) of the isolate was reported as the lowest concentration of antimicrobial that inhibited visible growth, apart from Trimethoprim/sulfamethoxazole were its end point is the well with 80% growth inhibition

compared to the positive. Most of the agents could be finalised and resulted at 72 hrs. except for Clarithromycin where it required a further 14-day incubation for its final interpretation. Clarithromycin was required to have a 72hr reading and a 14-day reading to check for inducible macrolide resistance. Please refer to table 3 for MIC interpretation, where S indicates susceptible, I for intermediate and R for resistance.

If purity plates that were set up in parallel to Sensititre plates were contaminated or had a colony count greater than 1000 the Sensititre plate were not interpreted and the culture isolate were repeated. If there was no growth in the positive growth well the plate was reincubated and read again after 24hrs, however if still no growth within 5 days it was invalid and the culture isolates were repeated.

Table 3-3 Antibiotic agents included and concentration range

Antibiotic	**MIC µg/mL**		
Trimethoprim/sulfamethoxazole	≤2/38	-	≥4/76
Ciprofloxacin	≤ 1	2	≥ 4
Moxifloxacin	≤1	2	≥4
Cefoxitin	≤ 16	32-64	≥128
Amikacin	≤ 16	32	≥64*
Doxycycline	≤1	2-4	≥8
Tigecycline	-	-	-
Clarithromycin	≤2	4	≥8
Linezolid	≤8	16	≥32
Imipenem	≤4	8-16	≥32
Cefepime	-	-	-

Amoxicillin/clavulanic acid 2:1 ratio	-	-	-
Ceftriaxone	-	-	-
Tobramycin	≤2	4	≥8

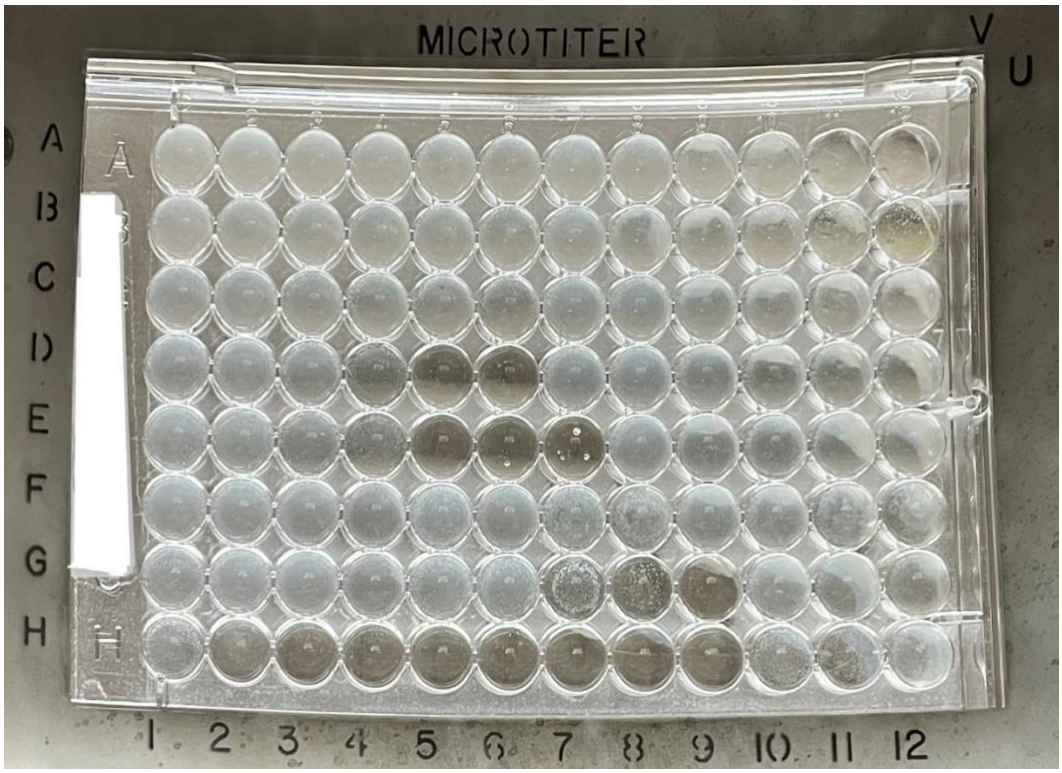

Figure 3-6 Analysing Sensititre Plate: The 96 wells are coated with different drugs at different MIC ug/ml levels. H12 contains positive control. A negative well is clear (H7) positive growth can be seen in A1 and H12.

This plate is growing M. abscessus isolate. The following wells are coated with the following drugs. A1- A6 Trimethoprim/sulfamethoxazole, A7- A12 Linezolid, B1-B6 Ciprofloxacin, B7-B12 Imipenem, C1-C6 Moxifloxacin, C7-C12 Cefepime, D1-D6 Cefoxitin, D7-D12 Amoxicillin/ clavulanic acid, E1-E7 Amikacin, E8-E12 Ceftriaxone, F1-F8 Doxycycline, F9-F12 Minocycline, G1-G9 Tigecycline, G10-G12 and H10-H11 Tobramycin, H1-H9 Clarithromycin.

3.2.9 DNA Extraction for Whole Genome Sequencing Extraction

Extraction was based on the publication of (86) DNA extraction was performed initially in PC3 with an initial inactivation of the live culture by boiling follow up by bead beating. After these steps the samples were safe to be processed in a PC2 lab.

Extraction Method One

PC3 laboratory steps

- Removal of human DNA

Samples that were identified as *Mycobacterium abscessus* from the above methods of HPLC and Real-time PCR were extracted. 1 mL of MGIT liquid culture or ¼ 10 µL loopful of colony from solid agar was transferred to a 2 ml screw top tube, colonies from solid culture was emulsified with 500 µL saline. These tubes were placed in a 99.9 ºC water bath for 10 minutes for cell inactivation, followed by 10-minute centrifugation (Eppendorf 5425) at 14500 rpm. Supernatant was removed without disturbing the pellet from each sample and the tubes were then placed in the freezer (-20 ºC) for a minimum of 15 minutes. The pellets from the sample were then resuspended with 700 µL of distilled water (ultrapure) by pipetting up and down. Tips were changed between each sample tube.

- Mechanical cell disruption

Pre aliquoted 0.25 ml of zirconia beads were added to each sample, the tubes were transferred to a mini-bead beater (Omni International) for 40 seconds at 4600 rpm twice and between each bead beading run the isolates were cooled down on ice for 30 seconds. After the last bead beading samples were centrifuged for 10 minutes at 14,500 rpm and 450 µL of the supernatant was transferred to new 2 mL lo bind tube.

PC2 laboratory steps

- Ethanol precipitation

40 µL of 3M sodium acetate was added to supernatant followed by 880 µL of cold 100% EtOH and mixed well by inverting tube several times (do not vortex). Tips should be changed between each sample. Samples were then incubated at -20 ºC for a minimum of 30 minutes to 1 hour. Samples were then centrifuged at 14500 rpm for 15 minutes and

supernatants removed followed by 1mL addition of freshly made 70 % ethanol to pellet. Samples were then centrifuged again at high speed for 3 minutes and supernatant removed without disturbing the pellet. The tubes were left open for 5 minutes to air dry and the pellet were re-suspended in 50 µL of Low TE buffer and placed on a shaking heating block at 55 ^{o}C for 10 minutes at 750 rpm to resuspend the DNA.

- DNA clean up

Transferred 45 µL of supernatant to low bind tube and added 81 µL of AMPure Xp magnetic beads (Beckman Coulter) and vortexed for 10 seconds, this was then incubated for 2 minutes at room temperature. Tubes were then placed on a magnetic tube holder (Invitrogen™) for 3 minutes followed by the removal of supernatant without disturbing the beads, then 200 µL of 80 % EtOH was added and incubated for 1 minute and EtOH was removed, this was done twice. After the second wash the tubes were pulsed down in the centrifuge for 5 seconds and any leftover EtOH was removed from the beads. The tubes were then air dried for not more than 5 minutes and 80 µL of Low TE buffer was added to the beads and mixed up and down until the beads were completely re-suspended. Spun down the tubes and placed it on the magnetic bead holder for 3 minutes and transferred 60 µL of supernatant to a new tube.

Extraction Method Two:

PC3 laboratory steps

- Removal of human DNA

Samples that were identified as *Mycobacterium abscessus* from the above methods of HPLC and Real-time PCR were extracted. 1 mL of MGIT liquid culture or ¼ 10 µL loopful of colony from solid agar was transferred to a 2 ml screw top tube, colonies from solid culture was emulsified with 1 mL of PBS saline. These tubes were placed in a 99.9 ^{o}C water bath for 8 minutes followed by 5-minute centrifugation at 14500 rpm and supernatant was removed without disturbing the pellet from each sample and the tubes were then placed in the freezer (-20 ^{o}C) for a minimum of 15 minutes. The pellets from the sample were then resuspended with 700 µL of distilled water (ultrapure) by pipetting up and down and ensuring that tips were changed between each sample tube.

- Mechanical cell disruption

Pre aliquoted zirconia beads were added to each sample, the tubes were transferred to a Omni bead ruptor 24 elite for 5m/s for 90 seconds three times and between each bead beading run the isolates were cooled down on ice for 90 seconds. After the last bead beading samples were centrifuged for 5 minutes at 14,500 rpm and 450 µL of the supernatant was transferred to new 2 mL lo bind tube.

PC2 laboratory steps

- Ethanol precipitation

40 µL of 3M sodium acetate was added to supernatant followed by 900 µL of cold 100% EtOH and mixed well. Tips should be changed between each sample. These samples were then incubated at -20 ^{o}c for a minimum of 30 minutes to 1 hour. Samples were then centrifuged at 14500 rpm for 15 minutes and supernatants removed followed by 1mL addition of freshly made 80 % ethanol to pellet. Samples were then centrifuged again at high speed for 3 minutes and supernatant removed without disturbing the pellet. The tubes were left open for 5 minutes to air dry and the pellet were re-suspended in 50 µL of Low TE buffer and placed on a shaking heating block at 55 ^{o}c for 10 minutes at 750 rpm to elute the DNA. 2 µL of RNase A (Thermo Fisher Scientific) was added to each sample, vortexed and pulsed down in the centrifuge. At this point it was safe to stop extraction method and continue another time or day within 6 months.

- DNA clean up

Transferred 45 µL of supernatant to low bind tube and added 81 µL of vortexed Bioline JetSeq™ beads and vortexed for 10 seconds, this was then incubated for 10 minutes at room temperature. Tubes were then placed on a magnetic tube holder for 3 minutes followed by the removal of supernatant without disturbing the beads, then 200 µL of 80 % ethanol was added and incubated for 1 minute and ethanol was removed, this was done twice. After the second wash the tubes were pulsed down in the centrifuge for 5 seconds and any leftover ethanol was removed from the beads. The tubes were then air dried for 5 minutes and 80 µL of Low TE buffer was added to the beads and mixed up and down until the beads were completely re-suspended. Spun down the tubes and placed it on the magnetic bead holder for 3 minutes and retrieved 60 µL of supernatant to a new tube.

- 96-well MIDI plate

Note: If there were more than 8 isolates extracted, DNA clean-up was performed using a 96-well MIDI plate.

Transferred 45 µL of supernatant to well with 81 µL of vortexed Bioline JetSeq™ beads and placed a Microseal 'B' adhesive film and shaked plate for 2 minutes at 1500 rpm. Plate was incubated for 10 minutes at room temperature. Plate placed on a magnetic plate stand for 3 minutes followed by the removal of supernatant without disturbing the beads, then 200 µL of 80 % ethanol was added and incubated for 1 minute and ethanol was removed, this was done twice. After the second wash the plate was pulsed down 5 seconds on plate spinner and any leftover Ethanol was removed from the beads using a P10 multichannel pipette. The plate was then air dried for 10 minutes and 80 µL of Low TE buffer was added to the beads and mixed up and down until the beads were completely re-suspended. Placed a Microseal 'B' adhesive film and shook plate for 2 minutes at 1500 rpm followed by placing the plate on magnetic holder for 3 minutes and retrieved 60 µL of supernatant to a 1.5mL Lo-bind tube

Extraction Method one and two used the same measure DNA concentration method

Measure DNA concentration

The supernatant was then prepared to measure for DNA concentration and quality by using the Qubit 2.0 Fluorometer (Inv**i**trogen™) as per manufacturer instructions, If DNA concentration was greater than 20 ng/µL the samples were diluted with TE buffer to get to measurement between 0.2-20 ng/µL. Samples were also measured for quality using the Allsheng Nano-300™ please see below.

Spectrophotometer A260:280	**Spectrophotometer A260:230**
1.7-2.2	1.5-2.3

Note: The 260:280 ratio detects for presence of proteins, phenol or other contaminants

The A260:230 ratio detects contaminants that are organic such as trizol, phenol or guanidine HCL (Salts)

3.2.10 Library preparation

Whole genome sequencing (WGS) of pure cultures was performed at the Microbial Genomics Reference Laboratory at the Centre for Infectious Disease Laboratory Services (CIDMLS), ICPMR, NSW Health Pathology. Sequencing libraries were prepared using Nextera XT DNA Library Prep Kit (Illumina) as per manufacturer instructions and sequenced on a NextSeq 500 instrument with a 150bp paired-end protocol using NextSeq 500/550 v2 Kits (Illumina). A minimum of 1.2 million reads of 150bp, paired-end reads per sample were sequenced.

3.2.11 Bioinformatics processing

Raw reads were quality assessed with FastQC v0.12.1 (https://github.com/sandrews/FastQC) and MultiQC v1.4 (https://github.com/ewels/MultiQC). FastQC analysis showed data with excellent quality (Phred score above 32), with no evidence of adapter contamination. Trimming of short reads was performed with Trimmomatic v0.36.

The Nullarbor pipeline (v2.0; https://github.com/tseemann/nullarbor) was used with *M. abscessus* ATCC 19977 (NC_010397) as a reference. This pipeline includes assembly using SKESA v.2.3.0, core SNP determination using snippy v.4.3.5, and generation of a maximum-likelihood phylogenetic tree from the the snippy core alignment file using IQTREE v.1.6.7 with parameters "-bb 1000 -alrt 1000 -m GTR+G4".

The relationship between genomes was examined further using core single nucleotide polymorphism (SNP) analysis where a SNP was defined as a substitution present in at least 90% of reads with a minimum depth coverage of 30. Isolates were regarded as likely linked when the SNP distance was ≤ 30 SNPS (10).

In house wrapping scripts were used to get mutations on genes of interest from vcf and csv files. Deletions were checked by inspecting bam files generated by Snippy in CLC Genomics Workbench v12.0. Discrepancies between phenotype and genotype were manually checked by inspecting possible heterogenicity in single nucleotide positions.

The Project number for submitted Fastq files is PRJNA989271.

3.2.12 Optimization of WGS extraction method

The methods involved in extraction method 1 for WGS isolates was slightly different from extraction method 2. Removal of human DNA in extraction method 1 involved saline as the dilute solvent and method 2 used PBS, 99. 9 ºC water bath and centrifugation was 5 minutes longer than method 2. The mechanical disruption was set for a shorter time at lower speed setting of 40 sec at 4600 rpm, the cooling of tube between each step was also at 30 seconds. While extraction method 2, bead beating step was performed 3 times at 5m/sec for 90 seconds and cooling step between each bead beading was 90 seconds. Extraction method 1 did not have RNase A added to the ethanol precipitation step. RNase A is used to eliminate RNA that possibly contaminated the extracted genomic DNA.

