Investigating *Mycobacterium abscessus* to inform treatment and drug discovery

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Doctor of Philosophy

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February 2021

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The environmental non-tuberculous mycobacteria, *Mycobacterium abscessus,* is quickly becoming a major health concern in developed countries in part to its extensive multi-drug resistance. Those at risk of contracting *M. abscessus* lung disease are primarily people who are immunocompromised or have pre-existing lung disorders such as Cystic Fibrosis. Treatment for this disease involves a lengthy regimen of several antibiotics, despite this, treatment failure rates remain unacceptably high. The background of this infectious disease, its clinical manifestation and management is discussed in detail in Chapter 1. The first results chapter of this thesis, chapter 2, makes use of genomic techniques to explore the subspecies of a panel of *M. abscessus* clinical isolates, alongside their drug susceptibility patterns, in order to elucidate a link between *M. abscessus* subspecies (*M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense*) and drug resistance. Given the high levels of treatment failure, it is essential to find new treatments for *M. abscessus* infections, that can be introduced into clinical practice in the near future. Chapter 3 of this thesis explores the repurposing of a new and approved β-lactamase inhibitor, relebactam, for inclusion into the *M. abscessus* chemotherapeutic regimen. Here, relebactam's efficacy against the endogenous *M. abscessus* β-lactamase, Bla_{Mab} is discovered, as well as its inhibitory activity when combined with the carbapenem, imipenem. This activity is further potentiated with by the addition of amoxicillin. This three-drug combination has widespread activity against a panel of clinical isolates, within a therapeutically achievable concentration range. Finally, an *in vitro* model of persistence in *M. abscessus* infection was developed and this was used to assess frontline drug susceptibilities, providing an insight into the possible causes of treatment failure for this infection. Overall, this body of work contributes to the knowledge of the organism, provides a greater understanding of the clinical challenge it represents and proposes a new treatment option for patients suffering with this deadly infection.

For my parents

Publications associated with this work

Lopeman, R., Harrison, J., Rathbone, D., Desai, M., Lambert, P. and Cox, J.A.G (2020). Effect of Amoxicillin in combination with Imipenem-Relebactam against *Mycobacterium abscessus*. *Nature Scientific Reports* 10(928): https://doi.org/10.1038/s41598-020-57844-8

Lopeman, R., Harrison, J., Desai, M. and Cox, J.A.G (2019). *Mycobacterium abscessus*: Environmental Bacterium Turned Clinical Nightmare. *Microorganisms* 7(3):90: 10.3390/microorganisms7030090.

Acknowledgements

I would like to thank Dr Jonathan Cox and Dr Maya Desai for giving me the opportunity, as well as their support, to pursue the studies presented in this thesis. For his unwavering support and expert knowledge, I would like to thank Professor Peter Lambert. I would also like to thank Aston University and the Birmingham Women's and Children's Hospital for funding me through my PhD. I would further like to thank Dr Dan Rathbone for his indispensable contribution to my third thesis chapter with his *in silico* modelling work. For her contribution to the anaerobic results of my fourth chapter, I would like to thank Savannah Gibson.

A special thanks goes to Dr James Harrison, for his invaluable help with every aspect of my PhD studies. His expert guidance on a variety of microbiological and biochemical techniques, particularly the enzyme kinetics shown in chapter 3, has helped me immeasurably during my PhD, not to mention his tirelessness in answering my many, many questions over the years.

To Federica, Savannah, Laura, Bella, Sarah, and everyone in lab 327, I would like to thank you all for your friendship and support, for providing the much-needed fun and laughter during my studies.

Finally, I would like to say a big thank you to my wonderful parents, Ann and Pete, my sister Alice, my partner Ryan, and my best friends Ellis and Melissa for their love and support through the ups and downs of the past four years. I truly could not have done it without you.

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List of abbreviations

% - Percent

AECB – acute exacerbation of chronic bronchitis

ADC – albumin-dextrose-catalase

AST – antibiotic sensitivity testing

ATS – American Thoracic Society

BAL – bronchoalveolar lavage

Bla_{Mab} – *M. abscessus* beta-lactamase

BMDM – bone-marrow-derived murine macrophages

BTS – British Thoracic Society

CAP – community-acquired pneumonia

CF – cystic fibrosis

CFU – colony-forming unit

CGH – comparative genomic hybridisation

CLSI – Clinical and Laboratory Standards Institute

CNS – central nervous system

DAP - diaminopimelic acid

DBO - diazabicyclooctanes

DDT – dithiothreitol

DNTPs – deoxyribonucleotide triphosphate

DSP – drug susceptibility pattern

DST – drug susceptibility testing

FDA – Food and Drug Administration

FEV1 – forced expiratory volume

GPLs – glycopeptidolipids

GTE – glucose-tris-EDTA

HPLC – high performance liquid chromatography

IMAC - Immobilised Metal Affinity Chromatography

MAC – *Mycobacterium avium* complex

mAGP – mycolyl-arabinogalactan-peptidoglycan

MBL – metallo-β-lactamase

MDR-TB – multi-drug resistant TB

MIC – minimal inhibitory concentration

MFS – Marfan syndrome

mL – millilitre

mm – millimetre

MRSA – methicillin-resistant *Staphylococcus aureus*

MS – mass spectrometry

MS/MS – tandem mass spectrometry

MSSA – methicillin-susceptible *Staphylococcus aureus*

MTBC – *Mycobacterium tuberculosis* complex

NAG - *N*-acetyl-glucosamine

NAM - *N*-acetyl-muramic acid

NRP – non-replicating persistence

NTM – non-tuberculous mycobacteria

NTM-PD – non-tuberculous mycobacteria pulmonary disease

OADC – oleic-acid albumin dextrose catalase

OD – optical density

ORF – open reading frame

PBP – penicillin binding protein

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PG - peptidoglycan

PIMs - phosphatidy-*myo*-inositol mannosides

PCR – polymerase chain reaction

TGF-β – transforming growth factor beta

TL -1 - phosphor-*N*-acetylmuramyl-pentapeptide-translocase

TNF-α – tumour necrosis factor alpha

OADC – oleic acid-albumin-dextrose-catalase

OL – outermost layer

ORF – open reading frame

RGM – rapidly-growing mycobacteria

RT-PCR – Reverse transcription polymerase chain reaction

SNP – single nucleotide polymorphism

SDW – sterile distilled water

SSTI – skin and soft tissue infection

TB - tuberculosis

TLC – thin-layer chromatography

TLR – toll-like receptor

VAP – ventilator-associated pneumonia

VNTR – variable number tandem repeat

UK – United Kingdom

USA – United States of America

UTI – urinary tract infection

WGS – whole genome sequencing

XDR-TB – extensively-drug resistant TB

ZOI – zone of inhibition

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Chapter 1: General Introduction

1.1. Mycobacterium abscessus

The *Mycobacterium abscessus* complex is a group of rapidly growing (RGM) non-tuberculosis mycobacteria (NTM) that was first described in 1953 (Moore & Frerichs, 1953) and has since come to be regarded as a major human pathogen that is responsible for a range of lung and soft tissue infections. *M. abscessus* is now considered one of the prominent mycobacteria involved in broncho-pulmonary infections in people with Cystic Fibrosis (CF) or chronic pulmonary disease such as bronchiectasis (Esther, et al., 2010) (Griffith, et al., 1993) (Sermet-Gaudelus, et al., 2003) (Radhakrishnan, et al., 2009)

NTM are mycobacteria that are not the causative agent of tuberculosis (*Mycobacterium tuberculosis*) or leprosy (*Mycobacterium leprae*). They are primarily environmental organisms that rarely cause disease in humans. Like all mycobacteria, they are aerobic, non-motile organisms that stain positively with acid-fast staining. Their cell wall is unlike other groups of bacteria in that it is unusually thick, lipid rich and hydrophobic. This unusual cell wall composition renders mycobacteria especially resistant to heavy metals, disinfectants and antibiotics (Johnson, 2014) (Jarlier, 1994). They are also resistant to high temperatures and somewhat resistant to low pH (Kirschner, et al., 1992).

NTM are ubiquitous in the environment, especially water sources and soil. They are prone to biofilm formation and this contributes to their ability to persist in harsh environments (Johnson, 2008). Their ubiquitous nature and hardiness pose a significant risk to human health, particularly in that NTM are able to persist in environments that are in close proximity to human populations, particularly human water sources, hospital water supplies (sinks, showerheads), and homes. The persistence and spread of NTM species within healthcare environments is increasingly becoming a serious threat to human health (Faria, 2015).

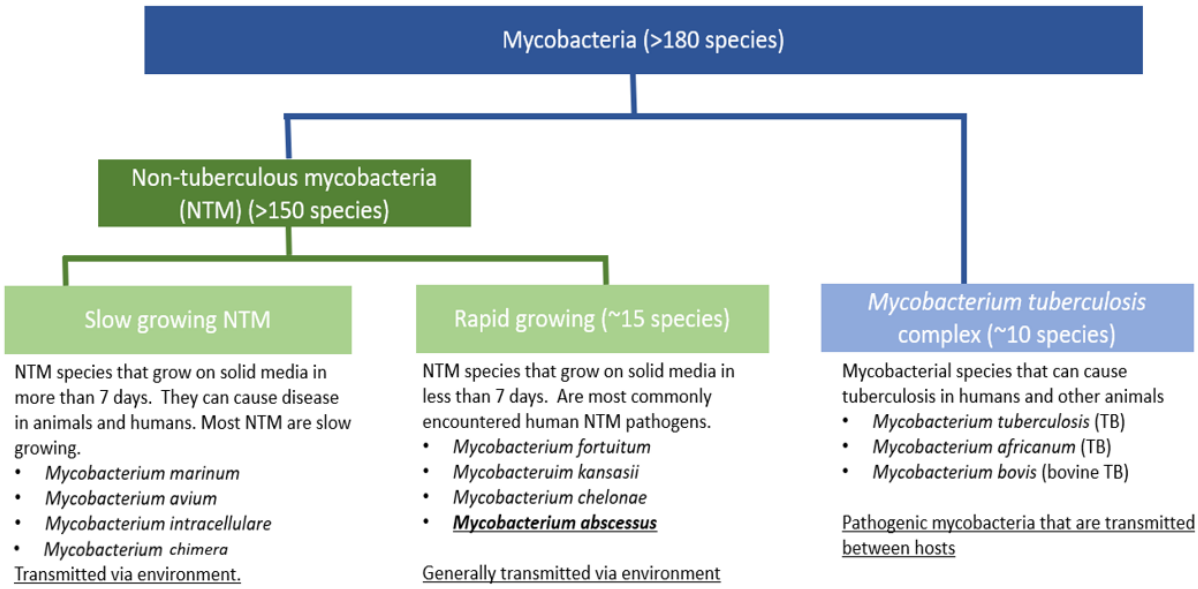


Figure 1.1. Flow chart showing the groups of species that make up the family of bacteria called Mycobacteria. Mycobacteria can be grouped into two overarching classifications: non-tuberculous mycobacteria (NTM), and the much smaller Mycobacterium tuberculosis complex (MTBC). NTM can be further classified based on its growth on solid media; either slow growing (more than 7 days), or fast growing (less than 7 days). The group of NTM that are rapidly growing (RGM) are considered to be the most clinically important species of NTM. Both types of NTM are generally transmitted via the environment and MTBC are exclusively transmitted between hosts.

RGM are grouped together on the basis that they all grow on solid media in 7 days or less (figure 1.1). They are only distantly related to the slow growing mycobacterial species, despite sharing some similarities, and possess a number of features that mimic that of the non-mycobacterial actinomycetes, such as susceptibility to antibiotics including macrolides, tetracyclines and aminoglycosides, and resistance to the first-line anti-TB drugs, isoniazid, rifampicin, ethambutol and pyrazinamide (Wallace, 1996).

Of all the RGM discovered, *M. abscessus* is undoubtedly the most dangerous because it is able to cause disease in immunocompetent patients and is resistant to all front-line anti-tuberculous drugs and almost all other chemotherapeutic agents (Medjahed, et al., 2010) (Sanguinetti, et al., 2001). Occasionally *M. abscessus* is grouped together with *M. chelonae*, these two species are often referred

to as the *M. chelonae-abscessus* group, and these mycobacteria account for 95% of disseminated cutaneous infections caused by RGM (Brown-Elliott and Wallace, 2002). However, pulmonary infection accounts for the majority of *M. abscessus* clinical isolates, with one study showing that 82% of RGM isolated from patients with pulmonary disease were *M. abscessus* species (Brown-Elliott and Wallace, 2002).

M. *abscessus* is a weakly staining Gram-positive mycobacterium and is, like other NTM, most often seen in soil and aquatic environments (Cortes, et al., 2010). The bacillus-shaped bacterium is 1-6µm long and 0.2-0.5µm in diameter, with curved ends and serpentine cords sometimes observed (Cortes, et al., 2010) (Howard, et al., 2006). On solid agar, *M. abscessus* can display either a rough or smooth morphotype with the rough morphotype displaying a more virulent phenotype than its smooth variant.

1.2. History and Taxonomy of *M. abscessus*

Between its first discovery and the present day, the taxonomy and nomenclature of *M. abscessus* has been unusually turbulent and wrought with inconsistencies (Figure 1.2). Due to the genetic similarity between different RGM species, defining what makes a species a species in these organisms has proven to be difficult. For two decades after its discovery in 1952 (Moore, 1953), *M. abscessus* enjoyed species status. In 1972, however, following a co-operative numerical analysis of RGM, it was determined that *M. abscessus* is in fact a subspecies of a closely related RGM, *M. chelonae* (Kubica, 1972). For a further two decades, researchers and clinicians treated *M. abscessus* as a pathogen belonging to the *M. chelonae* complex. This was a mistake because the clinical features, treatment options and treatment outcomes differ greatly between *M. abscessus* and *M. chelonae.* Firstly, *M. chelonae* primarily causes skin disease following corticosteroid therapy (Wallace, 1992), is highly resistant to cefoxitin (minimal inhibitory concentration (MIC) >32 µg/mL) (Swenson, 1985) (Wallace, 1991), and has never been implicated as a cause of otitis media (infection of the middle ear) (Wallace, 1997). *M. abscessus*, on the other hand, is more virulent in that it is able to cause disease in

immunocompetent hosts, is commonly associated with nosocomial disease, and has been reportedly responsible for over 90% of chronic otitis media following insertion of tympanostomy tubes (Franklin, 1994), ironically, tympanostomy tubes are primarily inserted into the middle ears of patients who suffer chronic or recurrent otitis media, but it appears that this increases the risk of *M. abscessus* otitis media. Furthermore, unlike *M. chelonae*, *M. abscessus* displays susceptibility to cefoxitin, but both *M. chelonae* and *M. abscessus* are susceptible to clarithromycin (Brown, 1992). Clarithromycin plus cefoxitin was a commonly used regimen against *M. chelonae* (and therefore *M. abscessus*), but this regimen was ineffective against *M. chelonae* (Wallace, 1997). Thankfully in 1992, genetic hybridisation studies revealed that *M. chelonae* and *M. abscessus* were indeed independent species, and the authors thus recommended that *M. abscessus* once again be elevated to species status (Levy-Frebault, 1986) (Kusunoki, 1992).

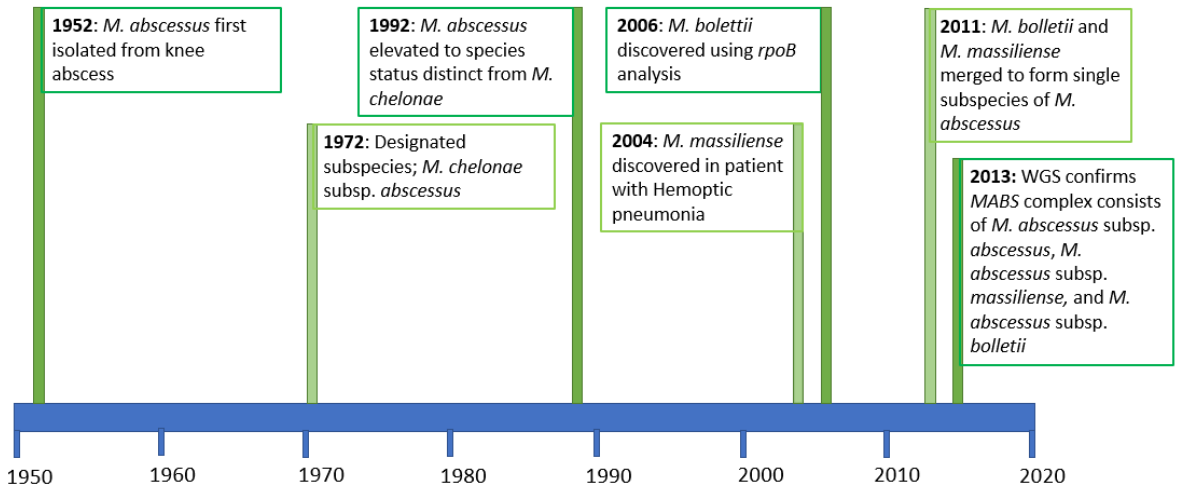


Figure 1.2. Timeline showing changes in taxonomy and species status of* M. abscessus *and its subspecies;* M. abscessus *subsp.* massiliense, M. abscessus *subsp.* bolletii, *and* M. abscessus *subsp.* abscessus. *From the time that* M. abscessus *was first isolated from a knee abscess in 1952, it took over 60 years for scientists to discover that not only is* M. abscessus *a species in its own right, but it encompasses a complex that displays varying antimicrobial susceptibilities and treatment outcomes.

The story of *M. abscessus* was far from over. In 2004, Adékambi *et al.* discovered a new species of rapidly growing *Mycobacteria.* The isolated strain was found to be similar in antimicrobial susceptibility profiles and biochemical profiles to both *M. abscessus* and *M. chelonae,* but with some major differences; for example, the β-galactosidase, β-*N*-acetyl-β-glucosaminidase, and β-glucuronidase activities were different in *M. abscessus* and this novel species. The use of 16S rRNA sequencing revealed 100% identity with *M. abscessus,* however partial *rpoB* and *recA* gene sequencing revealed 96% and 98% similarity, respectively. Subsequent phylogenetic analysis suggested that the new species, named *M. massiliense,* was perhaps recently derived from *M. abscessus,* and therefore was grouped in with the *M. abscessus-M. chelonae* group (Adekambi, 2004).

For many years, 16S rRNA sequencing was considered the first-line for differentiating species of mycobacteria from clinical samples (Pauls, 2003) (Tortoli, 2001) (Turenne, 2001). Traditional laboratory techniques such as phenotypic analysis and cell wall fatty acid composition were insufficient in distinguishing species due to similar patterns. Unfortunately, it became apparent that 16S rRNA sequencing was also not appropriate for this application, ambiguous results can stem from isolates harbouring two 16S rRNA sequences that are not homologous (Adekambi, 2004) (Ninet, 1996) (Reischl, 1998) (Turenne, 2001), furthermore, more closely related RGM cannot be differentiated due to highly conserved 16S rRNA identity, indeed it has been shown that 16S rRNA sequencing leaves 37% of NTM unclassified (Pauls, 2003).

In 2003, partial sequencing of the *rpoB* gene encoding the β-subunit of bacterial RNA polymerase was developed (Adekambi, 2003). The *rpoB* gene is a single copy gene that contains conserved regions flanked by highly variable regions (Boor, 1995), and the most variable region was determined (Adekambi, 2003); primer pairs for this variable region were designed, and a 764 bp *rpoB* region was amplified, from which a 723 bp amplicon was sequenced. Comparing these partial *rpoB* sequences of different RGM species showed higher sequence variability compared with 16S rRNA sequencing; *rpoB* variation was 84.3 – 96.6% and 16S rRNA variation was just 95.7 – 99.7% (Adekambi, 2003).

Then in 2006, this new tool was applied to investigate a collection of 59 RGM, 9 of which exhibited three original *rpoB* sequences, which the authors believed to be indicative of a novel species. One of these original *rpoB* sequences was found to have 95.6% identity with that of *M. abscessus*, but it shared 100% identity with its 16S rRNA sequence. This new species, subsequently named *Mycobacterium bolletii*, was determined to be closely related to *M. abscessus*, and was shown to be multidrug resistant, most notably to clarithromycin, and with intermediate resistance to amikacin (Adekambi, 2006).

In 2009, a comprehensive analysis involving biochemical tests, high performance liquid chromatography (HPLC), susceptibility testing, polymerase chain reaction (PCR) -restriction enzyme analysis of *hsp65*, gene sequencing of *rpoB* and *hsp65*, DNA-DNA hybridisation, phylogenetic tree construction, and analysis of the 16S rRNA sequencing suggested that *M. abscessus, M. massiliense,* and *M. bolletii* represent a single species, and the subspecies *M. abscessus* subsp. *abscessus*, and *M. abscessus* subsp. *massiliense* (where *M. massiliense* and *M bolletii* were merged), were proposed (Leao, 2009). In 2011, the same group of researchers declared that *M. abscessus* subsp. *massiliense* is not in accordance with the *Bacteriological Code* (1990 revision), and that the name *M. bolletii* has priority over the name *M. massiliense*, and therefore, *M. abscessus* subsp. *massiliense* should henceforth be named *M. abscessus* subsp. *bolletii.* The official line was that the *M. abscessus* complex now includes *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii* (Leao, 2011).

In 2013, whole-genome sequencing (WGS) analysis on *M. abscessus* isolates showed deep genetic divisions that correspond to the three *M. abscessus* subspecies, despite showing a high overall genetic similarity. The authors concluded that *M. abscessus* subsp. *massiliense* and *M. abscessus* subsp. *bolletii* are distinct subspecies and should not be grouped together as previously proposed (Bryant, 2013) (Leao, 2011).

1.3. Environmental niche and transmission

Like many other pathogenic RGM species, *M. abscessus* is an environmental pathogen that is particularly widespread in drinking water supplies and other water reservoirs (Falkinham, et al., 2001). *M. abscessus*, like other NTM, is able to survive in harsh, nutrient-starved environments where other competing microorganisms would not survive, such as in chlorinated water (McGrath, et al., 2010).

The presence of the lipid-rich cell wall results in a hydrophilic cell surface which facilitates the formation of biofilms, their slow growth, and adherence to surfaces thus aiding their survival (Primm, et al., 2004) (Brennan, 1995) (Bendinger, et al., 1993). Furthermore, many RGM are oligotrophic, requiring low levels of two carbon sources and minimal amounts of metal ions (Falkinham, 2010), further indicating their hardiness and persistence in many harsh environments. The impenetrable nature of the *M. abscessus* cell wall in comparison to other non-mycobacterial pathogens also contributes to its resistance against many antibiotics and disinfectants (Jarlier, 1990) (Nessar, et al., 2012). The ability of *M. abscessus* to survive in the human environment presents a huge problem for healthcare, and most studies up until this point suggest that NTM are predominantly acquired from the environment by patients with CF (Bryant, et al., 2013). In 2009, Feazel *et al.* demonstrated that showerheads provide an enriched environment for NTM biofilm formation; where the presence of human pathogens including NTM were >100 fold higher in showerhead biofilms compared to the background water contents (Feazel, et al., 2009). A study in Hawaii showed the prevalence of NTM in household plumbing; areas such as showerheads, sinks, taps, shower drains, and refrigerator water dispensers were swabbed by volunteers. The authors found that 69% of households surveyed had clinically significant NTM colonisation, of which 10% was *M. abscessus* (Honda, et al., 2016).

A major study was published in 2013 in which WGS was used to identify transmission of *M. abscessus* complex between patients at an adult CF centre in the UK between 2007 and 2011 (Bryant, et al., 2013). The authors found a high level of genetic relatedness between isolates of *M. abscessus* subsp. *abscessus*, but clusters were clearly segregated from one another, indicating that patients have

independently acquired either genetically diverse strains or a dominant circulating clone. In the case of *M. abscessus* subsp. *massiliense*, however, the authors found isolates from different individuals with almost identical genomic sequences, strongly indicating transmission between patients. Analysis of the environment revealed no NTM species isolated from the water supply to the clinic, showerheads, dish washers, bronchoscopes or the local River Cam or Papworth Hospital Pond. Further investigation into possible transmission routes revealed patients with isolates from the same genetic relatedness clusters were actually present in the clinic at the same time, further supporting the conclusion that *M. abscessus* subsp. *massiliense* is likely transmitted from patient to patient rather than independently from the environment, as previously believed. This is clinically important because if a pathogen is transmitted between CF patients it may require patients infected with *M. abscessus* to be segregated from *M. abscessus*-naïve patients to prevent transmission.

Following on from the localised retrospective study published in 2013 (Bryant, et al., 2013), worldwide WGS was performed on 1080 isolates from 517 patients from the UK, USA, Republic of Ireland, mainland Europe and Australia (Grogono, et al., 2017). They found that the majority of isolates were from densely clustered genotypes that were not diverse, suggesting a high level of human-human transmission. Phylogenetic analysis also revealed that there are 3 dominant circulating clones globally, and these clones were associated with higher virulence and poor clinical outcomes. The authors concluded that human-human transmission appears to have facilitated the evolution of *M. abscessus* from an environmental pathogen to a transmissible human pathogen (Grogono, et al., 2017).

In 2014, a small-scale study was performed on 27 *M. abscessus* isolates from 20 paediatric CF patients (Harris, et al., 2015). The authors used a combination of epidemiology, variable number tandem repeat (VNTR) profiling and WGS to find evidence of cross-infection between paediatric CF patients. They hypothesized that patients with strains that had identical VNTR profiles would have had intense exposure to each other compared with patients with strains that had different VNTR profiles. They found little evidence of transmission between patients, except for 2 patients who were siblings and

therefore had higher intensity of exposure. They concluded that cross-infection was uncommon in their cohort, and that transmission is most likely to be from a common environmental source, the authors noted that their findings contrasted with the study by Bryant, *et al*. in 2013. Further evidence of patient-patient transmission of *M. abscessus* was found in 2017 following genomic studies of 64 isolates from CF patients. They found there was significant genomic and phylogenetic evidence of the circulation of dominant clones between CF patients across a wide geographical area (Alateah, et al., 2017).

The current literature is generally lacking in information on the transmission of *M. abscessus.* There is however a growing body of evidence that human transmission is occurring between CF patients, but more research is needed. Nonetheless, in 2017 the Cystic Fibrosis Trust published guidelines for *M. abscessus* infection control (Jones, et al., 2017). The recommendations include general infection control measures such as hand washing, the segregation of patients with *M. abscessus* infection from all other CF patients and performing clinical procedures solely in the patient's room (Jones, et al., 2017).

1.4. Morphology, virulence and persistence

1.4.1 Morphology

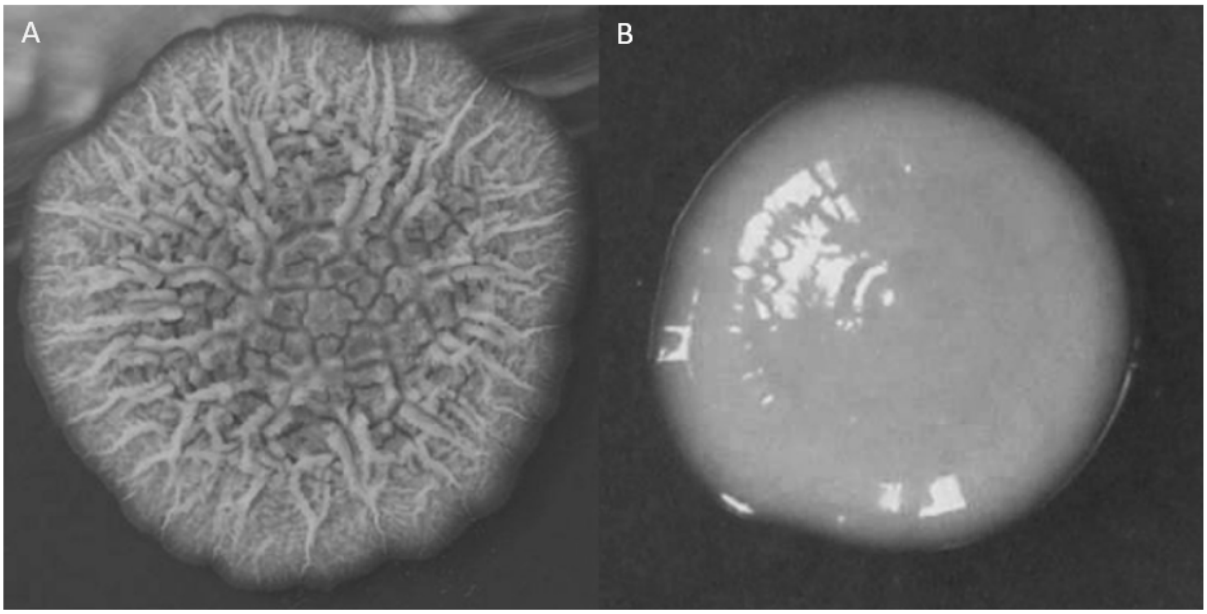


Figure 1.3a-b. Photographs of the two distinct colony morphotypes of M. abscessus. Figure 1.3a shows an M. abscessus colony displaying the rough morphotype, with distinct ridges and a dry appearance. Figure 1.3b shows an M. abscessus colony displaying the smooth morphotype, with no ridges, and a smooth, wet consistency. (Adapted from Ruger et al., 2013* (Ruger, 2013)*)

M. abscessus can display two distinct morphotypes on solid media; rough (*M. abscessus-R* and smooth (*M. abscessus-S*) (Fregnan, 1962) (Fig 1.3a-b). In 1999, Byrd and Lyons showed that *M. abscessus-R* is the more virulent of the two and whilst both variants are phagocytosed by human monocytes, there were marked differences in the phagosome; *M. abscessus-R* resides in a tight phagosome, which is typical for pathogenic mycobacteria; whereas *M. abscessus-S* resides in a 'loose' phagosome, where the cell wall is separated from the phagosomal membrane. Byrd and Lyons theorised that this may result in a difference between the intracellular trafficking of both variants, resulting in an inability of *M. abscessus-S* to prevent phagosome-lysosome fusion, which in turn exposes *M. abscessus-S* to the toxic lysosomal environment. Furthermore, *M. abscessus-R* forms microcolonies that display significant cording, whereas *M. abscessus-S* microcolonies are smaller, rounded and do not exhibit cording. Previous studies have shown that these characteristics can be used to differentiate virulent from avirulent *M. tuberculosis (MTb)* (Byrd, 1998). It has been suggested that cord formation enables *M. abscessus* to spread rapidly by allowing bacteria to grow in one direction, essentially travelling between host cells in a quicker fashion, and ultimately 'outrun' the immune response (Byrd, 1999). Live studies using zebrafish models show that *M. abscessus-R* forms extensive cords that are larger than the host immune cells, allowing them to physically avoid phagocytosis (Bernut, 2014). Furthermore, cords can also aid the formation of abscesses, which can disseminate throughout the central nervous system (of zebrafish), and can even disrupt blood vessels causing haemorrhage and death (Bernut, 2014). The inhibition of cording has been shown to induce an attenuated phenotype of *M. abscessus* (Bernut, 2014).

Mutations in *hadC*, which encodes a component of the HadBC dehydratase complex of mycolic acid biosynthesis, result in a loss of cording ability and subsequent attenuation in *MTb* (Slama, 2016);

similar genes have been identified in *Mycobacterium smegmatis* (*MSMEG_6754*) (Halloum, 2015). A blast analysis performed by Halloum *et al.* revealed a gene that was homologous to *MSMEG_6754* in *M. abscessus*, subsequently named *MAB_4780* (Halloum, 2016). *MAB_4780* was found to encode a mycolic acid-associated dehydratase similar to that of the *MTb hadC*. A *MAB_4780* deletion strain of *M. abscessus* was found to have lost all virulence traits typical of *M. abscessus-R* strains (cording, virulence, intramacrophage survival), indicating that *MAB_4780* and therefore cord formation, plays a critical role in the pathogenicity of *M. abscessus-R* (Halloum, 2016).

Colony morphology of NTM is known to be influenced by cell wall glycopeptidolipids (GPLs) (Barrow, 1982) (Eckstein, 2000), but in 2006, Howard *et al.*, during serial passage of a smooth variant of *M. abscessus* (named 390S), it was observed that one colony appeared to have spontaneously reverted back to the rough morphotype (subsequently named 390V). Using genetic techniques, the authors were able to show that the 390V strain did indeed arise directly from the 390S strain, and not via contamination of a rough variant of *M. abscessus.* They went on to show, using Thin-Layer Chromatography (TLC), that 390V, along with other *M. abscessus-R* strains, lacked lipid bands that were present in 390S and other *M. abscessus-S* strains. These lipid bands aligned with the GPLs from *M. smegmatis*, which suggested that only smooth variants of *M. abscessus* express GPLs. Because 390V was directly derived from 390S, this indicates that high level GPL expression is a reversible phenotype, with a reversion rate of $1:10^5$-10^6 (Howard, et al., 2006). This may have clinical implications, as people with underlying structural lung disorders are at risk of bacterial colonisation in the form of biofilms, meaning *M. abscessus* strains with a smooth morphotype may be able to cause chronic pulmonary infection in these patients, and a rough variant can then arise, resulting in a significantly more invasive infection. Genomic analysis was performed on smooth-to-rough transition in *M. abscessus* and it revealed a Y842H substitution in *Mmpl4a* gene that resulted in loss of GPL production in *M. abscessus-R* variants.

1.4.2. Virulence

For a pathogen to cause infection in its host, it must first evade or impair the host immune response. In healthy individuals, mucociliary clearance in the airways is sufficient to prevent *M. abscessus* colonisation, however, in some individuals with impaired mucociliary clearance, such as those with CF, *M. abscessus* can colonise and form biofilms in the airway (Rhoades, 2009) (Gibson, 2003). The next line of defence is the host's innate immune response mediated by toll-like receptors (TLRs) that are present on the cells in the airway, such as alveolar macrophages, dendritic cells, and mucosal epithelial cells (Bals, 2004) (Crevel, 2002). TLR interactions have been studied in *MTb,* where TLR2 binds to *MTb* TLR ligands called the phosphatidy-*myo*-inositol mannosides (PIMs) (Krutzic, 2004). In 2009 Rhoades *et al.* demonstrated that both *M. abscessus-S* and *M. abscessus-R* are able to stimulate TLR2 and lead to TNF-α release, however the GPLs present in *M. abscessus-S* effectively 'masks' the PIMs from the TLR2 receptors, allowing the bacteria to go unnoticed by the innate immune response (Rhoades, 2009). They also showed that temperature affects the expression of GPLs; rough variants of *M. abscessus* grown at 23 °C express the full GPL profile, but loses this expression at 37 °C, and smooth variants express GPLs at both temperatures (Rhoades, 2009). The reasoning behind this is unclear, however there may be some evolutionary benefit to high expression of GPLs at 37 °C. If *M. abscessus* is able to mask its PIMs from the host due to a change in GPL expression caused by a temperature of 37 °C, this may mean that *M. abscessus* has evolved to change its GPL expression and therefore virulence upon entering its host.

GPLs affect virulence in a myriad of ways; in 2016 Roux *et al.* investigated the effect of *M. abscessus* cell morphology on the host macrophage response (Roux, 2016). Bone-marrow-derived murine macrophages (BMDM) were infected with *M. abscessus-R* or *M. abscessus-S*, and phagocytic uptake, intracellular growth, phagosomal morphology and clearance were compared. They found that 80% of the phagosomes harbouring *M. abscessus-S* contained only one bacterium (lone phagosomes), whereas 60% of the phagosomes harbouring *M. abscessus-R* were social and contained multiple

bacteria. Furthermore, *M. abscessus-R* variants were highly aggregative and forms clumps/cords outside the cell membrane. They summarised that *M. abscessus-R* and *M. abscessus-S* can be regarded as two representatives of one isolate, which can evolve within the host in response to host immunity. It is likely that the infecting form is *M. abscessus-S* as it forms biofilm-like structures and has been shown to prevent TLR2 signalling in respiratory epithelial cells (Davidson, 2011). It is then able to survive within phagosomes that are unable to fuse with lysosomes, as well as being inefficient at inducing autophagy and apoptosis (Roux, 2016) (de Chastellier, 2009) (de Chastellier, 1997). This finding was unusual as other RGM (such as *M. smegmatis* and *M. fortuitum*) reside in phagolysosomes and are rapidly eliminated by the host macrophages (Roux, 2016), so the *M. abscessus-S* variant is said to resemble the phenotypic traits of SGM such as *M. marinum* and *M. avium* (Roux, 2016). The *M. abscessus-R* variant tends to form chains or large clumps that reside in phagocytic clumps rather than being internalised which are then processed into phagolysosomes. Unlike other species of bacteria, it is well established that mycobacteria can evade phagolysosome degradation (de Chastellier, 2009), and *M. abscessus-R* has further been shown to induce the formation of autophagic vacuoles, resulting in macrophage apoptosis (Roux, 2016). This ability of *M. abscessus-R* to induce apoptosis of the host macrophages may aid it in reaching the extracellular environment where it can form cords and ultimately abscesses (Roux, 2016) (Bernut, 2014), which is more in line with the phenotypic traits of other RGM that includes a particular extracellular state not seen in SGM or *MTb.* In summary, the ability of *M. abscessus* to adapt and survive a changing environment means that scientists should be aware of the rapid gene mutations that can potentially result in more pathogenic traits in *M. abscessus*.

1.4.3. Persistence

The high levels of similarities between NTM and *Mtb* pathology in pulmonary infections lends itself to the likelihood that similar factors that cause persistence are at play. In *Mtb* pathology, intracellular infection and caseum formation result in the pathogen being exposed to hypoxic and nutrient

deprived environments, which typically leads to the emergence of phenotypic drug resistance (Lanaerts, 2015). A well-known aspect of *Mtb* pathology is the formation of caseous granulomas, in which a distinct lack of vasculature results in a hypoxic environment (Wu, 2018). As NTM lung disease shares many pathological traits with *Mtb,* with nodular bronchiectatic and fibrocavity formation being a hallmark of NTM lung infection (Griffith, 2007), it is possible that *M. abscessus* may have evolved similar metabolic adaptations to oxygen and nutrient starvation in the human host as *Mtb*. Furthermore, comparative genomic studies have shown that NTM and *Mtb* harbour a conserved dormancy regulon, *dosR*, which has been shown to regulate dormancy in *Mtb* persistent infection (Gerasimova, 2011). Models of hypoxia and nutrient starvation in *M. abscessus* have already shown that this microorganism is indeed able to survive within these harsh environments, lying in a "dormant" state, not unlike that of *Mtb* (Yam, 2020). As NTM are obligate aerobes, requiring oxygen for growth, and many of the antibiotics used to target *M. abscessus* require actively growing cells, this may partly explain why *in vitro* drug susceptibility testing results are not always concordant with *in vivo* treatment outcomes.

1.5. Clinical features

1.5.1. Pulmonary Disease

The most common clinical manifestation of *M. abscessus* infection is pulmonary disease, and it primarily affects vulnerable patients who suffer from underlying structural lung disease such as bronchiectasis, CF and prior tuberculosis infection (Lee, et al., 2015) (Griffith, et al., 2007). Symptoms of NTM pulmonary disease vary and are often non-specific, however, the majority of patients present with a chronic or recurring cough. Other symptoms may include excess sputum production, malaise, dyspnea, fever, haemoptysis, chest pain and weight loss (Griffith, et al., 2007).

Understanding the pathophysiology of NTM lung disease has been a slow process taking over 30 years. It was long assumed that NTM infections were analogous to TB infections with the exception that TB is transmitted between humans whereas NTM infection was thought not to be transmitted between

humans. The similarities between TB and NTM were primarily thought to be clinical presentation and characteristics (Griffith, 2016), however it is now known that NTM is more likely to present as non-cavitary radiographic changes as opposed to upper lobe fibrocavity abnormalities usually seen with *MTb* pulmonary infection (Griffith, et al., 2007) (Prince, et al., 1989). Due to the fact that NTM species are ubiquitous in the environment, (unlike *MTb* and *M. leprae* which require a living host and are transmitted patient to patient or zoonotically), it is clear that NTM exposure is extremely common, whereas NTM disease is still relatively rare. Those with pre-existing lung diseases undoubtedly have some predisposition to NTM infection, leading some to describe a "two-hit" theory of NTM disease acquisition (Griffith, 2016).