Table 3-4 Comparing the two extraction methods for WGS

	Method 1			**Method 2**		
Isolate number	Concentration ng/µl	260:280 (1.7-2.2)	260:230 (1.5-2.3)	Concentration ng/µl	260:280 (1.7-2.2)	260:230 (1.5-2.3)
16-3027-0006	4.65	2.4	2.6	24.6	1.79	1.71
16-3027-0005	10.4	1.9	1.89	8.8	1.75	1.7
16-3027-0008	146 to 15.7	2.1	1.73	19.2	1.7	1.3
16-3027-0009	162 to 28.9	2	2	34.5	1.77	1.62
16-3027-0010	66.4 to 21.7	2.01	2.2	7.04	1.82	1.7
16-3027-0054	Failed	no record	no record	16.2	1.76	1.2
16-3027-0058	323 to 26.9	1.81	1.95	12.5	1.82	1.58
16-3027-0049	52.4	1.9	2.4	21.2	1.75	1.1
16-3027-0052	62	2.1	1.4	18	1.7	1.7

When comparing the results of DNA extracted for WGS, method 2 proved to be the best option for extraction. This is evident in Table 4; the concentration of DNA was at the ideal concentration since no further work needed to be done on the extracted sample to be acceptable for the WGS run. Compared to extraction method 1 there was 6 of the 10 isolates

that required further dilution to achieve a 20ng/µl DNA concentration. The quality at both ratio was consistent of 1.7 in extraction method 2 compared to extraction method 1 ranging from 1.9 – 2.6 in both the ratio.

CHAPTER 4

4 Results

4.1 Bacterial isolates included in the study

Amongst the 305 *M. abscessus* complex cultures received by the NSW MRL between January 2015 and December 2017, 146 cultures were excluded from the study (see **Figure 4.1.)**. Reasons for exclusion included: collection outside NSW in different States and Territories (n=35), recovery from animals (n=2), referred cultures from patients that were diagnosed with *Mycobacterium tuberculosis* (n=17) within a year of collection. Consecutive cultures collected within 90 days from the same patient were also excluded from the study (n=46).

The remaining culture isolates went through further review. Upon this review, the following isolates were also excluded from the analysis: (i) isolates with incomplete metadata (n=6), isolates provisionally identified as *M. abscessus* by a submitting laboratory but not confirmed as *M. abscessus* or identified as other rapid growing *Mycobacteria* using RT-PCR, HPLC and clinical investigation (n=8). Culture isolates that were identified as having mixed infection with other *Mycobacteria* (e.g., *M. intracellulare* and *M. abscessus* (n=11)) were confirmed through HPLC, PCR and clinical investigation.

Amongst the remaining 180 *M. abscessus* isolates, 8 isolates were excluded as they could not be retrieved from long-term storage. Further 13 culture isolates were found to be contaminated with other bacteria or fungal elements. The final set of *M. abscessus* isolates (n=159) was included in the analysis.

In the final 159 isolates recovered with M. *abscessus,* duplicates of samples were included. Duplicates were multiple (two to six) samples collected from a different source from the same patient or if recovered from the same patient and same source they were collected more than 90 days apart. There were 70 isolates considered as patient duplicates from 27 patients which were included in the analysis.

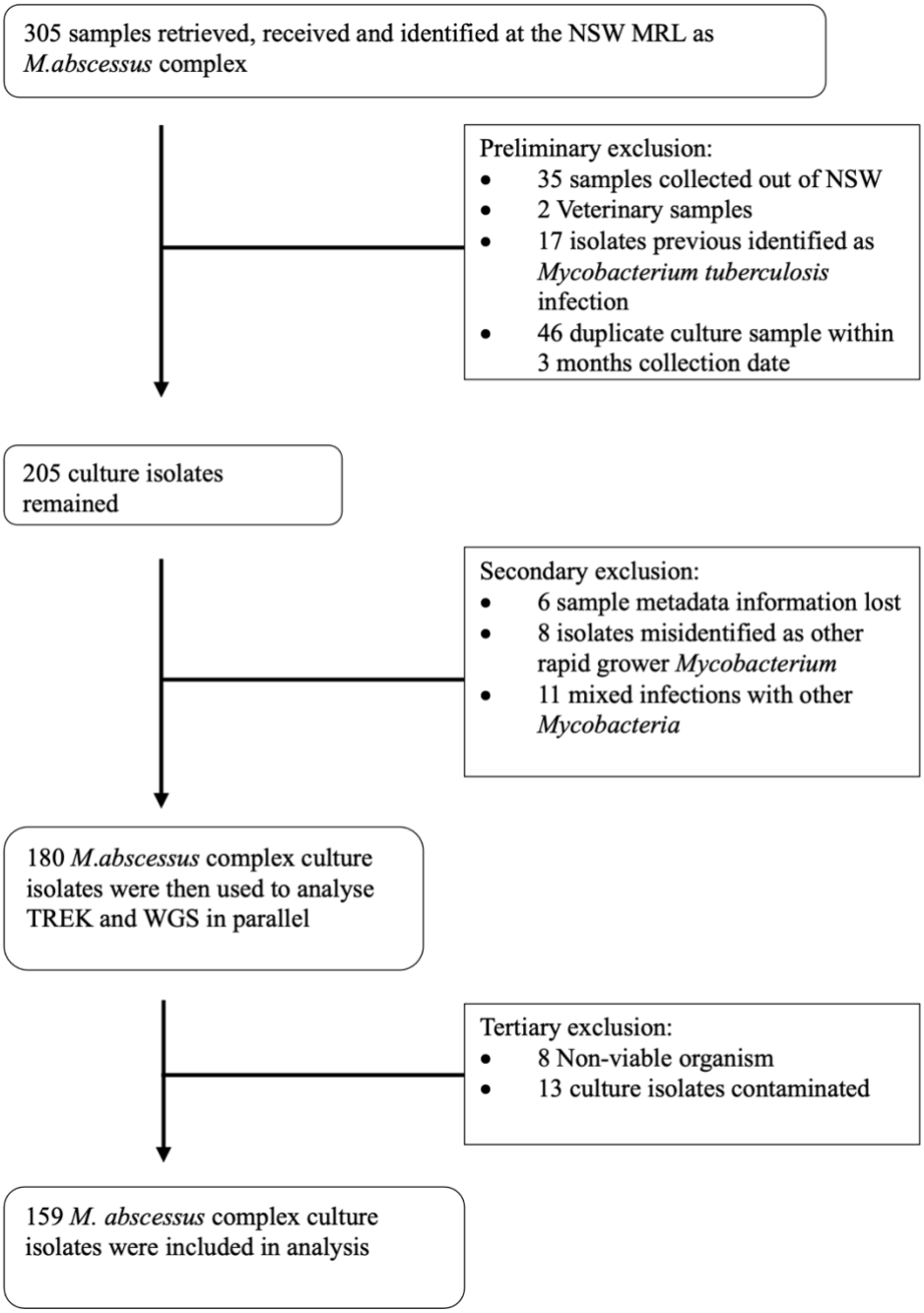

Figure 4-1 Process of selection of isolates for the study

Table 4-1 Species composition of the data set

Type	Variant	Count	Percentage %
Species	*M.abscessus* subsp. *abscessus*	123	77.4%
Species	*M. abscessus* subsp. *bolletii*	4	2.5%
Species	*M. abscessus* subsp. *massiliense*	32	20.1%

Table 4-1 above illustrates the proportion of different species within *M. abscessus* complex in the set of selected isolates. One hundred and fifty-nine *M. abscessus* isolates were collected and analysed by WGS, including 123 (77.4%) of *M. abscessus* subsp. *abscessus*, 32 (20.1%) of *M. abscessus* subsp. *massiliense,* and 4 (2.5%) of *M. abscessus* subsp. *bolletii.*

4.2 Patient demographics and sites of infection and colonization

From January 2015 to December 2017 the n=159 isolates analysed (Table 4.2) found that more isolates were collected from year 2016 n=64 (40.3%) compared to 2015 n=51 (32.1%) and 2017 n=44 (27.7%). The predominant source of *M. abscessus* was respiratory samples (Table 4.3). The majority of respiratory isolates (n=126) were cultured from sputum 71.1% (n=113) compared to 8.2 % bronchoalveolar lavage, n=13. Other isolates 20.8% (n=33) were recovered from skin and soft tissue specimens (n=5) (swab of injection site (n=1), knee swab (n=4)) and clinical fluids or aspirates (n=13). Specifically, breast fluid (n=6), bursa fluid (n=1), wrist fluid (n=1), leg fluid (n=2), pus (n=1), and fluid unknown source (n=2). A small proportion of isolates was cultured from surgical tissue samples (n=14) (i.e., breast tissue n=2, lung tissue n=1, gastric band n=2, abdominal tissue n=2, tissue unknown source n=7) and blood culture (n=1).

Table 4-2 Demographics and Characteristics of cases infected with M. abscessus complex included in the study

Year of isolation	variable	count	%
Year	2015	51	32.1%
Year	2016	64	40.3%
Year	2017	44	27.7%
Age_group	10-20	16	10.1%
Age_group	21-30	36	22.6%
Age_group	31-40	19	11.9%
Age_group	41-50	9	5.7%
Age_group	51-60	16	10.1%
Age_group	61-70	22	13.2%
Age_group	71-80	21	13.2%
Age_group	81-80	13	8.2%
Age_group	91-100	7	4.4%
Gender	Female	89	56.3%
Gender	Male	69	43.7%

CF_status	NO	96	60.4%
CF_status	YES	63	39.6%

As seen in Table 4-2 there were 89 (56.3%) reported isolates collected from females compared to 69 (43.7%) isolates from males. The age range of cases was 10 years to 98 years of age, with a median of 50 years. The age group most frequently infected with *M. abscessus* was the 21 to 30-year-old age group n=36 (22.6%) followed equally by 61- to 70-year-old n=21 and 71-80 n=21 (13.2%) with the lowest number of isolates collected from 91- to 100- year-old n=7 (4.4%). The greater proportion of isolates was collected from non-CF patients n=96 (60.4%) in comparison to 63 isolates recovered from cases with Cystic Fibrosis (39.6%), two isolates had no information provided to distinguish from Cystic Fibrosis to Non-Cystic Fibrosis.

Table 4-3 The sources of M. abscessus complex in NSW

Source of isolates	Count	Percentage %
BAL	13	8.2%
OTHER	33	20.8%
SPUTUM	113	71.1%

4.3 Duplicates

The n=27 duplicates equated to n=70 isolates, and the majority were recovered from respiratory source n=65; Sputum n= 59 being the main source followed by Lavage n=6 and

other (breast fluid) n=5. The main species identified was n= 37 *M. abscessus* subsp. *abscessus* n= 31 subsp. *massiliense* and n=2 subsp. *bolletii.*

Table 4-4 Comparison of CF cases and non-CF affected cases

Type	**Variant**	**CF**	**NonCF**	**Total**	**CF_label %**	**CF_non_label %**
Source	LAVAGE	1	12	13	7.7%	92.3%
Source	OTHER	0	33	33	0 %	100 %
Source	SPUTUM	62	51	113	54.9%	45.1%
Species	M.ABSCESSUS	55	68	123	44.7 %	55.4%
Species	M.BOLLETII	2	2	4	50%	50%
Species	M.MASSILIENCE	6	26	32	18.8%	81.2%
Year	2015	22	29	51	43.1%	56.9%
Year	2016	24	40	64	37.5%	62.5%
Year	2017	17	27	44	38.6%	61.4%
Age group	10-20	15	1	16	93.8%	6.2%
Age group	21-30	33	3	36	91.7%	8.3%
Age group	31-40	9	10	19	47.4%	52.6%
Age group	41-50	2	7	9	22.2%	77.8%
Age group	51-60	3	13	16	18.8%	81.2%
Age group	61-70	0	22	22	0%	100%
Age group	71-80	1	20	21	4.8%	95.2%
Age group	81-80	0	13	13	0%	100%
Age group	91-100	0	7	7	0%	100%
Gender	F	37	53	90	41.1%	58.9%
Gender	M	26	43	69	37.7%	62.3%

As expected, all 63 isolates recovered from patients with Cystic Fibrosis were sourced from respiratory samples, sputum 55.9% being the most common compared to 7.7% bronchial alveolar lavage sample (Table 4-4). 58.7% (n=37) were cultured from Female patients and 41.3% (n=26) from males. The age range of Cystic fibrosis cases was 10 years to 77 years, with a median age of 24 years. The most frequently collected sample age group was the 21to 30 – year old.

In comparison, the Non-Cystic fibrosis isolates were sourced from a variety of clinical samples. The majority still isolated from respiratory samples, Sputum n= 49 was the main source followed by Lavage n=12. However, n-33 isolates were sourced from non-respiratory isolates such as Tissue, Fluid, Skin and Soft Tissue and Blood. The age range of Non-Cystic Fibrosis patients' cases was 20 years to 98 years, with a median age 73 years. The most frequently collected sample age group was 61-to 70 -year-old n=21 and 71- to80-year -old n=20.

Of these 159 isolates n=63 isolates (39.6%) were from Cystic Fibrosis patients isolates and non-Cystic Fibrosis patients' isolates were n=96 (60.4%). Within the Cystic Fibrosis group

Female n=37 (58.7%) had a slight difference against Male n=26 (41.3%). This was also seen in the Non-Cystic fibrosis cohort. Where n=53 (55.2%) Female isolates was a little greater than Male n =43 (44.8%) isolates. Overall, it was observed that isolates collected from Female n=89 (56%) were greater in both cohorts.

Isolates recovered identified as *M. abscessus* subsp. *abscessus* were slightly more prevalent in patients without cystic fibrosis (n=68 (55.3%)) contrast in comparison to cases with cystic fibrosis (n=55 (44.7%)). However, *M. abscessus* subsp. *abscessus* was still the predominant species recovered in both cohorts. Twenty-six (81.3%) Subsp. *massiliense* isolates were recovered in patients without cystic fibrosis compares with n=6 (18.7%) isolates in cystic fibrosis patients. The study cohort had equal amounts of *M. abscessus* subsp. *bolletii* recovered with two cases per each group and it was also the less common subspecies to be recovered.

As previously mentioned, 27 duplicates were included in the 159 isolates collected. 18 duplicate isolates were recovered from cystic-fibrosis patients and 9 were recovered from patients without cystic fibrosis. Three of the 18 duplicated isolates were identified with two different subsp. *abscessus* and subsp. *massiliense*. They were also sourced from patients with cystic fibrosis and recovered from sputum, except for one duplicate which was recovered from a lavage and a sputum sample.

4.4 Phenotypic susceptibility and resistance among *M. abscessus complex* isolates in NSW

Table 4-5 . Phenotypic susceptibility and resistance of M. abscessus complex isolates in the study

Antibiotics	SubSpecies	Intermediate	Inducible Resistance	Resistant	Susceptible	Total	I_%	IR_%	R_%	S_%
Clarithromycin	*M. ABSCESSUS*	6	78	16	23	123	4.9	63.4	13	18.9
Clarithromycin	*M. BOLLETII*	0	1	3	0	4	0	25	75	0
Clarithromycin	*M. MASSILIENSE*	0	0	2	30	32	0	0	6.2	93.8
Amikacin	*M. ABSCESSUS*	2	0	5	116	123	1.6	0	4.1	94.3
Amikacin	*M. BOLLETII*	0	0	0	4	4	0	0	0	100
Amikacin	*M. MASSILIENSE*	0	0	0	32	32	0	0	0	100
Ciprofloxacin	*M. ABSCESSUS*	5	0	116	2	123	4.1	0	94.3	1.6
Ciprofloxacin	*M. BOLLETII*	0	0	4	0	4	0	0	100	0
Ciprofloxacin	*M. MASSILIENSE*	0	0	31	1	32	0	0	96.9	3.1
Linezolid	*M. ABSCESSUS*	37	0	25	61	123	30.1	0	20.3	49.6
Linezolid	*M. BOLLETII*	3	0	1	0	4	75	0	25	0
Linezolid	*M. MASSILIENSE*	13	0	4	15	32	40.6	0	12.5	46.9

The phenotypic antimicrobial susceptibilities for Clarithromycin, Amikacin, Ciprofloxacin and Linezolid for the 159 *M. abscessus* isolates allocated to the three subspecies are summarized in Table 4.5. As expected, of the 122 *M. abscessus* subsp. *abscessus* examined, the majority (78 (63.4%)), demonstrated inducible resistance (IR) to Clarithromycin 78, 16 appeared to be phenotypically resistant (R), 6 were intermediate and the remaining 23 (18.9%) isolates were susceptible. As for the 32 isolates identified as *M. abscessus* subsp. *massiliense* the set of isolates primarily comprised of 30 isolates (93.8%) susceptible to Clarithromycin, with one carrying inducible resistance and, unexpectedly, two (6.1%)

exhibiting *in vitro* resistance to Clarithromycin. Lastly, the 4 isolates identified as *M. abscessus* subsp. *bolletii* were resistant to clarithromycin, one had phenotype with inducible resistance and remaining three were resistant at 3 days as anticipated.