It is well documented that a risk factor for NTM pulmonary disease is in patients with low body fat. The mechanisms behind this are not well understood, however it is possible that leptin plays a role in NTM predisposition. Leptin has several immunomodulatory functions; Lord et, al., (1998) showed that leptin modulates the T-cell immune response, and that a falling leptin concentration acts as a signal of starvation, resulting in energy preservation. In this state, the human adaptive immune response, which requires large scale clonal expansion, is subdued due to lack of resources (Lord, et al., 1998). Further investigation specific to *M. abscessus* in animal models, performed by Ordway, *et al.* in 2008, showed that leptin-deficient ob/ob mice challenged with high-dose aersol of *M. abscessus* developed established infection and a pulmonary immune response consisting of an early influx of IFN-γ+ CD4+ T cells, resulting in successful clearance of *M. abscessus* infection (Ordway, et al., 2008).

Another patient group with no underlying pulmonary disease that appear to have a predisposition to NTM infection are those with thoracic cage abnormalities such as Marfan syndrome (MFS) (Honda, et al., 2015). MFS is a genetic disorder of the connective tissue caused by mutations in the extracellular matrix protein fibrillin 1. Many of the aspects of this disease are caused by abberant regulation of transforming growth factor beta (TGF-β), a cytokine known to suppress macrophage function (Judge, 2006), and studies on NTM infection using mouse models have shown that early elevated levels of

TGF-β1, along with downregulation of tumor necrosis factor alpha (TNF-α) receptors, leads to impaired anti-mycobacterial activity in the host (Champsi, et al., 1995).

1.5.1.1. CF and M. abscessus pulmonary disease

Before 1990, NTM infection was not often associated with CF. However, since then, reports of *M. abscessus* infection (along with other NTM species) have been increasingly common. Several wide-scale studies have been performed over the past decade or so, revealing an NTM prevalence in CF patients in some areas as high as 20% (Table 1.1)

Age is a strong correlator of NTM infection in this group, with 40% of CF patients over the age of 40 having NTM smear positive results, as opposed to 4-20% in the under 40s population (Griffith, et al., 2007). Other risk factors for NTM infection in CF patients appears to be lower BMI values, worse forced expiratory volume (FEV_1), current infection with *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*, experience of pneumothorax requiring chest drain, the use of inhaled antibiotics and other medical interventions (Viviani, et al., 2016). One study performed in Israel found a significant association between *Aspergillus* species and NTM species in sputum cultures of CF patients (Levy, et al., 2008).

Table 1.1: Prevalence of NTM lung disease in CF patients in differing geographical areas between 2004 and 2014.

Study	Location	Sample size	NTM prevalence in CF
Oliver, KN. (2004) (Olivier, 2004)	USA	750	13% (majority *M. avium* complex)
Roux, AL, et. al. (2009) (Roux, et al., 2009)	France	1582	6.6% (*M. abscessus* most common)
Seddon, P, et. al. (2013) (Seddon, et al., 2013)	UK	3805 adults 3317 children	5% adults 3.3% children
Adjemian, J, et. al. (2014) (Adjemian, et al., 2014)	USA	18,003	10-20%; depending on area
Mussaffi, H, et. al. (2005) (Mussaffi, et al., 2005)	Israel	139	8.6%

A study conducted across the United States in 2014 showed how along with individual host susceptibility, specific geographical area is a major determining factor in NTM prevalence. The study

used climatic data at the zip-code level to evaluate the effect of annual precipitation, temperature, wind speed, dew point, vapour pressure, saturated vapour pressure and humidity levels on NTM prevalence in the CF population. They found that saturated water vapour (vapour pressure is a measure of the amount of water in the air, and saturated vapour pressure is the maximum amount of water the air can hold at a given temperature) was the biggest environmental factor affecting NTM prevalence; in that areas with higher levels of moisture in the atmosphere had a higher prevalence of NTM pulmonary disease, as well as being linked to a higher prevalence of other pulmonary infections such as *P. aeruginosa* (Collaco, et al., 2011) (Adjemian, et al., 2014). It has been suggested that the reason for this link between atmospheric moisture and NTM prevalence is due to greater numbers of aerosolised bacteria circulating in air with a higher vapour pressure (Bowers, et al., 2013). Other studies have shown a link between residential dampness and mould and an increased risk of pulmonary infection, most likely due to excess water droplets and bacteria within them being deposited onto household surfaces and subsequent contact with these surfaces by CF patients (Fisk, et al., 2010). There is a distinct lack of data to support the findings from these studies, more research is needed to elucidate the link between climatic variations and NTM prevalence and to inform the clinical management of CF pulmonary infection in patients who live in areas with high saturated vapour pressure.

Despite uncertainty regarding its efficacy, CF patients with *M. abscessus* pulmonary infection are often given azithromycin, a macrolide antibiotic, in the initial phase of treatment. A study by Renna, *et al.*, (2011), however, revealed a worrying paradox concerning azithromycin usage in CF patients and an increased risk of NTM infection, particularly *M. abscessus* infection. They found that in primary human macrophages, the concentrations of azithroymcin achieved during therapeutic dosing inhibited autophagosome degradation and phagosome-lysosome fusion, which are vital components of an effective cellular response against mycobacteria (Vergne, et al., 2006). The inflammatory cytokines IFN-γ and TNF-α are important cytokines involved in the regulation of intracellular killing of mycobacteria, and the study showed that azithromycin significantly blocked IFN-γ and TNF-α-

dependant killing of *M. abscessus* in human macrophages. Furthermore, the authors found that azithromycin treatment in mice prior to challenging them with *M. abscessus* infection led to persistant pulmonary infection with granulatomous inflammation and a failure of macrophages to degrade intracellular mycobacteria. These findings raise an uncomfortable question regarding the treatment strategy of CF and NTM infection. It appears that azithromycin-mediated impairment in immune response to NTM infection outweighs any benefits of macrolide-based therapies. This conclusion is futher compounded by the fact that *M. abscessus* isolates often display an inducible *erm(41)* gene that results in macrolide resistance.

1.5.2. Skin and soft tissue infections (SSTIs)

SSTIs usually present following trauma or surgery (Wallace, et al., 1983) and *M. abscessus* is increasingly responsible for chronic pulmonary disease in immunocompromised patients, particularly patients with CF and people with bronchiectasis, especially elderly women (Griffith, et al., 2007) (Brown-Elliott and Wallace, 2002). There are several species of RGM that cause opportunistic infections in humans, however, *M. abscessus* is assuredly the most dangerous because it is capable of causing disease in immunocompetent individuals (Stout, et al., 2016).

There have been cases of *M. abscessus* outbreaks following the use of contaminated needles and other surgical instruments (Brown-Elliott & Wallace, 2002) and even, as was the case in a cohort of 'lipotourists' (i.e., people who travel abroad for cosmetic surgery for fat removal), severe outbreaks following cosmetic surgery (Furuya, et al., 2008). Interestingly, *M. abscessus* has also been linked to late-onset wound infections following crush trauma sustained by Swedish survivors of the 2004 tsunami that killed over 200,000 people and caused serious crush injuries in another >2000. It is known that the infections occurred as a result of the tsunami rather than during medical treatment as pulsed-field gel electrophoresis (PFGE) typing revealed all the *M. abscessus* isolates to be unrelated. RGM other than *M. abscessus* were found to have caused infection in the tsunami survivors, however their infections were not often severe, and in fact often healed without any intervention. The *M. abscessus*

infections, however, tended to cause multiple abscesses and required longer treatment times (Appelgren, et al., 2008).

M. abscessus is also responsible for serious disseminated infections following transplantation (Osmani, et al., 2018). A single case study involving post-transplant *M. abscessus* SSTI infection in a CF patient resulted in disseminated pulmonary infection and eventually the death of the patient, despite aggressive pre and peri-operative anti-mycobacterial therapy (Taylor, 2006). For this reason, many have recommended that *M. abscessus* colonisation should be viewed as a contraindication to lung transplantation. This suggestion, however, has been met with criticism. Some studies have shown that it is possible to perform a lung transplant on patients with *M. abscessus* colonisation and that subsequent clearance of infection is possible, albeit with a strong possibility of severe complications (Lobo, et al., 2013) (Gillijam, et al., 2010). Despite this uncertainty surrounding the outcome of lung transplantation in patients colonised with *M. abscessus*, it is increasingly clear that effective treatments for *M. abscessus* lung infection must be developed, as lung transplantation is a potentially life-saving therapy for end-stage lung disease caused by CF. If *M. abscessus* colonisation continues to be considered a contraindication to lung transplant, many unnecessary deaths will occur.

1.5.3. Atypical infections

Aside from the more commonly encountered pulmonary infections and SSTIs, *M. abscessus* rarely causes disease in other areas of the body. Central nervous system (CNS) infections can be caused by *M. abscessus,* commonly manifesting as meningitis and cerebral abscesses (Lee, 2012). Unlike *M. avium* complex (MAC) which causes the majority of NTM CNS infections, *M. abscessus* causes CNS infections in HIV-seronegative patients, particularly patients who have undergone neurosurgical procedures (Lee, et al., 2015). From the little data available on these types of infections, a combination therapy including clarithromycin offers the best chance of treatment success (Lee, 2012) (Lee, et al., 2015).

Bacteraemia infections caused by *M. abscessus* have been reported in immunocompetent hosts following heart surgery (Sarma, 2011), septic arthritis (Shoichi, 2015), and even following the administration of intravenous Cytokine-Induced Killer Cell Therapy for so-called "body beautification" (Liu, 2013). *M. abscessus* causes more serious infections in immunocompromised patients, with co-morbidities such as lymphoblastic leukemia (Kim, 2014), diabetes and conditions that require administration of steroids (Lee, 2014).

NTM have been known to cause ocular infections since 1965 when an ocular infection with *M. fortiutum* was reported (Turner, 1965). Ocular infections caused by *M. abscessus* have seen a rise in the past decade. One study reported a fourfold increase in the number of NTM eye infections between the 1980's and 2000's (Girgis, 2011). A systematic literature review performed by Kheir *et al.* brought to light the true prevalence of *M. abscessus* ocular infections; 11% of 420 NTM eye infection case reports were *M. abscessus.* The main other culprits were *M. chelonae* (42.6%) and *M. fortuitum* (14.8%) (Kheir, 2015). Almost half of eye infections caused by NTM follow some kind of intervention such as orbital reconstruction or orbital implants (Kheir, 2015). Treatment for these infections involves combination antibiotic therapy usually including macrolides, and surgical resection of infectious lesions (Kheir, 2015). Unfortunately, due to the indolent nature of *M. abscessus* ocular infections, delays in diagnosis and treatment often occur, however treatment outcomes are generally good, except in immunocompromised patients who are at higher risk of vision loss (Kheir, 2015).

1.6. Treatment

Treatment for *M. abscessus* infection is particularly difficult due to its intrinsic and acquired resistance to most of the commonly used antibiotics. Treatment for soft tissue or skin infections can take between 2 weeks and 4 months, and this extends to up to 6 months for serious bone infections. The treatment for lung infection is even longer, and clinical improvement often comes at a cost; high financial costs and increased morbidity (Griffith, 2007). Further complications in the treatment of *M. abscessus* infection is the lack of evidence that *in vitro* susceptibility of antibiotics corresponds to *in*

vivo efficacy in treating pulmonary disease (Griffith, 2007). Because chemotherapy-based treatment of *M. abscessus* infection is often unsuccessful, the American Thoracic Society (ATS) advise that certain patients may have the best chance of disease regression with resectional surgery, especially if the patient exhibits a poor response to drug therapy, if macrolide-resistance develops, or if the patient is experiencing disease-related complications such as haemoptysis (Griffith, 2007).

Before treatment is commenced, it needs to be determined whether or not the isolate demonstrates constitutive macrolide resistance or induced macrolide resistance/macrolide susceptibility (Haworth, 2017).

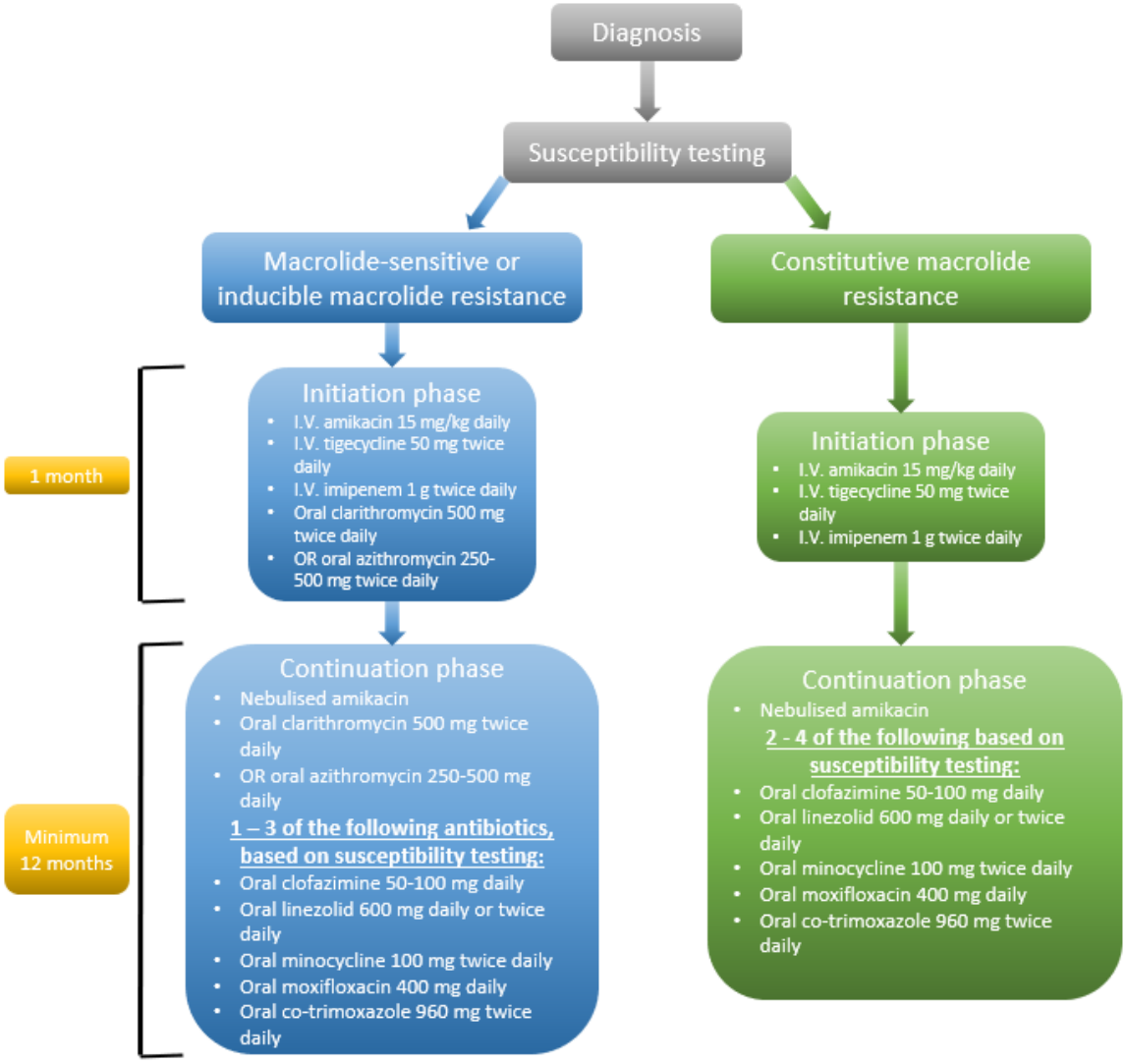


Figure 1.4. Guidelines for treatment of M. abscessus as given by the British Thoracic Society (BTS)

Current guidelines dictate that both regimens should consist of an initiation phase that lasts approximately 4 weeks, followed by a continuation phase that lasts at least 12 months (Figure 1.4). Patients with *M. abscessus* isolates that are clarithromycin sensitive or demonstrate inducible macrolide resistance are treated with intravenous amikacin, intravenous tigecycline, intravenous imipenem, and oral clarithromycin or oral azithromycin for the initial phase. For the continuation phase, patients are treated with nebulised amikacin, an oral macrolide, and then, guided by laboratory susceptibility results, 1-3 of the following: clofazimine, linezolid, minocycline or doxycycline, moxifloxacin or ciprofloxacin, and co-trimoxazole (Haworth, 2017). Patients whose isolates display constitutive macrolide resistance are treated with amikacin, intravenous tigecycline, and intravenous imipenem. Then for the continuation phase, patients are treated with nebulised amikacin, and then, guided by laboratory susceptibility results, 1-3 of the following: clofazimine, linezolid, minocycline or doxycycline, moxifloxacin or ciprofloxacin, and co-trimoxazole (Haworth, 2017). The BTS also notes that in the case of patients whose isolates are amikacin resistant (i.e. are known to have a 16S rRNA mutation that confers amikacin resistance), amikacin should be substituted for a suitable alternative oral antibiotic (Haworth, 2017). Genetic analysis of clinical isolates can provide information on the *erm*(41) and/or presence of 23S rRNA point mutation in clinical isolates of *M. abscessus*, which can then be used to inform treatment regimens specifically regarding macrolide therapy.

Side effects of *M. abscessus* treatment are common and can be severe. A retrospective analysis of 65 patients undergoing treatment for *M. abscessus* lung disease in South Korea (Jeon, 2009) revealed frequent adverse reactions to cefoxitin; 51% of patients developed leukopenia, 6% of patients developed thrombocytopenia, and 15% of patients experienced drug-induced hepatotoxicity. As a result of these side effects, cefoxitin was discontinued in 60% of patients and side effects resolved. Another common side effect they observed was gastrointestinal problems (nausea, anorexia, or diarrhoea), which affected 22% of patients and caused 4 patients (6%) to completely stop antibiotic

treatment. Recommendations were made by the authors to consider imipenem as an alternative to cefoxitin, however prolonged treatment with imipenem can cause neutropenia.

1.6.1. *Amikacin*

Amikacin is an aminoglycoside antibiotic that works by inhibiting protein synthesis by irreversible binding to 16S rRNA and the RNA binding S12 protein of the 30S subunit, changing the shape of the ribosome so it is unable to read mRNA codons correctly.

Reports suggest that 90% of *M. abscessus* isolates have low or intermediate MICS to amikacin, and is considered the most active of the parenteral agent against the pathogen (Griffith, 2007). Studies using a hollow-fiber model of infection have been performed to assess the efficacy of amikacin alone and in combination with other drugs from the *M. abscessus* treatment regimen. A 2016 study showed that whilst *M. abscessus* is susceptible in fixed concentration assays, the hollow fibre model revealed that doses failed to achieve the target in more than 75% of patients, which may explain the high levels of resistance to amikacin observed in the clinic (Ferro, 2016). Amikacin also has some undesirable side effects; hearing loss and tinnitus is common, and patients with renal impairment are at high risk of developing nephrotoxicity (NICE, 2020). When treating NTM-PD with amikacin, up to 39% of patients develop ototoxicity (related primarily to female sex and total dose of amikacin per bodyweight), and only around half can expect to see sputum conversion after one year (Aznar, 2019). Of course, treatment for *M. abscessus* involves a number of different antimicrobial agents that may exhibit synergistic properties, but considering that amikacin has limited efficacy on its own, and has high levels of reported side effects, it is clear that better drugs are needed.

1.6.2. *Tigecycline*

Tigecycline is a glycylcycline antibiotic closely related to the tetracycline class that was initially developed to overcome resistance to the tetracycline class of antibiotics (Tally, 1995). Tigecycline works by binding to the 30S bacterial ribosome, preventing the entry of tRNA, in turn inhibiting protein synthesis and ultimately bacterial growth (Greer, 2006) (Pharaceutics, 2005) (Garrison, 2005) (Zhanel,

2004). Tigecycline is structurally similar to minocycline, but the addition of an *N,N,*-dimethylglycyclamino group at the 9 position of minocycline molecule improves the affinity of tigecycyline for the ribosomal subunit up to five times compared with minocycline, increasing its potential spectrum of activity (Nathwani, 2005).

Tigecycline is effective against both Gram-positive and Gram-negative organisms, including multidrug resistant organisms, and is used to treat complicated skin infections, complicated intra-abdominal infections, and community-acquired bacterial pneumonia. Adverse effects are seen but are mild in most patients (and similar to placebo group); the most commonly reported being nausea (29.5%), vomiting (19.7%), and diarrhoea (12.7%) (Greer, 2006). These are likely to be down to tigecycline irritating the gastric mucosa (Greer, 2006).

In 2014, a large-scale clinical study was performed to determine the efficacy of tigecycline in a regimen against *M. abscessus* and *M. chelonae*. When tigecycline was administered alongside a regimen containing macrolides, amikacin and linezolid, clinical improvement was seen in 60% of patients, however 90% of patients also reported side effects, the most common being nausea and vomiting (Wallace, 2014). Then in 2016, Ferro *et al.* performed a dose-response experiment in a hollow-fibre model of *M. abscessus* infection. They found that tigecycline was "unprecedented" in its ability to produce a bacterial level of 1.0 $\log_{10}$ colony-forming unit/mL (CFU/mL) below pre-treatment inoculum, and this could be achieved in patients with a 200 mg/day dose (Ferro, 2016). Other studies have suggested that tigecycline is highly active against *M. abscessus*, with MICs ranging from 0.06 - ≤4 µg/mL (Fernandez-Roblas, 2008) (Kim, 2015). Therefore, it could be said that tigecycline is one of the most effective agents against *M. abscessus* and should be considered as a treatment option in most cases.

1.6.3. Imipenem

Imipenem is a member of the largest class of antibiotics in use today; the β-lactams. Like all β-lactams, imipenem works by inhibiting bacterial call wall production via binding to and inactivating

transpeptidases, also known as penicillin binding proteins (PBPs). Imipenem displays broad spectrum activity against many pathogens, and so can be administered to treat complicated infections involving mixed species with different susceptibility profiles. It is often indicated for use against ventilator-associated pneumonia (VAP), intra-abdominal infections, SSTIs, sepsis, endocarditis, amongst others (Rodloff, 2006).

A retrospective analysis of 244 cases of *M. abscessus* pulmonary disease by Chen *et al.* was performed in 2019. It was revealed that treatment regimens that include imipenem were associated with higher treatment success (also regimens including amikacin, tigecycline, and linezolid had a similar effect) (Chen, 2019). Imipenem has varying success in other studies, however. One study in Korea found that 60% of their *M. abscessus* subsp. *abscessus* isolates were resistant to imipenem (Li, 2017), and another indicated that only 50% of *M. abscessus* isolates show low MICs to imipenem (Griffith, 2007). However, laboratory determination of imipenem MIC can be unreliable, which hinders understanding of its efficacy. Common side effects of imipenem are diarrhoea, eosinophilia, skin reactions, vomiting, and thrombophlebitis, and rarer side effects seen include thrombocytopenia, psychiatric disorder, and seizures (Excellence, 2020).

1.6.4. *Clarithromycin/azithromycin*

The macrolide antibiotics; clarithromycin and azithromycin are mainstays of *M. abscessus* treatment. A more in depth discussion on the mechanism of action of macrolides and macrolide resistance can be seen in chapter 1.2: History and taxonomy of *Mycobacterium abscessus*. Briefly, macrolides work by binding to the bacterial 23S rRNA subunit and blocking bacterial protein synthesis.

Whether or not the patient with *M. abscessus* infection is given macrolides depends on its individual susceptibility; *M. abscessus* subsp. *massiliense* isolates are usually susceptible to macrolides due to a non-functional *erm*(41) gene, unless they carry a mutation in the 23S rRNA subunit, and *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *abscessus* often display inducible resistance to macrolides.

The ATS guidelines on NTM treatment does not indicate a preference for either azithromycin or clarithromycin in the treatment against *M. abscessus* (Griffith, 2007), thus the decision is left to clinicians based on preference and the potential for drug interactions (Stout, 2012). A 2011 study set out to compare the efficacies of clarithromycin and azithromycin against *M. abscessus*, specifically to compare treatment outcomes in mouse models and compare *erm*(41) induction between the two macrolides (Choi, 2012). They found that in all models of infection, azithromycin was more effective than clarithromycin, and clarithromycin exposure caused a significantly greater increase in *erm*(41) levels and levels of inducible resistance (except in *M. abscessus* subsp. *massiliense* isolates). Both macrolides were able to sufficiently accumulate in the lungs to inhibit *M. abscessus* growth, therefore it is highly likely that the azithromycin's superior activity against *M. abscessus* is due to differences in the *erm*(41) mRNA induction, and that levels of *erm*(41) rRNA are more important than the induction itself for macrolide resistance (Choi, 2012).

Furthermore, azithromycin is often administered to patients with CF on a long-term basis (Equi, 2002). Whilst this has been associated with clinical improvement, paradoxically it has also been implicated in the development of NTM infection (Renna, 2011). One study found that long-term azithromycin use is associated with increased incidence of *M. abscessus* infection, and showed that azithromycin at therapeutic dosing blocked autophagosome clearance in primary human macrophages by preventing lysosomal acidification (Renna, 2011). This leads to the failed intracellular killing of *M. abscessus* by human macrophages and facilitates the establishment of chronic infection. This leads to a catch-22, in which azithromycin appears to facilitate NTM infection, but is also superior to clarithromycin in that it is less able to induce *erm*(41) in *M. abscessus*. The fact that so little is understood about the relationship between the macrolides and *M. abscessus*, and yet it is still used as the mainstay of *M. abscessus* treatment, shows the importance of new effective and safe drugs against *M. abscessus.*

1.6.5. *Clofazimine*

Clofazimine was developed in the 1950's originally as an anti-tuberculous drug, but due to limited efficacy against *MTb,* its primary use is against *Mycobacterium leprae,* the organism that causes leprosy (Lechartier, 2015). The mechanism of action of clofazimine is not fully elucidated (Nugraha, 2021), however, studies have suggested that clofazimine potentiates the effect of bacterial phospholipase A2 and causes the release of lysophospholipids, which are toxic to mycobacteria (Bopape, 2004). Another suggested that clofazimine competes with the mycobacterial quinine cofactor, menaquinone, causing bacterial death (Lechartier, 2015). Other studies suggest that clofazimine affects the mycobacterial outer membrane by inhibiting the mycobacterial respiratory chain, resulting in the production of reactive oxygen species (Bopape, 2004) (Mirnejad, 2018)

As far back as 1986 it was shown that clofazimine was effective against *M. abscessus* (then known as *M. chelonae* subsp. *abscessus*) with MICs of 0.25-2 µg/mL (Ausina, 1986). A more recent study that utilised 117 *M. abscessus* isolates found that 99.1% of isolates were susceptible to clofazimine with MICs of ≤1 µg/mL (Shen, 2010). Furthermore, they demonstrated a synergistic effect with amikacin (Shen, 2010). Other similar studies have been published also showing similar findings. In 2016, Ferro *et al.* demonstrated that clofazimine alone is bacteriostatic against *M. abscessus* and that it acts synergistically with amikacin/clarithromycin (Ferro, 2016). Several more studies have attested to clofazimine's efficacy against *M. abscessus* and its synergistic capabilities with other antimicrobials, notably amikacin and clarithromycin (Singh S, 2014) (Yang, 2017). The safety profile of clofazimine in children and adults with CF and NTM pulmonary disease (NTM-PD) was studied in 2014, where Martiniano *et al.* found that generally, clofazimine was well tolerated, but 14% of patients did halt treatment due to adverse effects (Martiniano, 2017). There is little data on the severity and frequency of side effects reported with clofazimine, but some known side effects include abdominal pain, nausea, diarrhoea and other gastric problems (Excellence, 2020). The most commonly seen side effect (75-100% patients) is pink to brownish-black skin pigmentation (Kaur, 1987) (Ramu, 1976), and other skin disorders can arise too, such as rash, dry skin, and ichthyosis (Excellence, 2020).

1.6.6. Linezolid

Linezolid was the first antibiotic of a class of synthetic oxazolidinones, which were introduced in 1978 to be used to control plant diseases (Mohammad, 2018). Linezolid was considered a 'lead compound' i.e., a compound that displays promising pharmacological properties that warrant further investigation. In 2000 linezolid was approved for use against a range of bacterial pathogens including both methicillin-susceptible (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) by the U.S. Food and Drug Administration (FDA) (Mohammad, 2018). Linezolid works by binding to both the 30S and 50S bacterial subunit and preventing protein synthesis (Batts, 2000). It has also been suggested as a potential treatment against multi-drug resistant TB (MDR-TB) and extensively-drug resistant TB (XDR-TB) (Zhang, 2015). Side effects of linezolid include anaemia, diarrhoea, gastric discomfort, headache, renal failure and seizure, amongst others (Excellence, 2020).

Following FDA approval in 2000, linezolid was tested against a panel of RGM species (Wallace, 2001); 48% of 98 *M. abscessus* isolates tested were either susceptible or intermediately susceptible to linezolid. Other studies have shown conflicting results: a 2018 study with 32 *M. abscessus* strains and 32 *M. massiliense* strains showed 93.8% and 96.9% were susceptible to linezolid, respectively (Zhang, 2018), and a smaller study in 2007 with only 2 *M. abscessus* clinical isolates found both to be resistant to linezolid with MICs of >64 μg/mL (Cavusoglu, 2007). One 2018 study indicated a 3.2% resistance to linezolid (Zhang, 2018). A more recent study investigated the molecular basis of linezolid resistance in *M. abscessus* (Ye, 2019). Using 194 clinical isolates of *M. abscessus*, with the following susceptibilities to linezolid: 43.8% resistant and 56.2% susceptible (Ye, 2019), they performed whole genome sequencing (WGS) and extracted the entire 23S rRNA gene. They observed a total of 26 mutations in the 23S rRNA gene across the 194 isolates of *M. abscessus*, 9 of which were in linezolid resistant strains, suggesting a role of these mutations in linezolid resistance. They then investigated the role of efflux pumps in linezolid resistance in *M. abscessus*, showing that the MIC can be decreased when efflux pumps are inhibited, supporting the theory that efflux pumps play a big role in linezolid resistance in *M. abscessus* (Ye, 2019).

Several studies purporting to the efficacy of linezolid in combination with other antimicrobials have been carried out. Zhang *et al.* showed synergy between linezolid and tigecycline/amikacin, with linezolid plus amikacin being the most potent combination. They also found that linezolid rarely exhibits synergy with moxifloxacin or cefoxitin (Zhang, 2018). This goes against a 2008 study that indicated that linezolid exhibits the best synergy with clarithromycin, however this is at a time when clarithromycin resistance in *M. abscessus* was rare (Brown-Elliot, 2001). The usefulness of a linezolid-containing regimen to treat *M. abscessus* is still under debate. One case study regarding a 51-year-old male patient who had undergone a decade of treatment with for *M. abscessus* infection showed that a regimen containing meropenem, amikacin, and clarithromycin did not improve clinical symptoms, but the addition of linezolid to this regime resulted in improvement symptoms (Inoue, 2018). It is clear that whilst the efficacy of linezolid against *M. abscessus* is unclear, its usefulness within a treatment regimen containing amikacin, tigecycline and clarithromycin cannot be ignored.

1.6.7. Minocycline

Minocycline is a second-generation semi-synthetic tetracycline that has been used against both Gram-positive and Gram-Negative organisms for over 35 years (Yong, 2004). It works by binding to and inhibiting the bacterial 30S rRNA subunit, preventing protein synthesis (Garrido-Mesa, 2013). Studies on the efficacy of minocycline against *M. abscessus* are few and far between, and often have poor results. Ruth *et al.* showed a 5% susceptibility rate and no evidence of synergy between minocycline and a panel of other drugs including clofazimine, amikacin, linezolid and clarithromycin (Ruth, 2018). This supported a 2002 study that also indicated susceptibility to minocycline as low as 5% (Wallace, 2002). Side effects include nausea, vomiting, headache, hearing impairment and pancreatitis (Excellence, 2020). It seems clear that minocycline has only negligible benefits as being part of a regimen against *M. abscessus*. The long duration of treatment with an antibiotic that is only effective against 5% of isolates means many patients will be suffering with adverse effects with no clinical improvement.

1.6.8. *Moxifloxacin*

Moxifloxacin is a quinolone antibiotic with a broad spectrum of antibacterial activity, that is approved for use in treatments for acute exacerbations of chronic bronchitis (AECB), community-acquired pneumonia (CAP), bacterial sinusitis and uncomplicated SSTIs (Keating, 2004). Like all fluoroquinolones, its mechanism of action is the inhibition of DNA gyrase and topoisomerase IV, which are essential for bacterial survival. *In vitro* testing of moxifloxacin against *M. abscessus* yields conflicting results; with several studies reporting susceptibility to moxifloxacin to be 0% - 73%

(NIe, 2014) (Park, 2008) (Tang, 2015) (Hatakeyama, 2017). There is inadequate data that shows a consistent susceptibility pattern to moxifloxacin *in vitro,* and so Nie *et al.* tested moxifloxacin against *M. abscessus* in an *in vivo* zebrafish infection model. They found that there was no difference in growth between the moxifloxacin and control group, bringing into question the efficacy of moxifloxacin *in vivo* (Nie, 2020). This study highlights the importance of single-drug *in vivo* testing against *M. abscessus*. Moxifloxacin is generally well tolerated, with most commonly reported adverse effects being mild gastrointestinal symptoms (Keating, 2004).

1.6.9. *Co-trimoxazole*

Co-trimoxazole is a mixture of trimethroprim and sulfamethoxazole, both of which belong to the sulphonamide class of antibiotics. It is a broad spectrum antibiotic that is most commonly used to treat urinary tract infections (UTIs), and ear, nose and throat infections, acute exacerbation of chronic bronchitis (AECB), amongst others. *In vitro* it is active against many Gram-negative and Gram-positive bacteria, and some species of mycobacteria. It works by sequential inhibition of enzymes in the folic acid synthesis pathway, with sulfamethoxazole inhibiting the synthesis of tetrahydrofolic acid, and trimethoprim inhibiting the dihydrofolate reductase (Wormser, 2012). It is administered as two drugs because of its bacteriocidal activity when used together, as opposed to bacteriostatic properties of the drugs used alone (Patel, 2012), and optimum ratios for trimethoprim : sulfamethoxazole are between 1:5 – 1:40 (Patel, 2012). There is very little information of *in vitro* susceptibilities of *M.*

abscessus isolates against co-trimoxazole, despite it being recommended by the BTS and the ATS for treatment against *M. abscessus* infection (Haworth, 2017). One study showed a susceptibility rate of 8.2% for *M. abscessus* and 11.3% for *M. massiliense* (Cho, 2019), and another, albeit a case study involving a single isolate showed total resistance (Tiong, 2019). Sassi and Drancourt published a study showing the presence of genetic mutations conferring co-trimoxazole resistance in *M. abscessus* (Sassi, 2014). Little evidence of efficacy, combined with the knowledge of gene-mediated co-trimoxazole resistance in *M. abscessus* begs the question as to why this drug is still routinely administered. The most common adverse effects of co-trimoxazole are skin problems and gastrointestinal symptoms, both occurring in approximately 3% of patients, and less than 0.5% of adults will develop more severe haematological abnormalities, such as anaemia, eosinophilia, and thrombocytopenia (Wormser, 2012).

1.6.10. Vancomycin

Vancomycin is a glycopeptide antibiotic used to treat severe infections caused by Gram-positive bacteria, particularly MRSA, as well as for the use in patients with allergies to penicillins and cephalosporins (Bruniera, 2015). It's mode of action is said to be unique; it works by blocking the polymerisation of the *N*-acetyl-glucosamine (NAG) and *N*-acetyl-muramic acid (NAM) NAG/NAM peptides, resulting in inhibition of cell wall synthesis (Watanakunakorn, 1984). A 2017 study by Mukerjee *et al* showed that when tested alone, vancomycin has only weak inhibitory activity against the clinical isolate *M. abscessus* Bamboo. However when tested in combination with clarithromycin, synergy was present, resulting in a ≤0.5 and 4- fold decrease in MIC, including isolates that harbour an intact *erm(41)* gene. The authors conclude that while establishing a synergistic relationship between vancomycin and clarithromycin may be useful in improving the treatment of *M. abscessus,* limitations include vancomycin's requirement to be administered intravenously (Bruniera, 2015), as well as the lack of established breakpoints for *M. abscessus* infection.

1.7. Antimicrobial resistance in *M. abscessus*

M. abscessus is known for its intrinsic resistance to most chemotherapeutic agents, including all the anti-tuberculous drugs used to treat *MTb* infection (Brown-Elliott and Wallace, 2002) (Alcaide, et al., 1997). There are a number of natural resistance mechanisms displayed by *M. abscessus* (along with other mycobacteria), including a waxy and impermeable cell wall, drug export systems, antibiotic modifying/inactivating enzymes, and genetic polymorphism of target genes (Nessar, et al., 2012). In this chapter these mechanisms will be further discussed to build a detailed picture of how *M. abscessus* is able to tolerate most antibiotic treatment.

1.7.1. Cell wall

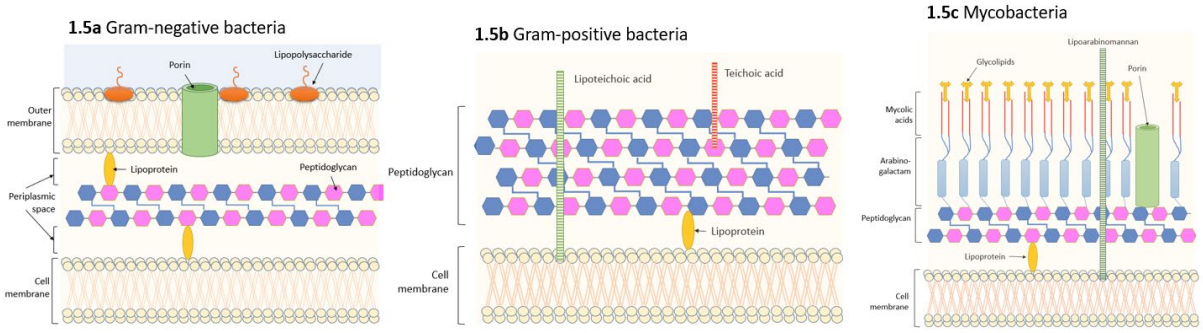


Figure 1.5a-c. ***Image showing structural differences between Gram-negative bacteria (a), Gram-positive bacteria (b) and mycobacteria (c). Like Gram-negative and positive bacteria, mycobacteria have a cell membrane and a peptidoglycan layer. However, in mycobacteria, this conventional peptidoglycan layer is covalently linked to arabinogalactan polysaccharides that are in turn esterified to branched, long chain fatty acids known as mycolic acids. Also can be seen are large porins that act to remove toxic compounds from the cell.***

The role of the mycobacterial cell wall in conferring antibiotic resistance has long been studied. One of the main characteristics of the mycobacterial cell wall is its high lipid content (Figure 1.5c), constituting 60% of the dry weight of the bacteria. This characteristic is considered to be the main factor involved in its unusually low permeability as it halts the passage of both hydrophobic and

hydrophilic compounds into the cytoplasm (Brennan, 1995). It is also thought that the arabinogalactan layer may limit the entry of hydrophobic molecules (Brennan, 1995)

The presence of porins in the mycobacterial cell wall also contributes to its intrinsic resistance. They enable the movement of lethal amounts of hydrophilic antibiotics through the cell wall. However, it is now known that these porins act synergistically with internal systems that are activated by the presence of intracellular antibiotics, and that the low permeability of the mycobacterial cell wall means that the bacteria has time to induce the expression of drug resistance genes (Nguyen & Thompson, 2006).

1.7.2. Antibiotic modifying/inactivating enzymes

M. abscessus produces enzymes that could potentially inactivate or modify antibiotics. Table 1.2 lays out these enzymes. Briefly, the Ambler class A β-lactamase, Bla_{Mab} is known to hydrolyse the β-lactam ring of β-lactam antibiotics, rendering them ineffective. There is also a putative gene coding for rifampicin ADP-ribosyl transferase, which acts to catalyse ADP-ribosylation of rifamycin antibiotics and inactivate them. Aminoglycoside resistance is mediated by two mechanisms; the presence of aminoglycoside 2'-N-acetlytransferase and aminoglycoside phosphotransferase, which transfer an acetyl and phosphate residues, respectively, onto key positions within the antibiotic, inhibiting their antimicrobial activity.

***Table 1.2. List of antibiotic modifying/inactivating enzymes that are present in* M. abscessus.**

Enzyme	Function
Amber class A β-lactamase, Bla_{Mab} (Soroka, et al., 2013)	Hydrolyses β-lactam ring of β-lactam antibiotics
Rifampicin ADP-ribosyl transferase, Arr_*Mab* (Rominski, et al., 2017)	Inactivates rifamycins such as anti-TB drug rifampicin
Aminoglycoside 2'-N-acetyltransferase (Maurer, et al., 2015)	Mediates susceptibility to aminoglycoside antibiotics
Aminoglycoside phosphotransferases (Ripoll, et al., 2009)	Mediates susceptibility to aminoglycoside antibiotics

1.7.2.1. Ambler class A β-lactamase, Bla_{Mab}

The most widely used class of antimicrobials in the world today, β-lactam antibiotics are a potent class of antibiotics that are used to treat a wide range of bacterial infections. They work by targeting and inhibiting the synthesis of the peptidoglycan (PG) layer of the bacterial cell wall. The peptidoglycan layer is a major component of the mycobacterial cell wall; it covalently bonds to the polysaccharide arabinogalactan which in turn is bound to the mycolic acid layer, a long fatty acid that is the hallmark of the mycobacterial cell wall (Marrakchi, et al., 2014).

Most β-lactams are ineffective against *M. abscessus*, this is likely due to the use of nonclassical transpeptidases, namely L,D-transpeptidase (also known as penicillin binding proteins or PBPs), in approximately 64% to 74% of its peptide linkages that form the PG layer (as opposed to the classical D,D-transpeptidase present in most other non-mycobacterial species) (Lavollay, et al., 2011). The L,D-transpeptidases in *M. abscessus* catalyse the linkage of the 3rd and 3rd residues of the peptide side chain, unlike the D,D-transpeptidases that catalyse the linkage of the 3rd and 4th residues of the peptide side chain. Most β-lactam antibiotics target the classical D,D-transpeptidases and have little to no activity against L,D-transpeptidases (Kumar, et al., 2017). Moreover, it has been shown that the PG of both the rough and smooth variants of *M. abscessus* are generated by L,D-transpeptidases, and therefore these L,D-transpeptidases could potentially be an attractive drug target for antimicrobials, if intrinsic β-lactamase-mediated resistance could be overcome (Lavollay, et al., 2011).

Recent studies have shown that a subclass of β-lactams, called carbapenems, are capable of inactivating mycobacterial L,D-transpeptidases (Kaushik, et al., 2015). One study found that two of the five highly conserved L,D-transpeptidases in *M. abscessus*, that are designated Ldt_{Mab1} and Ldt_{Mab2} can be inhibited by carbapenems (such as doripenem) and penems (such as faropenem), both of which are a subclass of β-lactams (Kumar, et al., 2017). Simulation studies on the molecular dynamics of β-lactam- Ldt_{Mab2} binding revealed that the β-lactams with higher activity against *M. abscessus* (such as

doripenem) had significantly lower binding free energy (ΔG_{bind}) to Ldt_{Mab2} than the other, less active β-lactams, meaning a tighter β-lactam- Ldt_{Mab2} bind and therefore better inhibition of this particular L,D-transpeptidase (Kumar, et al., 2017). Furthermore, studies on the interactions between Ldt_{Mab1} and Ldt_{Mab2} and faropenem, a penem antibiotic revealed that these L,D-transpeptidases preferentially bind to faropenem over both carbapenem and non-carbapenem β-lactams.

Despite the β-lactam agents imipenem, cefoxitin, doripenem and faropenem having moderate *in vitro* activity against *M. abscessus* (Huang, et al., 2010) (Kumar, et al., 2017), *M. abscessus* is known to be highly resistant to most other β-lactam antibiotics (Soroka, et al., 2013). The mechanisms behind this intrinsic resistance to β-lactams in mycobacteria is largely unexplored, however it is believed that the balance between β-lactamase production (i.e. intracellular inactivation) and the rate of β-lactam penetration into the cells through the outer membrane plays a key role (Jarlier, et al., 1991) (Nikaido, 1989). Ambler class A β-lactamases have been identified in several mycobacterial species, including *MTb* (Hackbarth, et al., 1997) and *M. smegmatis* (Basu, et al., 1997). In 2013, following analysis of the *M. abscessus* genome, a highly conserved gene encoding an Ambler class A β-lactamase was characterised and subsequently named Bla_{Mab} (Soroka, et al., 2013). Further analysis of the hydrolysis spectrum of Bla_{Mab} revealed its highly effective hydrolysis of all β-lactams, except cefoxitin (Soroka, et al., 2013). Clearly, the exact mechanisms involved in β-lactam hydrolysis by Bla_{Mab} are yet to be fully elucidated, because temocillin, a penicillin, was not hydrolysed by Bla_{Mab} yet this antibiotic shows no activity against *M. abscessus in vitro* (Soroka, et al., 2013). It is possible, however, that the varying binding affinities of *M. abscessus* L,D-transpeptidases to different β-lactams are involved in this phenomenon.

1.7.3. Macrolide resistance

One of the mainstays of *M. abscessus* treatment is a course of macrolide antibiotics, typically azithromycin or clarithromycin (Griffith, et al., 2007). Despite this, *M. abscessus* infections tend to respond poorly to macrolide therapy, even when they appear sensitive to clarithromycin *in vitro*

(Nash, et al., 2009). In other NTM species, macrolide resistance is associated with ribosome methylases, or *erm* genes (Nash, 2009).

Erm genes encode either N^6-monomethyltransferases or N^6- N^6-dimethyltransferases, which are specific to the nucleotide A2058 in 23S rRNA. It was in 1975 that a connection between N^6- N^6-dimethylation of adenine in 23S rRNA and macrolide resistance was first made (Tanaka, 1975), and in 1983, Skinner *et al.* demonstrated that the specific site of action was position A2058 (Skinner, 1983). It is now known that the mono- or di-methylation of this specific adenine residue sterically alters the binding site of macrolide antibiotics, disrupting hydrogen bonding between the macrolides and the rRNA, resulting in macrolide resistance (Maravic, 2004).

MTb, a distant genomic relative of *M. abscessus,* is known to be intrinsically resistant to macrolides (Hoffner, 1997) (Luna-Herrera, 1995) (Truffot-Pernot, 1995), and this resistance is mediated by the *erm*(37) gene (Buriankova, 2004) (Madsen, 2005). In 2006 it was found that this intrinsic resistance is inducible with clarithromycin and the ketolide HMR2004, and an increase in *erm*(37) mRNA levels appeared to mediate this phenotype (Andini, 2006). Similar genes were found in a number of other *Mycobacteria* species, including; *M. smegmatis* (Nash, 2006) (Madsen, 2005), *Mycobacterium wolinsky, Mycobacterium mageritense* (Nash, 2006), and *M. fortuitum* (Nash, 2005). All of these *erm* genes were found to be inducible (Andini, 2006) (Nash, 2006) (Nash, 2006) (Nash, 2005).

The idea that *M. abscessus* may also harbour a gene that confers inducible macrolide resistance may not have been new; a study on the activities of four macrolides, including clarithromycin and azithromycin, against *M. fortuitum,* "*M. chelonae*-like organisms", and *M. chelonae* (which at the time included *M. chelonae* subsp. *abscessus*) was performed in 1992 (Brown, 1992). Nash *et al.* clearly demonstrated a distinct difference between *M. chelonae* subsp. *chelonae* and *M. abscessus* (at the time known as *M. chelonae* subsp. *abscessus*). Whilst most *M. chelonae* isolates were macrolide susceptible (74%), 80% of the *M. abscessus* isolates were macrolide resistant after 3 days, and after 4 days these isolates showed between a 2 and 256-fold increase in MIC (Brown, 1992). At that time

however, this phenomenon was suggested to be perhaps down to a difference in the bactericidal activity of macrolides against both species (Brown, 1992).

Then, in 2009, Nash *et al.* demonstrated a novel *erm* gene in *M. abscessus,* when it was noted that despite intrinsic macrolide resistance in *M. abscessus* being commonly encountered in the clinic, mutations in the 23S rRNA gene conferring macrolide resistance (specifically a base change at position 2058 or 2059) were rarely encountered (Nash, 2009). In this study, 10 macrolide susceptible *M. abscessus* isolates and 2 macrolide resistant *M. abscessus* isolates with A2058G/C 23S rRNA gene mutations, were incubated either in medium alone or medium containing clarithromycin or erythromycin prior to MIC determination. On day 3 of MIC determination, all 10 macrolide susceptible isolates had clarithromycin/erythromycin MICs of ≤0.5 μg/mL, but on extended incubation, only 3 isolates were still susceptible, and 7 reached MICs of >32 μg/mL (Nash, 2009). This data suggests the presence of an inducible *erm*(41) gene in *M. abscessus* isolates. Nash *et al.* also demonstrated that preincubation with macrolides resulted in enhanced growth of the isolates displaying inducible macrolide resistance (Nash, 2009).

Further investigation into the molecular mechanisms behind inducible macrolide resistance in *M. abscessus* by Nash *et al.* revealed a 4.0-kbp region within the *M. abscessus* genome that conferred macrolide resistance (Nash, 2009). Within this region, the only complete known gene was *rspA* gene encoding the S1 ribosomal protein. Further analysis revealed a small open reading frame (ORF) downstream of the *rspA* gene which encoded a 173 amino acid polypeptide chain with conserved domains, characteristic of adenine rRNA methylase (Nash, 2009). Evidence of a transcriptional terminator between the *rspA* gene and the putative methylase gene indicated that the methylase gene must have its own promoter region, as opposed to being part of an operon downstream of *rspA* (Nash, 2009).

This novel *M. abscessus erm*(41) gene was compared with the *erm* genes of other RGM; it was found that *M. abscessus erm*(41) was 73-93 amino acids smaller than that of other RGM, and shared only

30-34% sequence identity. Indeed, the best gene alignment was with *MTb*, sharing 69% identity with the *MTb erm*(37) (Nash, 2009). Reverse transcription polymerase chain reaction (RT-PCR) analysis showed that exposure to macrolides leads to increases in *erm*(41) RNA levels, peaking 50-100 fold from the baseline, suggesting the presence of a promoter region within the *M. abscessus erm*(41) gene. Interestingly, the location of the *erm*(41) gene is rather different to other RGM, where the *erm* gene is proximal to the *folP* gene, 2 million bp away, and to *MTb erm*, which is in a region 400,000 bp away (Nash, 2009). This lead to Nash *et al.* to suggest that the *M. abscessus erm*(41) gene was acquired independently of other mycobacteria.

Since 2004 it has been reported that *M. massiliense* is markedly susceptible to clarithromycin, whilst *M. abscessus* and *M. bolletii* are not (Adekambi, 2004) (Kim HY, 2008) (Tortoli, 2008), and furthermore, it was reported that *M. massiliense* strains that do have high clarithromycin MICs exclusively have a 2058 or 2059 base change in the 23S rRNA gene (Kim HY., 2008). These incongruent MICs to macrolides within the *M. abscessus* complex was further investigated in 2010 (Kim, 2010). Kim *et al.* investigated the *erm* genes of 101 isolates from the *M. abscessus-M. chelonae* group, of which 46 were *M. abscessus,* 49 were *M. massiliense*, 2 were *M. bolletii* and one was *M. chelonae*. Using relevant primers, the full *erm*(41) gene in each isolate was amplified and then sequenced. Upon analysis of the sequences, it was revealed that there were two distinct sizes of *erm*(41) within the isolates; the *erm*(41) gene amplified from *M. abscessus* and *M. bolletii* was 673 bp long, whereas the same gene amplified in *M. massiliense* isolates were considerably smaller, at 397 bp (Kim, 2010). Susceptibility testing of the isolates showed that *M. massiliense* isolates were either markedly susceptible to clarithromycin (87.5% had MIC of ≤2 μg/mL), or totally resistant (23.5% had an MIC of >236 μg/mL), and the highly resistant isolates exclusively had a point mutation of either A2058G, A2058C, or A2059G of the 23S rRNA gene (Kim, 2010).

According to Kim *et al.,* the large deletion in the *M. massiliense erm*(41) gene is too large to allow for the production of a functioning methyltransferase, and accounts for the characteristic susceptibility

to macrolides seen in isolates of *M. massiliense* (Schluckebierm, 1999). Considering that the differentiation of RGM species is vital to effectively manage infection, molecular methods that target the *erm*(41) gene can be easily applied to the clinic to differentiate *M. massiliense* from *M. abscessus* and *M. bolletii.*

1.8. Future considerations/drug discovery

It perhaps goes without saying that there is an urgent, unmet need for safe and effective treatments against *M. abscessus* pulmonary disease. There have been instances of successful treatment of *M. abscessus* with already available antibiotics. One such case was reported in 2002, where a 63-year-old patient whose infection had not responded to the traditional regimen was prescribed a course of faropenem, a new member of the β-lactam antibiotic class. Treatment was successful and produced no adverse side effects (Tanaka, et al., 2002). It is not just antimicrobials that have potential in enhancing *M. abscessus* treatment. In 2012, Okazaki *et. al.* reported that the use of clarithromycin, amikacin and imipenem/cilastatin to treat a case of *M. abscessus* pulmonary was greatly enhanced with the addition of corticosteroids. The authors recommend that the presence of organising pneumonia (a non-specific inflammatory pulmonary process) or an allergic reaction may have helped to explain the poor response to antibiotic treatment alone in some patients, and that this possibility should be considered when applicable to improve treatment outcomes (Okazaki, et al., 2013).

Aside from these examples, very few case studies have reported successful treatment with repurposed antibiotics. Therefore, novel drug targets in *M. abscessus* must be discovered and elucidated, and novel compounds that safely and effectively inhibit these targets discovered.

There are potentially a wide variety of viable drug targets in *M. abscessus* (Figure 1.6) Many of the most promising leads against *M. abscessus* have come about as a result of concerted effort to find novel drugs for *MTb*, which a handful of researchers have applied to *M. abscessus* and other NTM species. Unfortunately, only a small percentage of the novel drugs that are active against *MTb* are

active against *M. abscessus,* further highlighting just how resistant and dangerous this pathogen is proving to be.

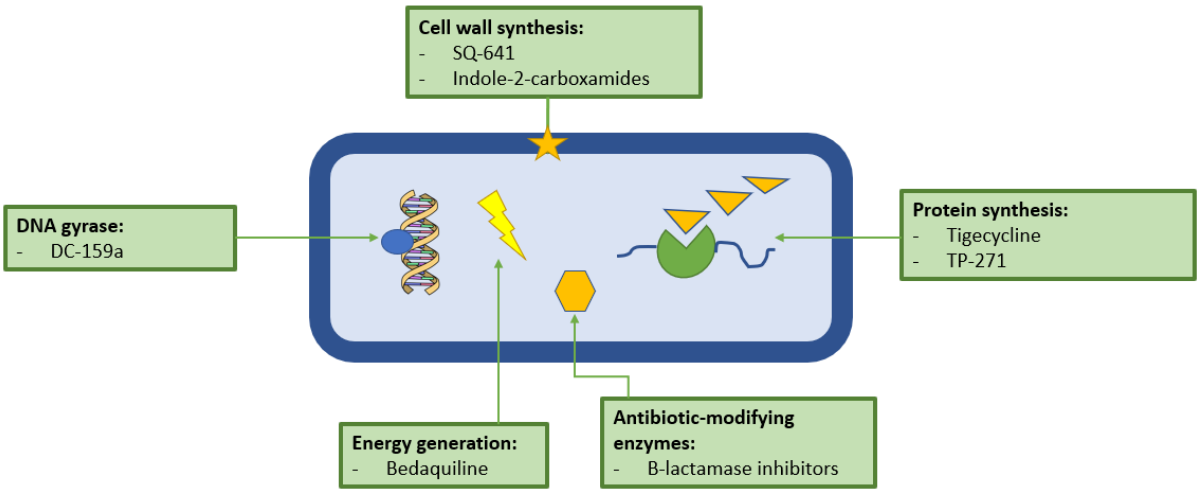


Figure 1.6. Graphical summary of the exploitable drug targets in M. abscessus. There are several potential target areas in M. abscessus including physiological, genomic, enzymatic and metabolic processes. Many of the drugs with potential to be used as part of M. abscessus treatment are old classes of antibiotics that have been repurposed, such as β-lactamase inhibitors, or have been discovered as part of the anti-tuberculous drug discovery pipelines, such as bedaquiline.

One potential target in *M. abscessus* is DNA gyrase, despite the fact that *M. abscessus* is naturally resistant to quinolones (Guillemin, et al., 1998), a novel fluoroquinolone, DC-159a was developed in 2010 as part of the Working Group on TB Drugs, and was found to be active against *M. abscessus* with an MIC of 16 µg/mL, which was 4 to 8-fold lower than the other already available quinolones tested (Disratthakit & Doi, 2010). The authors stressed the importance of *in vivo* testing of DC-159a, however, no publications attesting to the *in vitro* activity of DC-159a against *M. abscessus* have been released to date.

The mycobacterial cell wall, in all its complexity, can offer an attractive range of potential antibiotic targets. The three distinct layers of the mycobacterial cell wall: core peptidoglycan, arabinogalactan and mycolic acids are each essential to the pathogen and involve a number of exploitable processes (Abrahams & Besra, 2018). A 2010 study subjected several species of NTM to a capuramycin analogue

SQ641 (Dubuisson, et al., 2010). Capuramycins are a novel class of nucleoside antibiotics that work by targeting phosphor-*N*-acetylmuramyl-pentapeptide-translocase (translocase-1 or TL-1) which is essential for peptidoglycan synthesis. They found that the drug had an MIC of 0.25-1 μg/mL, as well as finding synergy between SQ641 and rifabutin and streptomycin. This drug has great potential as it is fast-acting and displays a long post-antibiotic effect (Reddy, et al., 2008). In 2017 a study was published in which several members of the newly synthesized MmpL3 inhibitors, indole-2-carboxamides, have shown potent activity against *M. abscessus*. These inhibitors have been shown to work by inhibiting the transfer of mycolic acids to their cell envelope acceptors in *M. abscessus* strains (Franz, et al., 2017). Further work has been done on this class of inhibitors; in 2019, Pandya *et al.* reported that oral administration of the inhibitors shows a statistically significant reduction in bacterial load in the lungs and spleens of *M. abscessus*-infected mice (Pandya, et al., 2019).

It has been demonstrated that *M. abscessus* displays high levels of intrinsic resistance to the tetracycline class of antibiotics through inhibition of the monooxygenase, MabTetX, a *WhiB7*-independent pathway (Rudra, et al., 2018). This is not the end of the road for this class of antibiotics. Tigecycline, the first developed glycylcycline, a new class of tetracycline antibiotics originally developed for SSTIs, was shown in 2014 to be highly effective *in vivo* against *M. abscessus* pulmonary disease (Wallace, et al., 2014). Further work in 2018 revealed that tigecycline is a poor substrate of MabTetX and is incapable of inducing its expression, explaining its high efficacy in comparison with other tetracycline antibiotics (Rudra, et al., 2018). Tigecycline is now part of the recommended treatment options for *M. abscessus* pulmonary disease, and is arguably one of the most effective, with one study citing clinical improvement in >60% patients with *M. abscessus* pulmonary disease when tigecycline is employed as part of the multi-drug regimen against *M. abscessus* (Wallace, et al., 2014). Tigecycline is not the only tetracycline showing activity against *M. abscessus*. A 2012 study tested the *in vitro* activity of a novel fluorocycline antibiotic, TP-271 (a tetracycline-related antibiotic) against 22 isolates of *M. abscessus*. They found all the isolates to have an MIC of ≤1 μg/mL with an average of

0.5 µg/mL, which is decidedly superior than that of the other orally available tetracycline antibiotics such as moxifloxacin and tetracycline (Cynamon, et al., 2012).

Bedaquilin, the latest drug indicated for the treatment of multi-drug resistant TB (MDR-TB) was approved by the FDA in 2011, and it works by targeting the ATP synthase of mycobacteria. Obregon *et al.* (Obregon-Henao, et al., 2015) demonstrated an MIC of 1.0 µg/mL against *M. abscessus* reference strain and then in 2017, Vesenbeckh and colleagues pointed to bedaquiline as a potential antimicrobial against *M. abscessus* after the drug exhibited MICs of ≤1 µg/mL against 20 *M. abscessus* clinical isolates *in vivo.* (Vesenbeckh, et al., 2017).

1.9. Summary

M. abscessus is increasingly being recognised as an important pathogen responsible for a wide range of infections and implicated in severe, and often untreatable pulmonary infections in people with CF and other structural lung disorders. Almost all of the currently available antibiotics are ineffective against the pathogen, with even official guideline treatment regimens having little to no evidence of *in vivo* efficacy. With such high treatment failure rates, clinicians are often forced to administer last-resort antibiotics in the hope of an effective treatment. Coupled with increasing prevalence and its already extensively drug resistant profile, it is glaringly obvious that novel, effective and safe treatments are needed. Many of the novel drugs mentioned in Chapter 1.8. are in various phases of clinical trial against *M. tuberculosis* and there is a significant paucity of data regarding their efficacy against *M. abscessus* and other NTM species. Furthermore, there is a startling lack of *in vivo* efficacy data for any of these drugs, which is particularly worrying considering the inconsistencies between *in vitro* and *in vivo* anti-*M. abscessus* activity. Whilst TB has many dedicated drug-discovery programmes, NTM has none. A dedicated NTM drug discovery pipeline is essential to ensure the disease burden of NTM does not become overwhelming. Priming this pipeline with new compounds is vital given the high attrition rate of newly discovered antibiotics. Considering the mounting evidence that *M. abscessus* infections are on the rise globally, the need for new treatments will only increase.

The main purpose of this thesis is to explore the relationship between *M. abscessus* subspecies and DSPs, as well as interrogate potential treatment avenues and drug targets for use against *M. abscessus.* Furthermore, this thesis will apply an *MTb*-like method for assessing the effect of latency and anaerobic conditions on *M. abscessus* DSPs. All of the data gathered in this thesis can be used to inform clinical management of *M. abscessus* disease.

Chapter 2: *Mycobacterium abscessus* clinical isolates

2.1. Aims and objectives

There is a paucity of information providing a full link between *M. abscessus* subspecies and DSPs. First, the research conducted in this chapter aims to elucidate the link between *M. abscessus* subspecies and drug susceptibility patterns. This will be done using a PCR-based assay to determine *M. abscessus* subspecies within a cohort of clinical isolates, along with an MIC assay to establish drug susceptibilities. Second, this chapter aims to assess the prevalence of *M. abscessus* subspecies within a cohort of clinical isolates using a novel PCR-based assay. There is no "gold-standard" rapid PCR-based assay available for the diagnosis and subspeciation of *M. abscessus* infection, so in this chapter the useability and reproducibility of a novel PCR-based assay will also be assessed. The data presented in this chapter can be used to inform clinical diagnosis and treatment regimes.

2.2. Introduction

The *M. abscessus* complex consists of 3 subspecies; *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* (Adekambi, 2006), and *M. abscessus* subsp. *massiliense* (Adekambi, 2004). For the purposes of brevity, subspecies will henceforth be referred to as *M. abscessus, M. massiliense,* and *M. bolletii.* Whilst it took decades for the scientific community to achieve congruence on the presence of three distinct subspecies, hindering the understanding of *M. abscessus,* it is now well established that the laboratory determination of subspecies is essential for achieving a positive clinical outcome.

2.2.1. Molecular identification of *M. abscessus* subspecies

A paper published by Griffith and colleagues in 2015 laid out the molecular differences between the three subspecies (Table 2.1) (Griffith, 2015). It is unfortunately not one simple gene mutation that results in the three different subspecies, rather small differences in various housekeeping genes that must be analysed together to reveal the subspecies. For example, the complete 16S gene sequence of each subspecies is identical, yet both *M. abscessus* and *M. bolletii* harbour a functional *erm*(41) gene conferring macrolide resistance, whereas *M. massiliense* does not. Using just these methods of

identification, it would not be possible to differentiate between *M. abscessus* and *M. bolletii.* Therefore, small base changes in *rpoB* gene sequences (3.5% difference between the subspecies) must also be analysed, as these are unique to each subspecies (Griffith, 2015). There is a clear clinical need for the differentiation of *M. massiliense* from the other two subspecies, particularly due to differences in macrolide susceptibility that if not taken into account will massively impact treatment. However, there is not a clear clinical difference between *M. abscessus* and *M. bolletii*; both harbour a functional *erm*(41) gene and therefore both demonstrate macrolide resistance, yet no evidence for differences in susceptibilities or outcomes is available in the literature.

Table 2.1: Table showing the various molecular similarities and differences between the three M. abscessus subspecies. Whilst 16S rRNA sequencing is often used to differentiate between pathogenic species in the clinic, it is clear that this method of identification is inadequate for M. abscessus subspecies. Rather, sequencing of the erm(41) gene, the partial rpoB sequence, and perhaps the Whole Genome are essential for subspeciation. This presents a hurdle for the already constrained clinical laboratories. Table modified from Griffith et al 2015 **(Griffith, 2015).**

Species	Complete 16S rRNA gene sequence	rpoB gene sequence	*Erm*(41) sequence	Functional *erm*(41)?	Whole-Genome Sequence
M. abscessus* subsp. *abscessus	Identical to *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *massiliense*	Unique to *M. abscessus* subsp. *abscessus*	Unique to *M. abscessus* subsp. *abscessus*	Yes – except in cases of T28C point mutation	Unique to *M. abscessus* subsp. *abscessus*
M. abscessus* subsp. *bolletii	Identical to *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*	Unique to *M. abscessus* subsp. *bolletii*	Unique to *M. abscessus* subsp. *bolletii*	Yes	Unique to *M. abscessus* subsp. *bolletii*
M. abscessus* subsp. *massiliense	Identical to *M. abscessus* subsp *.abscessus* and *M. abscessus* subsp *bolletii*	Unique to *M. abscessus* subsp. *massiliense*	Unique to *M. abscessus* subsp. *massiliense*	No	Unique to *M. abscessus* subsp. *massiliense*

2.2.2. Prevalence of the *M. abscessus* subspecies

The advancement of subspecies differentiation has allowed scientists and clinicians to make informed decisions on the treatment of patients infected with *M. abscessus* complex, most notably through determination of macrolide sensitivity and therefore whether or not the patient will respond to macrolide therapy (Lee, 2015) (Benwill, 2014). Whilst disease presentation, radiographic findings, and clinical signs are identical for all 3 subspecies, treatment regimens that include a macrolide have shown to result in negative sputum culture results in only 25% of *M. abscessus* infections, as opposed to 88% in *M. massiliense* infections (Koh, 2010). Furthermore, molecular subspeciation and gene sequencing of *M. abscessus* clinical isolates can provide information on transmission, as was shown in 2013 when WGS and subsequent phylogenetic analysis of *M. abscessus* clinical isolates revealed the presence of two clusters of outbreaks within the same hospital, strongly suggesting that patient-to-patient transmission was occurring (Bryant, 2013). Looking at global distribution rates of *M. abscessus* subspecies, it may provide researchers with vital information on how the infection spreads and therefore can inform infection control measures. It can be seen in Table 2.2 that *M. bolletii* is consistently the least prevalent subspecies across the globe, perhaps explaining the dearth of information on this subspecies.

Table 2.2. Table showing prevalence of the three M. abscessus complex subspecies by geographical area. *extra-pulmonary infections. ** one patient was co-infected with both M. abscessus and M. massiliense and was excluded from further analysis.

Location	Date range	Authors	No. of isolates	Subsp. prevalence		
				M. abs	*M. bolletii*	*M. massi*
France	2004	Roux *et al* (2009)	50	60%	18%	22%
Ireland	2006-2012	O'Driscoll *et al* (2016)	36	78%	-	22%
Seoul, South Korea	2010-2014	Jeong *et al* (2017)	20*	50%	-	50%
Ghangzhou, China	2013-2016	Tan *et al* (2018)	246	58.9%	-	41.1%
United Kingdom	2007-2011	Bryant *et al* (2013)	31**	41.9%	6.5%	48.4%

Seoul, South Korea	2005-2007	Kim *et al* (2008)	127	51.2%	1.6%	46.5%
Netherlands	1999-2005	van Ingen *et al* (2009)	39	64.1%	15.4%	20.5%
Milan, Italy	2005 - 2016	Teri *et al* (2020)	314	64%	16%	20%
China	2014-2018	Guo *et al* (2020)	129	75.2%	14.7%	10.1%

2.2.3. Macrolide resistance in *M. abscessus* clinical isolates

Several studies have been published demonstrating the difference between *M. abscessus* and *M. massiliense* in terms of clinical presentation and most importantly, macrolide resistance. Such studies highlight the importance of the *erm*(41) gene in the *M. abscessus* complex, as this gene confers resistance to macrolides (as discussed in section 1.6.4.) (Nash, 2009) (Lee, 2015) (Maurer, 2014) (Kim, et al., 2010). The question of which macrolide is superior for treating *M. abscessus* infections remains unanswered. One study published in 2020 showed using phenotypic assays and *in vivo* experiments that *erm*(41) induction in the *M. abscessus* complex occurs more rapidly upon exposure to azithromycin than clarithromycin (Richard, 2020). In 2014, however, Maurer *et al* subjected *M. abscessus, M. massiliense,* and *M. bolletii* clinical isolates to drug susceptibility testing (DST) against the two most commonly prescribed macrolides, azithromycin and clarithromycin (Maurer, 2014). Whilst they reported generally higher MICs for azithromycin than clarithromycin, both were equally efficient at inducing macrolide resistance, and in isolates in which resistance was induced, both macrolides displayed high MICs.

2.3. Materials and methods

2.3.1. Bacterial strains

A total of 23 clinical isolates were provided to this study from various sources. Of these, 16 clinical isolates were provided from Brighton and Sussex Medical School, UK and a further 8 were provided by Great Ormond Street Hospital, London, UK. One isolated was provided by Birmingham Children's Hospital, Birmingham, UK. The isolates from Brighton and Sussex Medical School and Great Ormond

Street Hospital had already undergone decontamination and were received as *M. abscessus* species in Middlebrook 7H9 broth supplemented with 10% Albumin-Dextrose-Catalase (ADC) and 1% glycerol. Clinical details for each isolate were requested however this data was not made available for the purpose of this thesis.

2.3.2. Growth media

Solid media used for the cultivation of *M. abscessus* was Middlebrook 7H11 agar supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC), and 1% glycerol. Liquid media used for the cultivation of *M. abscessus* was Middlebrook 7H9 media supplemented with 10% albumin, dextrose and catalase (ADC), 1% glycerol and 0.05% Tween 80. All filter sterilisation was performed using a 0.22 µm pore syringe filter (Millipore, UK).

Middlebrook 7H9 was prepared following manufacturer's instructions. This was subsequently autoclaved at 121 °C for 15 minutes for sterilisation. ADC was prepared by adding 0.85 g sodium chloride, 5 g bovine serine albumin, 2 g dextrose, and 0.003 g catalase to 100 mL of distilled water, and filter sterilising. Then 50 mL of ADC and 5 mL of 50% glycerol was added to the 7H9 and mixed gently. Lastly, 20% Tween 80 was prepared by mixing 20 mL Tween 80 with 80 mL distilled water. This was filter sterilised and 1.25 mL was added to the 7H9-ADC-glycerol mixture.

Middlebrook 7H11 was prepared following manufacturer's instructions. This was subsequently autoclaved at 121 °C for 15 minutes for sterilisation. OADC was prepared by adding 0.06 mL oleic acid, 0.85 g sodium chloride, 5 g bovine serine albumin, 2 g dextrose, and 0.003 g catalase to 100 mL of distilled water, and filter sterilising. Then 50% glycerol was prepared by mixing 50 mL of glycerol with 50 mL of distilled water and filter sterilising. Following this, 50 mL of ADC and 5 mL of 50% glycerol was added to the 7H11 and mixed gently. The 7H11 agar was immediately poured into petri dishes to a volume of approximately 20 mL per dish and were left to set at room temperature.

2.3.3. Isolation, growth, and storage conditions of *M. abscessus*

The clinical isolates from Brighton and Sussex Medical School and Great Ormond Street were inoculated onto Middlebrook 7H11 agar supplemented with 10% OADC and 1% glycerol and incubated at 30 °C and 37 °C for 3-5 days. Clinical details for isolates were unavailable. Once visible growth had occurred, 2 agar plates containing Middlebrook 7H11 agar supplemented with 10 % oleic acid-albumin-dextrose-catalase (OADC) and 1 % glycerol were inoculated with approximately 10 µL of culture each and incubated for around 7 days; in order to determine the optimum growth temperature of this isolate, one plate was incubated at 30 °C and one plate at 37 °C. Individual colonies from 7H11 agar plates were picked and inoculated into 10 mL of Middlebrook 7H9 broth supplemented with 10% OADC, 1% glycerol, and 0.05% Tween 80. Cultures were incubated with shaking for 3-5 days or until heavy growth had occurred. In a cryogenic vial (Thermoscientific, UK), 500 µL of filter sterilised 50% glycerol and 500 µL of *M. abscessus* culture was added and was flash frozen in liquid nitrogen, and subsequently stored at – 80 °C for future use. When preparing a new passage of *M. abscessus* culture, a small amount of frozen *M. abscessus* stock was inoculated into 10 mL Middlebrook 7H9 medium, or onto a Middlebrook 7H11 agar plate using a sterile loop.

2.3.4. Minimal inhibitory concentration (MIC) testing of *M. abscessus* clinical isolates against recommended antibiotic regimen

A total of 10 different antibiotics recommended by the British Thoracic Society/American Thoracic Society (Griffith, et al., 2007) (Haworth, et al., 2017) were used (Table 2.3). Breakpoints against RGM for 7 of these antibiotics were previously established by the Clinical and Laboratory Standards Institute (CLSI) (240) and can be seen in Table 2.3. Tigecycline does not have a widely accepted breakpoint for *M. abscessus*: The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends a breakpoint of 0.5 µg/mL for non-species specific testing, however *M. abscessus*, along with other RGM, tend to have higher breakpoints for most antibiotics, and so both 4 µg/mL (Broda, 2013) and ≥8 µg/mL (Wallace, 2002) have been recommended in the literature. In this study, a breakpoint of 4

µg/mL was used. Ceftazidime, an antibiotic traditionally indicated to treat multibacillary leprosy shows varying MICs against *M. abscessus in vitro,* and due to its limited applications, no official breakpoint has been determined for NTM. Breakpoints of 2 µg/mL have been suggested previously for ceftazidime (van Ingen, 2010), however as the breakpoints are contested, this study utilised a breakpoint of 4 µg/mL. Finally, the breakpoint for azithromycin was taken from the breakpoint of clarithromycin as the macrolide class representative (Woods, 2011).

***Table 2.3: List of the 10 antibiotics used in this study and the minimal inhibitory concentrations (in µg/mL) at which M. abscessus is either susceptible, intermediately susceptible (intermediate), or resistant. For amikacin, cefoxitin, imipenem, clarithromycin, linezolid, minocycline, and moxifloxacin, the CLSI provides breakpoints for M. abscessus* (240). **The breakpoints for azithromycin are taken from those of clarithromycin as both are macrolides* (240). *[a]Clofazimine breakpoint was determined by van Ingen et al (2010)* (van Ingen J, 2010). *[b]Tigecycline has no official breakpoints for M. abscessus or other NTM, and so in this study a breakpoint of 4 µg/mL was used in line with the literature* (Broda, 2013).**

Antimicrobial agent	Susceptible (µg/mL)	Intermediate (µg/mL)	Resistant (µg/mL)
Amikacin	≤16	32	≥64
Cefoxitin	≤16	32 - 64	≥128
Imipenem	≤4	8 - 16	≥32
Clarithromycin	≤2	4	≥8
Azithromycin*	≤2	4	≥8
Clofazimine[a]	-	-	≥1
Linezolid	≤8	16	≥32
Minocycline	≤1	2 - 4	≥8
Moxifloxacin	≤1	2	≥4
Tigecycline[b]	<4	-	≥4

Using the recommended breakpoints for each antibiotic, a 2-fold dilution was prepared for each antibiotic at the following concentration ranges: amikacin, 128 – 1 µg/mL; imipenem, linezolid, and cefoxitin, 64 – 0.5 µg/mL; clarithromycin and azithromycin, 32 – 0.25 µg/mL; minocycline and moxifloxacin, 16 – 0.125 µg/mL, clofazimine and tigecycline, 4 – 0.003 µg/mL. Amikacin, cefoxitin, imipenem and minocycline were all made up to 100x stock with sterile distilled water, and clarithromycin, azithromycin, clofazimine, linezolid, moxifloxacin and tigecycline were made up to

100x stock using filter sterilised DMSO. All antibiotics except imipenem were stored at -20 °C and imipenem was made fresh due to its instability in solution (Mendez, 1991).

Each antibiotic was tested in triplicate against all of the *M. abscessus* clinical isolates. For this, a sterile loop was used to inoculate approximately 10 µL of each clinical isolate in the form of frozen glycerol stocks into 10 mL of 7H9 middlebrook broth supplemented with 10% ADC, 1% glycerol and 0.05% Tween 80. Inoculates were then grown in a shaking incubator for 2-3 days at 30 °C for the NCTC strain and the optimal temperature of 37 °C for the clinical isolates. Once the isolates reached an OD_{600} of ~ 1.0, each isolate was diluted using 7H9 middlebrook broth supplemented with 10% ADC, 1% glycerol and 0.05% Tween 80 to achieve a final OD_{600} reading of ~ 0.1 for inoculation into 96-well plates. Subsequently, 1 µL of each 100x antibiotic stock was placed into the appropriate wells in a 96-well plate, then 99 µL of inoculate was added to each well for a final well volume of 100 µL. For the positive control growth wells, 100 µL of inoculate was used and 100 µL of of 7H9 middlebrook broth supplemented with 10% ADC, 1% glycerol and 0.05% Tween 80 was placed in the negative control wells, also to be used as a blank upon reading of the OD_{600}. Antibiotics were tested in triplicate. Plates were sealed to reduce risk of contamination and were incubated without shaking for a total of 336 hours for azithromycin and clarithromycin, and 96 hours for all other antibiotics. Plates were read every 24 hours, and for the macrolides, further readings at 168 hours (1 week) and 336 hours (2 weeks) were also performed. This step is recommended by the CLSI for detecting inducible macrolide resistance in *M. abscessus* clinical isolates (240).

2.3.5. Genomic DNA extraction of *M. abscessus* clinical isolates

M. abscessus clinical isolates were inoculated into 10 mL of 7H9 Middlebrook broth supplemented with 10% ADC, 1% glycerol and 0.05% Tween 80 and were grown in a shaking incubator at 30 °C until an optical density (OD) of >1.0 was reached. Cells were harvested by centrifugation at 4000 rpm for 15 minutes at room temperature and the supernatant was discarded. Pellets were then resuspended

in 5 mL Glucose-tris-EDTA (GTE) buffer (25 mM Tris HCl pH8, 10 mM EDTA pH 8, and 50 mM Glucose). Then, 5 mL of chloroform : methanol (2 : 1, v,v) was added to each sample and was mixed gently by hand for 5 minutes. The suspension was centrifuged at 3500 rpm for 20 minutes at room temperature. Cells are then suspended in the interphase layer, therefore the top and bottom solvent layers were carefully removed and cells were subsequently dried at 55 °C. Dry cells were then resuspended in 450 µL GTE + RNase A (10 µL of 10 mg/mL RNase A in 1 mL GTE buffer), and 50 µL of 10 mg/mL lysozyme solution was added to cells and was incubated at 37 °C overnight with no shaking. Following this, cells were transferred to a 2 mL Eppendorf tube and 100 µL of 10% (w/v) sodium dodecyl sulphate (SDS) and 15 µL of 20 mg/mL proteinase K was added to each sample and incubated for 3 hours at 55 °C. Then, 10 µL of RNase A (10 mg/mL) was added to each sample and was incubated at 37 °C for 30 minutes without shaking. Subsequently, 200 µL 5M NaCl was added before the addition of 1 mL chloroform : isoamyl alcohol (24 : 1, v/v) and the solution was gently mixed for 5 minutes. The samples were the centrifuged at 13,000 rpm on a benchtop microcentrifuge for 10 minutes, which produced an interphase layer of cell debris, which was subsequently removed and discarded. The samples were centrifuged again for 10 minutes at 13,000 rpm and the upper aqueous layers were placed into fresh tubes. Another 1 mL chloroform : isoamyl alcohol (24 : 1, v/v) was added to each tube and centrifuged again at 13,000 rpm for 10 minutes. The upper aqueous layer was then removed and placed in a fresh tube and a further 1 mL chloroform : isoamyl alcohol (24 : 1, v/v) was added and the centrifugation process was repeated once more. The upper aqueous layer was placed in a fresh tube and 0.7 volume (700 µL) of ice cold isopropanol was added to the retained aqueous layer, which was then mixed by gentle inversion. The samples were centrifuged at 13,000 rpm for 30 minutes at 4 °C. The supernatant was then removed and pellet was washed in 0.7 volumes (700 µL) of ice cold 70% ethanol and was again centrifuged at 13,000 rpm for 30 minutes at 4°C. The ethanol was carefully removed using a pipette and the remaining pellet was left to dry at room temperature. Once dried, the pellet was resuspended in 40 µL Nuclease-free water (Merck Life Science, Dorset, UK).