The high proportion of susceptibility to Amikacin was noticed in all 32 *M. abscessus* subsp *massiliense* (Table 4-5). There was 4.1% *M. abscessus* subsp. *abscessus* isolates displaying resistance to Amikacin and one strain exhibiting an intermediate result.

The tested isolates appeared to be predominantly resistant to Ciprofloxacin, in comparison to Amikacin. From the 123 isolates identified as *M. abscessus* subsp. *abscessus* 94.3% were resistant followed by 4.1% being intermediate. All the isolates identified as *M. abscessus* subsp. *bolletii* and 96.9% of the *M. abscessus* subsp. *massiliense* were resistant. There were only three *M. abscessus* isolates susceptible to ciprofloxacin; comprising of two isolates of *M. abscessus* subsp. *abscessus* and one *M. abscessus* subsp. *massiliense* (Table 4-5).

There were mixed results for the Linezolid. Almost half (49.6%) of *M. abscessus* subsp. *abscessus* isolates were in vitro susceptible, followed by 30.1 % of intermediate and 20.3% resistance phenotype to Linezolid. As for three *M. abscessus* subsp. *bolletii* isolates, they showed intermediate resistance and one was found to be resistant. Additionally, *M. abscessus* subsp. *massiliense* had 46.9% of isolates susceptible, 40.6 % intermediate and 12.5 % resistant. The ratio of susceptible and intermediate to Linezolid were quite similar in both *M. abscessus* subsp. *abscessus* and subsp. *massilliense*.

4.5 Epidemiology of *Mycobacterium abscessus* in New South Wales

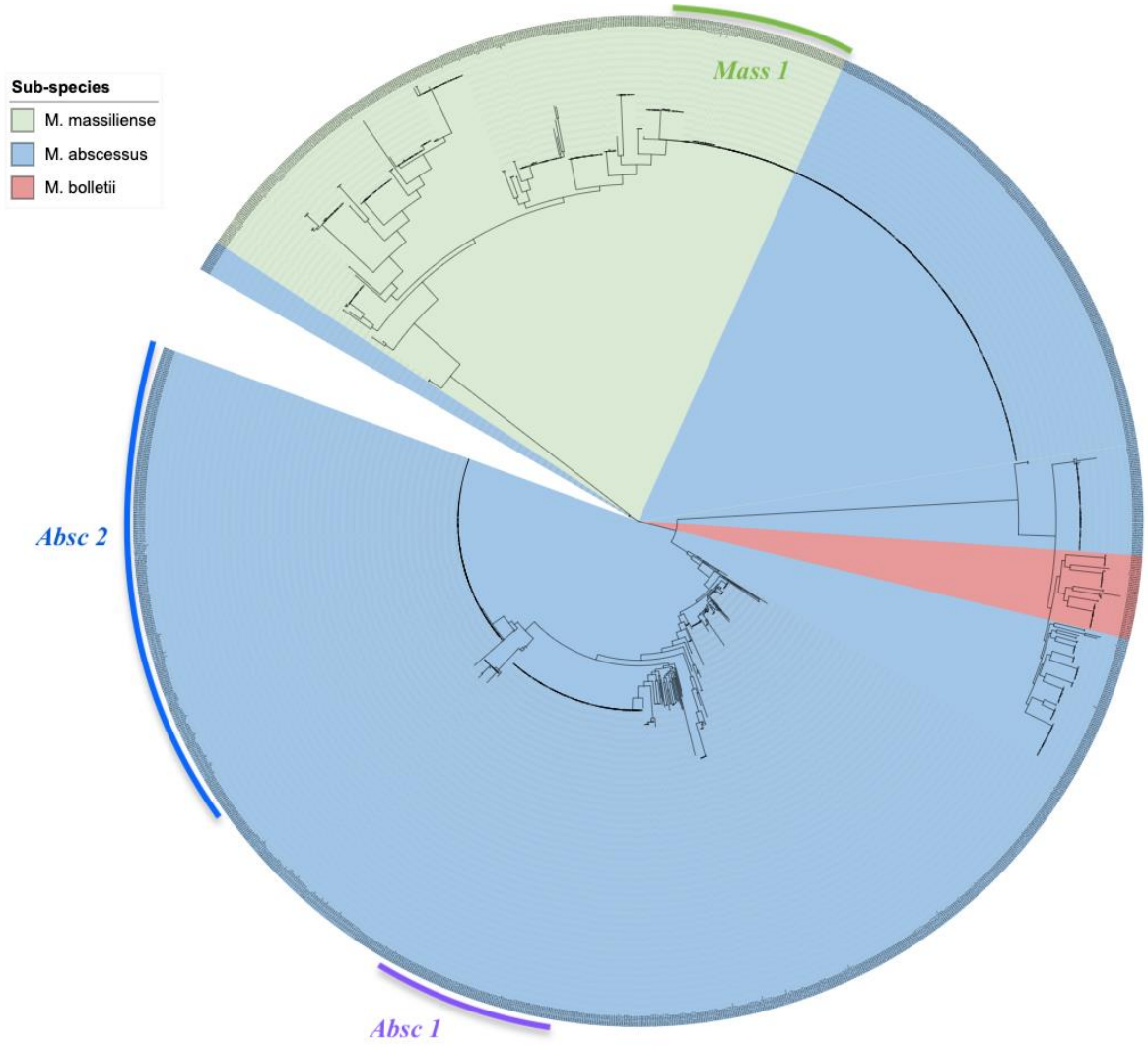

Figure 4-2 Phylogeny of 159 isolates with International M. abscessus strains from Bryant et. al, 2016

The phylogenetic tree includes the 159 isolates from New South Wales and the international M. abscessus isolates used in Bryant et. Al (2016) study (87) These isolates have been segregated into different colures for sub-speciation, *M. abscessus* subsp *abscessus* (Blue), *M. abscessus* subsp. *massiliense* (Green) and *M. abscessus* subsp. *bolletii* (Red). The outer ring shows the location of the clusters of the main dominating clone Absc 1, Absc2 and Mass 1.

4.5.1 Relatedness of clinical isolates of *M. abscessus* to the global clone

The genomes of the 159 isolates studied (Figure 4-2) were compared with the published international genomes from Bryant et al. (2016) (53). In this publication 3 dominant circulating clones were described Abscessus cluster 1 (Absc1), Abscessus cluster 2 (Absc2) and Massiliense cluster 1 (Masc1). As described by Davidson (2018) a dominant clone is large proportion of isolates that cluster in a phylogenomic tree due to high genetic similarities.

As per analysis of our data, it was found that some of the isolates analysed phylogenetically clustered among these dominant clones (see Figure 4-2). There were 17 isolates clustering to the same isolates in Absc1 clone, 40 isolates clustered with Absc2 including the reference strain and 14 isolates clustered with published isolates from the Masc 1 clone.

The isolates that were clustered among Absc1 clone consisted of respiratory site, (14, 82.4%) tissue site (1, 5.8%) and surgical devices (2, 11.8%). The 14 isolates that were respiratory were all sputum, of these isolates 11 were from confirmed CF patients and the remaining 3 were from non-CF patients. The isolate that was from tissue isolates were non-CF related and 2 surgical devices were related to lap band surgery.

Absc 2 clone consisted of the reference *M. abscessus* ATCC 19977 strain, respiratory isolates (34, 85%), punch biopsy (1, 2.5%), pus (2, 5%) and tissue (1, 2.5%). Of the 34 respiratory isolates, 30 were from sputum and 4 were bronchoscopy isolates. Nineteen of the sputum isolates were isolated from CF patients, 3 isolates were from lung transplant patients that were non-CF related and the rest were also from non-CF patients. As for the bronchoscopy 2 samples were from lung transplant patients, one non-CF patient and one lavage was from a confirmed CF patient. The reminding 4 isolates that were non-respiratory were also from non- CF related patients.

Thirteen of the 14 isolates that clustered in Masc 1 clone were from non-CF patients. Ten were from respiratory isolates consisting of bronchial wash (1, 7.1%) and 9 sputa, tissue

isolates (2, 14.2%) and one swab (1, 7.1%). The remaining isolate was a sputum from a confirmed CF patient.

Overall, the isolates identified in Absc1, Absc2 clone were predominantly respiratory samples from CF patients. However, the clones were not just restricted to CF patients, this was demonstrated in the Masc 1 clone where the majority of the isolates were from non-CF patients. The type of isolates in the clone were not just restricted to respiratory samples, as isolates from surgical devices, tissue and pus were found to cluster in these clones.

4.5.2 Clusters and epidemiological context

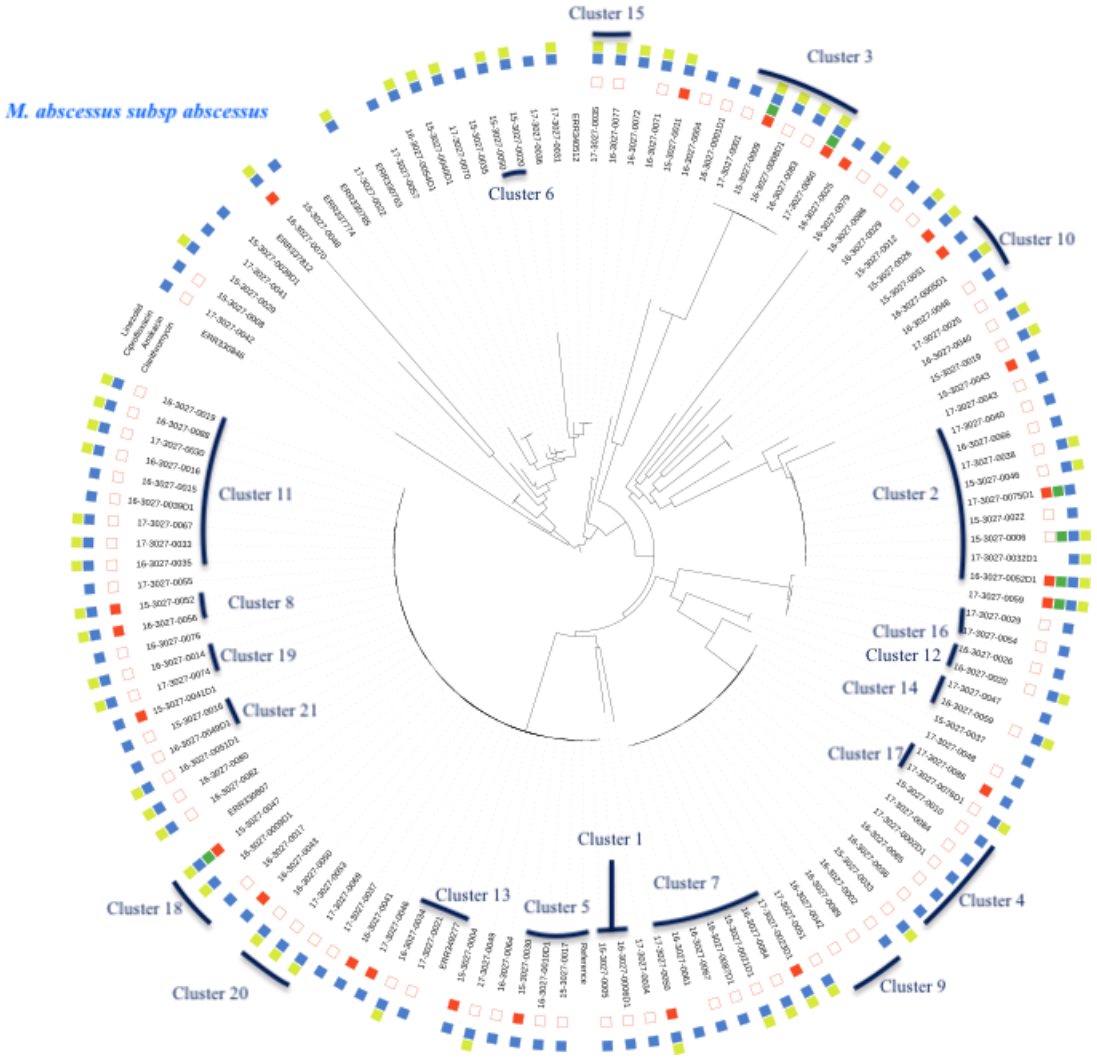

Figure 4-3 Phylogenetic tree for M. abscessus subsp. abscessus isolates with predicted phenotypic resistance and clusters isolated.

The tree above includes the 21 *M. abscessus* subsp. *Abscessus* clusters identified by 0-38 SNPs difference among isolates. The outer ring (inside to out) represents the predicted resistance for Clarithromycin, Amikacin, Ciprofloxacin and Linezolid.

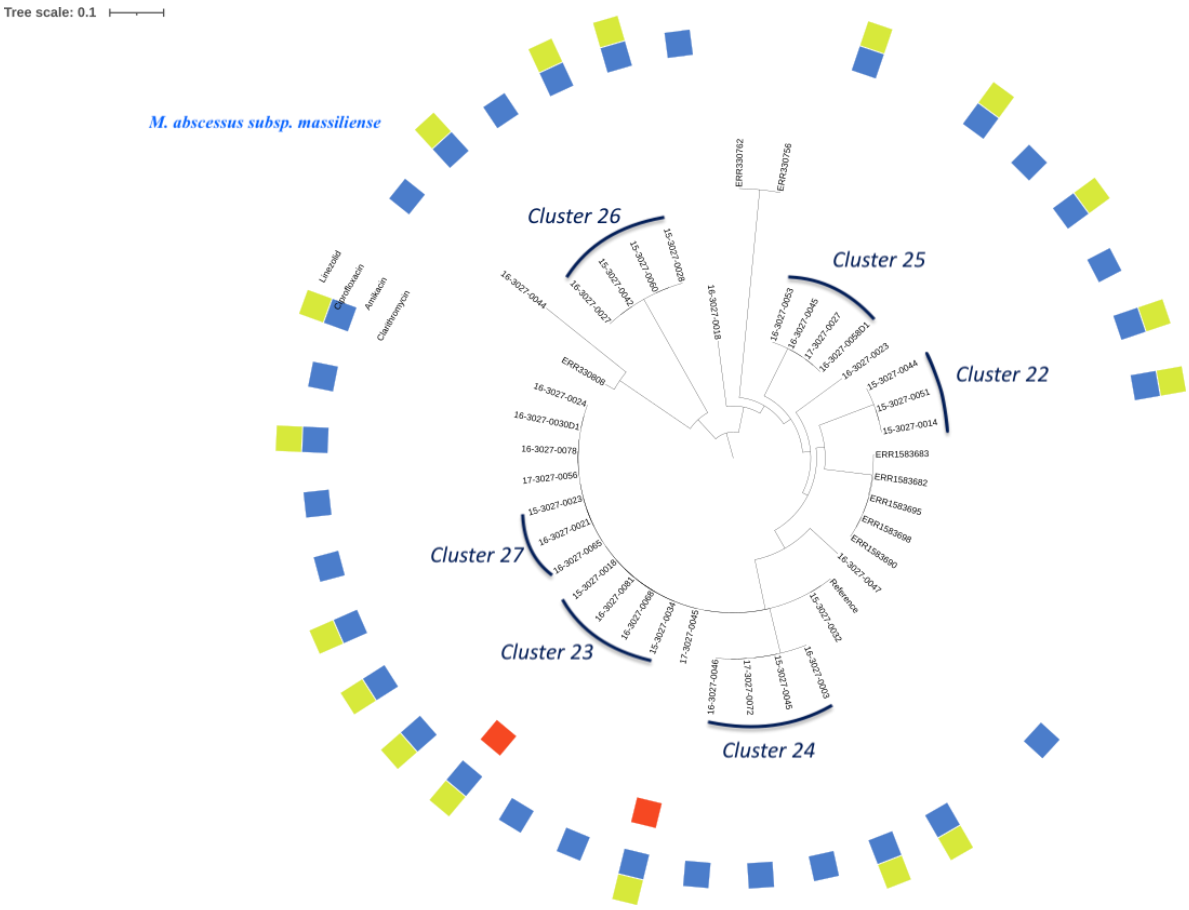

Figure 4-4 Phylogenetic tree for M. abscessus subsp. *Massiliense isolates with predicted phenotypic resistance and clusters isolated.*

The tree above includes the 6 *M. abscessus* subsp. *massiiense* clusters identified by 0-38 SNPs difference among isolates. The outer ring (inside to out) represents the predicted resistance for Clarithromycin, Amikacin, Ciprofloxacin and Linezolid.