2.3.7. PCR conditions

Polymerase chain reaction (PCR) was performed using 5 primer pairs as previously described by Shallom *et al* in 2015 (Shallom, 2013). Primers were selected based on discriminatory regions identified using comparative genomic hybridisation (CGH) with *M. abscessus* strain ATCC 19977 (Shallom, 2013). In this study, PCR was performed using 5 different primer pairs with 20 clinical isolates and one reference strain, using Q5 DNA Polymerase (New England Biolabs, Massachusetts, USA) in a 25 µL total reaction mixture volume with 0.6 µL DNTPs, 2.5 µL Q5 buffer (New England Biolabs, Massachusetts, USA), 3 µL extracted DNA suspension and deionised water. Table 2.4 shows the *M. abscessus* NCTC 13031 locus, the subsequent gene annotation and forward and reverse primers (Shallom, 2013).

Table 2.4: PCR primers that target regions in the DNA of M. abscessus, M. massiliense, and M. bolletii for subspecies differentiation.

***M. abscessus* NCTC**	**Gene annotation**	**Primers**	
13031 locus		**Forward**	**Reverse**
MAB_2396	Probable acetyltransferase	5′-AGGCGGCCACCGACGTCGCGATGGA-3′	5′-TGCGCCCGCCCAGCGCGTATCCG-3′
MAB_2697c	Hypothetical protein	5′-GACTCCGGTGGCCGCGGCGA-3′	5′-GCCGGAGCGCTGGGTGGGCT-3′
MAB_4751	Conserved hypothetical protein	5′-CCCGCATGCAGCTGGCCGCGCA-3′	5′-GCGCCAGTGGTGGGGCCACCCGT-3′
MAB_4792	Putative transcriptional regular	5′-GCGGTGACGACCGCGGGGGCGAT-3′	5′-TCGGGGCAGGCCAGGGCGCCTA-3′
***erm*(41)**	Erythromycin ribosomal methylase	5′-GACCGGGGCCTTCTTCGTGAT-3′	5′-GACTTCCCCGCACCGATTCC-3′

The PCR cycling conditions were adapted from Shallom *et al* (2013) (Shallom, 2013) and involved an added optimisation step to determine the appropriate annealing temperature for each primer pair. Briefly, there was initial denaturation at 95 °C for 2 minutes, 35 cycles of denaturation at 95 °C for 1 minute, annealing at a temperature gradient of +/- 6 °C from 72 °C, and extension at 72 °C, with a final extension at 72 °C for 10 minutes. PCR products were visualised by 1% agarose gel electrophoresis that was run for 40 minutes at 100 V.

2.3.8. Determination of subspecies of *M. abscessus* clinical isolates

It is possible to determine the subspecies of *M. abscessus* by comparing the sizes of each PCR product produced using the PCR primers in Table 2.4. Table 2.5 shows the putative size(s) of each PCR product and the associated *M. abscessus* subspecies. Methods adapted from Shallom *et al* (2013) (Shallom, 2013) in which the laboratory strain *M. massiliense* (CCUG 48898) and the laboratory strain *M. bolletii* (CCUG 50184) were used to confirm the correct PCR product sizes for each clinical isolate; in this study, CCUG 48898 and CCUG 50184 were unavailable and so putative PCR product sizes were determined using the previous study (Shallom, 2013).

Table 2.5 (adapted from Shallom et al (2013)* (Shallom, 2013) *shows PCR product sizes for M. abscessus subspecies and putative PCR product sizes for M. abscessus subspecies clinical isolates using PCR primers for various genetic regions laid out in Table 2.4.

***M. abscessus* locus**	**Predicted PCR product size (bp)**					
	M. abscessus		*M. massiliense*		*M. bolletii*	
	NCTC 13031	Clinical isolates	CCUG 48898	Clinical isolates	CCUG 50184	Clinical isolates
MAB_2396	~1500 and 500	~1500 and 500	~750	~750	~750	~750
MAB_2967c	~1000	~1000 and/or 500 faint	~450	~450	~1000	~1000
MAB_4751	~1400 faint and 500	~1400 faint and/or 500	~450	~450 or 375	~500 and 400	~500 and 400 or~500
MAB_4792	~1200	~1200 or 650	~600	~600	~1200	~1200
***Erm*(41)**	~700	~700	~350	~350 or 700	~700	~700

2.4. Results

2.4.1. MIC testing of *Mycobacterium abscessus* clinical isolates against recommended antibiotic regimen

Following broth microdilution using 8 non-macrolide antibiotics, MICs were determined for each of the 24 clinical isolates and the NCTC 13031 *M. abscessus* strain in triplicate. Table 2.6 shows the MICs (in µg/mL) for each of the drugs against each isolate. It can be seen using the colour-coded key, where red equals resistant, yellow equals intermediate and green equals susceptible, that there is a high

degree of resistance across the whole regimen within this cohort of *M. abscessus* clinical isolates, as well as the *M. abscessus* NCTC strain.

Table 2.6. MICs (µg/mL) of each antibiotic in the recommended regimen against 25 M. abscessus clinical isolates including the NCTC 13031 strain. Table 2.6 shows the MIC at 96 hours of imipenem (IMI), amikacin (AMI), tigecycline (TIG) linezolid (LIN), clofazimine (CLOF), cefoxitin (CFX), minocycline (MIN), and moxifloxacin (MOX) against each M. abscessus isolate. Using CLSI breakpoint analysis, red squares indicate the isolate is resistant to the antibiotic, yellow squares indicate an intermediate MIC, and green squares indicate susceptibility to the antibiotic.

	Antibiotics (µg/mL)							
Isolate ID	IMI	AMI	TIG	LIN	CLOF	CFX	MIN	MOX
NCTC	2	16	1	64	>4	32	16	8
DC088 A	4	64	>4	>64	>4	64	32	32
DC088 B	4	>64	2	>64	>4	32	32	2
DC088 C	8	>64	>4	>64	>4	32	>32	8
DC088 D	4	16	1	32	>4	64	8	4
DC088 E	2	4	0.5	64	>4	32	32	4
DC088 Ref	4	64	0.5	>64	>4	32	32	2
211666	4	4	>4	>64	>4	16	8	8
137071	2	2	0.25	>64	>4	16	16	16
199277	4	>128	4	>64	>4	16	>16	>16
194891	4	>128	1	>64	>4	16	8	16
159544	4	16	>4	>64	>4	16	16	>16
186433	2	8	2	64	>4	16	16	16
186144	8	16	0.5	ND	>4	16	ND	ND
186154	16	4	0.25	>64	>4	32	8	16
147028	4	16	>4	>64	>4	16	>16	16
GOSH1BAL2	32	>128	>4	64	>4	>64	>16	>16
GOSH2	32	4	>4	>64	>4	>64	8	8
GOSH3	8	>128	>4	>64	>4	8	16	16
GOSH4	16	16	4	>64	>4	>64	>16	16
GOSH5	4	2	4	16	>4	>64	2	2
GOSH6	8	32	4	>64	>4	>64	8	8
GOSH7	8	8	>4	>64	>4	>64	16	16
GOSH8	>32	2	4	>64	>4	>64	16	8
BCH1	4	>128	4	>64	>4	>64	8	8

Imipenem (IMI) was the most effective drug against the panel of clinical isolates, with 60% (15/25) being susceptible (MIC range 2-4 µg/mL), 28% (7/25) being intermediate (MIC range 8-16 µg/mL), and only 12% (3/25) showed resistance, with MICs of 32 µg/mL or higher. Amikacin (AMI) was the second-

most effective antibiotic; 60% (15/25) of the isolates were susceptible after 96 hours (MIC range 2-16 μg/mL), 4% (1/25) was intermediate (MIC 16 μg/mL) and 36% (9/25) were resistant (MIC ≥64 μg/mL). The next most effective drug was the β-lactam, cefoxitin (CEF), where 36% (9/25) were susceptible (MIC range 8-16 μg/mL), 32% (8/25) were intermediate (MIC range 16-64 μg/mL), and a further 36% (8/25) were resistant (>64 μg/mL).

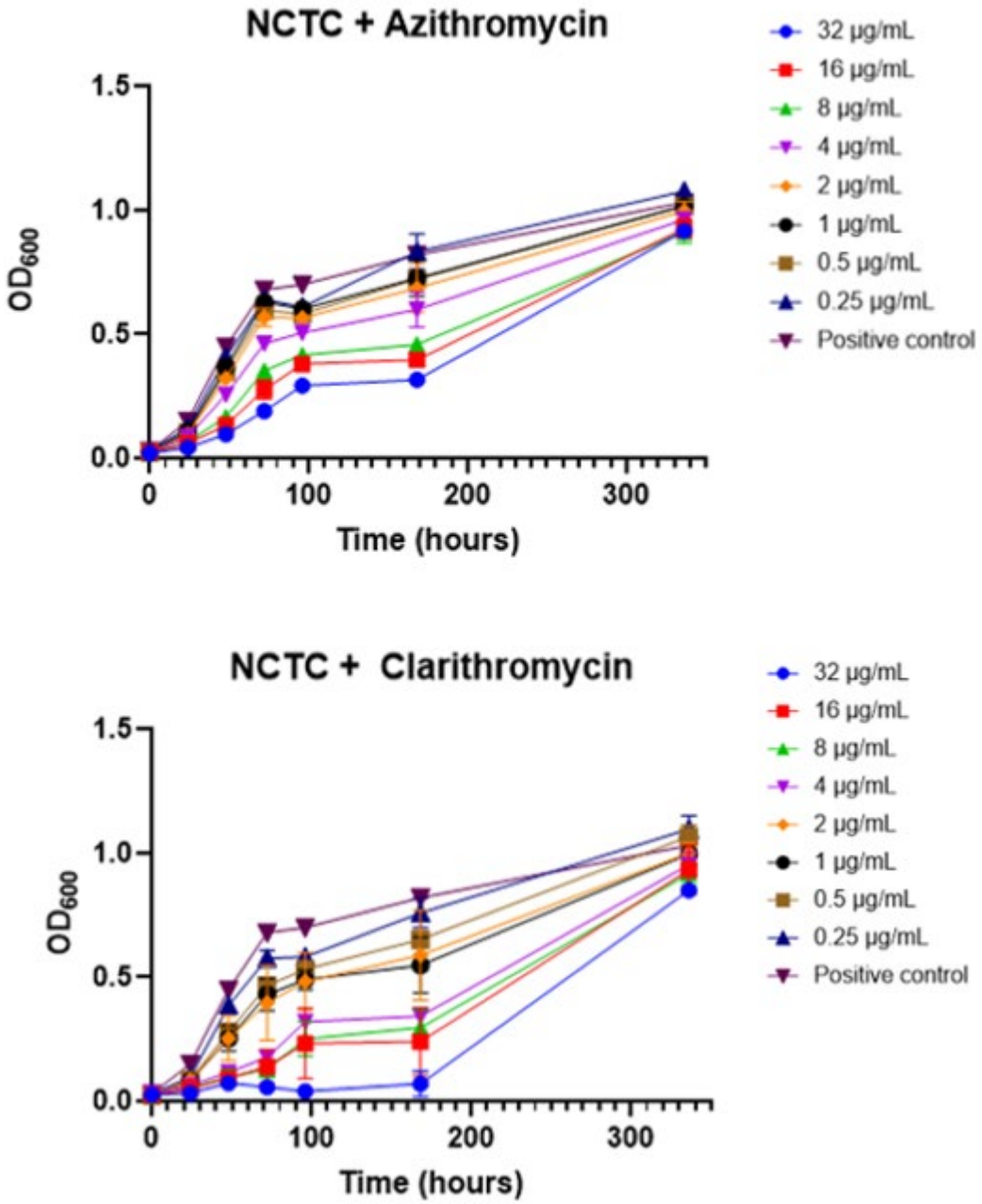
Tigecycline (TIG) showed rather mixed results, with MICs ranging from 0.25 to >4 μg/mL (Table 2.6). The breakpoint for susceptibility in this experiment was determined to be 4 μg/mL, with no intermediate concentration. Therefore, if an isolate had an MIC of lower than 4, it was deemed susceptible, and isolates with an MIC of equal to or higher than 4 μg/mL would be deemed resistant. In this cohort, 40% of the isolates were susceptible (MIC range 0.25 – 2 μg/mL) and 60% of the isolates were resistant with MICs equal to or higher than 4 μg/mL.

For Linezolid (LIN), minocycline (MIN), and moxifloxacin (MOX) one isolate (186433, see Table 2.6) did not grow efficiently during the experiment and therefore will be excluded from further analysis. Linezolid showed poor activity against the 24 isolates, with no isolates showing susceptibility and only 4.2% (1/24) were intermediate. The other 95.8% (23/24) were resistant to linezolid with MICs equal to or higher than 32 μg/mL. Moxifloxacin (MOX) also performed relatively poorly, with no susceptible isolates, and only 12.5% were intermediate (MIC of 2 μg/mL) with the remaining 87.5% (21/24) showing total resistance with MICs of 4 μg/mL or higher. Minocycline (MIN) also showed poor activity; again no isolates were susceptible and only 4.2% (1/24) was intermediate with an MIC of 2 μg/mL, with the remaining 95.8% (23/24) being totally resistant with MICs equal to or higher than 8 μg/mL. The worst performing drug within the regimen against the panel of clinical isolates was clofazimine, with 100% resistance (MIC >4 μg/mL).

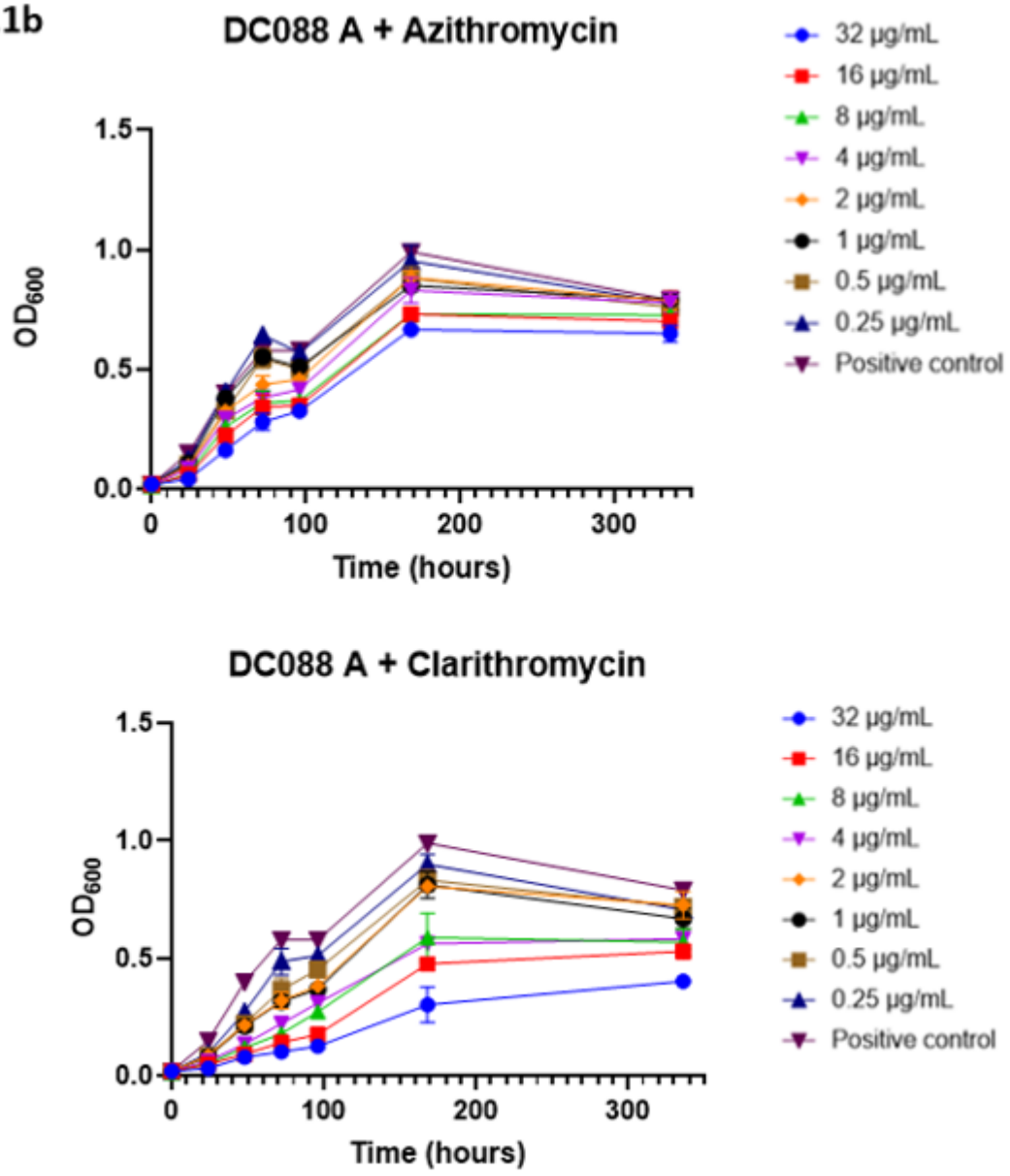
2.4.2. Determination of phenotypic inducible macrolide resistance in *M. abscessus* clinical isolates.

Using the broth microdilution method based on current CLSI recommendations (Wayne, 2015), MIC ranges were determined for the macrolide antibiotics azithromycin and clarithromycin against 24 *M. abscessus* clinical isolates and the NCTC 13031 *M. abscessus* strain. The data in Figures 2.1a-z shows that both azithromycin and clarithromycin display high MICs against the *M. abscessus* clinical isolates, with clarithromycin generally performing better than azithromycin over the 14-day testing period.

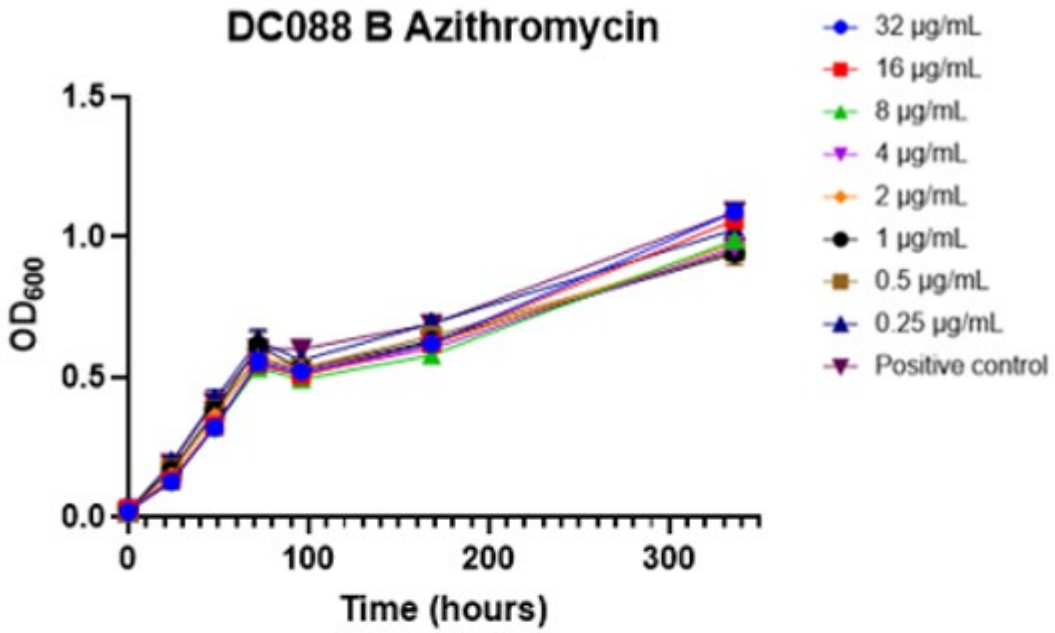
2.1a

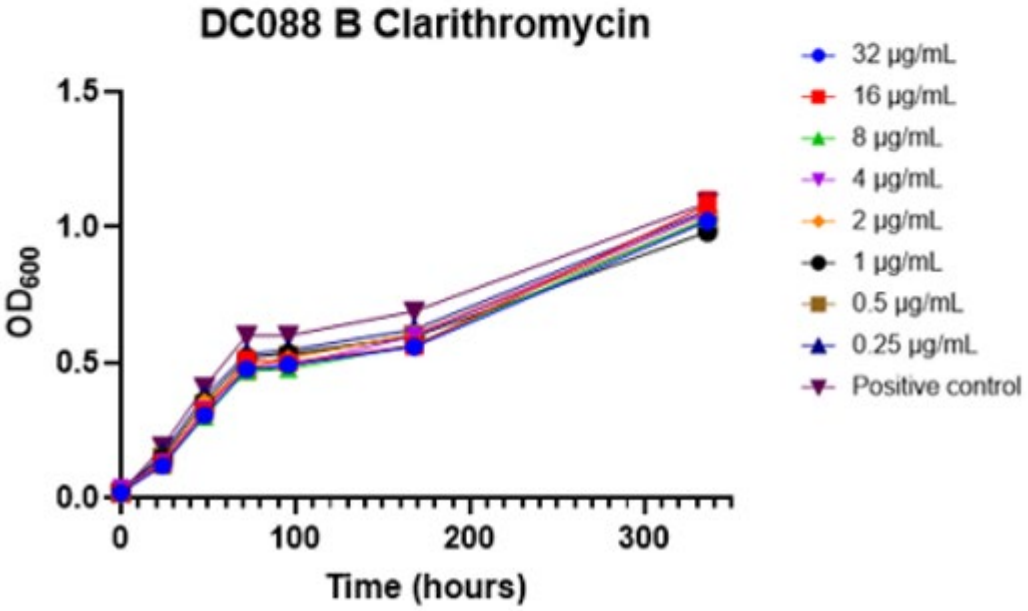

NCTC + Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
NCTC + Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1b

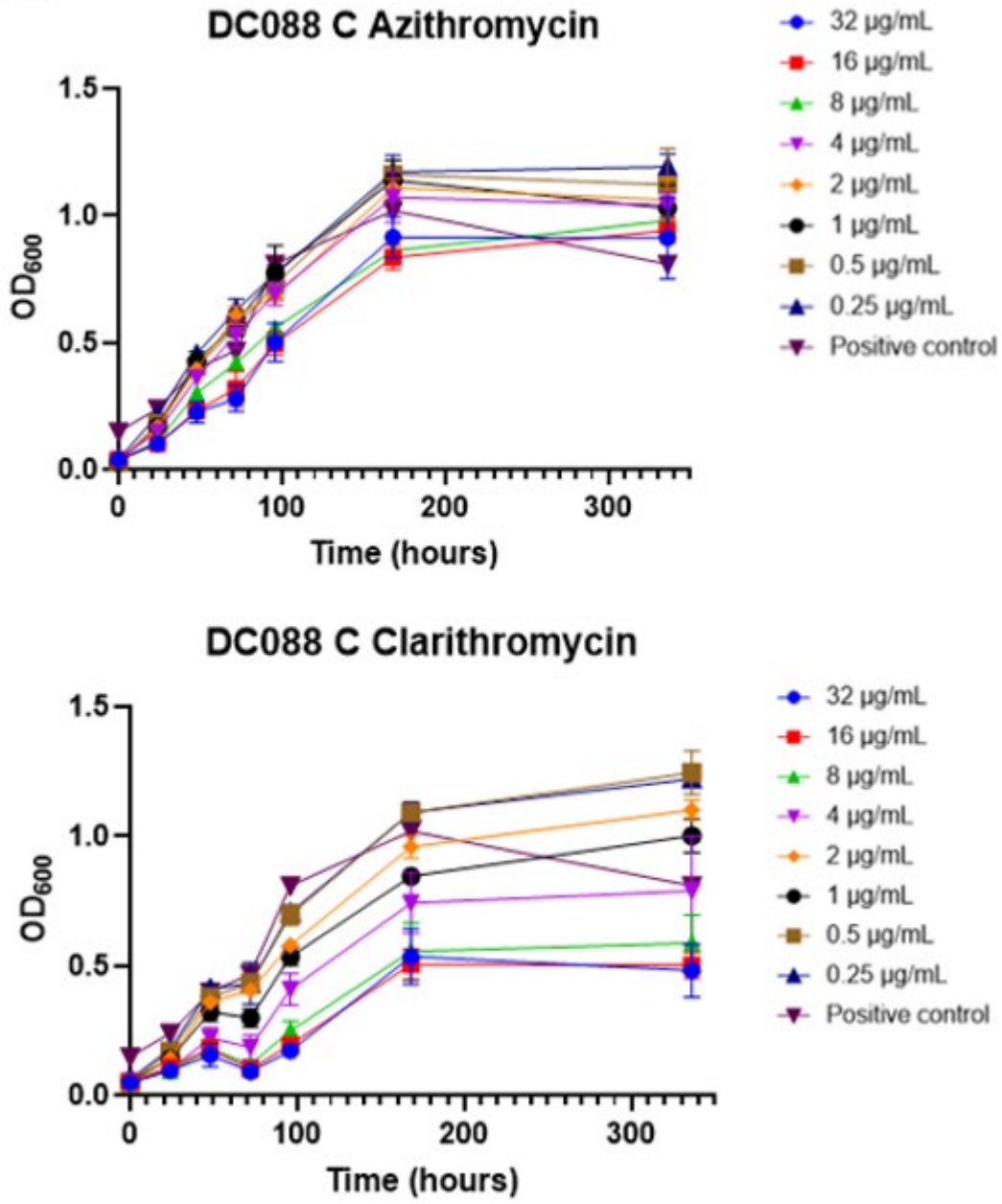

DC088 A + Azithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
DC088 A + Clarithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)

2.1c

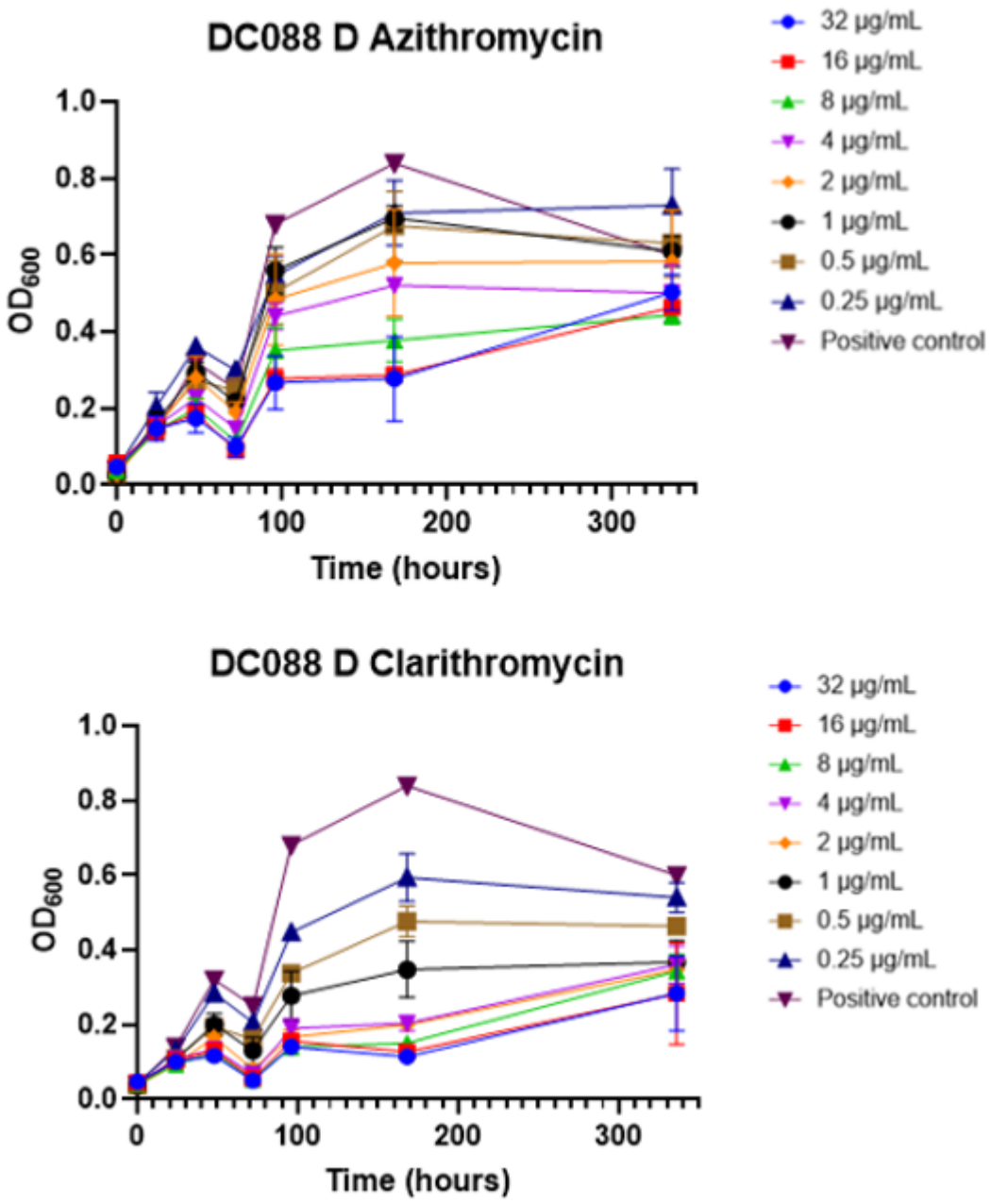

DC088 B Azithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)


DC088 B Clarithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)

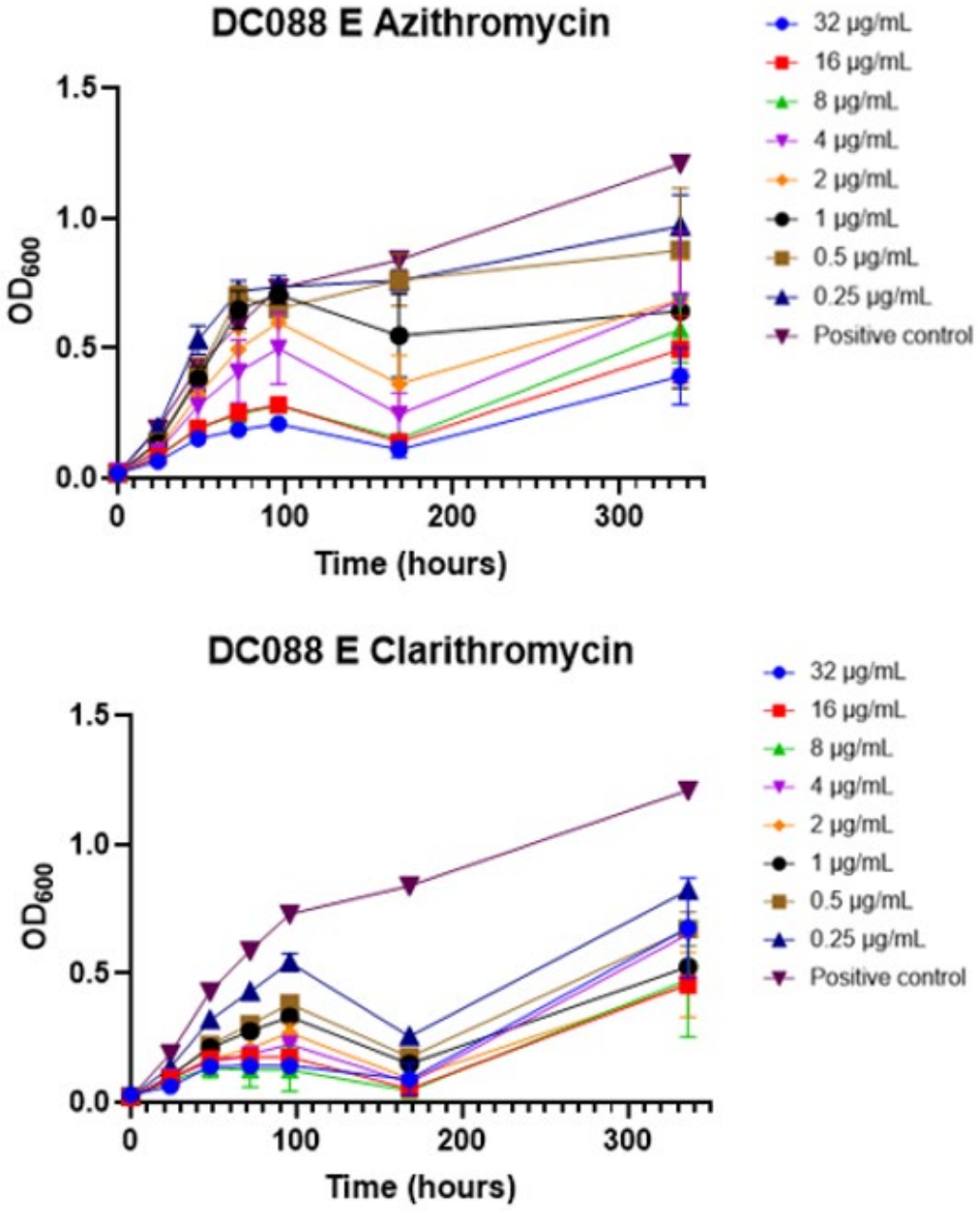
2.1d


DC088 C Azithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
DC088 C Clarithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)

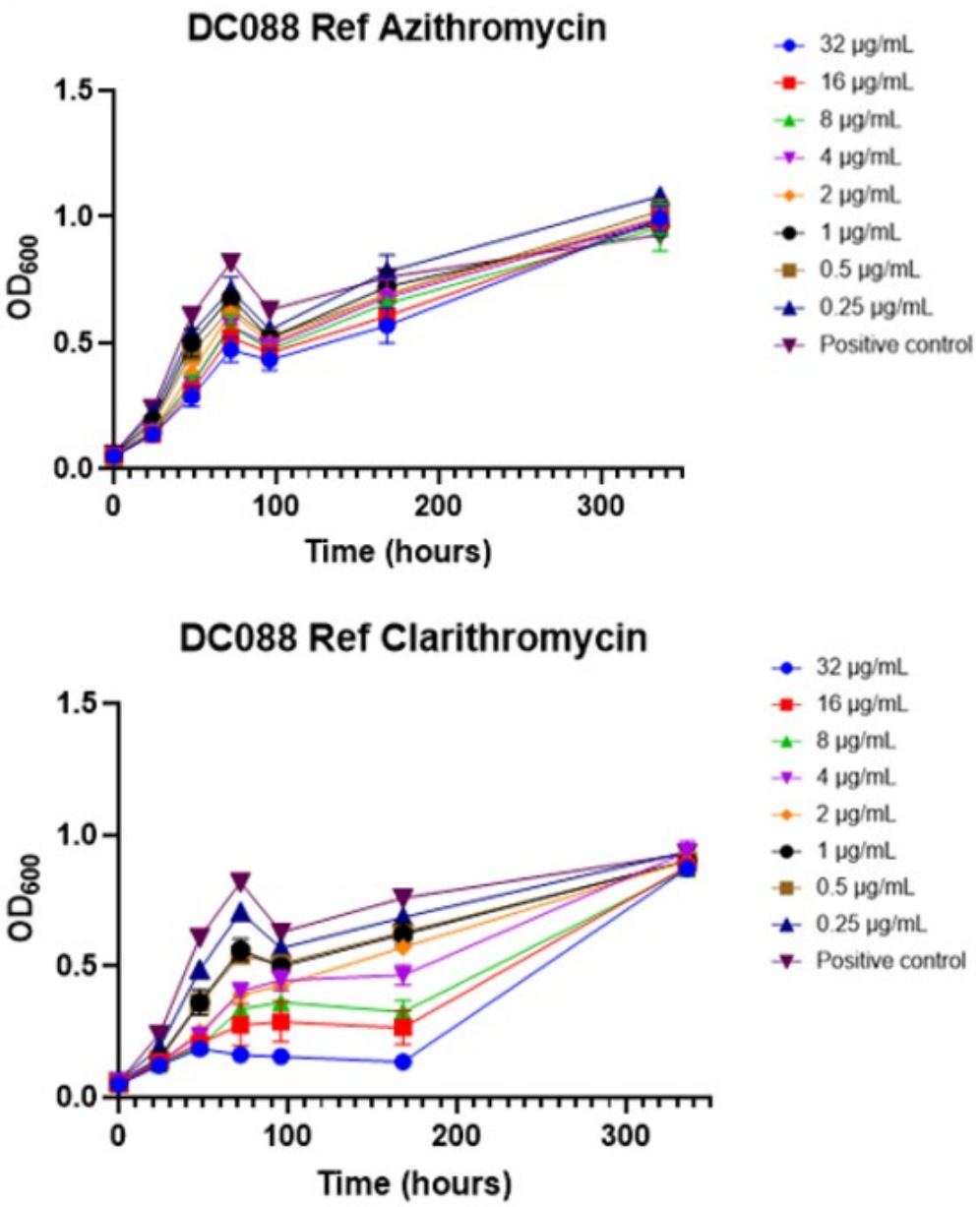
2.1e


DC088 D Azithromycin
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
DC088 D Clarithromycin
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

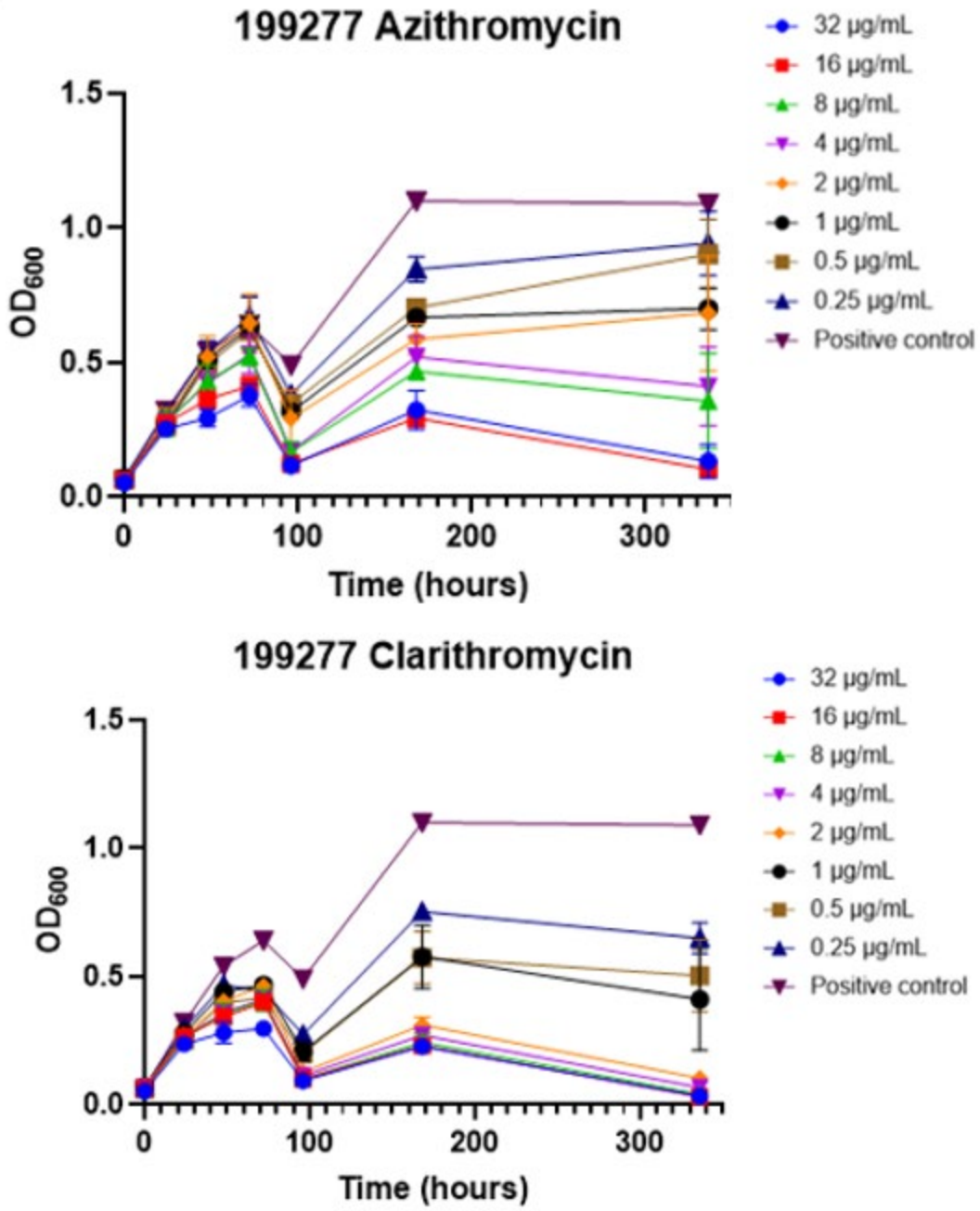
2.1f


DC088 E Azithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
DC088 E Clarithromycin
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)

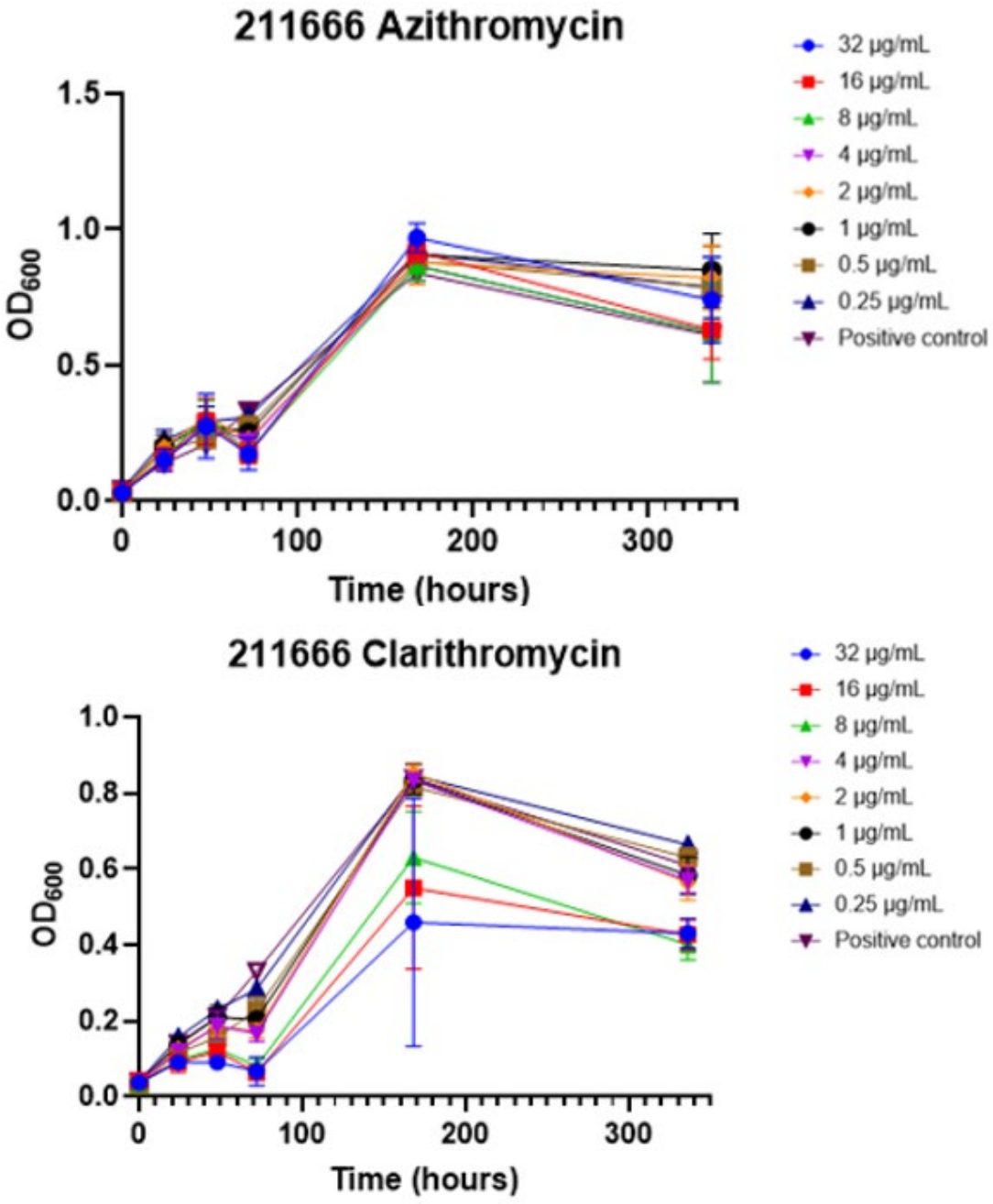
2.1g


DC088 Ref Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
DC088 Ref Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

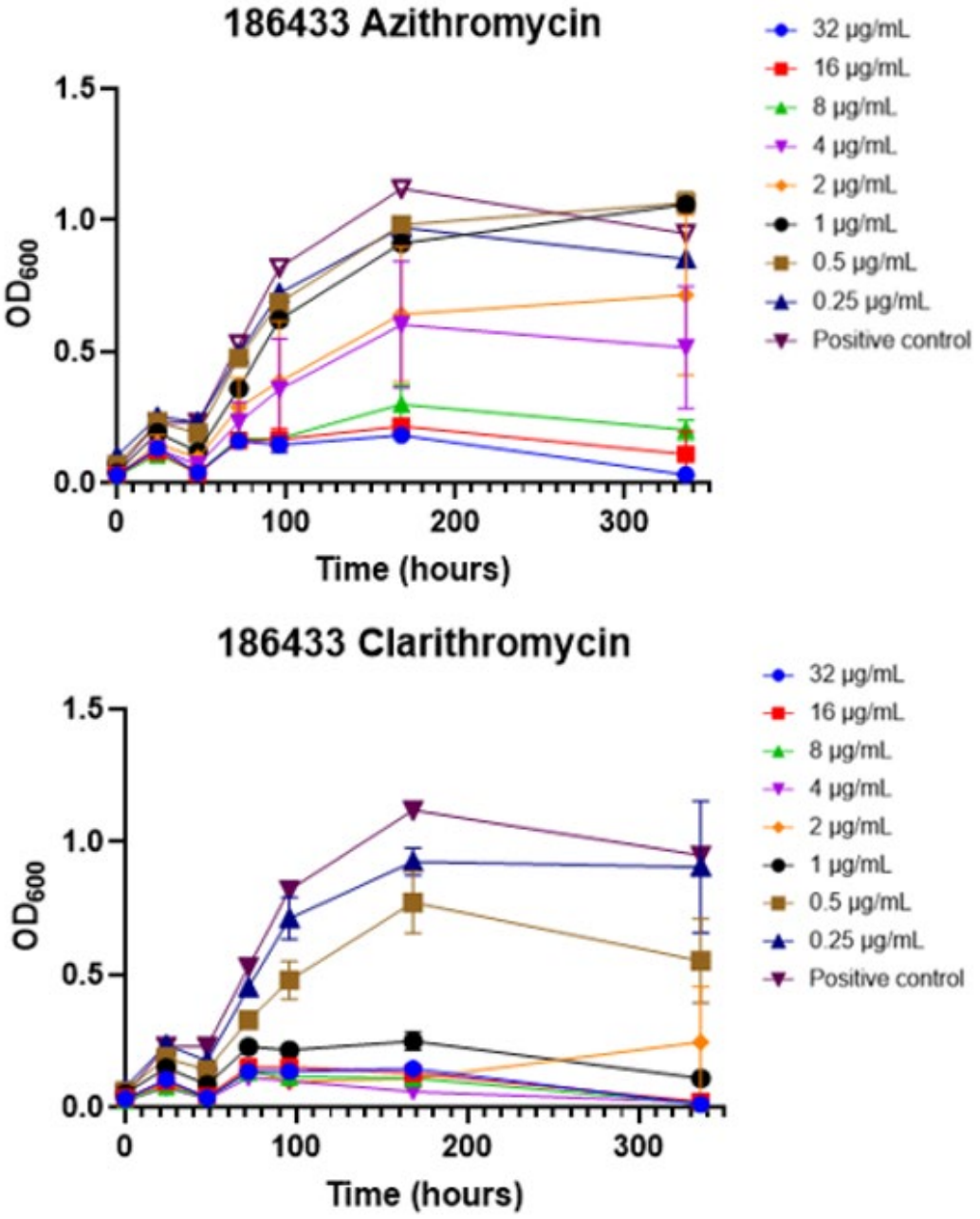
2.1h


199277 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
199277 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

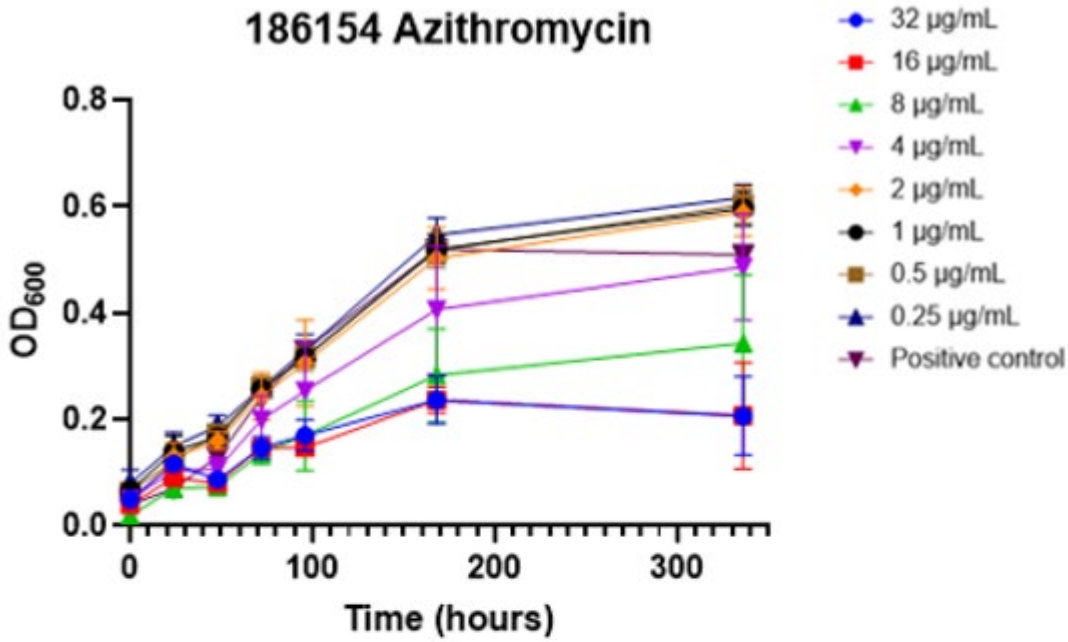
2.1i

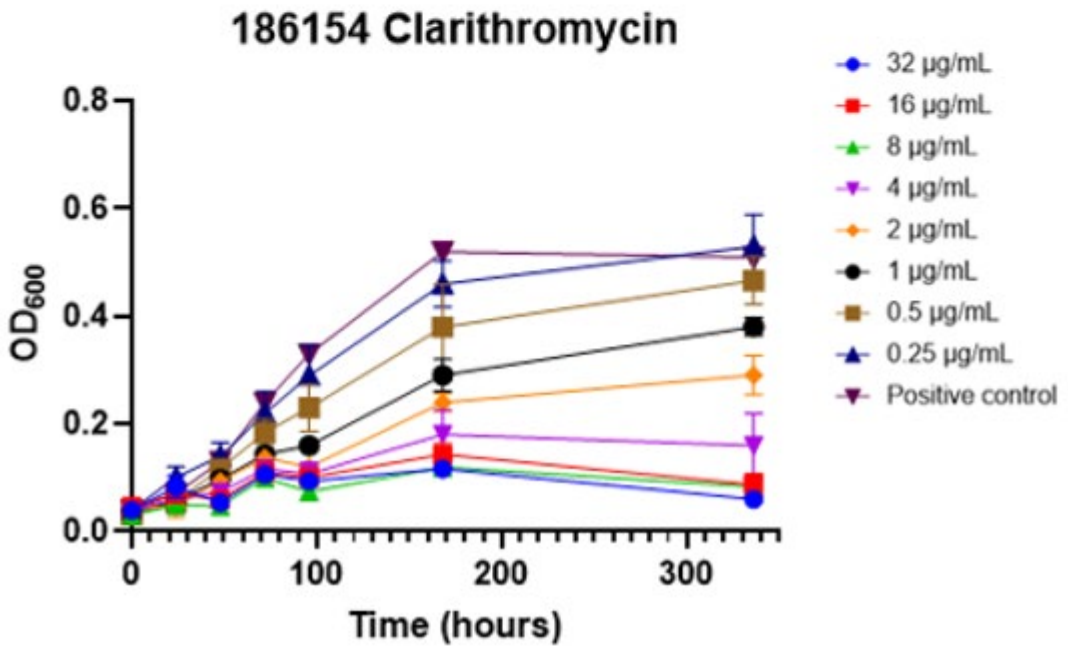

211666 Azithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
211666 Clarithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1j

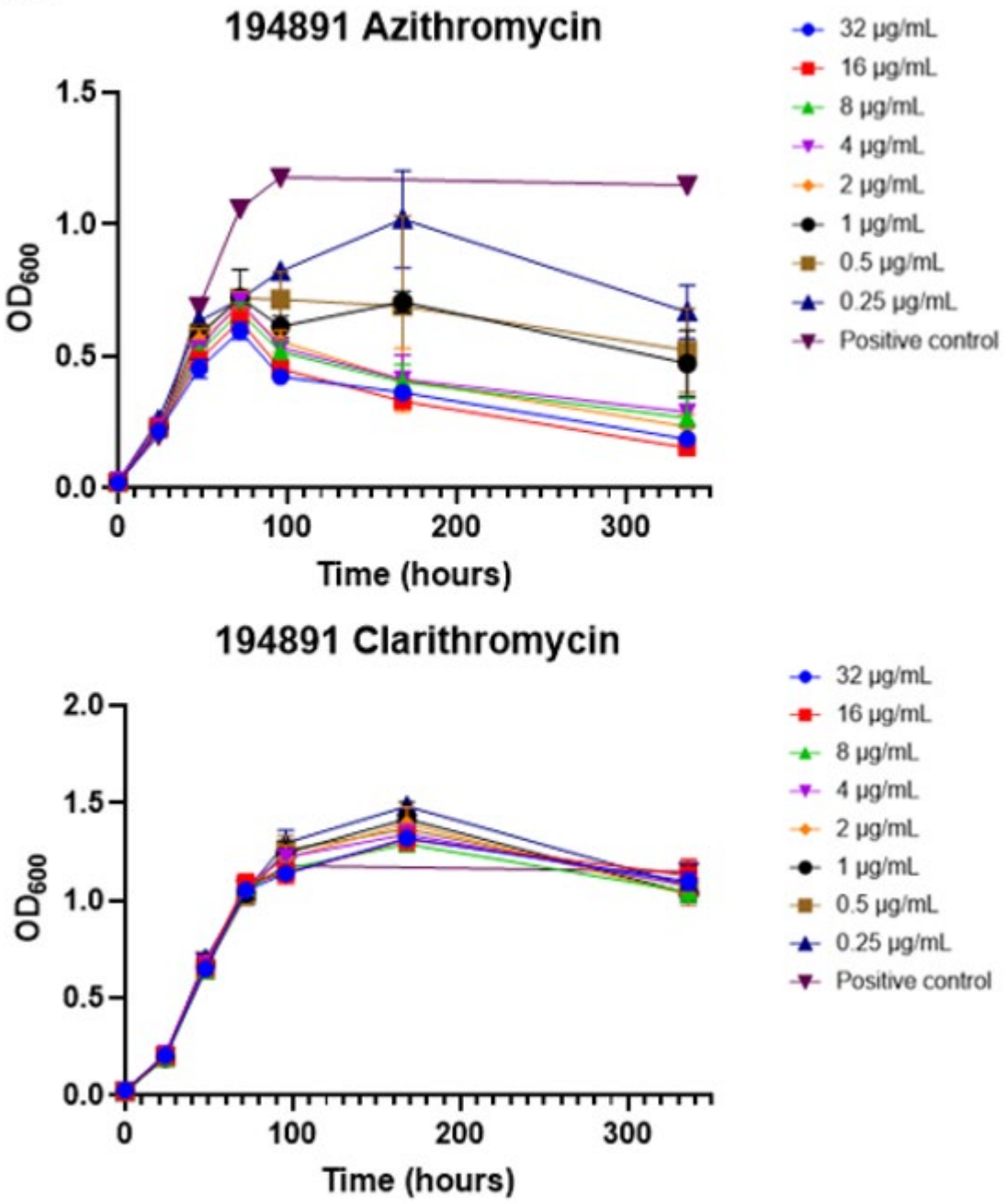

186433 Azithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
186433 Clarithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1k

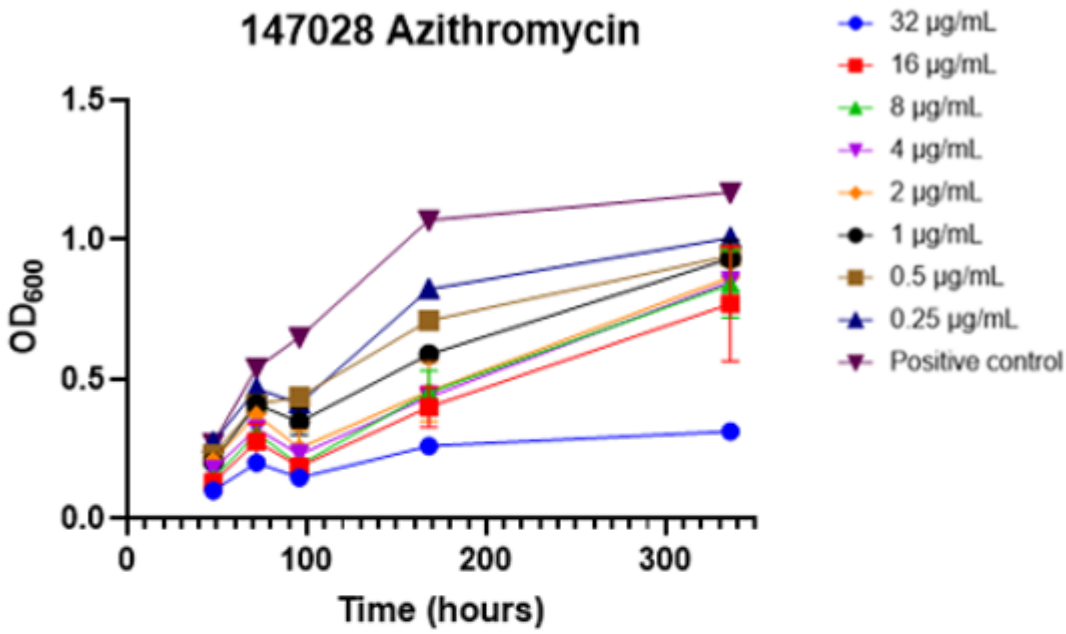

186154 Azithromycin
OD600
0.0
0.2
0.4
0.6
0.8
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

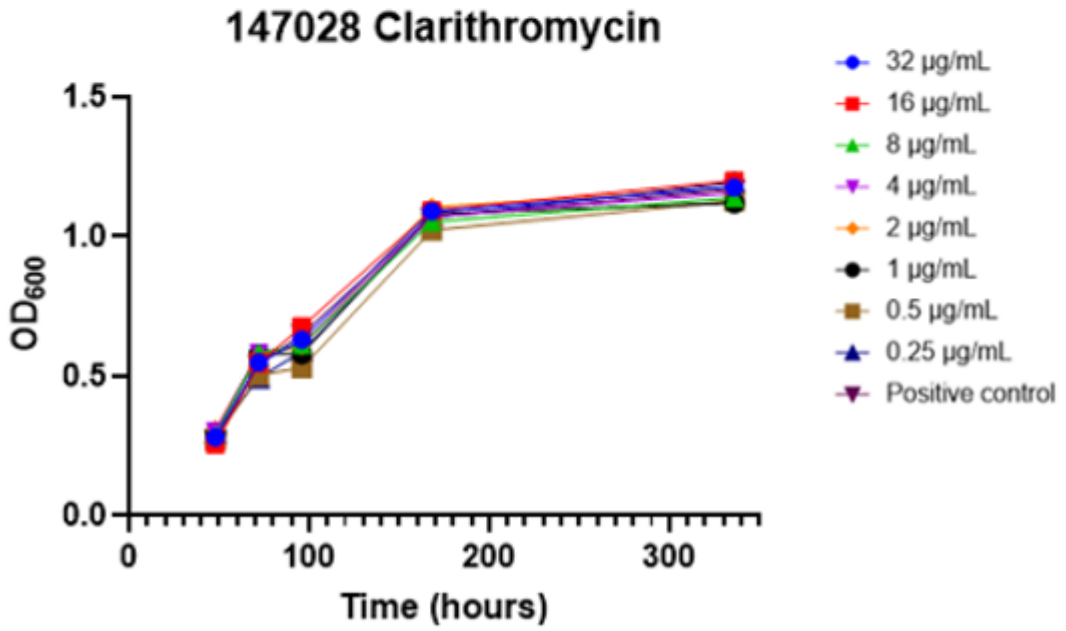

186154 Clarithromycin
OD600
0.0
0.2
0.4
0.6
0.8
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1l

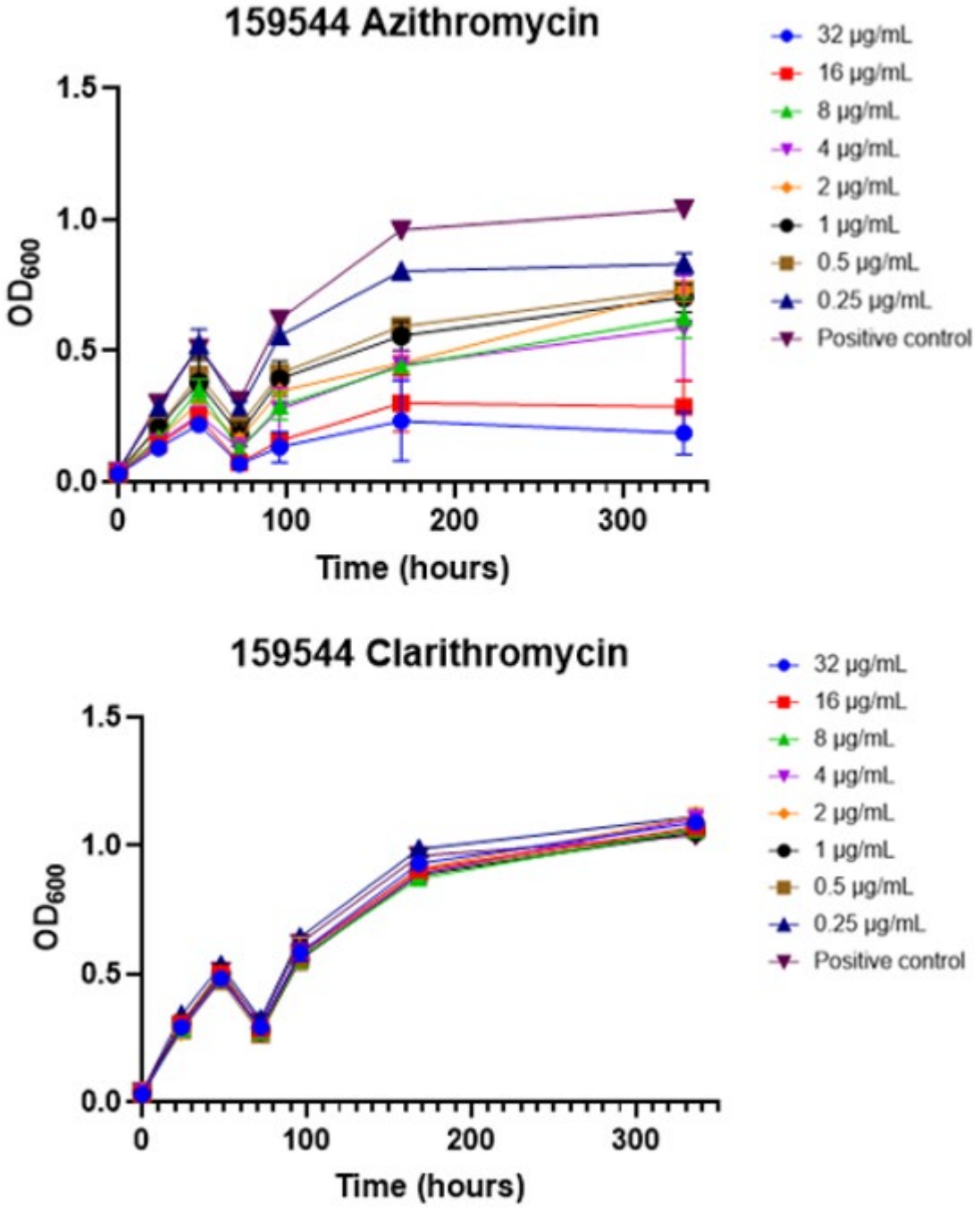

194891 Azithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
194891 Clarithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1m

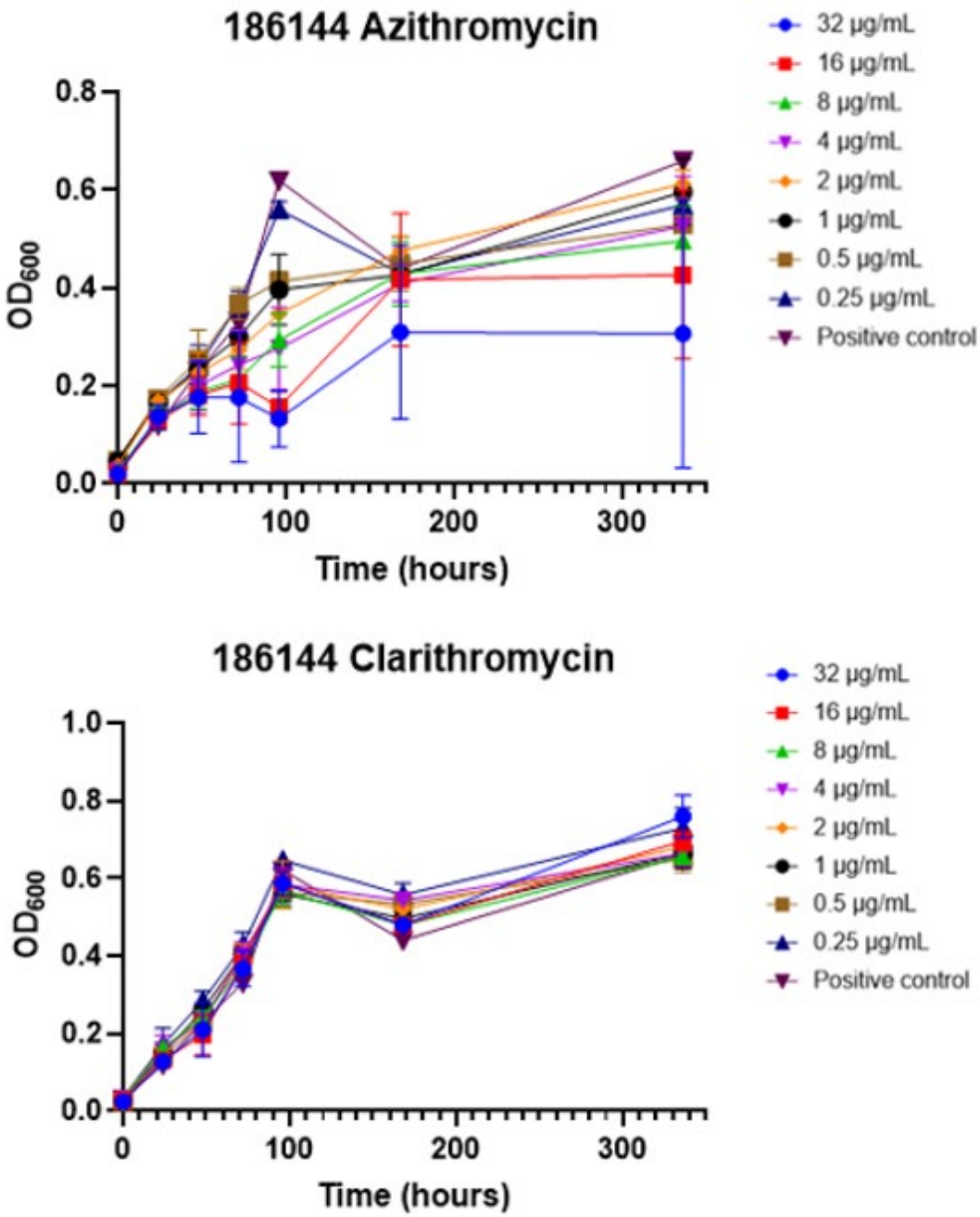

147028 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control


147028 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

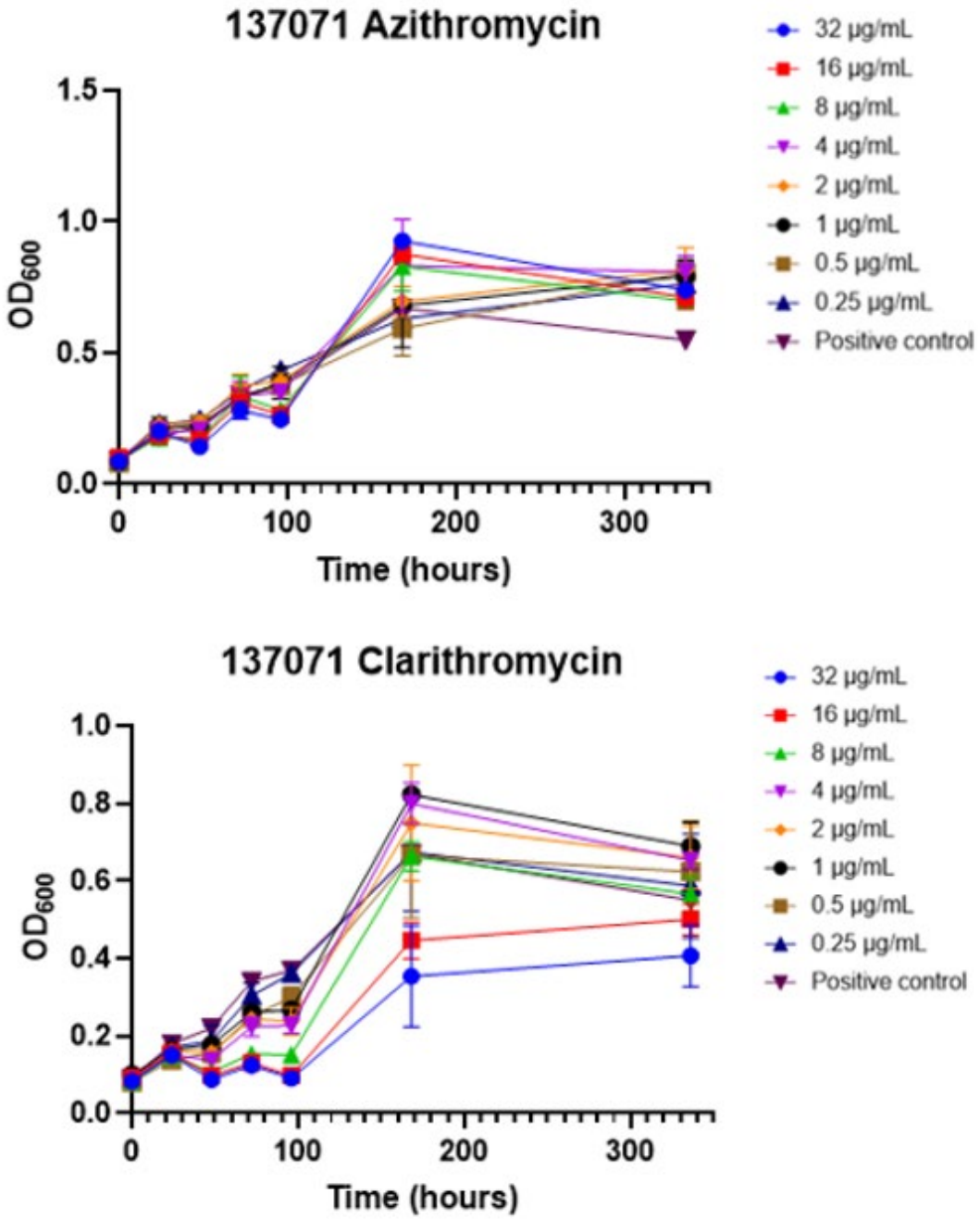
2.1n


159544 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
159544 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

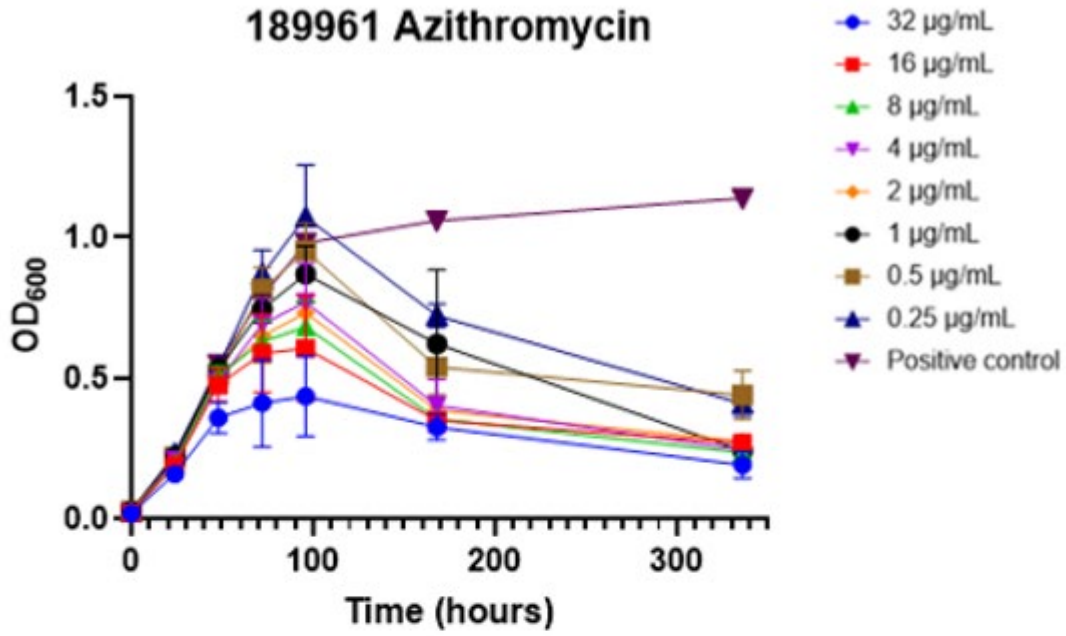
2.1o

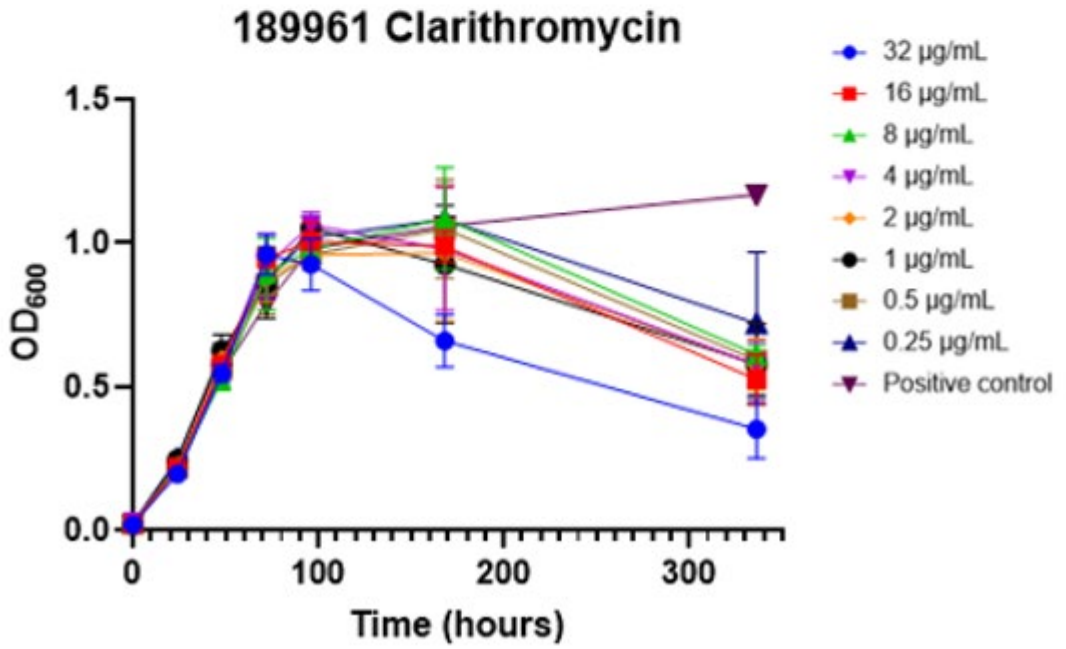

186144 Azithromycin
OD600
Time (hours)
0.0
0.2
0.4
0.6
0.8
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
186144 Clarithromycin
OD600
Time (hours)
0.0
0.2
0.4
0.6
0.8
1.0
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1p

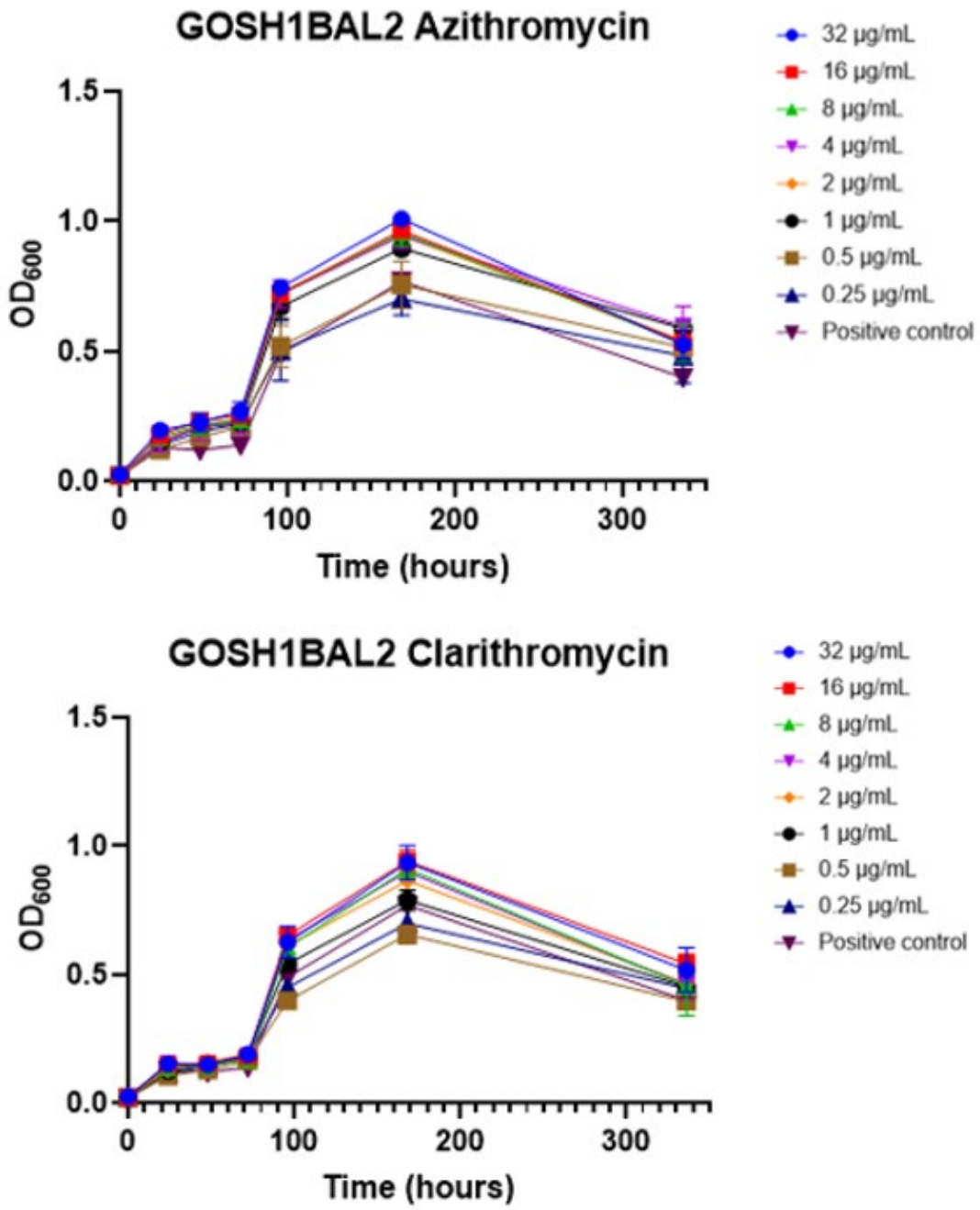

137071 Azithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
137071 Clarithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1q