When analysing the clusters in this cohort, we defined clusters as a group of isolates that clustered with 20-38 SNPs difference indicated probable transmission and less than 20 SNPs indicating possible transmission as per study from Bryant et al (2016) (87).These clusters were than further examined for epidemiological contact. Our definition of epidemiological contact was based on contact with another patient that attendant the same hospital within the year before proven infection.

Nighty-eight isolates were part of a genomic cluster, there were a total of 28 clusters (appendix B) identified in the cohort. Twenty-one clusters identified as *M. abscessus* subsp. *abscessus* (Figure 4-3), 6 clusters identified as *M. abscessus* subsp. *massiliense* (Figure 4-4) and one cluster identified as *M. abscessus* subsp. *bolletii.*

Eleven clusters consisted of 31 (31.6%) isolates from only CF patient, of these 5 clusters identified as *M. abscessus* subsp. a*bscessus* (25 isolates) one cluster identified as *M. abscessus* subsp. *massiliense* (4 isolates) and *M. abscessus* subsp. *bolletii* (2 isolates). Nine of these clusters consisted of isolates from CF patients with multiple samples (duplicates) that were isolated at different days or hospital site.

Three clusters (Cluster 3,17 and 25) consisted of isolates from multiple CF patients. These clusters could be identified as possible transmission with other CF patients as there was 2-5 SNPs between isolates (appendix B). On further analysis cluster 3, 17 and 25 consisted of isolates that had individual samples collected from different hospital sites. Indicating that there could be possible transmission from a different source as it is most likely the patients were clearly separated from each other when isolates were collected. It was also noticed that Cluster 17 belong to one of the common dominate circulating clone Absc1. There were also 11 clusters of 32 non-CF isolates (32.7%), 6 clusters recognised as *M. abscessus* subsp. a*bscessus* (14 isolates) and 5 clusters identified as *M. abscessus* subsp. *massiliense* (18 isolates). Six clusters (Cluster 6,13,14,15,20 and 27) consisted of isolates of patients that had multiple samples collected (appendix B). Analysing further Cluster 13 and 20 belonged to the common circulating clone Absc2 and Cluster 27 in Masc1.

The remaining four clusters (Clusters 9, 22, 23 and 24) with only non-CF isolates where collected from multiple patients, some from the same hospital site and other from different hospital site. Cluster 9 was identified as *M. abscessus* subsp. a*bscessus* dominant clone Absc1 it consisted of 3 isolates all collected from the same hospital site in the same year (2016) and from two different patients, they had 18-19 SNPs between the two patients. Cluster 22 had no SNPs difference between all 3 isolates collected from multiple patients from the same hospital site indicating a high probability of possible transmission. Further

analysis demonstrated that these patients had wounds from injection site, indicating a possible transmission from an unknown source.

Cluster 23 identified as *M. abscessus* subsp. *massiliense* dominant clone Masc1 had multiple isolates from 4 different patients and all collected from different hospital sites within a year with 12-24 SNP's between the isolates. Cluster 24 contained 4 isolates collected from 3 different patients, two isolates were from the same patient and collected within a year from each other and had no SNPs difference between them. The remaining two isolates were collected from two different hospital sites with at least 10-30 SNPs difference. These two cluster a likely not to have any transmissible isolates since no strong epidemiologically link could be established.

Six clusters that had a total of 35 isolates (35.7%) consisted of mix isolates from CF (21 isolates) and non-CF patients (14 isolates). The isolates identified as *M. abscessus* subsp. a*bscessus.* Three of these clusters (Cluster 5, 8 and 11) were clustered among Absc2 dominating circulating clone. Cluster 5 included 5 multiple isolates, three isolates were from the same CF patient collected at different times within a year, two of these isolates were collected from the same hospital and the other from a different hospital site. The remaining isolate was collected from a different hospital site and was not CF related and also included in this data was the ATCC 19977 reference strain. There was high probable epidemiologically link between the isolates from one patient from multiple sites but no strong linkage of the non-CF isolate. However, these isolates were identified as the global dominant circulating clone (88).

Three isolates completed cluster 8, two of the isolates were collected from the same CF patient at least 1 year apart and had no SNPs difference. The other isolate was collected from a non-CF patient at a different site with at least 7 SNPs difference. Unfortunately, no epidemiolocal link could be established.

Nine isolates with 0-26 SNP's difference contribute to cluster 11. Four of the 11 were collected from the same hospital and isolated from CF patients, two of the isolates were

from the same patient with 0 SNP's difference and isolated 24 days apart. Whereas the other two isolates were from two different CF patients with only 10-14 SNP's difference and collected 5 months apart from each other and 6 months from the previous sample. The 4 isolates have a high probability of transmission link. The remaining 5 isolates were from non-CF patients, 3 of the 5 were from same patients with a history of lung transplant and collected from different hospital to the CF isolates. The other two isolates were collected from different hospitals from the CF and Non-CF isolates mentioned before, most likely have no epidemiological link.

Seven isolates contribute to Cluster 7. Six of those isolates were duplicate isolates from 3 different patients at different collection points and collected from 2 different sites. Two of the patients were CF related and both CF patients had isolates collected from different hospital site. The other 2 isolates related to Non-CF patient were collected from the same hospital of one of the patients that had CF, with 10-11 SNP's difference. Therefore, a possible transmission was likely. The remaining one isolate was also collected from a CF patient but from a different hospital site. All these isolates were clustered among the Absc 1 circulating clone.

Cluster 2 comprises of 5 isolates collected from the same CF patient at different isolation time and at 3 different hospital sites. The remaining 4 isolates were from multiple non-CF patients and collected from different hospital site. No identifiable epidemiological link could be established between these 4 isolates and the 5 isolates collected from CF patients.

The last of the mix clusters was cluster 10. Cluster 10 included three isolates, two of the three were from the same CF patient collected at different time and from the same hospital. The other isolate was non-CF related and collected from a different hospital site to the CF patient. However, they had 0-1 SNPS difference between these samples highly suggesting possible transmission but because the sample were collected from different hospital site there was no epidemiological link established. Another explanation could be cross-contamination from processing the sample.

Further analysis noticed that there was an isolate from a CF patient (case 8) clustered in cluster 7, cluster 3 and cluster 17 among the CF clusters. This will require further epidemiological investigations to determine if there was possible transmission of the isolate from this individual being at different hospital sites.

Overall, clustered isolates were equally consisted in CF (49.1%) and non-CF patients. Three clusters were clustered among the international isolates belonging to Absc1 clone, two clusters corresponded to the Masc1 clone and nine clusters had isolates that positioned with the dominant global Absc2 clone. The isolates belonging to these clones were mixed with CF and non-CF isolates.

4.6 Comparison of phenotype and genotype in consecutive isolates (duplicates) of *M. abscessus*

In this study we had 159 isolates collected. Some of these isolates were collected from the same patient multiple times at different collection dates. In this section, the patients that had multiple samples will be discussed as cases and separated by CF patient and non CF-patients.

4.6.1 Isolates recovered from cases with Cystic Fibrosis

Case 1: Two Sputum isolates collected four months apart were both identified as *M. abscessus* subsp. *bolletii.*

Phenotypic susceptibility and resistance profile: Both samples were resistant to Clarithromycin and Ciprofloxacin, intermediate for Linezolid and susceptible to Amikacin.

Evaluation of resistance-conferring mutations: There were no specific mutations in *erm*41, the *erm*(41) T28T was intact. They had no specific mutations related to resistance in *rrl*, *gyrA*, gyrB, *rrs, rplC and rplD* gene.

SNP's analysis (see appendix B): There were 16 SNPs difference among the two isolates and clustered together.

Case 2: Five Sputum isolates collected, 3 isolates collected in 2015 three to six months apart, 1 isolate collected in 2016 and the last one collected 14 months apart in 2017. All 5 isolates identified as *M. abscessus* subsp. *abscessus*.

Phenotypic susceptibility and resistance profile: All 2015 isolates were inducible resistant, 2016 and 2017 isolate were resistant to Clarithromycin. The 5 isolates were also resistant to Ciprofloxacin. Amikacin 2015 isolates started as intermediate and then susceptible and isolates from 2016 and 2017 demonstrated resistance. There were mixed results for Linezolid. Isolates from 2015 had mixed results of intermediate, susceptible and resistance to Linezolid and 2016 was intermediate and 2017 was susceptible.

Evaluation of resistance-conferring mutations: There were no specific mutations in *erm*41, the *erm*(41) T28T was intact. Most isolates had no *rrl* gene mutation besides the isolate collected in 2016 with *rrl* A2271G which confers clarithromycin resistance. When evaluating *rrs* gene the 2016 isolate demonstrated a mutation at A1374G which confers to resistance and the other four collected isolates exhibited no mutation change. The *gyrA* gene had no mutations but all isolates had C-41G promoter *gyrB* mutation. Mutations in gene *rplC*, *rplD* and *rrl* related to linezolid resistance were not evident.

SNP's analysis: Among the five samples there were 4-16 SNPs differences, all isolates were clustered together in Cluster 2 among other isolates that were not CF related.

Case 3: Six sputum isolates were collected, first isolate collected in 2015, two isolates in 2016 six months apart and 3 isolates in 2017 ranging 4 -7months apart. All isolates were identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: All isolates had the same phenotypic antibiotic profile. The isolates were inducible resistant to Clarithromycin, susceptible to Amikacin and Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: All the isolates had no *rrl* gene mutation, the *erm*(41) T28T was intact but one isolate collected in 2017 had promoter mutation at A31 in *erm*(41). No mutation was displayed in the *rrs* gene associated with Amikacin resistance for all isolates. The *gyrA* gene had no mutations but all isolates had C-41G promoter *gyrB* mutation. Mutations in gene *rplC*, *rplD* and *rrl* related to linezolid resistance were not evident.

SNP's analysis: There were 0-4 SNP between the 5 Samples and clustered together as cluster 4. One sample didn't cluster among these samples or any other sample. Sample known as P3.5 had 117 SNPs difference.

Case 4: Two sputum collected 8 months apart in 2015. The first collected sputum was identified as *M. abscessus* subsp. *abscessus* and the second *M. abscessus* subsp. *massiliense*
Phenotypic susceptibility and resistance profile: The first isolate was inducible resistant to Clarithromycin while the second isolate was susceptible. Both isolates had similar phenotypic results to Amikacin as susceptible, Ciprofloxacin resistant and Linezolid intermediate.
Evaluation of resistance-conferring mutations: The first isolate had an *erm*(41) T28T profile while the second isolate showed a truncated *erm*(41). None of the isolates had *rrl* gene mutation. No mutation was displayed in the *rrs* gene associated with Amikacin resistance for both isolates. The *gyrA* gene had no mutations, however *gyrB* gene C1727G mutation and in the promoter region A-78G was seen in the second isolate. First isolate didn't have any mutations in *gyrB.* Mutations in gene *rplC*, *rplD* and *rrl* related to linezolid resistance were not evident. However, the second isolate had a mutation in *rrl* A1717G and T3001C with no known association.
SNP's analysis: No data available.

Case 5: Two sputum isolates collected 10 months apart. Both identified as *M. abscessus* subsp. *abscessus.*
Phenotypic susceptibility and resistance profile: Both samples had the same phenotypic antibiotic profile. The isolates were inducible resistance to Clarithromycin, susceptible to Amikacin and Linezolid and resistant to Ciprofloxacin.
Evaluation of resistance-conferring mutations: All the isolates had no *rrl* gene mutation, the *erm*(41) T28T was intact. They had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyrA*, gyrB, *rrs, rrl, rplC and rplD* gene.
SNP's analysis: There was no SNPs difference between the two isolates and this clustered as cluster 21.

Case 6: Three sputa collected, two collected in 2015 at 4 months apart and the third isolate collected in 2016 almost 12 months apart from the second isolate. All isolates identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: The first and third isolate collected were inducible resistant to Clarithromycin in contrast to the second isolate which was resistant at 72hr. All 3 isolates were susceptible to Amikacin, Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: All the isolates had no *rrl* gene mutation, the *erm*(41) T28T was intact. They had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyr*A, gyrB, *rrs, rrl, rplC and rplD* gene.

SNP's analysis: There were 1-3 SNP between 3 Samples. These samples clustered among the ATCC 19977 reference strain and another non-CF related isolate. Both of these isolates had 23-28 SNPs difference between Case 6. These isolates were among cluster 5.

Case 8: Four sputa collected. 1st isolate collected in 2015, followed by one 14 months later in 2016, then 7 months after the 3rd isolate was collected in 2017, the last one collected 9 months after. All four isolates identified as *M. abscessus* subsp. *abscessus***.** *Phenotypic susceptibility and resistance profile:* All 4 isolates were inducible resistant, susceptible to Amikacin and resistant to Ciprofloxacin. As for Linezolid the second and last collected isolate was intermediate, the first isolate was resistant and the third isolate susceptible.

Evaluation of resistance-conferring mutations: All the isolates had no *rrl* gene mutation, the *erm*(41) T28T was intact. No mutation in *rrs*, *rplC,* rplD and rrl gene in respect to Amikacin and Linezolid. No mutation in *gyrA* gene but mutation detected at the promoter *gyr*B gene C -41G for three of the isolates. The third isolate didn't have associated mutations in *gyr*B gene.

SNP's analysis: Two of the sputa were clustered in cluster 7 showing no SNPs difference between them. One isolate was clustered among other CF isolates in Cluster 3, they had 3-5 SNPS difference with case 13. The last isolate was clustered among another CF isolate from cluster 17, have 2 SNP's difference.

Case 10: Two sputum collected 16 months apart. Both isolates identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: The 1st collected isolate was resistant to clarithromycin and the second isolate was inducible resistant. Both isolates were susceptible to Amikacin and Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: All the isolates had no *rrl* gene mutation, Both had T28T *erm* (41) intact. Both isolates no mutation in *rrs*, *rplC*, *rplD* and *rrl* and *gyrA* and *gyrB* gene

SNP's analysis: Among the two isolates there were no SNPs difference.

Case 11: All three isolates were collected from sputum; the first two isolates were collected one year apart and the last isolate collected 5 months later. All three isolates were identified as *M. abscessus* subsp. *abscessus*.

Phenotypic susceptibility and resistance profile: The first isolate was resistant, second isolate susceptible and last isolate inducible resistant to Clarithromycin. The second and last isolate were susceptible to Amikacin and the 1st collected isolate was intermediate. All three isolates were resistant to Ciprofloxacin. As for linezolid the 1st isolate was resistant, second isolate intermediate and the last isolate was susceptible to Linezolid.

Evaluation of resistance-conferring mutations: No mutation in *rrl* gene and *erm*(41) T28T intact for all 3 isolates. No mutations in *rrs, gyrA, rplC, rplD* gene. The last collected isolate had a C1727G mutation while the previous two displayed no mutations in *gyrB*.

SNP's analysis: There were no SNPs difference in the three isolates.

Case 12: Two sputum isolates collected 8 months apart and both identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: Both isolates had similar susceptible patterns. Clarithromycin and Ciprofloxacin resistant and susceptible to Amikacin. The 1st collected isolate was intermediate to Amikacin and the second isolate resistant.

Evaluation of resistance-conferring mutations: No mutation in *rrl* gene and *erm*(41) T28T intact for both isolates. They had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyr*A, gyrB, *rrs, rrl, rplC and rplD* genes.

SNP's analysis: Among the two isolates there were no SNPs detected. However, the isolates clustered with another non-CF isolate showing 7 SNPs difference.

Case 13: 4 sputa collected 6 months apart from each other. All identified as *M. abscessus* subsp *abscessus.*

Phenotypic susceptibility and resistance profile: The 1st and last isolate were resistant to Clarithromycin, Amikacin and Ciprofloxacin. They differed in Linezolid where the 1st was intermediate and the last sample was resistant. The second and third collected isolate had the same susceptibility profile, both inducible resistant to Clarithromycin, susceptible to Amikacin and resistant to Linezolid.

Evaluation of resistance-conferring mutations: All isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. The first and second isolate had a mutation in *rrs* gene at C1462T this is a potential association to resistance. Only one isolate had a mutation at the promoter region C-41G of *gyrB*, the rest had no mutations in *gyrA* and *gyrB*. All 4 isolates had no mutations in *rplC, rplD* and *rrl* gene associated with Amikacin resistance.

SNP's analysis: There were 2-5 SNPs difference in the four isolates. There was also another isolate (case 8.3) clustering among these 4 isolates having 3-5 SNPs difference.

Case 14: Two sputum isolates collected 3 months apart both identified as *M. abscessus* subsp. abscessus

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin and Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. They had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyrA*, *gyrB*, *rrs, rrl, rplC and rplD* gene.

SNP's analysis: There were 1 SNPs difference between the two isolates.

Case 15: Two sputum isolates collected less than 3 months apart both identified as *M. abscessus* subsp. abscessus.

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin and Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. They had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyrA*, *gyrB*, *rrs, rrl, rplC and rplD* gene.

SNP's analysis: There were No SNPs difference between the two isolates. They were also clustered with 7 other isolates.

Case 16: Two sputum isolates collected 10 months apart and identified as *M. abscessus* subsp. *abscessus***.**

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin, resistant to Ciprofloxacin. However, the Linezolid was different. The first isolate was intermediate and the second isolate was resistant to Linezolid.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutations found in *rrs, gyrA, gyrB, rplC, rplD* gene in both isolates. A mutation in *rrl* gene C3042T was detected, and this mutation have no associated resistance.

SNP's analysis: There were 0 SNPs difference between the two isolates.

Case 18: Two sputum isolates collected 10 months apart and identified as *M. abscessus* subsp. *abscessus***.**

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin, resistant to Ciprofloxacin. However, the Linezolid was different. The first isolate was intermediate, and the second isolate was susceptible to Linezolid.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutations found *in rrs, gyrA, gyrB, rplC, rplD* gene in both isolates. A mutation in *rrl* gene C3042T was detected in both isolates, and it has no associated resistance.

SNP's analysis: There were 12 SNPs difference between the two isolates.

Case 21: Three sputum isolates collected from 3 to 6 months apart. All 3 isolates identified as *M. abscessus* subsp *massiliense*.

Phenotypic susceptibility and resistance profile: All isolates were susceptible to Clarithromycin and Amikacin. The 1st isolate was also susceptible to Ciprofloxacin and Linezolid. The second and third isolate were resistant to Ciprofloxacin. As for Linezolid the second isolate was intermediate and the last collected isolate intermediate Linezolid.

Evaluation of resistance-conferring mutations: All the isolates had a truncated *erm*(41) gene. The second isolate had a A1374G mutation in *rrs* gene. The first and third isolate had similar *gyrB* mutations; A-78G at promoter region and C1727G. The second isolate also displayed the same *gyrB* C1727G mutation but the promoter mutation was C-41G. The first and third isolate displayed mutation at A1717G and C3042T and the second isolate had a A2271C mutation in *rrl* gene.

SNP's analysis: There were 1-4 SNPs difference between the three isolates. Sample also clustered among another CF isolate with 1-3 SNPs difference.

Case 26: Two isolates collected from sputum at 3 months apart. Both isolates identified as *M. abscessus* subsp *abscessus*.

Phenotypic susceptibility and resistance profile: First isolate was resistant and the second isolate inducible resistant to Clarithromycin, susceptible to Amikacin, Intermediate to Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No *rrs*,*gyrA,rplC* and *rplD* gene mutation seen in both isolates. Both isolates had a C-41G mutation at the *gyrB* promoter region.

SNP's analysis: Among the two isolates there were no SNPs detected.

Case 27: Two isolates collected from sputum at 6 months apart. Both isolates identified as *M. abscessus* subsp *abscessus*.

Phenotypic susceptibility and resistance profile: Both isolate inducible resistant to Clarithromycin, susceptible to Amikacin and Linezolid. The Ciprofloxacin result varied between both isolates, 1st isolate was Intermediate and 2nd isolated resistant.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No *rrs,gyrA,gyrB rplC* and *rplD* gene mutation seen in both isolates. Both isolates had C3042T mutation in *rrl* gene associated to Amikacin.

SNP's analysis: There was 1 SNPs difference between the two isolates.

4.6.2 Isolates recovered from non-Cystic Fibrosis Patients

Case 7: Two breast fluid collected 2 months apart, the first collected sample was from the right breast and the second from the left breast. Both samples were identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: The same antibiotic profile was seen in both isolates. Clarithromycin and Amikacin susceptible, Linezolid intermediate and Ciprofloxacin resistant.

Evaluation of resistance-conferring mutations: Both isolates had a mutation at *erm*(41)T28C related to sensitivity to Clarithromycin and no mutation in *rrl* gene was evident. No mutation found in *rrs*, *gyrA*, *rplC*, *rplD* and rrl gene. A promoter mutation at C-41G evident in both samples at *gyr*B gene.

SNP's analysis: There were 9 SNPs difference between the two isolates.

Case 9: Three isolates collected, 1st isolate collected was a swab from unknown source and the last 2 were sputum collected 7 months apart. All three isolates were identified as *M. abscessus* subsp. *massiliense.*

Phenotypic susceptibility and resistance profile: Clarithromycin, Amikacin susceptible and Ciprofloxacin resistant for all 3 isolates. The 1st isolate was susceptible, 2nd isolate intermediate and 3rd isolate resistant to Linezolid.

Evaluation of resistance-conferring mutations: No mutation in *rrl* gene but there was a truncated *erm*(41) gene in all 3 isolates. No mutation in *rrs* gene associated to Amikacin. The gene *gyr*A had no mutations but all 3 isolates displayed mutation in the *gyr*B promoter region A-78G and C1727G in *gyr*B gene. No mutations evident in *rplC, rplD* and *rrl* gene with known resistance for all three isolates. However, isolate 1 had two mutations a A1717G and T3001C and isolate 2 displayed a mutation at T3001C in the *rr*l gene. These mutations have no associated resistance.

SNP's analysis: There were 1 SNPs difference between the three isolates.

Case 17: Two isolates collected; the first isolate was from a lavage and the second isolate collected 17 months apart was a sputum. Both isolates identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin, resistant to Ciprofloxacin. However, the Linezolid was different. The first isolate was intermediate, and the second isolate was resistant to Linezolid.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. Both had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyrA*, *gyrB*, *rrs, rrl, rplC and rplD* gene.

SNP's analysis: There were 1 SNPs difference between the three isolates.

Case 19: Two isolates collected; the first isolate was from a lavage and the second isolate collected 22 months apart was a sputum. First isolate identified as subsp. abscessus and second as *M. abscessus* subsp. *massiliense.*

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both resistant to Clarithromycin, susceptible to Amikacin, resistant to Ciprofloxacin. However, the Linezolid was different. The first isolate was susceptible and the second isolate intermediate to Linezolid.

Evaluation of resistance-conferring mutations: First isolate had no *rrl* gene mutation and *erm*(41) T28T intact. The second isolate had a truncated *erm*(41) gene and a mutation A2271G in *rrl* gene. Both isolates had no mutation in *rrs* and *gyrA* gene. 1st isolate displayed a C-41G mutation in the *gyrB* promotor region and the second isolate at A -78G it also had C1727G mutation in *gyrB*. A mutation C3042T in *rrl* gene was detected in both isolates and it no known association to resistance to Linezolid.

SNP's analysis: No data available

Case 20: Two isolates collected from different source 5 months apart. 1st isolate was a tissue with unknown origin and second was Pus-thoracotomy site. Both isolates identified as *M. abscessus* subsp. *abscessus*.

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin, resistant to Ciprofloxacin. However, the Linezolid was different. The first isolate was intermediate and the second isolate was susceptible to Linezolid.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. Both had no specific mutations related to resistance for Ciprofloxacin, Amikacin and Linezolid in *gyrA*, *gyrB*, *rrs, rrl, rplC and rplD* gene.

SNP's analysis: There were 2 SNPs difference between the two isolates.

Case 22: Two sputum isolates collected 11 months apart both identified as *M. abscessus* subsp. *abscessus*.

Phenotypic susceptibility and resistance profile: Both isolates have similar antibiotic patterns. They were both inducible resistant to Clarithromycin, susceptible to Amikacin, Linezolid and Ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutations found in *rrs, gyrA, rplC, rplD* gene in both isolates. Both had C-41G promoter *gyrB* mutation.

SNP's analysis: Among the two isolates there was 1 SNPs difference. There was another isolate clustered among the two isolates with 18-19 SNPs difference

Case 23: Two sputum isolates collected 7 months apart and identified as *M. abscessus* subsp. *massiliense.*

Phenotypic susceptibility and resistance profile: Isolates susceptible to Clarithromycin, Amikacin, Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates displayed truncated *erm*(41) gene and no *rrl, rrs, gyrA* mutation. The first collected isolate had mutation A-78G in *gyrB* promoter gene and C1727G *gyrB* mutation. The first isolate also found to have mutation in the *rrl* gene associated with Linezolid at A1717G and T3001C and the second isolate T3001C.

SNP's analysis: There were 0 SNPs difference between the two isolates.

Case 24: One lavage isolate collected in 2016 followed by two sputum isolates. The second isolate collected 8 months from the 1st and the 3rd isolate collected 7 months apart. All isolates identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: All isolates were inducible resistant to Clarithromycin, susceptible to Amikacin, intermediate to Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: All 3 isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutation in *rrs*,*gyrA,rplC* and *rplD*. The second isolate had a A-78G mutation at *gyrB* promoter region.

SNP's analysis: There were 0 SNPs difference between the two isolates.

Case 25: Two sputum isolates collected 15 months apart and both identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: All isolates were inducible resistant to Clarithromycin, susceptible to Amikacin, intermediate to Linezolid and resistant to Ciprofloxacin.

Evaluation of resistance-conferring mutations: All 3 isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutation in *rrs*, *gyrA, rplC* and *rplD*. The second isolate had a C-41G mutation in the *gyrB* promoter.

SNP's analysis: There were 6 SNPs difference among the two isolates.

Case 28: Two tissue isolates collected from two surgical devices related to lap band collected 5 months apart identified as *M. abscessus* subsp. *abscessus.*

Phenotypic susceptibility and resistance profile: The 1st isolate was susceptible and the second isolate was inducible resistant to Clarithromycin, both were susceptible to Amikacin, Linezolid and resistant to ciprofloxacin.

Evaluation of resistance-conferring mutations: Both isolates had no mutation in *rrl* gene and *erm*(41) T28T intact. No mutation in *rrs*, *gyrA, rplC* and *rplD*. Both isolate had a C-41G mutation located in *gyrB* promoter.

SNP's analysis: There were 1 SNPs difference between the two isolates. The isolates also clustered (Cluster 7) among 5 CF isolates having 1 to 10 SNPs difference.

In our analysis of the 28 duplicate samples, most of the duplicate samples had reproduced the same subspecies in their repeat collection. The average SNPs difference between most of the duplicates were between 0-18 SNP. There was one duplicate case in CF cohort (Case 4) and non-CF cohort (case 19) that isolated two different subspecies from different collection dates.

4.6.3 Genomic analysis of the genes associated with in vitro resistance

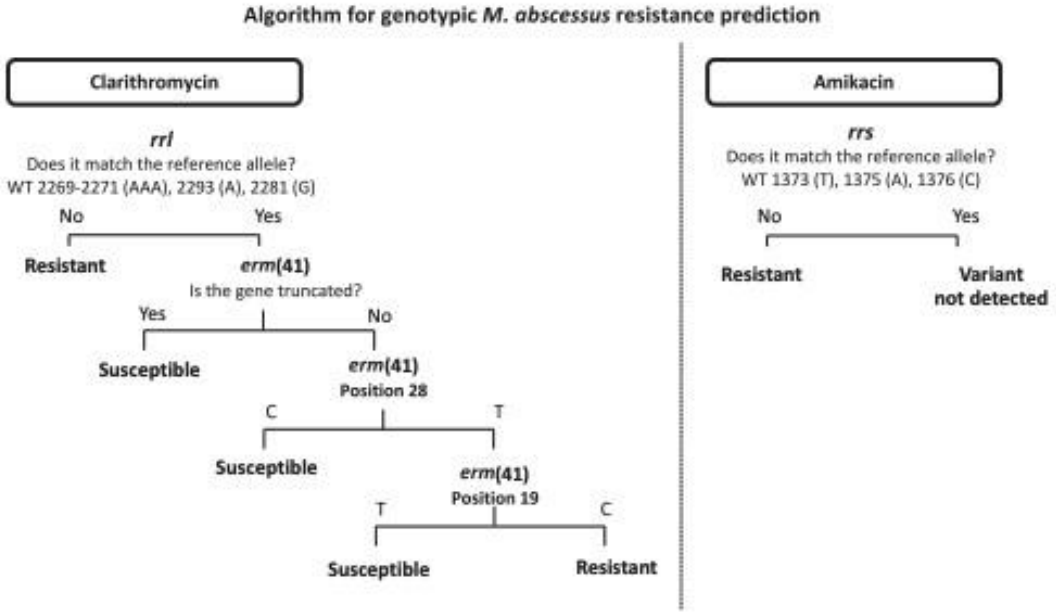

Figure 4-5 *The Algorithm used to analyse Clarithromycin and Amikacin genotype data. Reprinted from Realegeno S, et al, 2021. Clinical Whole Genome Sequencing for Clarithromycin and Amikacin Resistance Prediction and Subspecies Identification of Mycobacterium abscessus The Journal of Molecular Diagnostics. doi:10.10.16/j.moldx.2021.07.023.*

Clarithromycin

Of the 159 *M. abscessus* isolates retrieved from 116 patients the following algorithm (Figure 4-5) was followed when analysing the data for Clarithromycin mutation (64) . When analysing the *rrl* gene, there were 4 (2.5%) isolates that confer *rrl* A2271G mutation possibly indicating acquired resistance. Two isolates identified as *M. abscessus* subsp. *abscessus* and the other two isolates was subsp. *massiliense.*

Truncated *erm*(41) gene was visible in 32 (20.1%) isolates, the majority identified as *M. abscessus* subsp. *massiliense* (31/32) and unpredictable one isolate identified as *M. abscessus* subsp. *abscessus*. There were 16 (10.1%) *M. abscessus* subsp *abscessus* isolates that had a C28 substitute in the *erm*(41) gene. The remaining 111 (69.8%) had an intact *erm*(41) gene because of the T28 SNP; 96.4% (107/111) *M. abscessus* subsp *abscessus* and 3.6% (4/111) *M.abscessus* subsp *bolletti.* Within the isolates with intact *erm*(41), interestingly there were two isolates that also had a A-31 *erm*(41) promoter mutation one identified as *M.abscessus* subsp *bolletti* and the other *M. abscessus* subsp *abscessus.*

Amikacin

Only 3 isolates were found to have A1374G mutation that predicted resistance in the *rrs* gene (23 S rRNA) from the 159 *M. abscessus* complex isolates analysed. Two were *M. abscessus* subsp *abscessus* and one identified as M. *abscessus* subsp. *massiliense.* There was also probable mutation C1462T related to resistance in two *M. abscessus* subsp *abscessus isolates.*

Ciprofloxacin

Analysing both *gyrA* and *gyrB* gene and corresponding promoters. It was noticed that all of the isolates that had reported mutations, possibly related to resistance was only seen in *gyrB* gene and its promoter region. Almost half of the *M. abscessus* isolates 46.5% had no reported or probable mutations related to resistance in *gyrB* and the main species 65/74 *M. abscessus* subsp *abscessus* isolated. Followed by 5/74 M. *abscessus* subsp. *massiliense* and 4/74 *M. abscessus* subsp *bolletti.*

The remainder of the 85 isolates had mutations in *gyrB* and promoter region. 56 isolates had a C-41 mutation which were from *M. abscessus* subsp *abscessus* isolates. Isolates that had both C-41 and C1727G which existed only 2 identified as M. *abscessus* subsp. *massiliense*. The next known mutation was A-78 (25/85) combined with C1727G *gyrB*, these isolates were identified as 23 M. *abscessus* subsp. *massiliense* and 2 *M. abscessus* subsp *abscessus.* There were only two isolates with a A-21 mutation and they included M. *abscessus* subsp. *massiliense* and *M. abscessus* subsp *abscessus* identification.