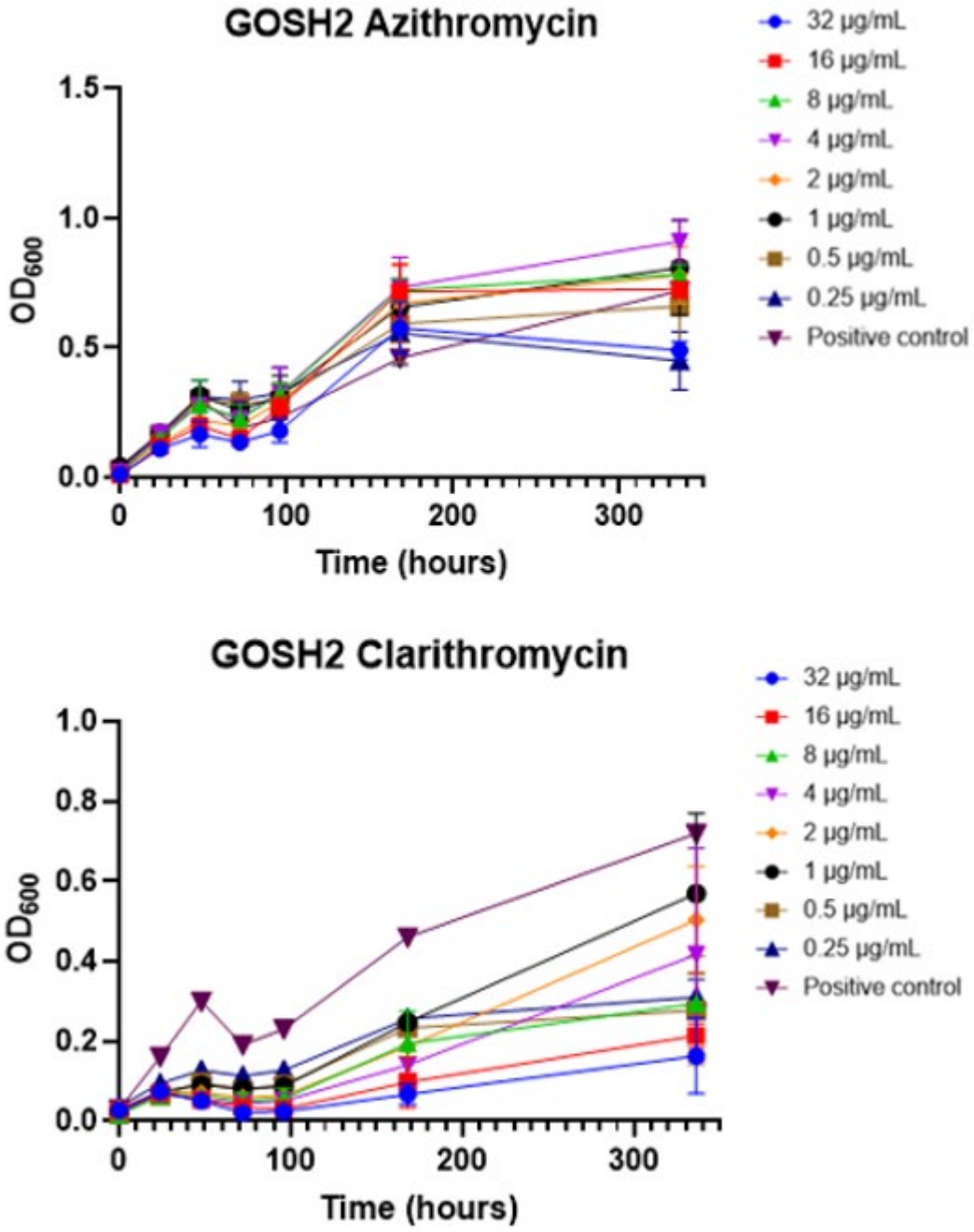

189961 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control


189961 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

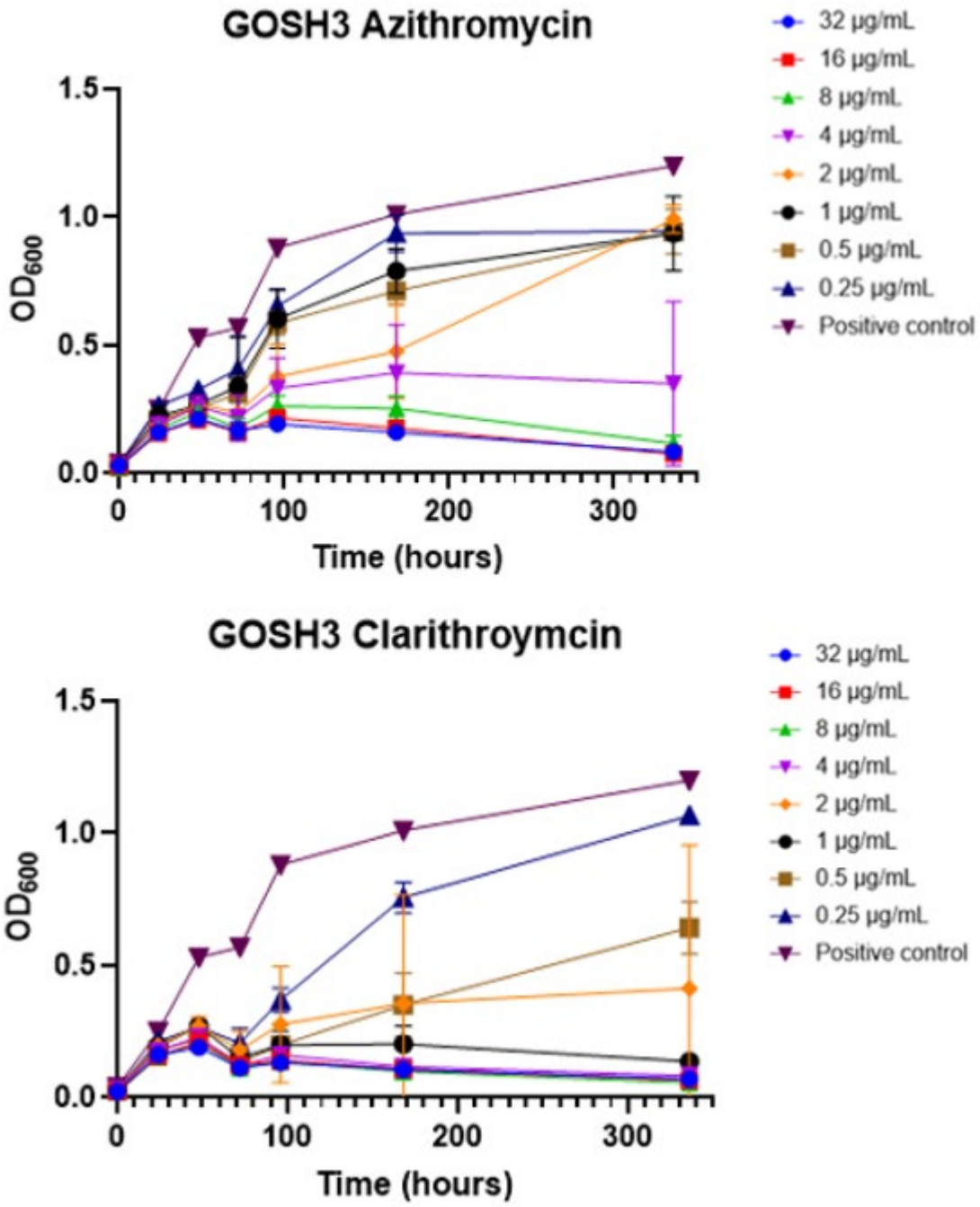
2.1r


GOSH1BAL2 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH1BAL2 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

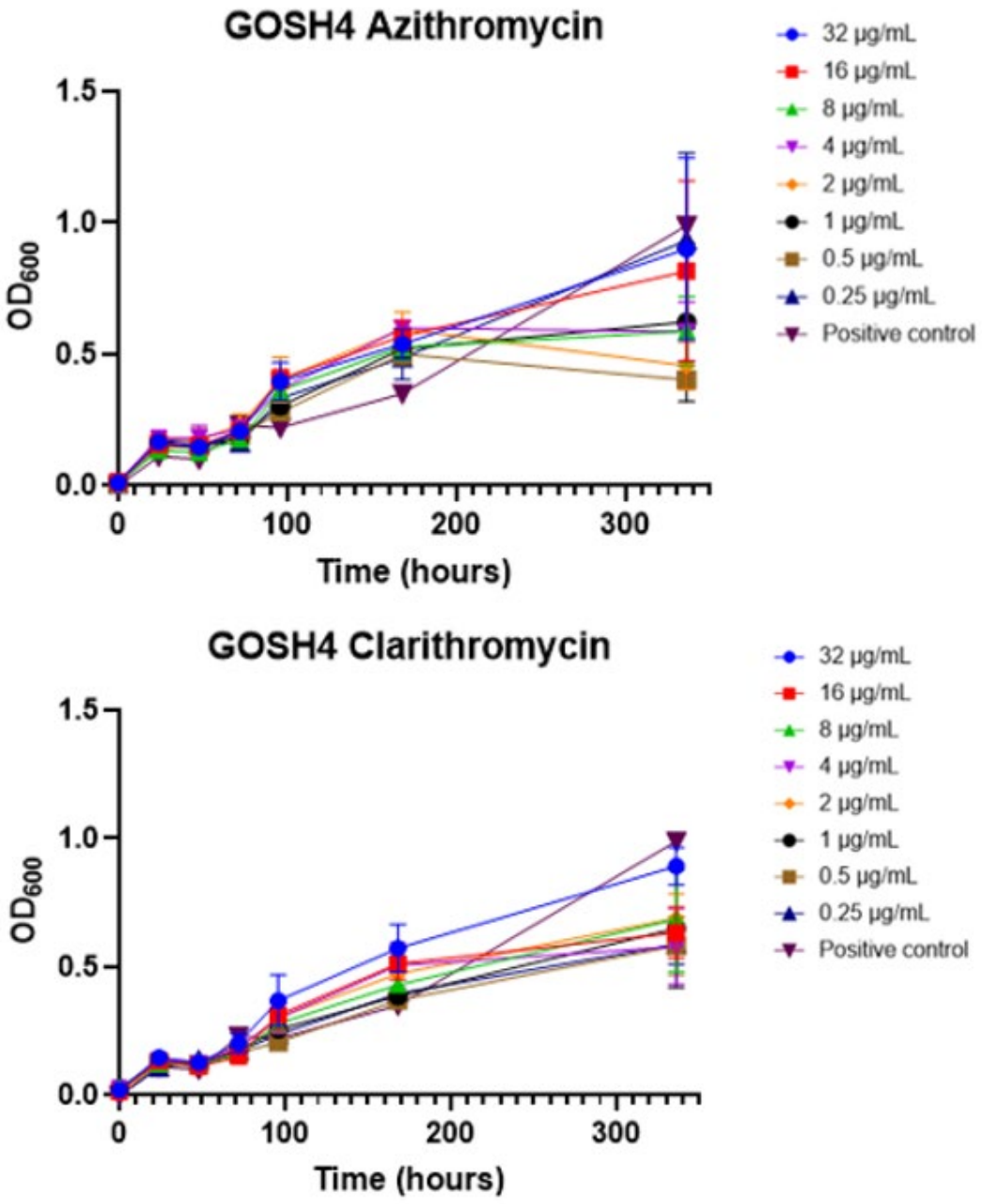
2.1s


GOSH2 Azithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH2 Clarithromycin
OD600
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

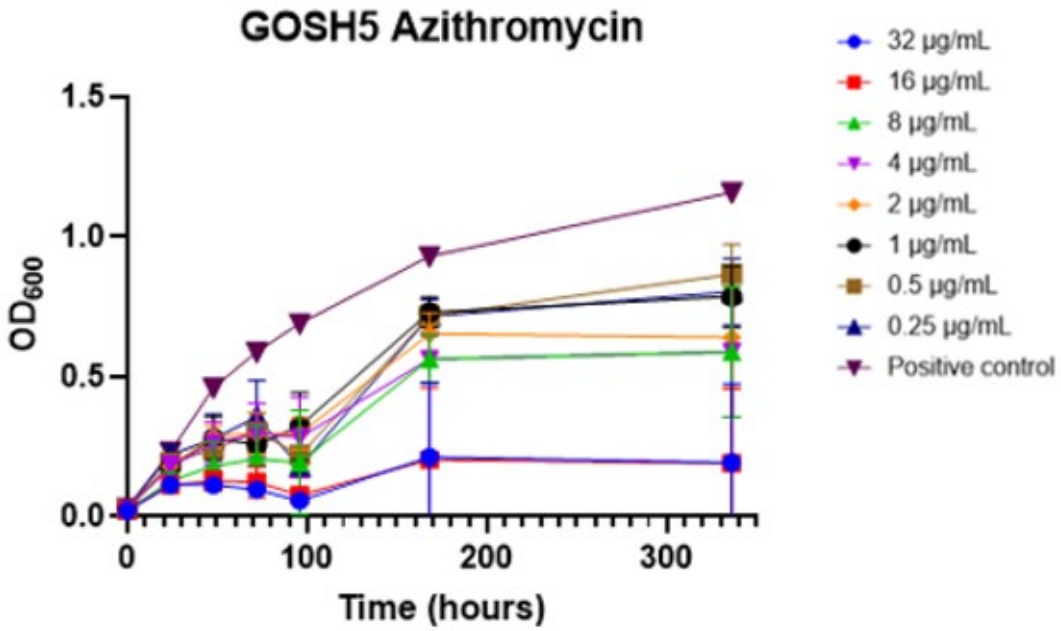
2.1t

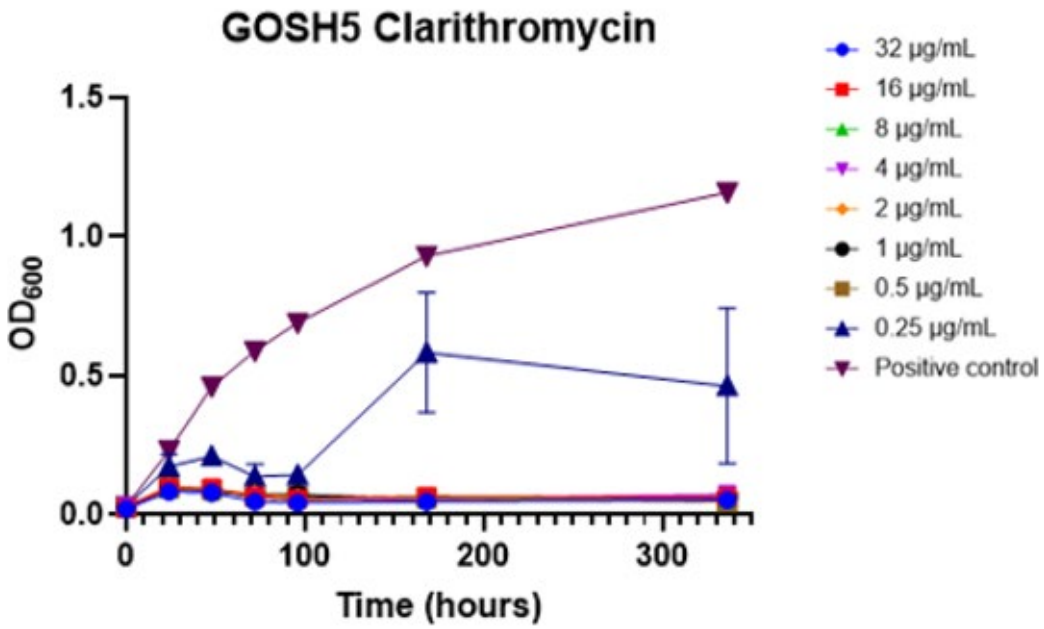

GOSH3 Azithromycin
OD600
Time (hours)
0.0
0.5
1.0
1.5
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH3 Clarithroymcin
OD600
Time (hours)
0.0
0.5
1.0
1.5
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1u

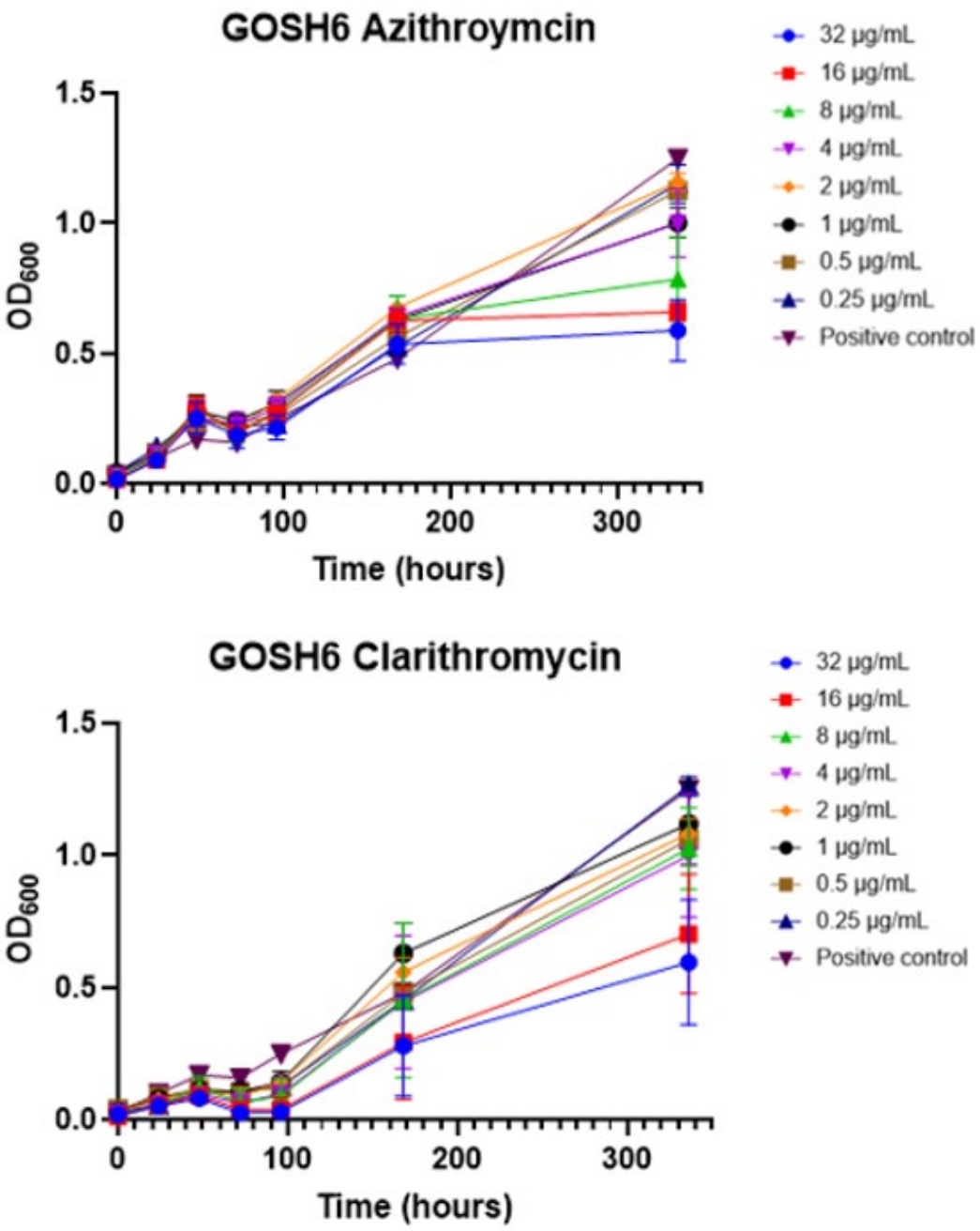

GOSH4 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH4 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1v

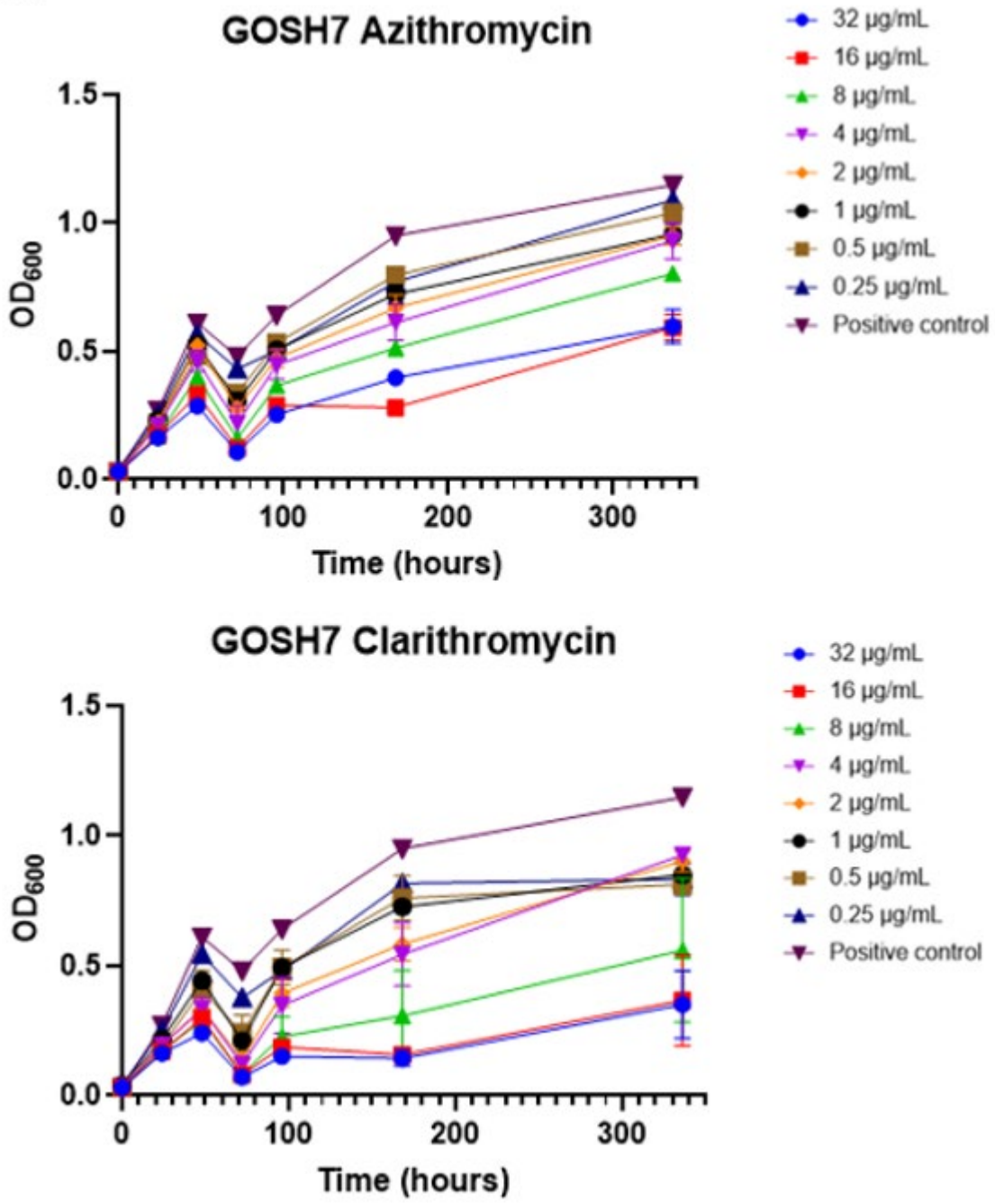

GOSH5 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control


GOSH5 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

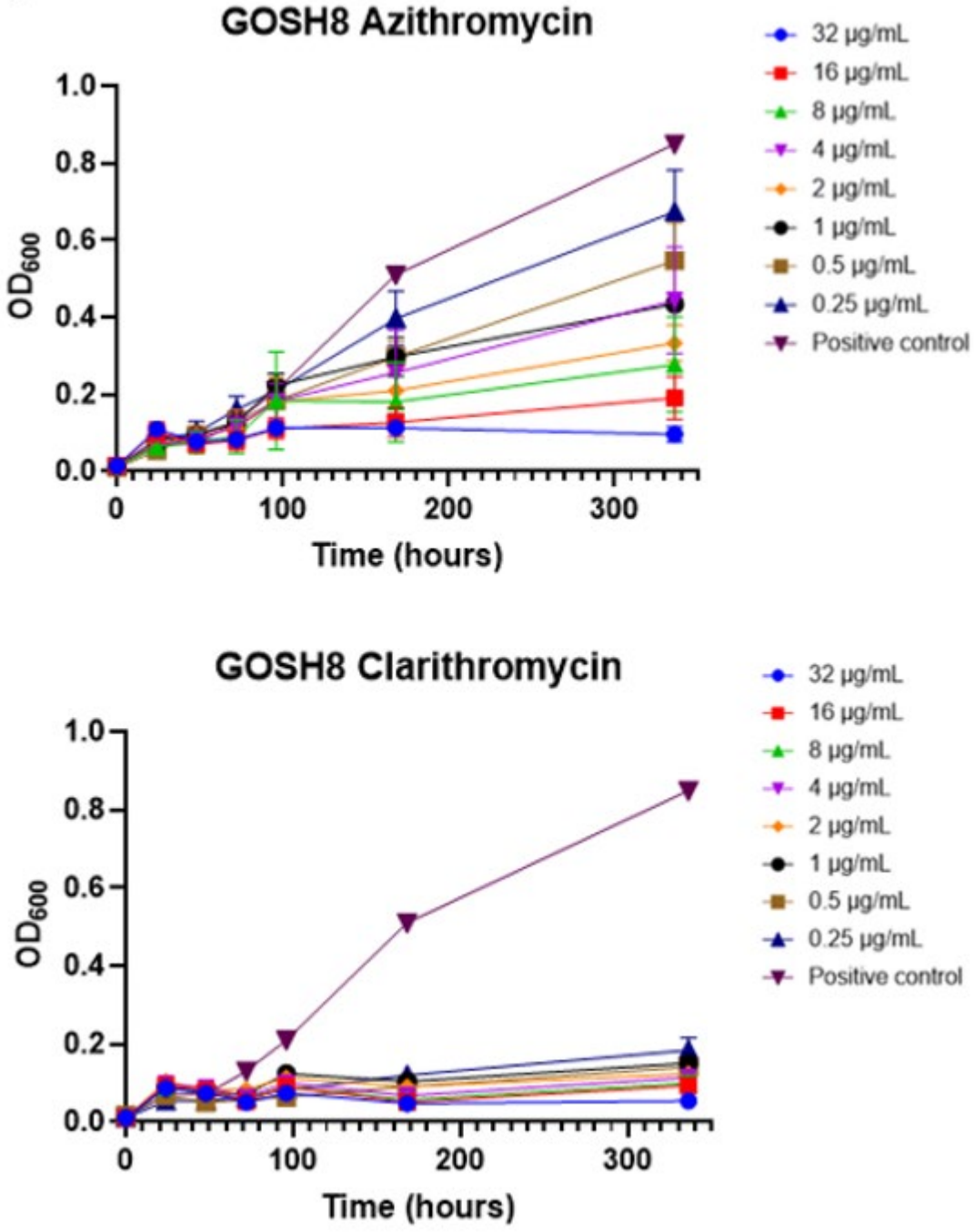
2.1w


GOSH6 Azithroymcin
OD600
Time (hours)
0.0
0.5
1.0
1.5
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH6 Clarithromycin
OD600
Time (hours)
0.0
0.5
1.0
1.5
0
100
200
300
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1x


GOSH7 Azithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH7 Clarithromycin
OD600
1.5
1.0
0.5
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

2.1y


GOSH8 Azithromycin
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control
GOSH8 Clarithromycin
OD600
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
Time (hours)
32 µg/mL
16 µg/mL
8 µg/mL
4 µg/mL
2 µg/mL
1 µg/mL
0.5 µg/mL
0.25 µg/mL
Positive control

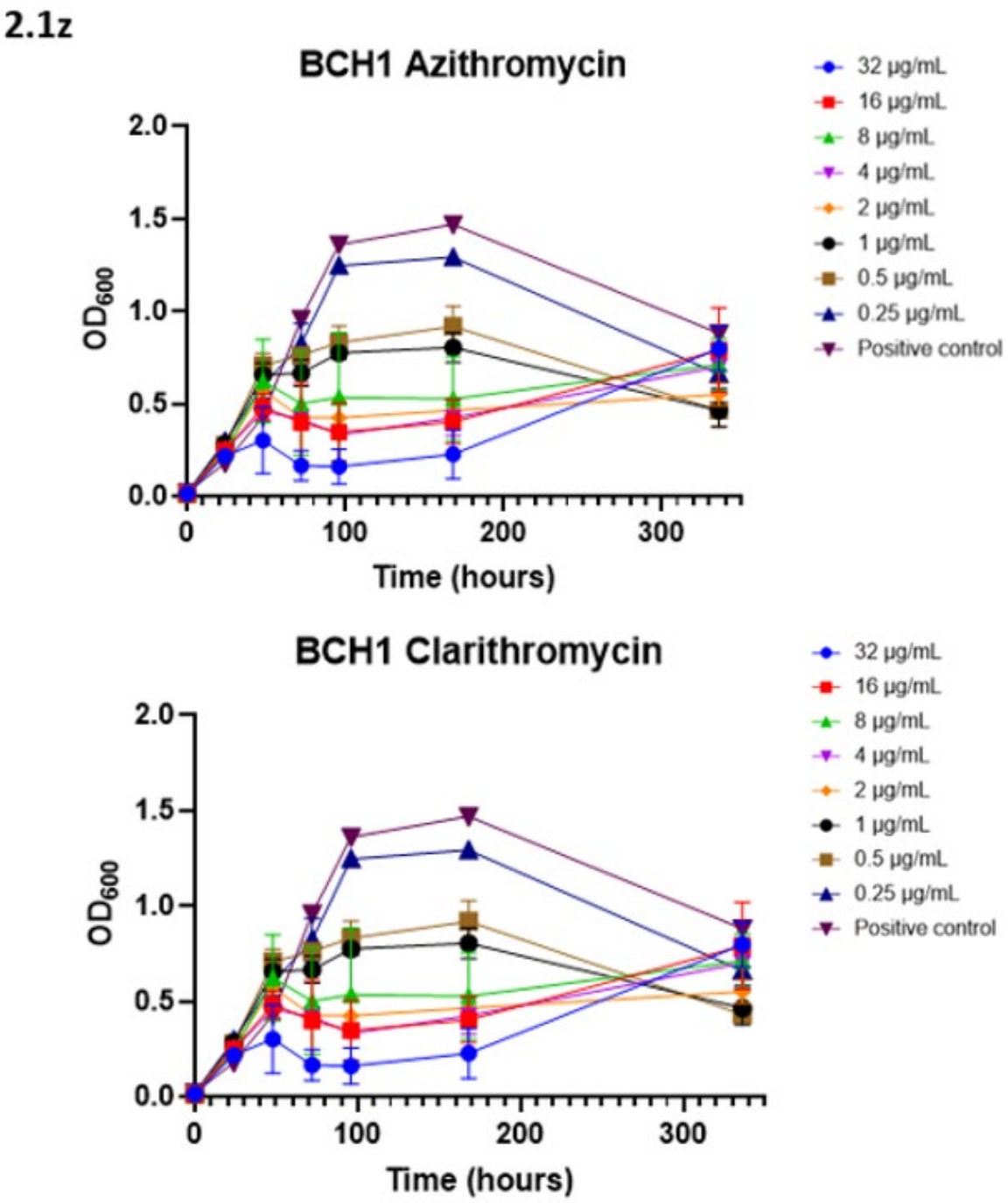


Figure 2.1a-z shows the growth curves over 336 hours (14 days) of 25 M. abscessus clinical isolates including NCTC 13031 (n=3) against the macrolides, azithromycin and clarithromycin. Many of the isolates display evidence of inducible macrolide resistance, with clarithromycin generally showing superior inhibition of growth than azithromycin.

Clarithromycin clearly performed better than azithromycin on several isolates, where the growth curves indicate that clarithromycin has superior inhibition compared with azithromycin: fig 2.1a, NCTC 13031 strain shows growth from around 48 hours with azithromycin at the highest concentration, whereas clarithromycin was able to inhibit growth until around 168 hours at the same concentration. Similarly, with Figure 2.1b, DC088 A growth was inhibited to a larger degree with clarithromycin than azithromycin, which had no inhibitory effect at the highest concentration tested. Figure 2.1d shows the growth curves of DC088 C, where again it can be seen that clarithromycin, at concentrations of ≥8 μg/mL, shows relative inhibition of growth compared with azithromycin at the same concentrations. Figure 2.1e, DC088 D, shows enhanced inhibition with clarithromycin compared with azithromycin at the same concentrations. It can be seen in Figure 2.1f, that inhibition of growth of isolate DC088 E is enhanced in clarithromycin; generally lower concentrations of clarithromycin were needed to inhibit growth, however azithromycin performs slightly better at the highest concentration tested (32 μg/mL). For DC088 Ref (Figure 2.1g), azithromycin has no inhibitory affect, whereas a concentration-dependant inhibitory affect can clearly be seen with clarithromycin. Both azithromycin and clarithromycin have an initial inhibitory effect on isolate 211666 (Figure 2.1i) until about 96 hours, where bacterial growth was not affected with azithromycin, but a concentration-dependent inhibitory effect can be seen with clarithromycin. Isolate 186433 (Figure 2.1j) is inhibited by both azithromycin and clarithromycin, but the growth curves reveal that clarithromycin can inhibit growth to around 2 μg/mL, whereas azithromycin is only able to produce the same effect at concentrations of 8 μg/mL and higher. Both macrolides had a similar effect on isolate 186154 (Figure 2.1k), but clarithromycin, at concentrations of ≥8 μg/mL produced a larger inhibitory effect than azithromycin at those same concentrations. Azithromycin had no inhibitory effect against isolate 137071 (Figure 2.1p), but clarithromycin produced a small reduction in the bacterial growth at 32 μg/mL. For isolate GOSH2 (Figure 2.1s), it can be seen that both azithromycin and clarithromycin produce a mild concentration-dependent inhibition, but with clarithromycin showing enhanced inhibition over azithromycin at concentrations of 8 μg/mL or higher. A rather similar effect is seen in isolate GOSH3 (Figure 2.1.t)

where it is clear that concentration-dependent inhibition is occurring, but inhibition occurs at clarithromycin concentrations of 4 – 32 μg/mL, whereas azithromycin is only able to inhibit at concentrations of 8 μg/mL and above. Isolate GOSH5 (Figure 2.1v) displayed susceptibility to both macrolides, but again enhanced inhibition can be observed with clarithromycin, where total inhibition is seen at concentrations of ≥0.5 μg/mL, whereas azithromycin can only inhibit growth at the highest concentrations of 16 – 32 μg/mL. In isolate GOSH6 (Figure 2.1w), both macrolides perform almost the same, however in the first 96 hours it can be seen that clarithromycin inhibits growth more effectively than in azithromycin, following which both macrolides are unable to inhibit growth. Both macrolides performed similarly in isolate GOSH7 (Figure 2.1.x), with a clear dose-dependent response, but at concentrations of ≥16 μg/mL, clarithromycin was able to inhibit growth more effectively than azithromycin, particularly after 96 hours. Isolate GOSH8 (Figure 2.1y) clearly shows a dose-dependent response with azithromycin, with only the highest concentration of 32 μg/mL only being able to inhibit growth. In contrast, clarithromycin is highly active against this isolate with total inhibition seen at all concentrations tested.

In isolate 194891, azithromycin had more of an effect on bacterial growth; Figure 2.1l shows growth is uninhibited by clarithromycin, yet azithromycin appears to have enacted a mild concentration-dependent inhibition particularly at higher concentrations. This effect is not sufficient, however, to totally inhibit growth. Azithromycin performed better against isolate 147028 (Figure 2.1m), where relative growth inhibition occurred at 32 μg/mL, and a concentration-dependant inhibition was seen with the lower concentrations, in contrast with clarithromycin which failed to inhibit growth at all. A very similar pattern can be seen with isolate 159544 (Figure 2.1n), in which azithromycin causes concentration-dependent inhibition, with the most inhibition seen at 32 μg/mL, whereas clarithromycin produces no inhibitory effect at all. For isolate 186144 (Figure 2.1o), a very small concentration-dependent inhibitory effect can be seen with azithromycin, where the highest concentration tested caused a small reduction in rate of growth, whereas clarithromycin produced no reduction in growth across all concentrations tested. Isolate 189961 (Figure 2.1q) also appears more

susceptible to azithromycin than clarithromycin; growth was uninhibited by any concentration of clarithromycin until around 96 hours, after which only the highest concentration caused a small reduction of bacterial growth, whereas azithromycin, in a concentration-dependent manner, saw a relative reduction in bacterial growth from 24-336 hours of testing.

There were some isolates in which no macrolide could be said to be superior to the other; in isolate DC088 B (Figure 2.1c), constitutive resistance was seen for both azithromycin and clarithromycin at all concentrations. Isolate GOSH1BAL2 (Figure 2.1r) also showed constitutive resistance to both macrolides at all concentrations. A third isolate showed total resistance to both macrolides, isolate GOSH4 (Figure 2.1u) with growth unaffected by any concentration of azithromycin or clarithromycin. Some isolates showed equal susceptibility to both macrolides; isolate BCH1 (Figure 2.1z) shows a concentration-dependent response to both macrolides equally.

20% (5/25) of the isolates displayed phenotypic evidence of constitutive macrolide resistance, where the isolate appears phenotypically resistant to the macrolides throughout the whole 14-day testing period. A total of 24% (6/25) of the isolates were susceptible to the macrolides until the end of the testing period, suggesting these isolates harbour a truncated *erm*(41) gene and therefore are likely *M. abscessus* subsp. *massiliense.* Evidence of inducible macrolide resistance and therefore a functional *erm*(41) gene was present in 44% (11/25) of the isolates. There is substantial variation between the efficacy of azithromycin and clarithromycin amongst these isolates; within the 11 isolates displaying inducible macrolide resistance, 36.4% (4/11) were resistant to azithromycin throughout the entirety of the experiment, and showed initial susceptibility to clarithromycin until a resistant phenotype appeared around the 7-day mark, and the other 63.6% (7/11) of the isolates displayed initial susceptibility and inducible resistance to both azithromycin and clarithromycin. In all of the 63.6% of isolates that displayed inducible resistance to both the macrolides, clarithromycin still shows enhanced inhibition compared to azithromycin. For example, Figure 2.1a shows the growth curve of the 13031 NCTC strain of *M. abscessus*, where it can be clearly seen that clarithromycin displays

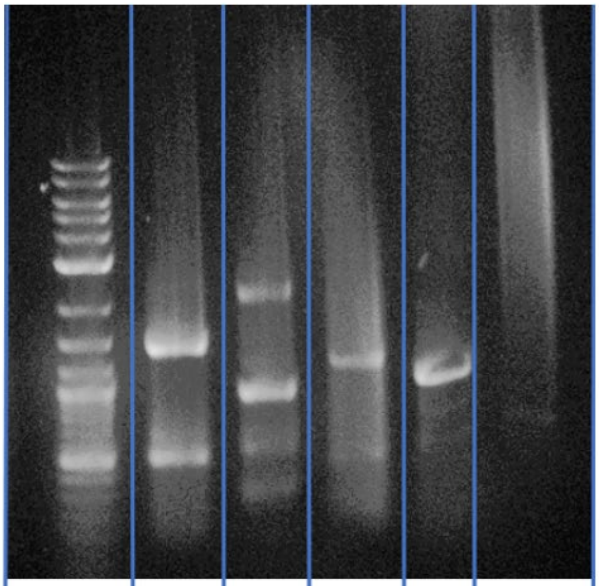
enhanced inhibition of growth, particularly within the first 168 hours of testing, compared with azithromycin.

2.4.3. PCR

2.4.3.1. Subspeciation of Mycobacterium abscessus isolates using PCR-based genotyping

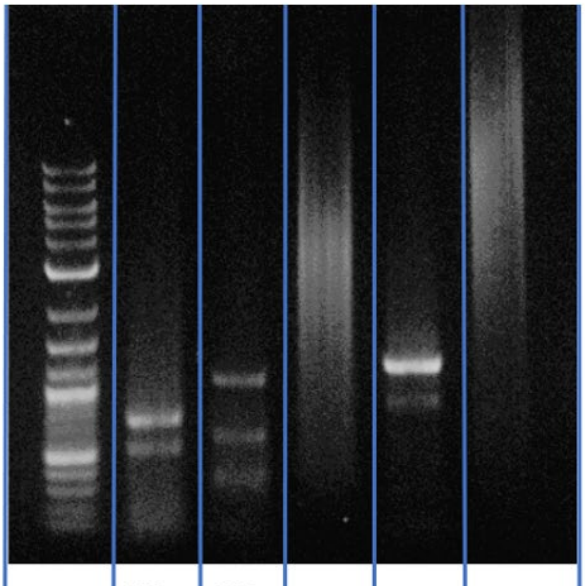
Of the 25 isolates subjected to PCR analysis, it was possible to successfully identify the subspecies of 24 isolates. This was done by matching the size of the bands present to the subspecies as laid out in Table 2.5.

2.3a. NCTC



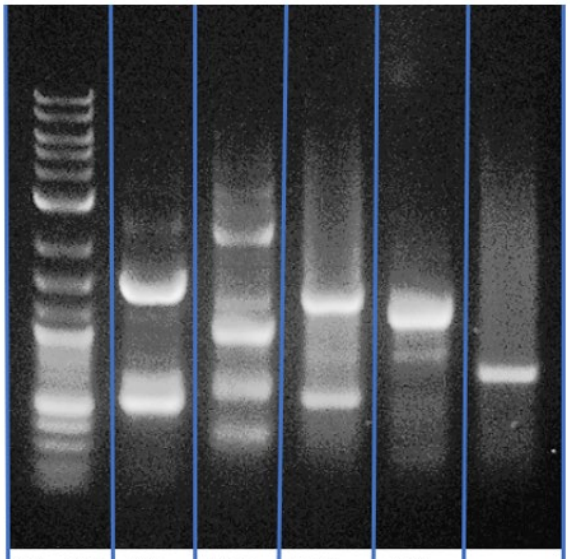
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 500	1000 ~2300	1400 500 f	1200	750 f
Subsp.	*M. abscessus*				

2.3b. DC088 A



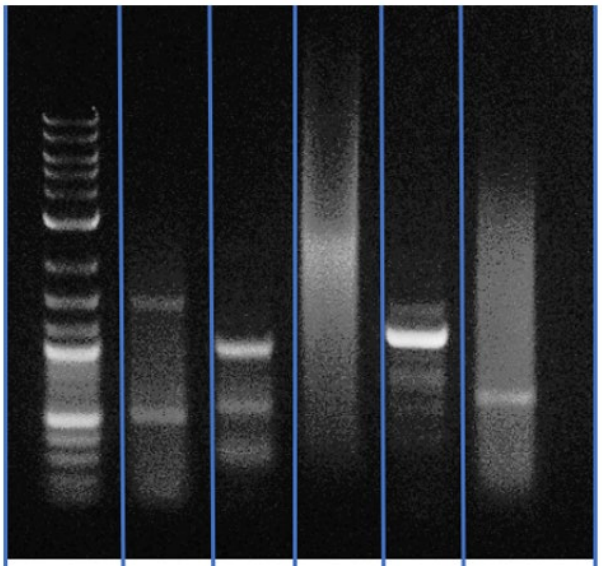
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~750 ~600	1050 ~700 ~300	ND	1200	ND
Subsp.	*M. bolletii*				

2.3c. DC088 B



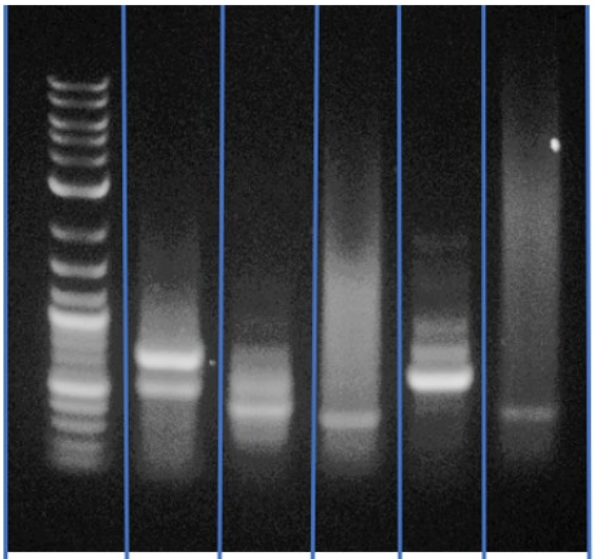
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 500	1000 ~2300 ~600	1400 500	1200	750
Subsp.	*M. abscessus*				

2.3d. DC088 C



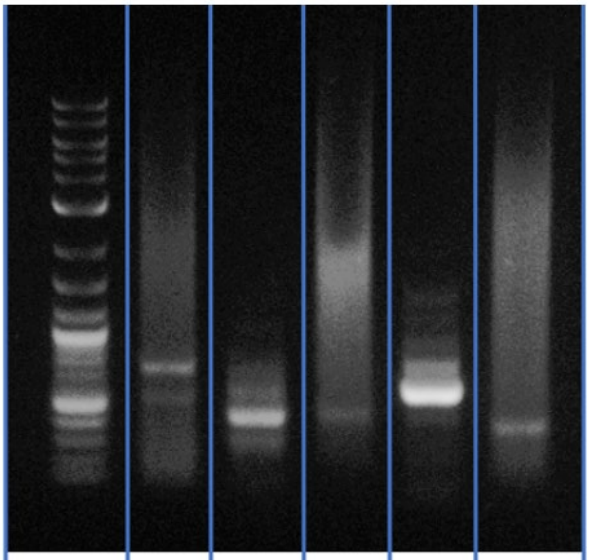
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~500 1500	1000 ~600	ND	1200 ~600	~700
Subsp.	*M. abscessus*				

2.3e. DC088 D



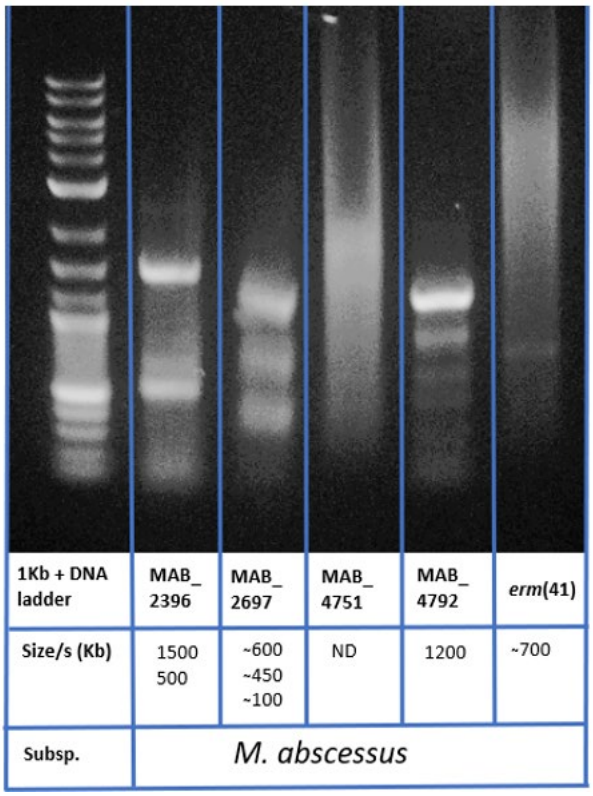
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~750 500	~450	~375	~600	350
Subsp.	*M. massiliense*				

2.3f. DC088 E



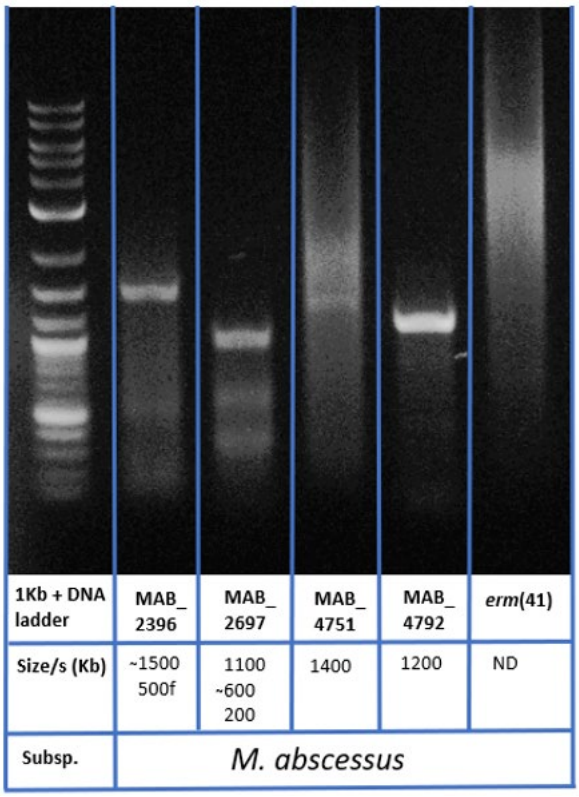
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~750 500 f	~450	450	~600	350
Subsp.	*M. massiliense*				

2.3g. DC088 Ref



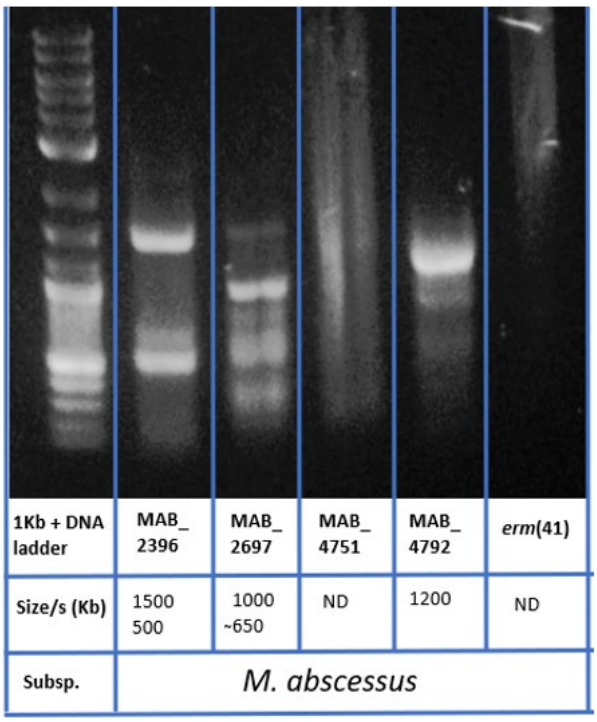
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	~600 ~450 ~100	ND	1200	~700
Subsp.	*M. abscessus*				

2.3h. 186433



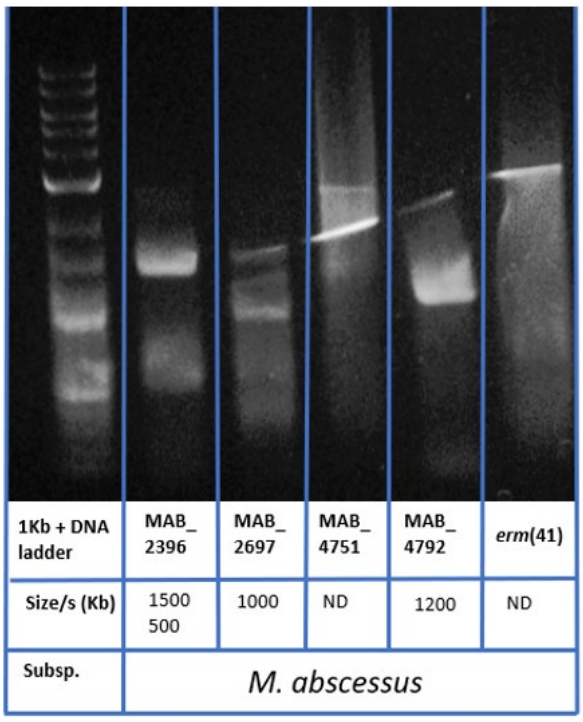
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 500f	1100 ~600 200	1400	1200	ND
Subsp.	*M. abscessus*				

2.3h. 194891



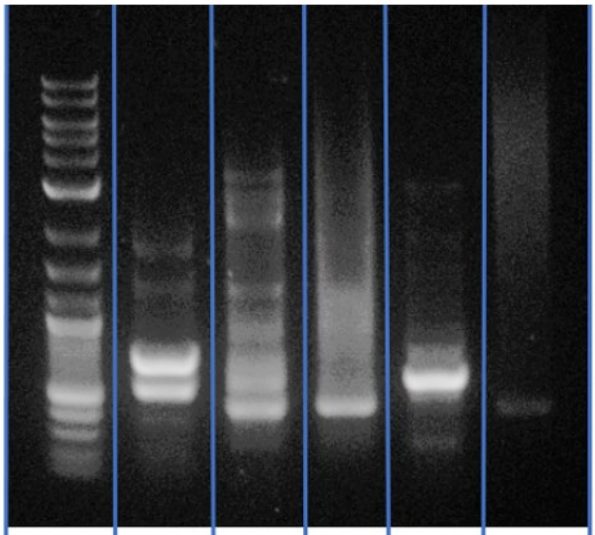
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000 ~650	ND	1200	ND
Subsp.	*M. abscessus*				

2.3i. 199277



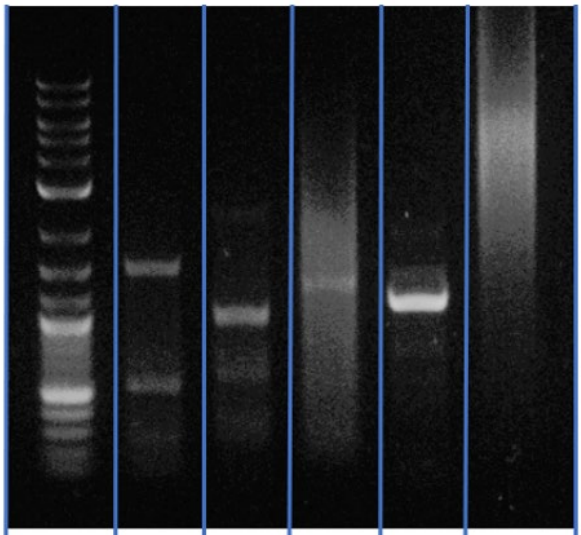
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000	ND	1200	ND
Subsp.	*M. abscessus*				

2.3j. 186154



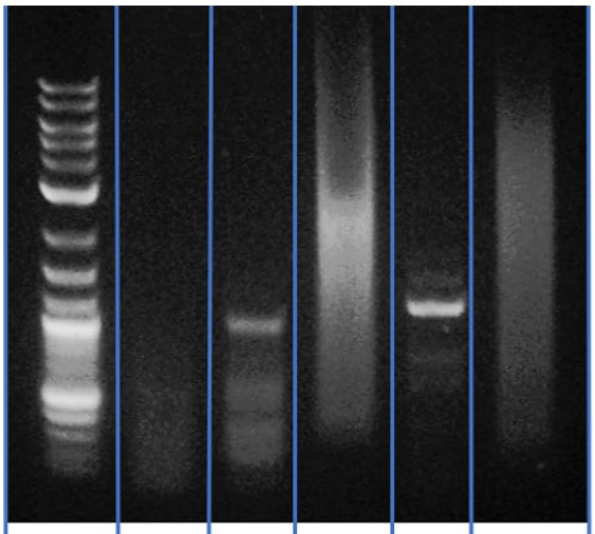
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~750 ~600	~550 ~450	450	600	350
Subsp.	*M. massiliense*				

2.3k. 211666



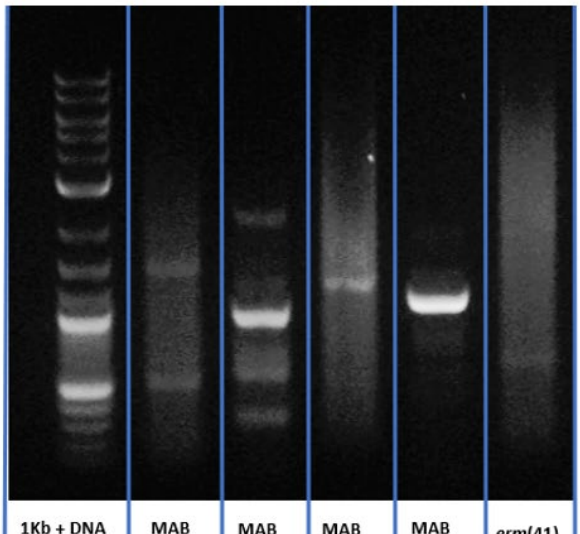
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 ~600	1000 700 f	1400	1200	ND
Subsp.	*M. abscessus*				

2.3k. 159544



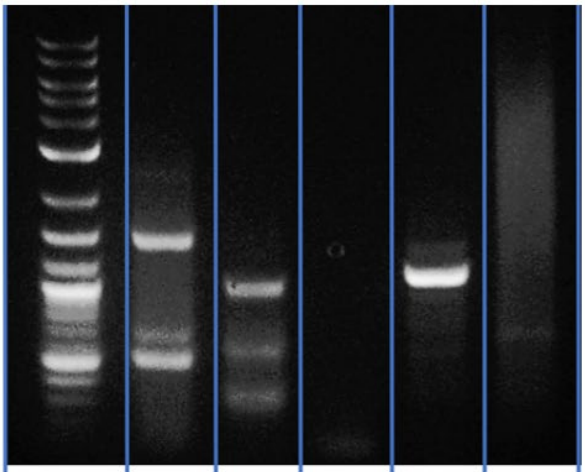
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	ND	1000	ND	1200 650	ND
Subsp.	*M. abscessus*				

2.3l. 137071



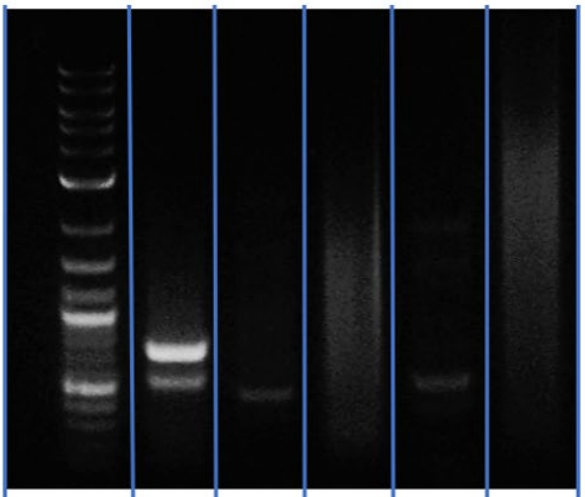
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000 600 f 300 f	1400	1200	750
Subsp.	*M. abscessus*				

2.3m. 147028



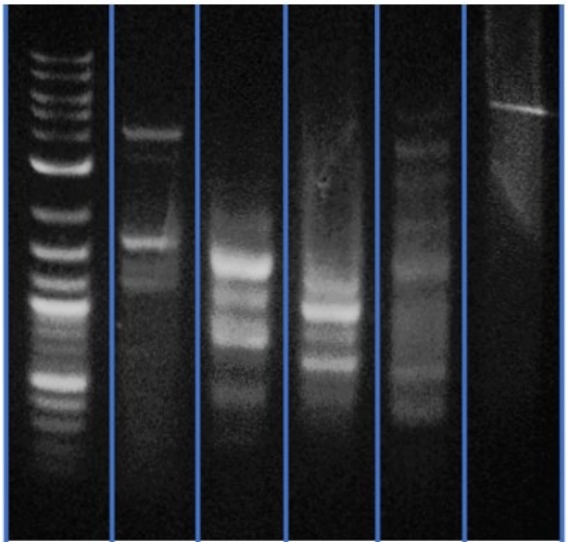
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000 600 f 300 f	ND	1200	750
Subsp.	*M. abscessus*				

2.3n. 186144



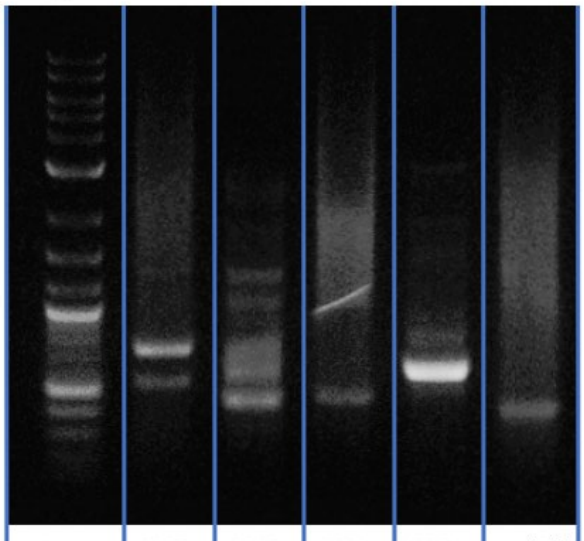
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	750 550	450	ND	~600	ND
Subsp.	*M. massiliense*				

2.3o. 189961



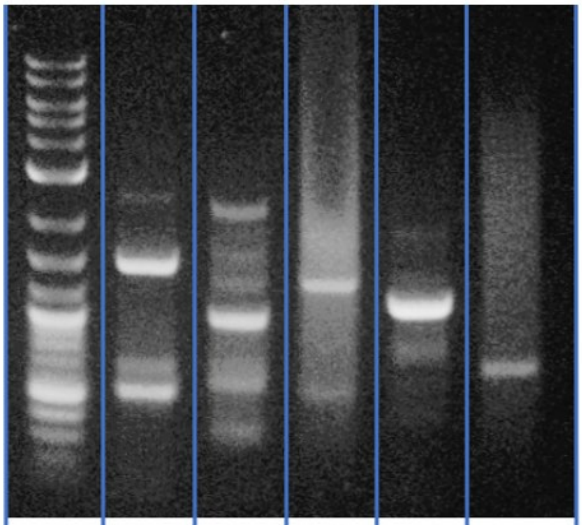
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 >4000	~1200 ~700	1000 650	600 ~1300	ND
Subsp.	ND				

2.3p. GOSH1BAL2



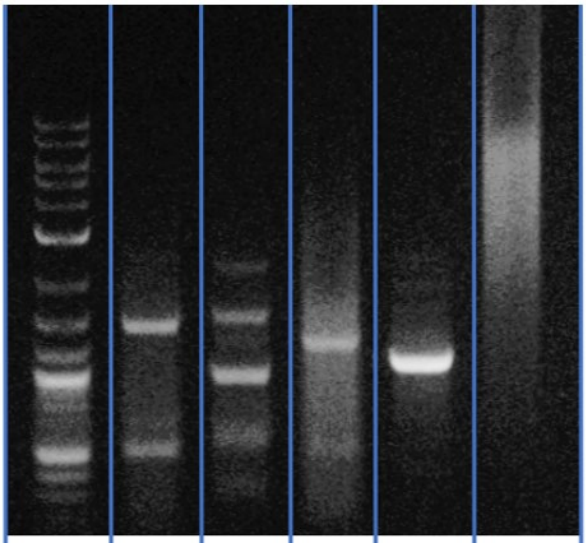
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~600 ~750	~450	~450	~600	350
Subsp.	*M. massiliense*				

2.3q. GOSH2



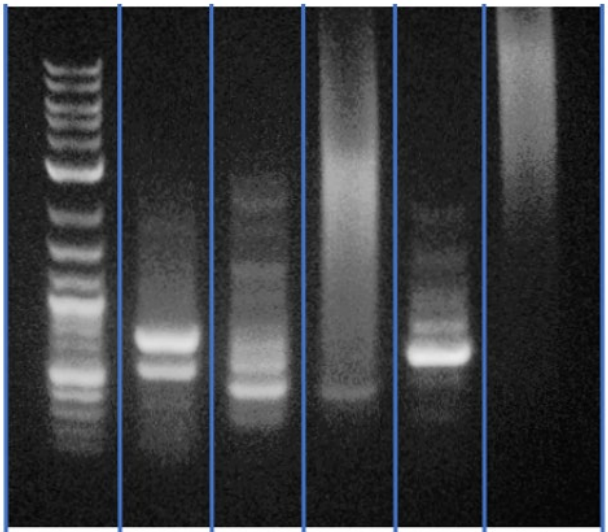
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 750 500	2500 1000 ~550	1400 500 f	1200	750
Subsp.	*M. abscessus*				

2.3r. GOSH3



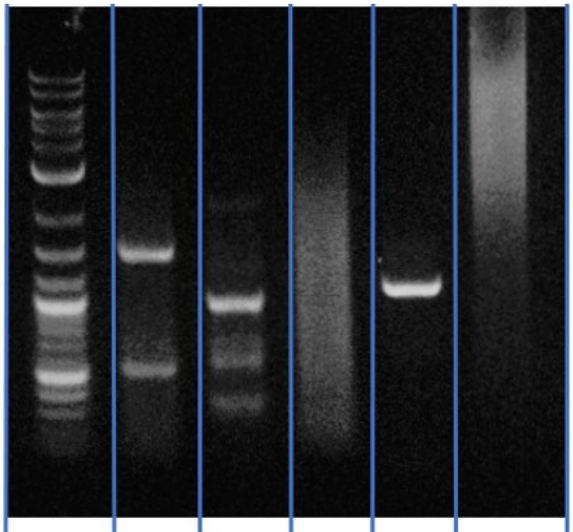
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000 ~1550	1400 500 f	1200	ND
Subsp.	*M. abscessus*				

2.3s. GOSH4



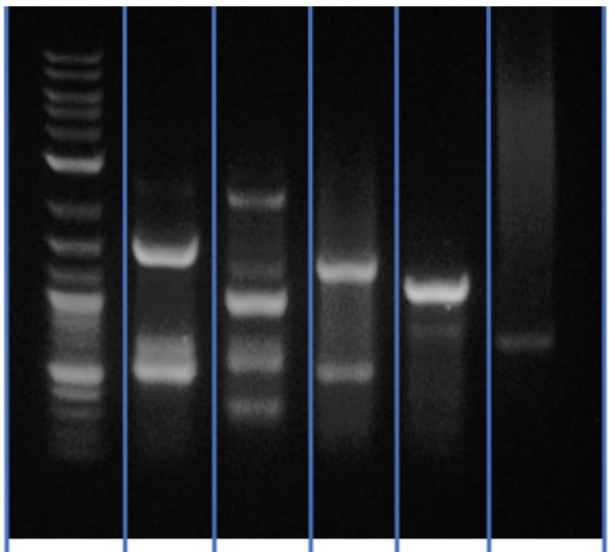
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	750 ~600	450	450	~600	ND
Subsp.	*M. massiliense*				

2.3t. GOSH5



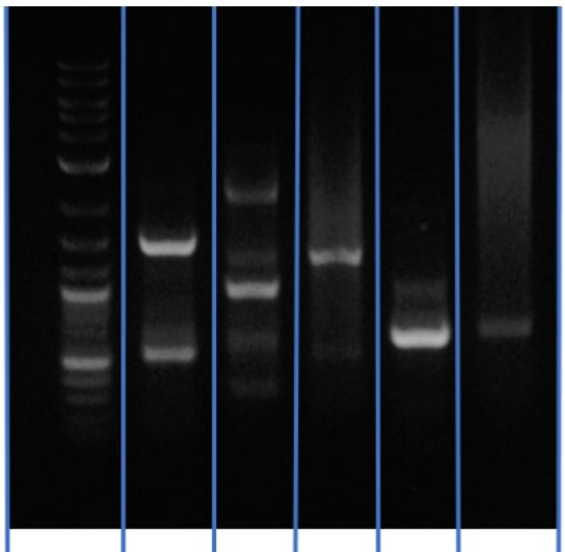
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 600 300	1000 ~2300	ND	1000	ND
Subsp.	*M. abscessus*				

2.3u. GOSH6



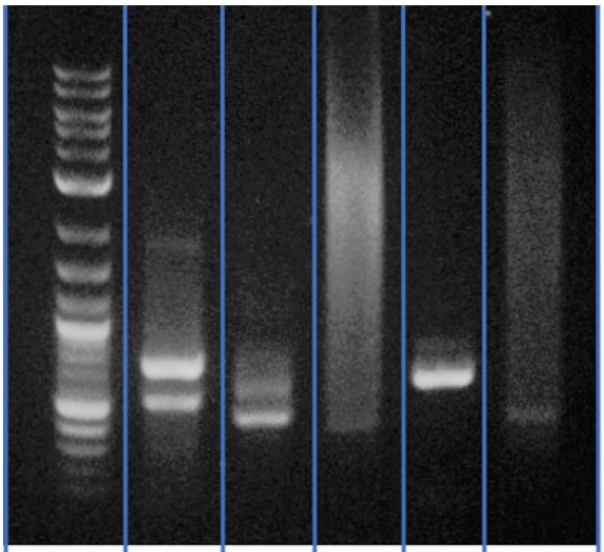
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 500	1000 ~2500 ~600	1400 650	1200	750
Subsp.	*M. abscessus*				

2.3v. GOSH7



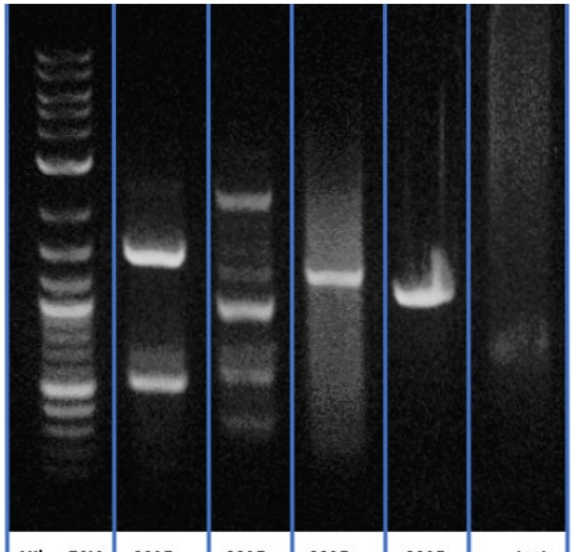
1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	~1500 500	1000 ~2500 ~600	1400 650 f	600	700
Subsp.	*M. abscessus*				

2.3w. GOSH8



1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	750 ~600	450	400 f	600	~500
Subsp.	*M. massiliense*				

2.3x. BCH1



1Kb + DNA ladder	MAB_ 2396	MAB_ 2697	MAB_ 4751	MAB_ 4792	*erm*(41)
Size/s (Kb)	1500 ~500	1000 ~600 ~2500	1400	1200	750
Subsp.	*M. abscessus*				

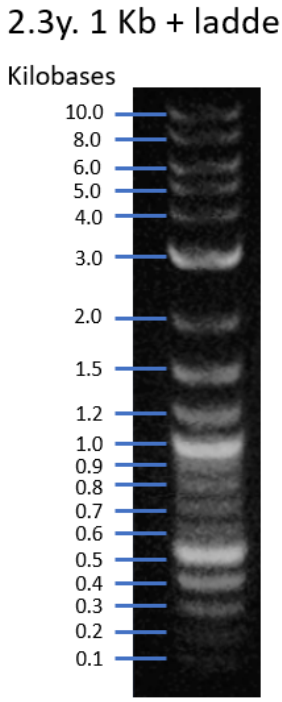


Figure 2.2a-y. Figures 2.2a-x show photographs of the gel electrophoresis using 5 PCR products, MAB_2396, MAB_2697, MAB_4751, MAB_4792, and erm(41) for 25 M. abscessus clinical isolates and the NCTC 13031 strain. Each gel shows the DNA band size observed for each primer set, and using the information in table 2.5, the putative subspecies for each isolate is also shown in the Figures. Figure 2.2y shows the key for the 1 Kb Plus ladder as provided by New England Biolabs. Each band is labelled with the respective size of DNA present in that band.

Photographs of the agarose gels following PCR and gel electrophoresis of the NCTC 13031 strain and 23 clinical isolates, as well as the corresponding ladder key can be seen in Figure 2.2a-y. On each gel, the 1Kb plus DNA ladder can be seen on the far left band, then subsequent bands show PCR products for MAB_2396, MAB_2697, MAB_4751, MAB_4752, and *erm*(41). By analysing the sizes (in Kb) of the bands for each gene (Table 2.5) it is possible to determine the subspecies for each clinical isolate.

Figure 2.2a shows the 13031 NCTC strain, which was indeed expected to be *M. abscessus* subsp. *abscessus*, shows band sizes (Kb) of 1500 and 500, 1000 and 2300, 1400 and 500 (faint), 1200, and 750 (faint) for MAB_2396, MAB_2697, MAB_4751, MAB_4752, and *erm*(41) respectively. It was not possible to determine the subspecies of isolate 189961 (Figure 2.2o) based on genotypic testing.

MAB_2396 showed a band at 1500 Kb, in line with *M. abscessus*, however MAB_2697 showed band sizes of 1200 and 700, where it would be expected to be 1000 and/or 500 Kb for *M. abscessus*. Furthermore, MAB_4751 would be expected to be 1400 and 500 Kb for *M. abscessus,* whereas this isolate showed 1000 and 650 Kb, MAB_4792 showed very weak bands which appear to be 1300 and 600 Kb, which could be indicative of any of the 3 subspecies (see Table 2.5), therefore isolate 189961 was excluded from any further analysis.

The following isolates displayed the correct band sizes (Kb) for *M. abscessus* (1500 and 500, 1000, 1400 and 500 (faint), 1200, and 700 for MAB_2396, MAB_2697, MAB_4751, MAB_4752, and *erm*(41) respectively: DC088 B (Figure 2.2c), 137071 (Figure 2.2l), GOSH2 (Figure 2.2q), GOSH6 (Figure 2.2u), GOSH7 (Figure 2.2v), and BCH1 (fig 2.2x). Several of the isolates failed to show one or more bands, however analysis of the visible bands could still indicate the subspecies where visible bands were specific to one subspecies. There are 10 isolates determined to be *M. abscessus* based on visible bands but with missing bands. MAB_4751 and *erm*(41) were the most common primer sets to not produce any bands in the gel electrophoresis; 30% of the 10 isolates had MAB_4751 missing (DC088 C, Figure 2.2d.; DC088 Ref, Figure 2.2g.; 147028, Figure 2.2m.), 30% of the isolates had *erm*(41) missing (186433, Figure 2.2h.; 211666, Figure 2.2k.; GOSH3, Figure 2.2r), and 30% of the isolates had both MAB_4751 and *erm*(41) missing (194891, Figure 2.2h.; 199277, Figure 2.2i.; GOSH5, Figure 2.2t). Furthermore, 10% of the isolates (159544, Figure 2.2k) had MAB_2396, MAB_4751 and *erm*(41) missing. Of the isolates with DNA bands missing, it was still possible to determine the subspecies as *M. abscessus* because the remaining bands correlated with *M. abscessus* and not any of the other 2 subspecies (Table 2.5). Specifically with isolate number 159544 (Figure 2.2k.), with 3 missing bands; it was still possible to determine the isolate as *M. abscessus* because a band size of 1000 Kb for MAB_2697 is indicative of either *M. abscessus* or *M. bolletii*, and band sizes 1200 and 650 Kb for MAB_4792 is indicative of *M. abscessus*, as *M. bolletii* is not expected to have a band at 650 Kb whereas this can be expected in *M. abscessus* clinical isolates (see Table 2.5.)

Isolates that displayed band sizes aligning with *M. massiliense* (750, 450, 450 or 375, 600, and 350 or 700 for MAB_2396, MAB_2697, MAB_4751, MAB_4752, and *erm*(41) respectively) were as follows: DC088 D (Figure 2.2e), DC088 E (Figure 2.2f), 186154 (Figure 2.2j), GOSH1BAL2 (Figure 2.2p), and GOSH8 (Figure 2.2w). A further 2 isolates had bands missing but had visible bands suggesting *M. massiliense.* One isolate (GOSH4, Figure 2.2s) was missing a band for *erm*(41), and the other isolate (186144, Figure 2.2n) was missing both MAB_4751 and *erm*(41). GOSH4 displayed band sizes of 750 and 600, 450, 450, and 600 for MAB_2396, MAB_2697, MAB_4751, and MAB_4752 respectively, which indicates *M. massiliense.* Isolate 186144 showed band sizes of 750 and 550, 450, and 600 for MAB_2396, MAB_2697, and MAB_4792 respectively, which again suggests this isolate can only be *M. massiliense* (Table 2.5).

For Isolate DC088 A (Figure 2.2b), only MAB_2396, MAB_2697, and MAB_4792 had visible DNA bands following gel electrophoresis, however this isolate was determined to be *M. bolletii* as the band sizes that were visible (MAB_2396: 750 and 600, MAB_2697: 1050, 700, 300, and MAB_4792: 1200) all correlate with *M. bolletii* only (see table 2.5).

Overall, of the 24 clinical isolates tested (excluding the NCTC 13031 lab strain and isolate 189961), 66.7% (16/24) were *M. abscessus,* 29.2% (7/24) were *M. massiliense* and 4.2% (1/24) were *M. bolletii.*

2.5. Discussion

The determination of *M. abscessus* subspecies is important not only for informing treatment regimens and predicting patient outcomes, but also for tracking transmission from the environment and/or from other patients. Whilst *M. abscessus* complex can be divided into 3 subspecies, drug susceptibility patterns can vary wildly from isolate to isolate. Determination of subspecies, particularly in differentiating *M. massiliense* from *M. abscessus* and *M. bolletii*, can be useful in a clinical or epidemiological sense, however, it is still the extreme levels of drug resistance and variation in that drug resistance that truly needs investigating. Furthermore, there is mounting evidence that the

current treatment regimen is insufficient for *M. abscessus* infection of any subspecies, so it is vital that new, effective and safe treatments for *M. abscessus* are discovered and investigated.

In this study, a high level of resistance was found within all clinical isolates to the main treatment regimen given; only 3 drugs (imipenem, amikacin, and cefoxitin) showed any consistent activity against more than 50% of the isolates. Some of drugs indicated in the treatment regime for *M. abscessus* had no inhibitory effect against most or all of the isolates, namely clofazimine, minocycline, moxifloxacin and linezolid. Finally, tigecycline was effective against just 40% of the isolates but showed to be very effective at growth inhibition within those susceptible isolates with MICs of 0.25 – 2 µg/mL.