Linezolid

There were 4 isolates with A2271G *rrl* gene mutation which is known to be related to resistance. The isolates divided equally between *M. abscessus* subsp *abscessus* and M. *abscessus* subsp. *massiliense*. It was also noticed that one of the M. *abscessus* subsp. *massiliense* isolate had another two mutation A1717G and T3001C *rrl* gene which is also probably associated with resistance.

When analysing the *rrl* gene it was noticed that there were 21 isolates with the combined A1717G and T3001C mutation that is possibly related to resistance. Twenty identified as M. *abscessus* subsp. *massiliense* and one as *M. abscessus* subsp. *abscessus.* There were also 4 isolates with single T3001C mutation, and they were identified as M. *abscessus* subsp. *massiliense.*

4.6.4 Comparison of predicted genomic markers of susceptibility to phenotypic testing results

The following genomic changes in genes conferring resistance increase in the MIC to relevant antimycobacterial agents have been observed.

Clarithromycin

The following (figure 4-6) compares antimicrobial resistant markers of clarithromycin with the isolates phenotypic results by species.

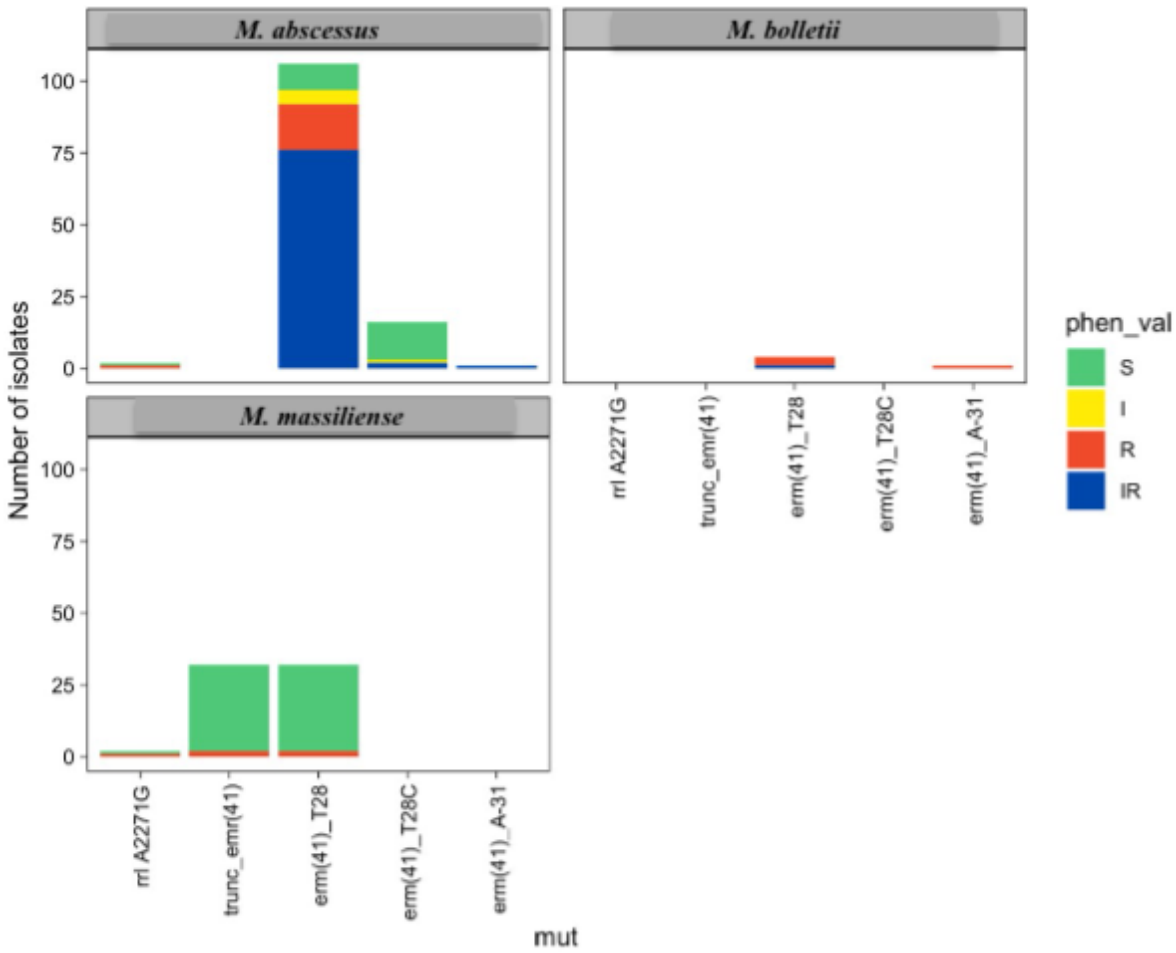

Figure 4-6 :Isolates with Antimicrobial resistant markers related to Clarithromycin and there corresponding phenotypic results by Mycobacterium abscessus complex sub-species.

The coloured bar represent the isolates phenotypic susceptibility result. Green is susceptible, yellow intermediate, red is resistant and blue inducible resistant. These bars were aligned with the predicted resistant markers of Clarithromycin.

Only two isolates from the 23 isolates of *M. abscessus* complex that were phenotypically resistant to Clarithromycin (n=23) had A2271G *rrl* gene mutation, which has been reported in association with acquired resistance to this drug. Additionally, two isolates identified as M. *abscessus* subsp. *massiliense* and *M. abscessus* subsp. *abscessus* had a deletion of 270 bp seen within the gene becoming a truncated *erm*(41) gene. There was also one phenotypically resistant isolate identified as M. *abscessus* subsp. *massiliense* that displayed a truncated *erm(*41) gene and had no other mutations associated with Clarithromycin resistance. An isolate identified as *M. abscessus* subsp *bolletti* with phenotypic resistance acquired a A-31 mutation in the *erm*(41) promoter region that has association to resistance (89) (90).The rest of the phenotypically resistant isolates (n=19) had an intact *erm(41)* gene. Of these three were identified as *M. abscessus* subsp. *bolletii* and sixteen as *M. abscessus* subsp. *abscessus.*

The majority of phenotypically resistant isolates of *M. abscessus* complex presented with inducible resistance (IR) to the drug (n=79), most (n=76) had an intact *erm(*41) gene with T28 and were identified as *M. abscessus* subsp. *abscessus.* Three of the isolates contained other mutations, one isolate acquired a mutation A-31 in the *erm(*41) promoter that is related to resistance and the other two acquired T28C mutation in the *erm(*41) this should be phenotypic sensitive to Clarithromycin. Out of the six isolates with intermediate resistance to Clarithromycin, five had an intact *erm(*41) gene with T28 and one isolate had a *erm*(41) T28C SNP change which has been usually documented in Clarithromycin susceptible strains.

Fifth-one isolates in our collection (32.1%) were reported as susceptible to Clarithromycin in vitro, however, some of them have contained drug resistance conferring changes in their genomes. Two of these isolates had A2271G mutation in *rrl* gene suggesting acquired resistance (90) (91) (92) one of the two, which was identified as M. *abscessus* subsp. *massiliense,* also possessed a truncated *erm* (41) gene. Deletion of the *erm*(41) gene was detected in 29 isolates, majority identified as M. *abscessus* subsp. *massiliense* and one

identified as *M. abscessus* subsp. *abscessus*. Mutation T28C in *erm(*41) gene was found in 13 *M. abscessus* subsp. *abscessus* strains. All of these mutations were concordant with the phenotypic result.

The remainder 7 isolates had an intact *erm(*41) T28 gene.

Through analysing the gene associated with clarithromycin, there were 109 isolates that were predicted to be inducible resistance. Of these isolates 102 were phenotypic resistant and 7 were susceptible by traditional gold standard drug testing. It was determined that the sensitivity of genotype analysis was 85.7% with a specificity of 96.2% (Table 4-6). The positive predictive value (PPV) for this analysis was 91.3% and negative predictive value (NPV) at 93.7%

Table 4-6 WGS prediction against Clarithromycin phenotype MIC interpretations

Clarithromycin				
		Phenotype		
Genotype		Sensitive	Resistant	**Total**
	Sensitive	42	4	**44**
	Resistant	7	102	**109**
	Total	**49**	**106**	
	Sensitivity		85.7 %	
	Specificity		96.2 %	
	PPV		91.3 %	
	NPV		93.7 %	

Table 4-6 Didn't include the isolates that had a *rrl* gene SNP (n=4). Isolates reported with an interpretation of intermediate were treated as resistant when calculating sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV). Isolates that were interpreted as inducible resistance were also treated as resistant.

Amikacin

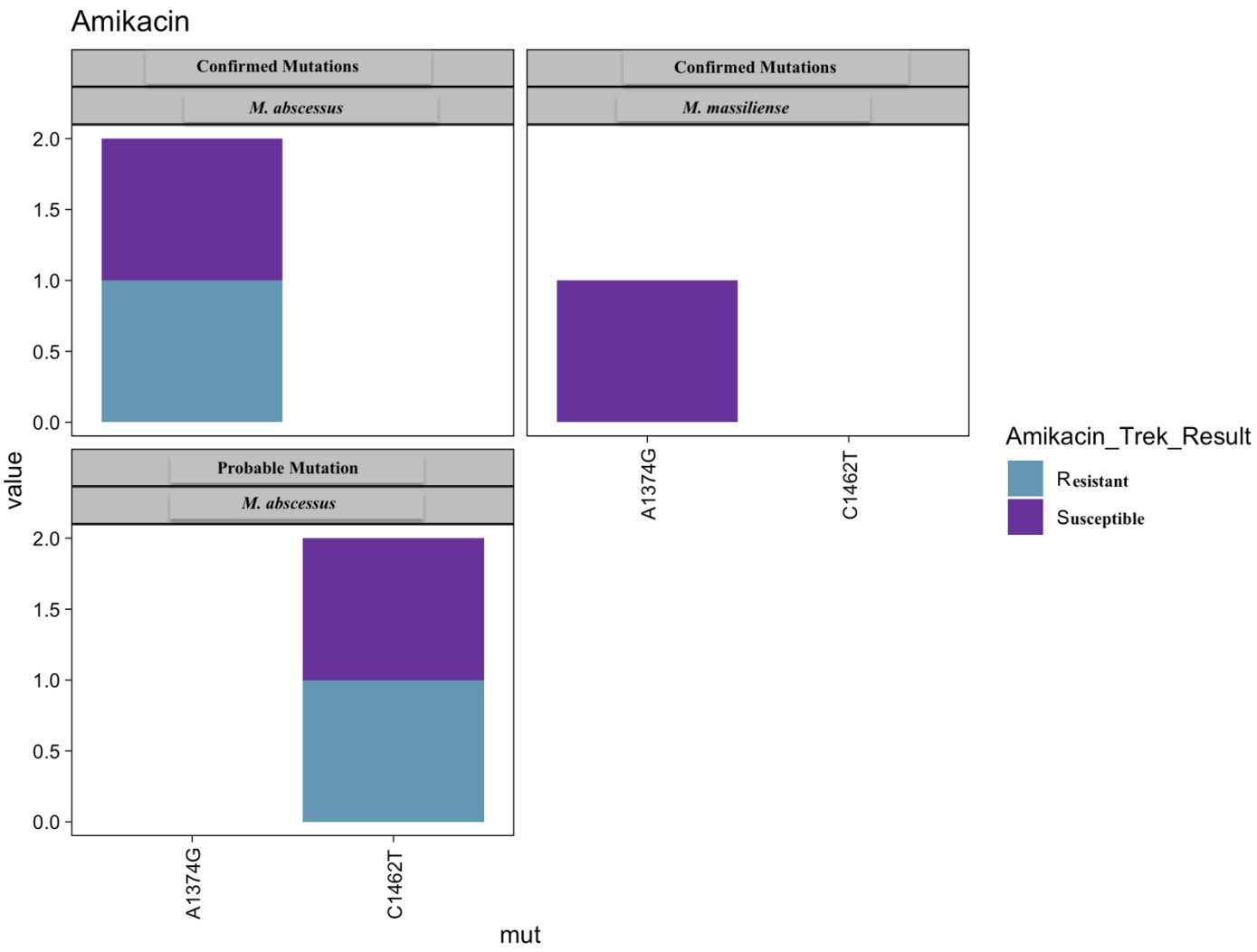

Figure 4-7 Isolates with known Antimicrobial resistant markers related to Amikacin and there corresponding phenotypic results by Mycobacterium abscessus complex sub-species.

Only two isolates in our set had both genotypic and phenotypic resistance to Amikacin. One isolate had a A1374G *rrs* gene mutation with known association with resistance (Figure 4-7) and the other carried C1462T *rrs* gene mutation which has been published as potentially associated to resistance (82) (64). The remainder of the isolates that were phenotypically resistant had no known or potential drug resistant mutations in *rrs* gene.

There was a small number of potentially discrepant results between phenotype and genotype. Out of the 152 phenotypically susceptible isolates one isolate had C1462T *rrs* mutation and two isolates contained a recognised A1374G mutation in *rrs* gene which should lead to phenotypic resistance. There were no mutations seen in phenotypic intermediate isolates.

Analysing the *rrs* gene associated with amikacin resistance it was predicted that 154 of the isolates would be sensitive and 5 would be resistant. However, there was only 152 isolates susceptible and 7 were phenotypic resistant (Table 4-7). The PPV was at 97.4% but the NPV to detect resistance was at 60%.

Table 4-7: WGS prediction against Amikacin phenotype MIC interpretations

Amikacin				
		Phenotype		
		Sensitive	Resistant	**Total**
Genotype	Sensitive	150	4	**154**
	Resistant	2	3	**5**
	Total	**152**	**7**	
	Sensitivity	98.7 %		
	Specificity	42.9 %		
	Positive predictive value	97.4%		
	Negative predictive value	60 %		

Table 4-7 The isolates with an interpretation of intermediate were treated as resistant when calculating sensitivity, specificity, PPV and NPV.

Ciprofloxacin

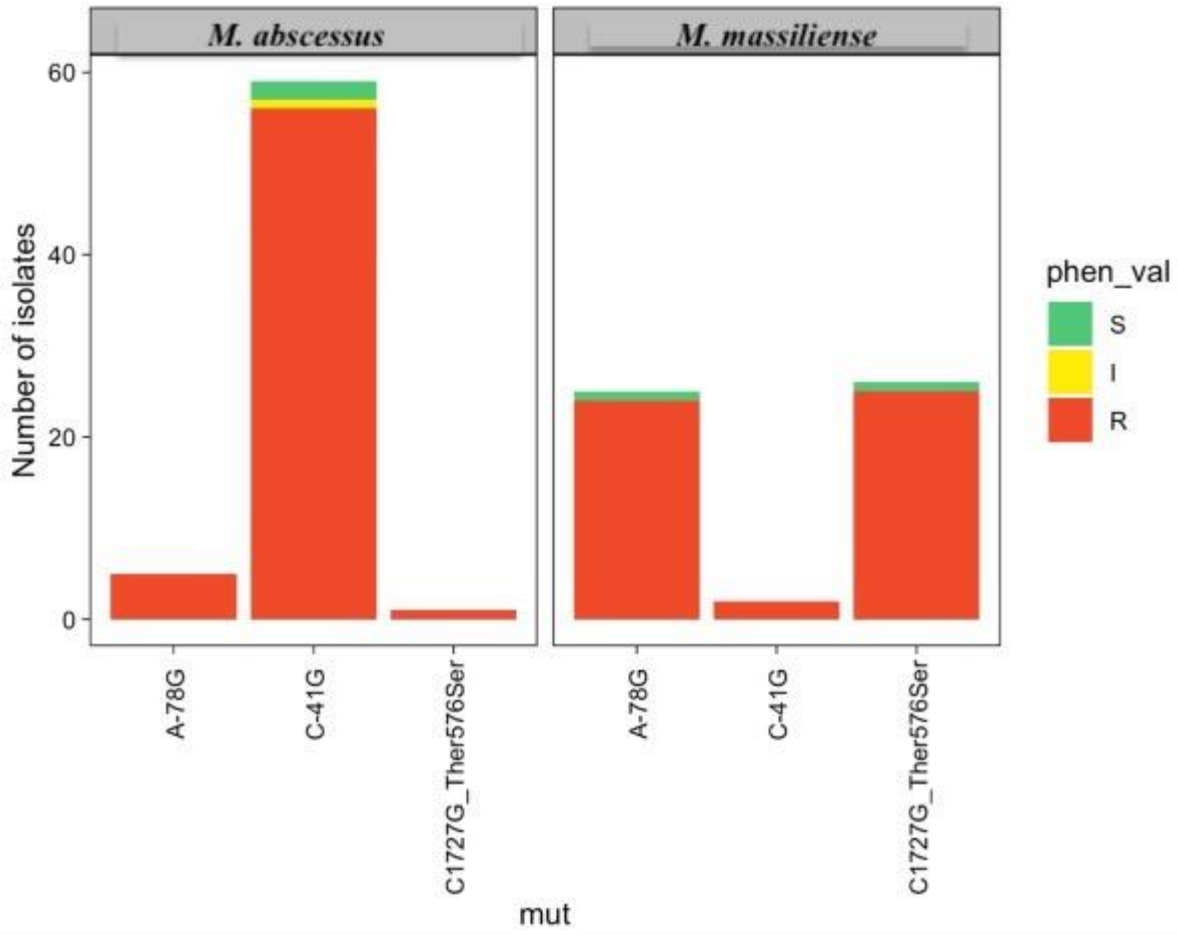

Figure 4-8 Isolates with Antimicrobial resistant markers related to Ciprofloxacin and there corresponding phenotypic results by Mycobacterium abscessus complex sub-species.

Predominant majority of *M. abscessus* complex isolates in our dataset were reported as phenotypically resistant. However, only 81 isolates (54%) contained high-confidence mutations related to resistance. Mutation C-41G located at the promoter region of *gyrB* gene was evident in 35.1% (53/151) of isolates identified as *M. abscessus* subsp. *abscessus*. There were two *M. abscessus* subsp. *massiliense* isolates that also had this mutation and additional C1727Gmutation in *gyrB* gene. Another common *gyrB* promoter mutation was A-78G, two isolates had this but 24 isolates (15.9%) had a combination of A-78G and C1727G *gyrB* mutations (22 isolates of *M. abscessus* subsp. *massiliense* and 2 of *M. abscessus* subsp. *abscessus)*. No mutations associated with resistance was seen in the remanding n=70 isolates.

Intermediate resistance was documented in 5 *M. abscessus* subsp. *abscessus* isolates, one of which had a C-41G mutation and the other four had no mutations.

Isolates phenotypically susceptible to Ciprofloxacin were uncommon (n=3) but C-41G mutation was evident in two isolates identified as *M. abscessus* subsp. *abscessus* and one *M. abscessus* subsp. *massiliense* isolate had a combination of mutations in *gyrB* gene: A78G and C1727G. There were no mutations within *gyrA* and *gyrA* promoter gene that predicted or associated resistance to ciprofloxacin seen in any of the isolates.

Linezolid

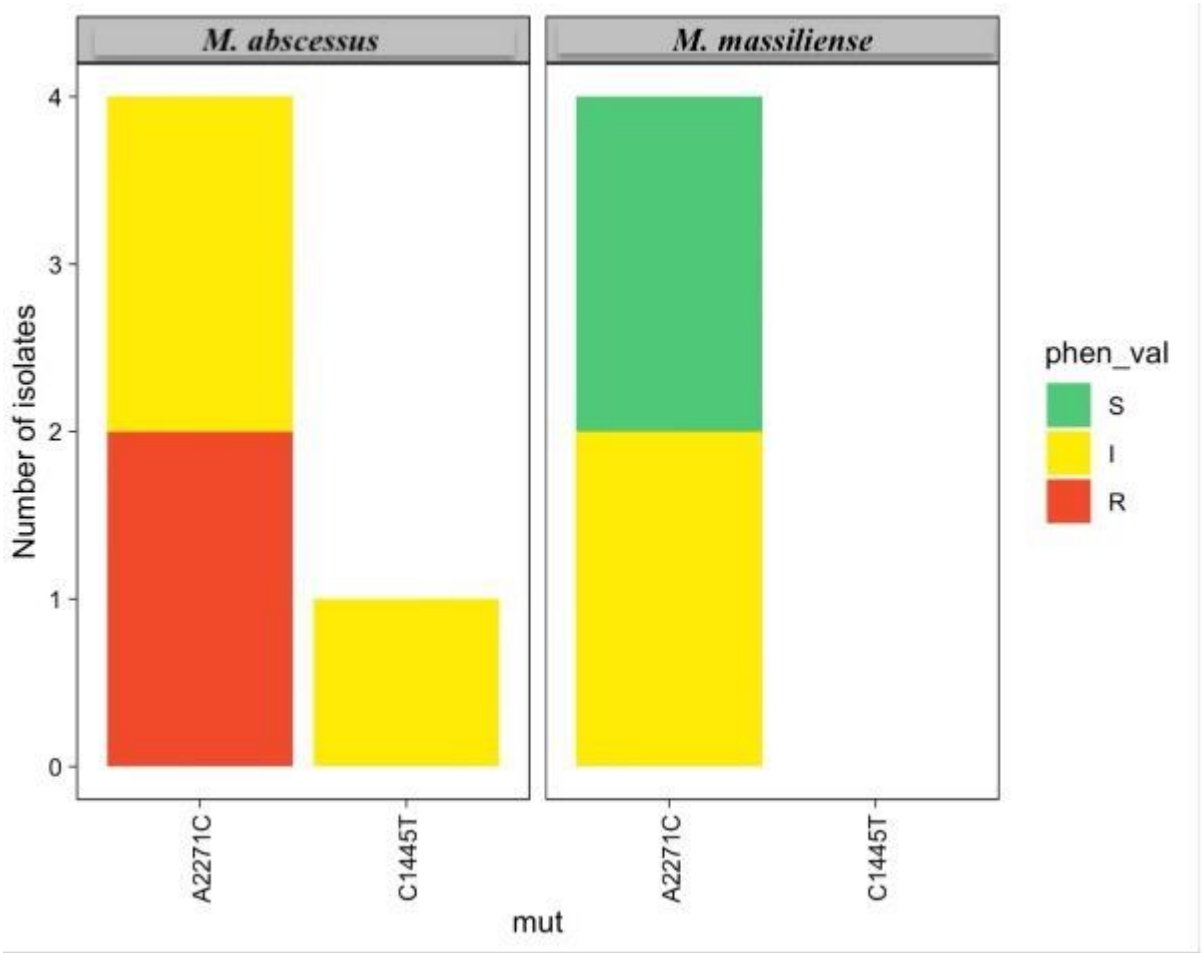

Figure 4-9 Isolates with Antimicrobial resistant markers related to Linezolid and there corresponding phenotypic results by Mycobacterium abscessus complex sub-species.

There were 30 isolates in our set which were reported as phenotypically resistant to Linezolid. Only one isolate (1/30) contained a high-confidence A2271C mutation in *rrl* gene associated with resistance (Figure 4.9). The other six isolates had mutations which could explain the phenotypic resistance, three of them shared C3042T *rrl* mutation and the rest had a combined A1717G and T3001C mutation in *rrl* gene. The remainder of resistant isolates (n=23) had no common mutations related to resistance.

Linezolid isolates with intermediate resistance (n=53) also had confirmed mutations. There were 3 isolates with mutations related to resistance. A mutation C1445T in *rrl* gene was found in one isolate, another isolate had A2271C *rrl* change and the last isolate had combined A2271C, A1717G and T3001C mutations in *rrl* gene; this isolate was identified as *M. abscessus* subsp. *massiliense* while the previous two were identified as *M. abscessus* subsp. *abscessus.* Eleven other isolates that were considered intermediate to Linezolid contained mutations that were probably related to resistance. Combined mutations of C1445T and T3001C in the *rrl* was evident in nine *M. abscessus* subsp. *massiliense* isolates. Single mutation T3001C was also found in one *M. abscessus* subsp. *massiliense* isolates and C3042T *rrl* change in another *M. abscessus* subsp. *abscessus* isolate. The rest of the 39 *M. abscesssus* isolates that were intermediate via phenotypic testing conferred no mutations in their genomes.

Overall, 76 isolates were phenotypic susceptible to Linezolid. One isolate contained the A2271C mutation in *rrl* gene associated to resistance and 15 isolates carried mutations probably related to resistance. 53.3% (8/15) isolates contained a combined A1717G and T3001C mutation in *rrl* gene. 20% (3/15) of isolates had a single T3001C mutation and were identified as M. *abscessus* subsp. *massiliense* and 26.7% (4/15) contained C3042T *rrl* mutation and were identified as *M. abscessus* subsp. *abscessus.* The remainder of susceptible isolate (n=60) had no mutations related or probably associated with resistance, where 57 was identified as *M. abscessus* subsp. *abscessus* and rest as M. *abscessus* subsp. *massiliense.*

The gene analysed for the prediction of resistance and susceptibility were not strongly associated with the phenotypic results. The specificity of the gene analysed versus phenotypic result was at 4.8% (Table 4-8) and PPV value was 48.7%.

Table 4-8: WGS prediction against Linezolid phenotype MIC interpretations

Linezolid				
		Phenotype		
		Sensitive	Resistant	**Total**
Genotype	Sensitive	75	79	**154**
	Resistant	1	4	**5**
	Total	**76**	**83**	
	Sensitivity	98.7 %		
	Specificity	4.8 %		
	Positive predictive value	48.7 %		
	Negative predictive value	80 %		

CHAPTER 5

5 Conclusions

This study investigated for the first time the molecular epidemiology and genomic data of 159 *M. abscessus* isolates recovered from CF and non-CF cases in NSW, Australia collected between 2015 to 2017. The findings suggested that infection and colonisation with *M. abscessus* complex was more prevalent in female patients (58.7% in CF cohort and 55.2% in non-CF cases). The mean age was 24 years in CF patients which was consistent with recent reports from Germany (93).

Through WGS analysis we were able to identify all the subspecies of *M. abscessus.* The most common pathogen diagnosed in NSW was *M. abscessus* subsp. *abscessus*. The prevalence of *M. abscessus* subsp, *abscessus* in isolates associated with respiratory infections was higher than other *M. abscessus* subspecies and consistent with other studies from Australia, USA, Japan, England (26) (91) (89) (94). This study was the first to identify and report *M. abscessus* subsp. *bolletii* in NSW, previous reports from our laboratory were unable to produce this level of resolution as no subspecies description existed at the time of their analysis (26).

The major strength of this study was the direct comparison of cohorts of *M. abscessus* infected cases with and without CF as major predisposing condition for *M. abscessus* colonisation. In our findings we confirmed that *M. abscessus* subsp. *abscessus* (87.3%) were the dominant subspecies isolated in CF patients as reported internationally (53) (13) (87). Similar to previous reports two other subspecies were present in both cohorts: *M. abscessus* subsp. *massilience and M. abscessus* subsp. *bolletii* (10).

Similary, our phylogenetic tree with the international dataset were able to identify the three global circulating clones. The predominant isolates from our data set identified in the global dominant circulating clone Absc2 were CF isolates, they were highly genotypic and phenotypic resistant likewise to previous studies (87) (89) (94). This clone is also believed to spread globally in the CF community and have no epidemiological link (95) observed in some of our clusters.

Contrary to previous studies the isolates from these clones in our study were not limited to CF patients. Isolates were from different sites and not limited to non- respiratory sample type and from patients with no CF history were genetically near identical to the circulating

clones. These observations suggested that dominating clones can colonise other patients with no history of CF and are consistent with recent reports by Lipworth et al (2021) (83) and Bronson et al (2021) (96).

The drug resistance-conferring mutations in *M. abscessus* were analysed against the MIC interpretations instead of MIC values. We were able to predict the genetic markers for Clarithromycin using WGS analysis with high confidence similarly to other studies in different contexts (82) (64). This was evident as clarithromycin susceptibility or resistance was predicted with positive predictive value (PPV) of 91.3% and 93.7% (NPV). We also found by WGS analysis, that two isolates identified as *M. abscessus* subsp. *massilience* obtained acquired resistance and Kaewprasert et al (2022) also discovered this in their report (82).

The genetic markers for amikacin, ciproflaxcain and linezolid did not correlate well with phenotypic results. In the *rrl* and *rrs* gene the coverage for some isolates when analysing was low and could contribute to the discrepancies (64). When analysing the *gyrA* and *gyrB* gene we found mutations that may contribute to resistance (13) (74) however these observations lacked consistency. There were also other mutations that were seen in the *rrl* gene that could be associated to resistance but lacked evidence for confirmation.

Several limitations of our research have to be acknowledged. We studied *M. abscessus* complex in one jurisdiction of a large country, with relatively limited number of cases under genomic study, a specific laboratory testing and surveillance environment and microbial ecology for relatively short period of time. The relatively small number of sequenced genomes could bias our observations of genomic changes and inferences about genomic clusters within hosts and between hosts. However, we believe that the complexity of multi-factorial epidemiology of *M. abscessus* complex and cost of WGS made, for this thesis purposes, an overarching study of genome dynamics unfeasible and the research of genomic variations under controlled conditions of experimental models and defined clusters is the way to tackle the relationships between genomic markers and phenotypes. A larger study may be warranted to further examined our observations about *M. abscessus* complex genotype-antimicrobial susceptibility correlations.

In conclusion, this study described the molecular epidemiology of *M. abscessus* subspecies co-circulating in NSW and evaluated and quantified the relevance of resistome for predicting susceptibility or resistance of this important opportunistic pathogen to relevant antimycobacterial drugs. The findings of this study have been implemented for surveillance of *M. abscessus* in NSW Mycobacterium Reference Laboratory at the NSW Health Pathology. They will assist in early detection and recognition of *M. abscessus* cases amongst patients with cystic fibrosis and other chronic conditions. Our study findings can be translated into better public health and infection control outcomes because of more targeted interventions and treatments.

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Appendix A

Isolate	Reads	AvgQual	Depth
16-3027-0078	3,847,316	34.8	107
16-3027-0087D1	1,258,326	32	34
16-3027-0077	4,661,696	32.2	124
17-3027-0053	2,441,838	33.3	68
ERR337812	4,395,486	35.8	84
16-3027-0053	2,849,812	33.4	79
15-3027-0006	3,234,326	32.1	87
16-3027-0051D1	9,940,252	34.8	276
16-3027-0036	3,271,266	33.4	91
16-3027-0089	1,461,712	31.8	39
17-3027-0036	5,043,060	33.2	139
15-3027-0060	3,335,914	33.2	93
16-3027-0052D1	4,918,884	34.9	137
16-3027-0019	2,975,814	33.3	83

16-3027-0054D1	9,685,434	34.9	269
17-3027-0049	2,499,206	33.2	70
16-3027-0023	3,696,566	33.5	103
15-3027-0041D1	7,955,820	34.9	221
15-3027-0035	2,609,324	32.2	70
16-3027-0030D1	5,565,550	34.9	154
15-3027-0043	3,114,992	32.4	83
15-3027-0029	8,901,724	33.4	241
16-3027-0024	4,004,456	32.7	111
17-3027-0022	2,514,408	33	69
17-3027-0051	2,458,798	33.2	69
16-3027-0088	1,475,320	33.5	40
15-3027-0042	3,089,924	32.3	82
15-3027-0012	5,235,820	34	145
ERR330807	4,310,396	36	83
16-3027-0079	3,955,724	34.8	110
ERR1583683	1,306,768	36.4	37
15-3027-0044	2,263,576	32.2	60
15-3027-0014	4,685,908	33.9	130

16-3027-0005D1	4,729,660	34.8	131
15-3027-0028	10,006,796	33.5	266
ERR330850	4,871,716	35.6	93
16-3027-0021	2,450,032	33.2	68
ERR340512	4,484,244	35.3	86
17-3027-0021	3,073,576	33.3	84
17-3027-0054	4,672,502	33.4	131
16-3027-0049D1	5,692,302	34.9	158
17-3027-0023D1	8,139,948	35	220
16-3027-0065	5,020,976	34.9	139

17-3027-0030	3,307,220	32	87
17-3027-0047	2,697,512	33.4	75
17-3027-0048	3,543,590	33.1	98
16-3027-0017	3,424,662	33	93
16-3027-0048	1,860,330	33.1	51
15-3027-0011	4,616,262	33.8	128
16-3027-0018	3,699,148	32.9	101
16-3027-0047	3,559,596	31.9	95

17-3027-0086	2,557,834	33.1	64
16-3027-0025	2,832,574	33.4	79
16-3027-0003	2,447,598	32.2	66
17-3027-0076D1	1,424,510	32.9	39
16-3027-0001D1	5,944,340	34.9	165
ERR330808	4,862,260	35.7	93
16-3027-0086	2,178,888	33.4	60
17-3027-0025	3,181,292	33.3	85
ERR330767	4,983,486	35.7	96
15-3027-0009	2,972,498	31.9	79
15-3027-0045	2,933,304	33.7	74
16-3027-0010D1	7,149,980	34.8	199
15-3027-0033	2,495,138	32.1	66
15-3027-0026	10,593,212	33.6	273

17-3027-0070	2,599,306	33.3	72
17-3027-0002D1	8,973,286	34.9	248
17-3027-0055	2,891,596	33.4	81
16-3027-0064	3,142,486	32.1	84

16-3027-0070	4,202,314	34.9	117
17-3027-0046	2,972,488	33	83
ERR330786	2,990,820	35.6	57
16-3027-0046	3,737,850	32	100
16-3027-0016	2,935,264	32.9	80
ERR1583695	1,152,364	36.2	32
16-3027-0063	3,972,052	32.2	106
ERR337774	3,947,568	35.7	76
16-3027-0076	9,463,450	34.8	262
16-3027-0039D1	9,313,600	34.5	265
17-3027-0001	2,688,444	33	74
15-3027-0020	3,421,154	32.2	92
15-3027-0008	2,942,288	32	79
15-3027-0031	3,727,872	33.1	102
16-3027-0040	3,643,438	31.5	95
ERR330763	3,049,818	35.6	58
17-3027-0040	2,689,430	33	74
ERR330836	5,183,884	35.6	99

17-3027-0037	4,012,700	33	111
ERR343178	5,426,010	35.3	104
17-3027-0038	4,203,780	33	116
16-3027-0080	4,483,344	33.3	105
16-3027-0002	1,797,976	33.6	49
ERR330785	3,035,128	35.7	58
15-3027-0050	3,609,120	33.2	100
16-3027-0004	5,088,680	34.3	145
ERR337792	3,717,968	35.8	71
15-3027-0034	2,997,518	32.3	80
15-3027-0032	2,526,264	32.2	67
ERR340519	4,032,520	35.2	77
ERR349277	2,936,180	35.5	56
16-3027-0066	4,043,670	32.1	108
ERR1583682	1,228,128	36.2	34
17-3027-0057	2,452,612	33.2	68
15-3027-0022	2,571,062	32.2	69
16-3027-0081	4,927,424	34.8	137
16-3027-0006D1	4,459,900	34.9	124

15-3027-0051	3,529,826	33.3	98
16-3027-0014	3,915,168	32.6	104
17-3027-0042	3,649,354	33.1	101

15-3027-0036D1	8,680,550	34.9	241
16-3027-0044	3,231,748	33.3	90
16-3027-0042	2,922,696	33.1	81
16-3027-0084	2,357,342	33.5	65
15-3027-0052	2,557,002	33.3	71
17-3027-0027	2,551,456	33	69
ERR1583690	1,214,618	36	34
16-3027-0082	3,622,660	34.9	101
16-3027-0027	3,134,440	33.3	87
17-3027-0084	2,678,152	33.7	74
16-3027-0041	2,735,910	31.6	71
15-3027-0018	2,607,784	32.2	70
15-3027-0047	3,921,490	33	108
15-3027-0030	10,592,004	33.6	272
15-3027-0017	2,538,090	32.1	68

15-3027-0048	3,885,888	33.8	99
17-3027-0041	4,607,920	33.2	127
16-3027-0058D1	8,936,840	34.9	248
16-3027-0071	5,149,704	34.9	143
16-3027-0083	3,829,500	32.1	102
16-3027-0059	10,248,730	34.8	284
17-3027-0059	2,925,538	33.4	78

15-3027-0023	2,216,836	32.5	59
15-3027-0005	3,040,312	32	81
17-3027-0060	2,803,678	33.4	73
17-3027-0074	2,182,300	33.9	57
16-3027-0072	4,171,066	32.2	111
15-3027-0021D1	5,744,026	34.8	159
16-3027-0091	4,240,134	34.3	121
15-3027-0019	3,286,546	32.2	88
17-3027-0072	2,504,774	33.3	70
15-3027-0049	3,507,172	33.4	98
17-3027-0035	3,821,314	33.2	104

17-3027-0043	3,777,136	33.1	104
16-3027-0029	4,389,464	32.9	121
16-3027-0043	4,425,852	32	118
17-3027-0029	4,542,260	32.2	121
16-3027-0035	3,583,818	33.4	99
16-3027-0020	2,678,516	33.3	74
15-3027-0001	2,426,070	32	65
15-3027-0039D1	7,653,272	34.8	212
17-3027-0031	1,180,856	31.2	30
16-3027-0009D1	4,842,426	34.8	135
17-3027-0069	2,967,514	33.3	83
15-3027-0037	2,553,700	32.2	68
15-3027-0010	3,130,152	31.8	83
15-3027-0004	2,595,630	32	69
16-3027-0050	2,403,918	32.2	64
15-3027-0002	2,673,688	31.9	71
17-3027-0050	2,573,738	33.3	72
ERR337783	3,906,764	35.9	75

17-3027-0034	4,942,572	33.3	136
ERR330846	5,053,796	35.5	97
16-3027-0034	3,223,948	33.3	89
16-3027-0068	9,384,506	34.9	260
16-3027-0067	4,205,260	32.2	112
17-3027-0056	1,107,040	32.7	30
17-3027-0067	4,715,276	33.5	129
16-3027-0056	2,670,312	33.3	74
16-3027-0008D1	8,608,836	34.9	239
ERR330756	5,122,208	35.7	98
16-3027-0015	4,362,832	32.5	116
15-3027-0040D1	5,802,354	34.8	161
16-3027-0045	3,318,870	31.9	89
17-3027-0045	2,724,556	33.2	76
17-3027-0033	4,857,464	33.3	133
16-3027-0085	2,083,972	33.4	57
ERR1583698	1,318,406	36.2	37
16-3027-0026	3,025,168	33.3	84

17-3027-0075D1	1,352,090	33	37
15-3027-0016	3,087,446	33	84
ERR330762	2,967,898	35.7	57
15-3027-0046	3,215,584	32.9	89
17-3027-0032D1	1,213,508	33	33

Appendix B

Clustering of isolates as per subspecies

M. abscessus subsp. *abscessus*, SNP cut off of < 30 SNPs

				CF isolates	Non-CF isolates					
Cluster 1										
	15-3027-0005	16-3027-0006D1								
15-3027-0005	0	1	P14.1							
16-3027-0006D1	1	0	P14.2							
Cluster 2										
	15-3027-0006	15-3027-0022	15-3027-0046	16-3027-0052D1	17-3027-0075D1	16-3027-0066	17-3027-0032D1	17-3027-0038	17-3027-0040	
15-3027-0006	0	4	5	8	4	16	11	13	26	P2.3
15-3027-0022	4	0	1	4	0	12	7	9	22	P2.2
17-3027-0075D1	4	0	1	4	0	12	7	9	22	P2.5
15-3027-0046	5	1	0	5	1	13	8	10	23	P2.1
16-3027-0052D1	8	4	5	0	4	16	3	13	26	P2.4
17-3027-0032D1	11	7	8	3	7	19	0	16	29	
17-3027-0038	13	9	10	13	9	17	16	0	27	
16-3027-0066	16	12	13	16	12	0	19	17	24	
17-3027-0040	26	22	23	26	22	24	29	27	0	
Cluster 3										
	15-3027-0009	16-3027-0008D1	16-3027-0083	17-3027-0001	17-3027-0060					
15-3027-0009	0	2	3	5	4	P13.1				
16-3027-0008D1	2	0	1	3	2	P13.2				
16-3027-0083	3	1	0	2	1	P13.3				
17-3027-0060	4	2	1	3	0	P13.4				
17-3027-0001	5	3	2	0	3	P8.3	it is >35k SNPs to P8.1			
Cluster 4										
	15-3027-0010	16-3027-0036	16-3027-0085	17-3027-0002D1	17-3027-0084					
15-3027-0010	0	2	2	2	4	P3.1	117 SNPs to P3.5 other members			
16-3027-0036	2	0	0	0	2	P3.3				
16-3027-0085	2	0	0	0	2	P3.2				
17-3027-0002D1	2	0	0	0	2	P3.4				
17-3027-0084	4	2	2	2	0	P3.6				
Cluster 5										
	15-3027-0017	15-3027-0030	16-3027-0010D1	ATCC19977	17-3027-0046					
15-3027-0017	0	2	3	22	27	P6.1				
15-3027-0030	2	0	1	22	27	P6.2				
16-3027-0010D1	3	1	0	23	28	P6.3				
17-3027-0046	27	27	28	25	0					
ATCC19977	22	22	23	0	25					

Cluster 6

	15-3027-0020	15-3027-0050	
15-3027-0020	0	9	P7.2
15-3027-0050	9	0	P7.1

Cluster 7

	15-3027-0021D1	16-3027-0061	16-3027-0067	16-3027-0084	16-3027-0087D1	17-3027-0023D1	17-3027-0051	
15-3027-0021D1	0	10	10	0	11	11	11	P8.1
16-3027-0084	0	10	10	0	11	11	11	P8.2
16-3027-0061	10	0	0	10	1	9	9	P28.1
16-3027-0067	10	0	0	10	1	9	9	
17-3027-0023D1	11	9	9	11	10	0	0	P26.1
17-3027-0051	11	9	9	11	10	0	0	P26.2
16-3027-0087D1	11	1	1	11	0	10	10	P28.2

Cluster 8

	15-3027-0052	16-3027-0056	16-3027-0076	
15-3027-0052	0	0	7	P12.1
16-3027-0056	0	0	7	P12.2
16-3027-0076	7	7	0	

Cluster 9

	16-3027-0002	16-3027-0042	16-3027-0089	
16-3027-0002	0	18	19	
16-3027-0042	18	0	1	P22.2
16-3027-0089	19	1	0	P22.1

Cluster 10

	16-3027-0005D1	15-3027-0031	16-3027-0048	
16-3027-0005D1	0	0	0	
16-3027-0048	0	0	0	P10.2
15-3027-0031	1	1	1	P10.1

Cluster 11

	16-3027-0015	16-3027-0016	16-3027-0019	16-3027-0035	16-3027-0039D1	16-3027-0088	17-3027-0030	17-3027-0033	17-3027-0067	
16-3027-0015	0	17	18	26	0	18	19	10	14	P15
16-3027-0039D1	0	17	18	26	0	18	19	10	14	P15
17-3027-0033	10	13	14	22	10	14	15	0	10	
17-3027-0067	14	17	18	26	14	18	19	10	0	
16-3027-0016	17	0	3	23	17	3	4	13	17	
16-3027-0019	18	3	0	24	18	0	1	14	18	P17
16-3027-0088	18	3	0	24	18	0	1	14	18	P17
17-3027-0030	19	4	1	25	19	1	0	15	19	P17
16-3027-0035	26	23	24	0	26	24	25	22	26	

Cluster 12

	16-3027-0020	16-3027-0026	
16-3027-0020	0	12	P18.1
16-3027-0026	12	0	P18.2

Cluster 13

	16-3027-0034	17-3027-0021	
16-3027-0034	0	2	P20.1
17-3027-0021	2	0	P20.2
ERR349277	29	29	

Cluster 14

	16-3027-0059	17-3027-0047
16-3027-0059	0	1
17-3027-0047	1	0

Cluster 15

	16-3027-0077	17-3027-0035	
16-3027-0077	0	6	P25.1
17-3027-0035	6	0	P25.2
ERR340512	26	32	

Cluster 16				
	17-3027-0029	17-3027-0054		
17-3027-0029	0	1	P27.1	
17-3027-0054	1	0	P27.2	
Cluster 17				
	17-3027-0076D1	17-3027-0086		
17-3027-0076D1	0	2	P8.4	is 35 SNPs to P8.1
17-3027-0086	2	0		
Cluster 18				
	15-3027-0047	16-3027-0009D1	16-3027-0017	
15-3027-0047	0	0	0	P11.1
16-3027-0009D1	0	0	0	P11.2
16-3027-0017	0	0	0	P11.3
Cluster 19				
	16-3027-0014	17-3027-0074		
16-3027-0014	0	0	P16.1	
17-3027-0074	0	0	P16.2	
Cluster 20				
	16-3027-0050	17-3027-0053	17-3027-0069	
16-3027-0050	0	0	0	P24.1
17-3027-0053	0	0	0	P24.3
17-3027-0069	0	0	0	P24.2
Cluster 21				
	15-3027-0016	16-3027-0049D1		
15-3027-0016	0	0	P5.1	
16-3027-0049D1	0	0	P5.2	

***M. abscessus* subsp. *massiliense*, SNP cut off of < 30 SNPs**

Cluster 22

	15-3027-0014	15-3027-0044	15-3027-0051	
15-3027-0014	0	0	0	
15-3027-0044	0	0	0	
15-3027-0051	0	0	0	

Cluster 23

	15-3027-0018	15-3027-0034	16-3027-0068	16-3027-0081
15-3027-0018	0	24	22	19
16-3027-0081	19	25	23	0
16-3027-0068	22	12	0	23
15-3027-0034	24	0	12	25

Cluster 24

	15-3027-0045	16-3027-0003	16-3027-0046	17-3027-0072	
15-3027-0045	0	22	10	10	
16-3027-0046	10	30	0	0	P23.1
17-3027-0072	10	30	0	0	P23.2
16-3027-0003	22	0	30	30	

Cluster 25

	17-3027-0027	16-3027-0045	16-3027-0053	16-3027-0058D1	
16-3027-0045	1	0	4	1	P21.1
16-3027-0058D1	0	1	3	0	P21.2
17-3027-0027	0	1	3	0	P21.3
16-3027-0053	3	4	0	3	

Cluster 26

	15-3027-0028	15-3027-0042	15-3027-0060	16-3027-0027
15-3027-0042	26	0	18	8
16-3027-0027	26	8	18	0
15-3027-0060	26	18	0	18
15-3027-0028	0	26	26	26

Cluster 27

	15-3027-0023	16-3027-0021	16-3027-0065	
15-3027-0023	0	0	1	P9.1
16-3027-0021	0	0	1	P9.2
16-3027-0065	1	1	0	P9.3

***M. abscessus* subsp. *bolletti*, SNP cut off of < 30 SNPs**

Cluster 28			
	15-3027-0002	15-3027-0049	
15-3027-0002	0	16	P1.1
15-3027-0049	16	0	P1.2