Within the cohort of clinical isolates, 66.7% were genotypically determined to be *M. abscessus*, 29.2% *M. massiliense* and 4.2% *M. bolletii.* A low prevalence of *M. bolletii* is consistent with similar studies in the literature, where prevalence of *M. abscessus* is between 42-78%, prevalence of *M. massiliense* is between 20-48.4%, and prevalence of *M. bolletii* is between 0-18% (Roux, et al., 2009) (O'Driscoll, 2016) (Jeong, 2017) (Tan, 2018) (Bryant, 2013) (Kim, 2008) (van Ingen, 2009) (Teri, 2020). There is a dearth of information in relation to the differences in levels of drug resistance and treatment outcomes between *M. bolletii* and *M. abscessus,* this may be due to, as in these investigations, a low prevalence of *M. bolletii* impeding thorough investigation. One study performed in China on 129 *M. abscessus* complex isolates was able to show differing rates of resistance between the three subspecies (Guo, 2020). They found an overall high rate of resistance (>70%) to minocycline, linezolid, and moxifloxacin, similar to this study, and found that only amikacin and tigecycline were the most effective with 65.1% and 87.6% susceptibility, respectively. The authors purport that resistance rates between the three subspecies differ, and not just in macrolide susceptibility. Rates of cefoxitin resistance were 34.0% for *M. abscessus,* 46.2% for *M. massiliense,* and 63.2% for *M. bolletii* (Guo, 2020). In this cohort, there appears to be no correlation between subspecies and cefoxitin resistance, in fact the only *M. bolletii* isolate present in the cohort had an intermediate MIC (64 µg/mL), and several of the *M. massiliense* isolates were in fact resistant (GOSH1BAL2, GOSH4, and GOSH8) and

several others were intermediate (DC088 E, DC088 E, and 186154). GOSH3 had the lowest MIC for cefoxitin (8 µg/mL), and this particular isolate was determined to be *M. abscessus*. This correlates with a 2018 study which found no difference between *M. abscessus* and *M. massiliense* in their susceptibilities to cefoxitin (Ananta, 2018).

There is some evidence that suggests that *M. massiliense* may display higher levels of resistance to tigecycline compared to *M. abscessus.* A study published in 2018 utilised 37 *M. massiliense* isolates and 31 non-*M. massiliense* isolates (i.e., *M. abscessus* and *M. bolletii*) and compared tigecycline susceptibility patterns (Ananta, 2018). They found that *M. massiliense* isolates showed 37.84% resistance, and non-*M. massiliense* subspecies showed only 16.13% resistance. The cohort of isolates in this study showed a differing pattern; 64.7% of the *M. massiliense* isolates showed resistance to tigecycline, compared with just 50% of non-*M. massiliense* isolates (including the one *M. bolletii* isolate). Whilst the previously mentioned authors concluded that *M. massiliense* has generally higher resistance rates to *M. abscessus* and *M. bolletii,* this study suggests the opposite. One major limitation of this study is the sample size, which was considerably smaller than the author's. Cheng *et al* in 2019 compared the drug susceptibility rates of *M. abscessus* isolates to *M. massiliense* isolates (Cheng, 2019), they concluded that apart from macrolide susceptibility, there is no difference between the two subspecies in their susceptibilities to other drugs in the *M. abscessus* regimen, including tigecycline as well as clofazimine, cefoxitin, and imipenem. It must be noted that this cohort did not include any *M. bolletii* isolates and so does not tell us the whole story.

A larger sample size of *M. abscessus* complex isolates for retrospective drug susceptibility analysis is required to adequately assess the effect of subspecies on tigecycline susceptibility.

In the 2020 Chinese study (Guo, 2020), resistance to imipenem was *M. abscessus* 41.2%, *M. massiliense* 23.1%, and *M. bolletii* 41.2%. This is in stark contrast to this study, where imipenem displayed superior performance than any other drugs tested, with an overall susceptibility rate of 60%, and the one *M. bolletii* isolate (DC088 A) included in this study had an MIC of 4 µg/mL. Furthermore,

2 out of the 3 resistant isolates were later determined to be *M. massiliense.* One study in 2019 compared the drug susceptibility patterns of 277 *M. abscessus* clinical isolates and 269 *M. massiliense* clinical isolates (Cho, 2019). When looking at moxifloxacin, they found that 90.3% of the *M. abscessus* isolates were resistant to moxifloxacin, whereas 83.3% of *M. massiliense* were resistant to the same drug. The results of this study show an overall resistance rate of 87.5% across all subspecies, with *M. abscessus* and *M. massiliense* isolates showing 82.2% and 100% resistance, respectively.

A treatment regimen that includes imipenem is associated with higher rates of treatment success in *M. abscessus* infections. Several studies have been published highlighting the efficacy of imipenem against *M. abscessus* complex *in vitro* (Chen, 2019) (Kwak, 2019) (Cheng, 2019) and *in vivo* (Lefebvre, 2016). Furthermore, it has been shown that the combination of imipenem alongside other drugs can exert an additive or synergistic effect. Drugs such as rifabutin (an antibiotic primarily used to treat *M. tuberculosis* and *M. avium* complex disease), ceftazidime (Lopeman, 2020) and avibactam (a non-β-lactam β-lactamase inhibitor) show synergy alongside imipenem (Le Run, 2018), as well as moxifloxacin and clarithromycin (Miyasaka, 2007). The data from these studies indicates that the anti-*M. abscessus* effects of drugs such as moxifloxacin, minocycline, clofazimine, and linezolid (drugs that when tested alone *in vitro* had extremely high rates of resistance in this study) may need to be tested with imipenem to assess its synergistic capabilities, as alone they may appear ineffective, but may be enhanced with administered alongside imipenem.

Genomic differentiation of the *M. abscessus* subspecies presents a significant challenge for clinical microbiology laboratories. Single gene sequencing is insufficient for accurately determining *M. abscessus* subspecies, as this is typically done in microbiology laboratories by sequencing 16S rRNA gene sequences, which are identical within the *M. abscessus* complex (Zelazny, 2009). Sequencing of the *rpoB* gene encoding the β-subunit of bacterial RNA polymerase has historically been useful for distinguishing between *M. abscessus* and *M. chelonae* (Arnold, 2012). But since the discovery that *M. abscessus* is taxonomically distinct from *M. chelonae,* and that *M. abscessus* complex also

encompasses the species *M. massiliense* and *M. bolletii*, the use of *rpoB* sequencing alone for *M. abscessus* has fallen out of favour. Furthermore, evidence has come to light that there is horizontal gene transfer of *rpoB* genes between the three subspecies (Macheras, 2011), further nullifying the use of single gene sequencing. More recently, multi-locus gene sequencing has become the gold standard for effectively differentiating between *M. abscessus* subspecies. Typically, this includes partial sequencing of the housekeeping genes *rpoB, hsp65,* and *secA,* however this has also proven to be insufficient for some isolates, and further sequencing and phylogenetic analysis is sometimes required (Zelazny, 2009). Sequencing and analysis of eight housekeeping genes, *argH, cya, glpK, gnd, murC, pgm, pta,* and *purH* on 120 *M. abscessus* complex isolates was able to accurately distinguish between the three subspecies (Macheras, 2011). A glaring problem with this highly sensitive method for distinguishing *M. abscessus* subspecies is that the gene sequencing of eight housekeeping genes is extremely time-consuming and costly, and is particularly unsuitable for clinical microbiology laboratories, and indeed was outside the scope of this study.

In 2013 a novel PCR-based scheme for detecting *M. abscessus* subspecies was developed (Shallom, 2013), in which comparative genomic hybridisation (CGH) was employed to identify discriminatory regions in the *M. abscessus* complex. Shallom *et al* were able to distinguish between *M. abscessus* subspecies using only PCR and gel electrophoresis, a technique that is widely employed in microbiology laboratories and is considerably more time and cost effective than multi-locus gene sequencing. The methods described by the authors were employed in this study to accurately and rapidly identify the subspecies within the cohort of clinical isolates. Its robustness in distinguishing subspecies is down to the analysis of the combination of product sizes for each region (Shallom, 2013). This scheme is also able to elucidate subgroups within the subspecies, for example *M. massiliense* can be divided into two subgroups based on the size of the *erm*(41) product; 375 or 450 bp, and *M. abscessus* can be divided into two subgroups based of MAB_4792 product sizes (Shallom, 2013). In this study, the subspecies of 23/24 of the clinical isolates were identified. Of the seven *M. massiliense*

isolates in the cohort, five showed bands for *erm*(41). Of those, four (DC088 D, DC088 E, 186154, and GOSH1BAL2) had band sizes of 375 bp, and one (GOSH8) showed a band size of ~500 bp (475 bp), suggesting the presence of subgroups of *M. massiliense* in the cohort. It is not clear if these subgroups differ in drug susceptibility patterns, as the sample size is too small to form any correlation. The genomically heterogenous nature of *M. massiliense* isolates is certainly useful for phylogenetic analysis that can be then exploited for tracking disease transmission and informing infection control measures.

Essentially, the employment of rapid phenotypic profiling (determination of DSPs), in concurrence with genotyping to the subspecies level, could significantly enhance the way in which *M. abscessus* are clinically managed. This study highlights the efficacy of this approach and further establishes its value in terms of influencing clinical decisions and ultimately patient treatments and outcomes.

Chapter 3: Imipenem relebactam and amoxicillin

3.1. Aims and objectives

This chapter aims to address the paucity in consistent and effective drug regimens for the treatment of *M. abscessus* infection. Recent discoveries have identified the *M. abscessus* β-lactamase as an exploitable drug target for antibiotic discovery. This chapter will assess the activity of the novel non-β-lactam β-lactamase inhibitor, relebactam, alongside the carbapenem, imipenem. The chapter will also assess the of the addition of the penicillin, amoxicillin to this combination. All of the drugs used in this chapter are already approved for use, with imipenem in particular being a mainstay of the *M. abscessus* treatment regimen. The results gathered in this chapter can be used to inform knowledge of the *M. abscessus* β-lactamase as an exploitable drug target, and any effective treatments established within this chapter can be rapidly implemented in the clinic.

3.2. Introduction

Following on from the discovery of penicillin and the subsequent boom of antibiotic research and development, a worrying trend was starting to appear; antimicrobial resistance and the selection of resistant organisms meant that antibiotics that had been effective for decades were now useless against a wide range of bacterial pathogens. An unmet need for antimicrobials with broad-spectrum activity, rapid bactericidal activity and limited opportunity for resistance was beginning to become apparent, and carbapenems, such as imipenem were the answer.

The discovery of imipenem came about following the discovery of *Streptomyces cattleya,* a Gram-positive bacterium that produces cephamycin, penicillin and thienamycin. It was shown that thienamycin displayed potent, broad spectrum activity, however, chemical instability lowered its potential as an antimicrobial drug. This chemical instability was overcome with the development of the N-formimidoyl derivative, imipenem, by scientists at Merck in the mid-1970s (Rodloff, 2006). Furthermore, this novel carbapenem had a good safety profile and displayed activity against Gram-

positive organisms, as well as against Enterobacteriaceae, *Pseudomonas aeruginosa,* and Bacteroides (Rodloff, 2006) (Birnbaum, 1985).

The development of imipenem was hindered when it was discovered that imipenem undergoes extensive renal metabolism by dehydropeptidase-I (Birnbaum, 1985). To counteract this, a drug called Cilastatin, shown to inhibit renal dehydropeptidase, was developed. When administered in a one-to-one ratio, cilastatin provides prolonged and reversible inhibition of imipenem metabolism (Birnbaum, 1985).

Imipenem has been recommended as an option in the treatment of *M. abscessus* pulmonary disease since at least 2007 when the American Thoracic Society published recommendations of NTM treatment (Griffith, 2007). Traditionally, macrolides have been considered the backbone of *M. abscessus* treatment, but the presence of a functional *erm*(41) in the subspecies *bolletii* and *abscessus* confers inducible macrolide resistance (Nash, 2009), meaning macrolides potentially demonstrate limited efficacy against some isolates of *M. abscessus*. Subspeciation is recommended to guide treatment options, however some CF centres are not equipped to perform this, and so often prescribe a regimen containing intravenous amikacin and cefoxitin or imipenem instead of a macrolide (Philley, 2016).

3.2.1. *M. abscessus* peptidoglycan

Peptidoglycan (also known as murein), is an essential component of the bacterial cell wall, forming the cell membrane of almost all species of bacteria, its primary function to maintain cell integrity (Vollmer, 2008). It also acts as a scaffold for anchoring cell surface components such as proteins (Dramsi, 2008). An essential component involved in cell growth and division, the inhibition of biosynthesis, or degradation of the peptidoglycan will certainly result in cell lysis by osmotic rupture, and death (Vollmer, 2008).

The building block of peptidoglycan consists of alternating disaccharides, namely NAG and NAM, in repeating units (forming the glycan strand) that are crosslinked together with a stem peptide

comprised of 4-5 amino acids. The peptide stems are attached to the NAM component by substitution of the D-lactoyl group present in NAG. The polymerisation of the disaccharides by transglycosylases, and polymerisation of the stem peptides by transpeptidases results in the formation of a three-dimensional macromolecule i.e. the peptidoglycan (Figure 3.1.).

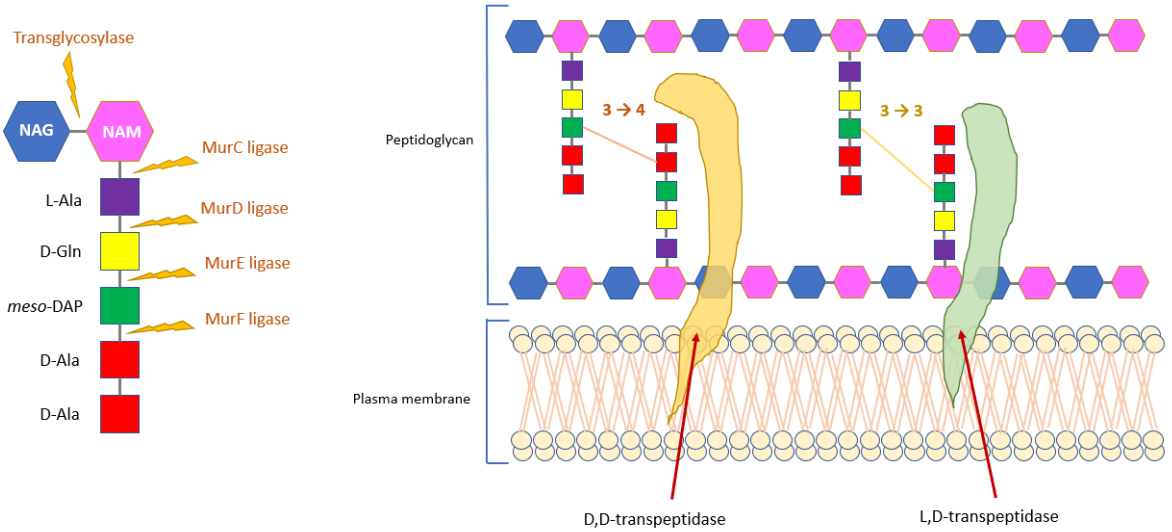


Figure 3.1. Model of the M. abscessus peptidoglycan. The left figure shows the polymer NAG and NAM, which form the glycan strand that is catalysed by transglycosylase, and the five amino acids found in M. abscessus: L-Ala, D-Gln, meso-DAP, and D-Ala-D-Ala which are added to the peptide chain by the enzymes MurC ligase, MurD ligase, MurE Ligase and MurF ligase, respectively. The right figure depicts the macromolecule of M. abscessus peptidoglycan, showing its position above the plasma membrane. 3 → 4 crosslinks are catalysed by D,D-transpeptidase, and 3 → 3 crosslinks by L,D-transpeptidase. The predominant cross-links observed in M. abscessus peptidoglycan are catalysed by L,D-transpeptidase, which is the main target of the carbapenem subclass of the β-lactam antibiotics.

The amino acids that form the cross-links often differ from species to species but generally will contain L-Ala, which is attached to the glycan strand by MurC ligase. In all bacterial species, the next amino acid added to the chain is D-Glu, which is catalysed by MurD ligase (Vollmer, 2008). The third amino acid sees the most cross-species variation: but in general it is a diamino acid of either *meso*-diaminopimelic acid (DAP) (notably found in most Gram-negative bacteria and Mycobacteria) or L-Lys (found in most Gram-positive bacteria). This step is catalysed by the MurE enzyme (Vollmer, 2008).

Positions 4 and 5 on the peptide chain are most commonly added as a dipeptide, usually D-Ala-D-Ala (Vollmer, 2008). Synthesis of this dipeptide is catalysed by the Ddl enzyme, and its addition to the peptide chain catalysed by MurF ligase (Vollmer, 2008). Some species, for example those with vancomycin resistance, may incorporate D-Lac or D-Ser in position 5 (Vollmer, 2008).

There is further variation within the cross-linking of the peptide chains with bacterial species. It was previously believed that only 3 → 4 crosslinks could be formed in bacterial peptidoglycan synthesis. However in 2005, a novel transpeptidase that catalysed non-canonical cross-links (3 → 3) within the peptide stem was discovered (Mainardi, 2005). This transpeptidase was subsequently named L,D-transpeptidase and was said to be β-lactam resistant (Mainardi, 2005). The vast majority of bacterial species use D,D-transpeptidases to form D-Ala → *meso*-DAP (or 3-4 crosslinks) (Magnet, 2008). However a small subset of species may employ the use of L,D-transpeptidases which catalyse a link between 3-3 amino acid residues (*meso*-DAP → *meso*-DAP) (Vollmer, 2008) (Magnet, 2008).

Until relatively recently, the exact structure of the peptidoglycan of *M. abscessus* was unknown, and so in 2011 Lavollay *et al.* determined the structure of both rough and smooth variants of *M. abscessus* (Lavollay, et al., 2011). Using high performance liquid chromatography (HPLC), mass spectrometry (MS) and tandem mass spectrometry (MS/MS), the authors were able to show that L,D-transpeptidases, that form 3→3 cross-links, were the predominant contributors to peptidoglycan cross-linking in *M. abscessus*, comprising of 64% to 74% of the peptidoglycan structures. Further studies since that have suggested that at least 5 putative L,D-transpeptidases exist in *M. abscessus*; subsequently named Ldt_{Mab1-5} (Mattoo, 2017). Furthermore, Lavollay *et al.* also discovered that the peptide chain in the *M. abscessus* peptidoglycan consists of L-Alanyl-D-Glutaminyl-*meso*-diaminopimelyl-D-Alanyl-D-Alanine (Figure 3.1.)

3.2.2. Imipenem: structure and mechanism of action

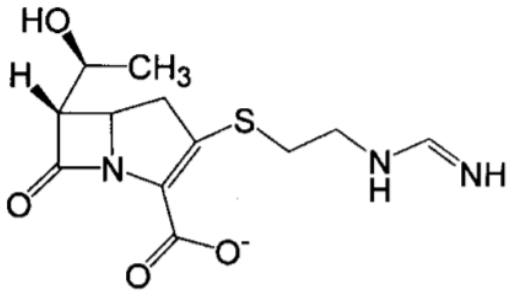


Figure 3.2. Chemical structure of imipenem, adapted from Beadle *et al* (2002) (Beadle, 2002).

Imipenem works like other β-lactams by inhibiting synthesis of the peptidoglycan component of the bacterial cell wall, specifically the last cross-linking step of peptidoglycan synthesis (Lavollay, et al., 2011). Imipenem can inhibit both classes of transpeptidases; the classical D,D-transpeptidases, which catalyse the formation of 3→4 cross-links (Sauvage, 2008), and the L,D-transpeptidases, which form 3→3 cross-links (Mainardi, 2005).

Evidence began to emerge within the literature that *M. abscessus* is more susceptible to carbapenems than other classes of β-lactam (Kaushik, et al., 2015), and further studies suggested that is down to the unique ability of the carbapenems to inhibit both the classical D,D-transpeptidases and more importantly, the non-classical L,D-transpeptidases of mycobacterial species (Kumar, et al., 2017) (Cordillot, 2013).

It has long been established that β-lactams, like imipenem, mimic the C-terminal end of the native stem peptide, therefore is able to bind to the active site of the transpeptidases, irreversibly inhibiting their activity (Park, 1957) (Story-Roller, 2018). Previous studies have suggested that the penicillins and cephalosporins (except cefoxitin) are ineffective against *M. abscessus*, while carbapenems such as imipenem, meropenem, and doripenem have moderate activity against the organism (Soroka, et al., 2014). Further studies have determined that imipenem is superior to cefoxitin against *M. abscessus,*

and furthermore, suggested that inhibition of the β-lactamase enzyme (see chapter 3.1.3.) could further extend the efficacy of the carbapenems and cefoxitin against the organism (Lefebvre, 2016).

3.2.3. *M. abscessus* β-lactamase

The story of bacterial β-lactamases begins in the early days of antimicrobial discovery; when Alexander Fleming noted that penicillin produced by certain *Penicillium* moulds could inhibit the growth of *Staphylococcus* variants, but could not inhibit some bacteria, notably members of the coli-typhoid group and other intestinal species such as *Escherichia coli* and *Haemophilus influenzae* (Fleming, 1929) (Kong, 2010). In 1940, Abraham and Chain took an extract of *Escherichia coli* (*E. coli*), a bacteria known to be resistant to penicillin at this point, and demonstrated that this extract contained a substance capable of obstructing the antimicrobial properties of penicillin (Abraham, 1940). The authors concluded that this substance was likely an enzyme, and considering that a number of bacteria possessed the newly discovered 'penicillinase', they also concluded the enzyme was likely essential for bacterial metabolism (Abraham, 1940). Whilst the discovery of the first β-lactam, Penicillin G, by Fleming in 1929 was undoubtedly world-changing, from relatively humble beginnings as a treatment for allied soldiers during World War II to its current status as the most widely prescribed antimicrobial in the world today, it also lead to the rapid emergence of β-lactamases in many pathogenic species.

β-lactamases inactivate penicillin drugs by hydrolysing the β-lactam ring (Kong, 2010). To date, over 300 β-lactamase enzymes have been described in the literature (Majiduddin, 2002). There are currently four classes of β-lactamases; of which the mechanism of action is driven by either a serine active site (class A, C and D) or by divalent metal cations (Zn^{++}) (class B), and the classification is based on peptide sequences.

Early genetic analysis of class A enzymes showed that these β-lactamases share a phylogenetic origin, and likely evolved from penicillin-sensitive enzymes involved in bacterial cell wall synthesis (Ambler, 1980). Class A β-lactamases are by far the largest group of β-lactamases, and work by binding to the substrate (β-lactam antibiotic) to form a Henri-Michaelis complex, from which the active-site serine

performs a nucleophilic attack on the carbonyl of the substrate resulting in a high-energy acylation intermediate. The high-energy intermediate converts to a more stable covalent acyl-enzyme by protonation of the β-lactam nitrogen and cleavage of the C-N bond, then an activated water molecule attacks the covalent acyl-enzyme complex, resulting in another high energy deacylation intermediate. Finally, the hydrolysis between the β-lactam carbonyl and the oxygen of the nucleophilic serine releases the active β-lactamase enzyme and the inactivated β-lactam (Drawz, 2010).

The production of β-lactamase by mycobacteria has long been known to contribute to intrinsic β-lactam resistance (Jarlier, 1991). As early as 1972 mycobacterial β-lactamase were being described in *M. tuberculosis* and *M. smegmatis* (Thompson, 1972) and in the 1990s, *M. fortuitum* (Amicosante, 1990). Whilst no attempt at characterising the *M. abscessus* β-lactamase had yet been done, it was known that mycobacterial β-lactamases display a broad hydrolysis spectrum (Sauvage, 2006), and that each species has its own species-specific β-lactamase with corresponding chromosomally located genes (Soroka, et al., 2014). They are also generally inactivated by the β-lactamase inhibitor, clavulanate (Hugonnet, 2007). Finally in 2013, Soroka *et al* characterised the *M. abscessus* β-lactamase and subsequently named it Bla_{Mab} (Soroka, et al., 2014). They found that Bla_{Mab} was similar to other mycobacterial β-lactamases, displaying a broad hydrolysis spectrum, but found that Bla_{Mab} has a higher catalytic efficiency compared with the *M. tuberculosis* β-lactamase, BlaC (Soroka, et al., 2014). Another glaring distinction was that Bla_{Mab} was not inhibited by the β-lactamase inhibitors, clavulanate, tazobactam, and sulbactam, in fact it efficiently hydrolysed clavulanate (Soroka, et al., 2014). Considering that the conventional β-lactam β-lactamase inhibitors were useless against Bla_{Mab}, it was vital that other inhibitors be developed.

3.2.4. β-lactamase inhibitors

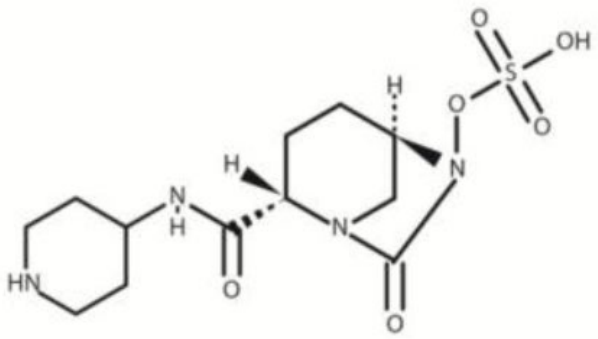


Figure 3.3. Chemical structure of avibactam. Adapted from Wong and van Duin, 2018 (Wong, 2018).

The rise in β-lactamase-mediated resistance to β-lactam antibiotics significantly threatens the efficacy of these antibiotics, so researchers must either develop novel β-lactam antibiotics that are able to evade the β-lactamase activity of its target organism, or inhibit the β-lactamases so the partner β-lactam drug can reach its target (Drawz, 2010). β-lactamase inhibitors were developed in the 1980's as partner drugs for β-lactams that can block inhibition by β-lactamase. The earliest β-lactamase inhibitors, clavulanate, tazobactam and sulbactam were considered a revolutionary advancement in infectious disease research, and were able to form stable intermediates with β-lactamases, thus allowing the β-lactam agent to bind with its target (Wong, 2017). Clavulanate, also known as clavulanic acid, was first isolated from *Streptomyces clavuligerus* in 1977. At that time it was observed that this novel β-lactamase inhibitor structurally resembled penicillin with some notable differences such as lacking an acylamino side chain and containing oxygen instead of sulphur (Reading, 1977). It was also shown at the time that clavulanate was a potent inhibitor of a variety of β-lactamases, particularly those of (plasmid mediated) *E. coli, Klebseilla aerogenes,* and *S. aureus,* but was found to be less potent against the β-lactamases of (chromosomally mediated) *Staphylococcus aureus, P. aeruginosa* and *Enterobacter cloacae* (Reading, 1977). Initial testing confirmed that the addition of clavulanate reduced the MICs of ampicillin and cephaloridine against several β-lactamase producing strains (Reading, 1977).

Early studies assessing the efficacy of clavulanate against the *M. abscessus* β-lactamase suggested that clavulanate is able to reduce the MIC_{90} of meropenem, a carbapenem, from 80 µg/mL (not susceptible) down to 5 – 10 µg/mL, a 16-fold decrease (Kaushik, et al., 2015). They also demonstrated, however, that clavulanate has no effect on the MICs of imipenem, ertapenem, and panipenem (Kaushik, et al., 2015). Evidence that contradict these findings published in 2013 showed that clavulanate has no activity against the *M. abscessus* β-lactamase (Soroka, et al., 2014). It is clear that a broadly effective β-lactamase inhibitor and partner β-lactam is urgently required to effectively treat *M. abscessus* infection.

3.2.4.1. Avibactam

Previously known as NXL104, avibactam (Figure 3.3) is a member of the relatively new class of non-β-lactam β-lactamase inhibitors called diazabicyclooctanes (DBO). This class of inhibitors differs from the classical β-lactamase inhibitors in that they do not contain a β-lactam ring. Additionally, its mechanism of inhibition differs somewhat to that of the classical β-lactamase inhibitors; in lieu of irreversible hydrolysis and turnover of the β-lactamase inhibitor compound, avibactam is able to rapidly acylate a wide range of β-lactamases in a reversible reaction, limiting hydrolysis of the avibactam molecule. This results in inhibitor regeneration, as opposed to inhibitor destruction that is seen in β-lactam β-lactamase inhibitors (Ehmann, 2012) (Lahiri, 2013). Activity of avibactam against *M. abscessus* β-lactamase has been well studied, with several studies showing that avibactam is a potent inhibitor of Bla_{Mab} (Lefebvre, 2016) (Dubee, et al., 2015). Avibactam was developed to be only administered alongside ceftazidime, a β-lactam antibiotic that has no activity against *M. abscessus* (Dubee, et al., 2015), but evidence has suggested that avibactam can reduce the MICs of other β-lactams including amoxicillin (Dubee, et al., 2015) and imipenem (Lefebvre, 2016) against *M. abscessus*. This was indeed an encouraging step forward for *M. abscessus* treatment options, however with avibactam only approved for use alongside ceftazidime, at this stage avibactam has limited potential to be used to treat *M. abscessus* infections.

3.2.4.2. Relebactam

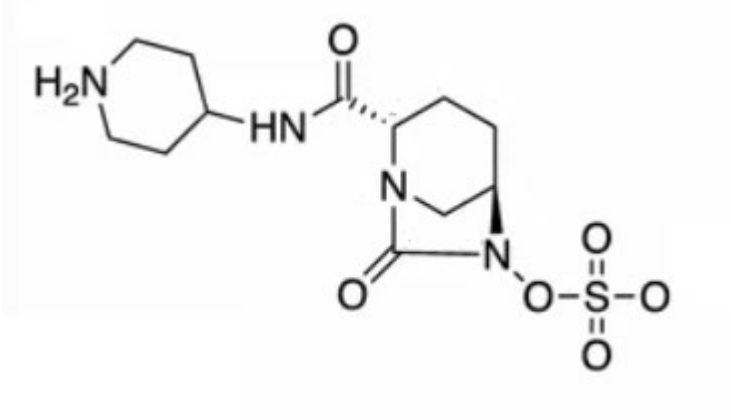


Figure 3.4. Chemical structure of relebactam. Adapted from Zhanel et al. (2017) (Zhanel, 2017)

Relebactam (previously named MK-7655) (Figure 3.4), was developed and evaluated in 2014 as a potential partner drug to imipenem to overcome class A and class C β-lactamase-mediated antimicrobial resistance (Blizzard, 2014). Like avibactam, it contains a diazabicyclooctane core, except the R1 side chain includes a piperadine ring, where avibactam contains a carboxyamide (Figure 3.3). More promisingly, relebactam was developed to be used in conjunction with Primaxin®, simply known as imipenem-cilastin (Blizzard, 2014). Early studies suggested that imipenem/relebactam may be useful in the treatment of *M. abscessus* (Kaushik, 2019) (Le Run, 2020), but testing a wide panel of clinical isolates and testing imipenem/relebactam alongside other β-lactam agents is yet to be performed.

3.2.5. Amoxicillin

Amoxicillin is a β-lactam antibiotic that was developed in the 1970's used to treat a wide range of bacterial infections (Geddes, 2007). Despite clinical success, the emergence of β-lactamase-mediated resistance resulted in widespread resistance to amoxicillin; this was overcome with the discovery and development of clavulanate, of which amoxicillin was chosen to be co-administered (Geddes, 2007). Amoxicillin/clavulanate regained activity against β-lactamase producing organisms such as *E. coli, H. influenzae,* and *Moraxella catarrhalis*, greatly broadening its spectrum of activity (White, 2004). A

2016 study showed that *M. abscessus* is highly resistant to amoxicillin *in vitro,* however, in a Δbla_{Mab} strain of *M. abscessus,* susceptibility is restored, with an MIC of just 4 µg/mL (Lefebvre, 2016). This suggests that β-lactamase production by *M. abscessus* may be driving resistance to amoxicillin (Soroka, et al., 2014). As clavulanate is purported to be ineffective at hydrolysing BlaMab, it therefore stands to reason that amoxicillin/clavulanate combination will be ineffective against *M. abscessus* infection.

3.2.6. Summary

In this study, the non-β-lactam β-lactamase inhibitor relebactam was tested alongside imipenem and/or amoxicillin against a panel of clinical isolates of *M. abscessus.* It was hypothesised that it would be possible to reduce the MIC of imipenem with the addition of relebactam, previously shown to be an effective inhibitor of BlaMab (Kaushik, 2019) (Le Run, 2020). Furthermore, this study aimed to determine whether it is possible to induce susceptibility to amoxicillin in *M. abscessus* when co-administered with relebactam. It has been confirmed that imipenem and relebactam prove to be an effective combination against *M. abscessus* clinical isolates, whilst also showing for the first time that it is possible to induce susceptibility to amoxicillin with the addition of relebactam, and furthermore, it was shown that a combination of imipenem, amoxicillin and relebactam was able to inhibit growth in 100% of the clinical isolates *in vitro.* In doing this, synergy was established between the 3 compounds, where the MICs of each component of the combination saw a significant reduction. From a clinical perspective this shows that it may indeed be possible to treat *M. abscessus* infection with relatively low concentrations of antibiotics, reducing the burden on healthcare and providing an improved treatment regimen with lower risk of adverse effects for patients, representing a significant step forward in the treatment options for *M. abscessus* infection.

3.3. Materials and Methods

3.3.1. Bacterial isolates

A total of 16 *M. abscessus* clinical isolates from Brighton and Sussex Medical School and *M. abscessus* NCTC 13031 were used in this study. Stock solutions of the isolates were kept in 50% glycerol (Sigma, Dorset, UK) and Middlebrook 7H9 Broth and stored at −80 °C. Isolates were grown in Middlebrook 7H9 medium supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC), 1% glycerol (50% w/v) and 0.05% Tween 80 (v/v) prior to testing. *Escherichia coli* Top 10 cells were used for propagation of plasmid DNA. These cells were routinely grown in nutrient broth, or nutrient agar (Oxoid, UK) at 37 °C. *E. coli* BL21 (DE3) cells were used for the overproduction of recombinant protein, grown in Terrific Broth (Melford, UK) at 37 °C.

3.3.2. Antimicrobials

The antimicrobial agents meropenem (MEM), amoxicillin (AMX) and phenoxymethylpenicillin (Penicillin V/PenV) were obtained from Sigma Aldrich (Dorset, UK) and relebactam (REL), imipenem (IMI) and avibactam (AVI) were obtained from Carbosynth (Compton, UK). Stock solutions were prepared in sterile de-ionised water and stored at −20 °C until use.

3.3.3. Disk diffusions

For the disk diffusion assays, *M. abscessus* clinical isolates were grown in Middlebrook 7H9 Broth to logarithmic phase and 100 µL of bacterial culture was inoculated into 10 mL Middlebrook 7H9 Broth supplemented with 0.7% bacteriological agar. This was poured as a layer in agar plates on top of 15 mL Middlebrook 7H11 Agar supplemented with 10% OADC and 1% glycerol. Once the top layer of agar had sufficiently set, 6 mm sterile filter paper diffusion disks were placed on the agar and were subsequently impregnated with antibiotic at the following concentrations in sterile distilled water: relebactam 1 µL of 10 mg/mL, amoxicillin 3.3 µL of 3 mg/mL and meropenem 1 µL of 10 mg/mL. Plates were incubated at 30 °C for 5 days or until clear zones of inhibition were visualised. Zones of inhibition were measured across the diameter to include the disk itself in mm. Disk diffusions were also

performed on *M. abscessus* pVV16-*bla*$_{Mab}$ and *M. abscessus* pVV16 (see chapter 3.2.5) and were performed in triplicate.

3.3.4. Broth Microdilution Assay

The broth microdilution assay was performed as described previously with alterations appropriate to this study (Wayne, 2015) (Caleffi-Ferracioli, 2013). Plates were prepared by serially diluting amoxicillin (128 µg/mL–0 µg/mL) or meropenem (128 µg/mL–0 µg/mL) in the *x*-axis and either relebactam (32 µg/mL–0 µg/mL) or relebactam: imipenem (32: 64 µg/mL–0 µg/mL) or avibactam: ceftazidime (128: 128 µg/mL–0 µg/mL) in the *y*-axis. Then, 80 µL of *M. abscessus* NCTC 13031 suspension adjusted to an OD_{600} of 0.1–0.2 was inoculated in each well to reach a final well volume of 100 µL. Assay plates containing amoxicillin and/or imipenem with or without relebactam were also tested against 15 other clinical isolates and *M. abscessus* pVV16 *bla*$_{Mab}$ and *M. abscessus* pVV16 (see chapter 3.2.5). Each combination plate was performed in triplicate.

Plates were sealed and incubated at 30 °C for 3–5 days. The minimum inhibitory concentrations (MICs) of the various combinations were determined by optical density measurement using a spectrophotometric plate reader and identified as the well that had the lowest concentrations of both compounds and exhibited no bacterial growth (for imipenem susceptibility testing, CLSI breakpoints were utilised (Institute, Clinical and Laboratory Standards 2012). The relevant well was retroactively plotted into a growth curve over time, and this growth curve was compared to the wells containing no drug, and the wells containing either relebactam, relebactam-imipenem or ceftazidime-avibactam only and amoxicillin only. The broth microdilution assay was also performed on *M. abscessus* pVV16-*bla*$_{Mab}$ and *M. abscessus* pVV16 (see chapter 3.2.5).

3.3.5. Generation of *M. abscessus* pVV16-bla$_{Mab}$ overexpressor strain

The *bla*$_{Mab}$ gene (MAB_2875) from *M. abscessus* NCTC 13031 was amplified by polymerase chain reaction (PCR). Primers used were as follows (with restriction site underlined): Forward primer AAAAAA<u>GGATCC</u>GCGCCGGACGAACTCGCC and Reverse primer

AAAAAAAAGCTTAGCGCCGAAGGCCCGCAG (Eurofins Genomics). Amplicons were purified and cloned into pVV16 using *Bam*HI/*Hin*DIII restriction sites and the correct sequence was confirmed by DNA sequencing (Eurofins Genomics). Both the plasmid pVV16 and the construct pVV16-bla_{Mab} were inserted into *M. abscessus* NCTC 13031 cells by electroporation (2.5 kV, 25 µF and 1000 Ω). The pVV16 vector was selected due to its ability to induce ectopic expression of mycobacterial proteins. pVV16 also contains the gene required for kanamycin resistance and a His tag on N terminal on the plasmid backbone (Parikh, 2013).

3.3.6. Expression and purification of recombinant Bla_{Mab}

The bla_{Mab} gene, with the first 90 base pairs omitted (resulting in a −30 residue N-terminal truncated protein), was amplified by PCR using the following primers (with restriction sites underlined): Forward primer AAAAAAGGATCCGCGCCGGACGAACTCGCC and Reverse primer AAAAAAAAGCTTTCAAGCGCCGAAGGCCCG (Eurofins Genomics). Amplicons were purified and cloned into pET28a using *Bam*HI/*Hin*DIII restriction sites and the correct sequence was confirmed by DNA sequencing (Eurofins Genomics). Bla_{Mab} was expressed in *E. coli* BL21 (DE3) cells by addition of 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation at 25 °C for 18 h. Bla_{Mab} (6×-His tagged) was purified by Ni^{2+} Immobilised Metal Affinity Chromatography (IMAC) and dialysed into 25 mM Tris HCl pH 7, 100 mM NaCl.

3.3.7. Thin-Layer Chromatography (TLC) activity assay

Relebactam (2 mg/mL) was added to recombinant Bla_{Mab} (0.01 mg/mL) and incubated for 5 min at room temperature, before addition of Penicillin V (4 mg/mL) for a further 10 min incubation at room temperature. Alongside appropriate control reactions (Figure. 3.6a), 1 µL of the reaction was spotted onto aluminium backed silica gel plates (5735 silica gel 60 F_{254}, Merck) and dried before being subjected to Thin Layer Chromatography (TLC) using ethyl acetate:water:acetic acid ($C_4H_8O_2$:H_2O:CH_3COOH) (3:1:1, v/v/v). Once dry, plates were visualised by being dipped into $KMnO_4$ TLC stain with light charring.

3.3.8. Biochemical analysis of Bla_{Mab} inhibition by relebactam

Recombinant Bla_{Mab} (0.25 nM) was mixed with an increasing concentration of relebactam (0, 0.1, 1, 10 and 100 µM) and 100 µM nitrocefin (Carbosynth, UK). The hydrolysis of nitrocefin was monitored at 486 nm using a Multiskan Go plate reader (Thermo Scientific). This was repeated using a varying concentration of nitrocefin (1–500 µM) with a shorter range of relebactam concentrations (0, 0.5, 0.75, 1 and 2.5 µM) and initial velocities (v_i) were plotted against substrate concentration. Data analysis was performed as previously described (Story-Roller, 2019) (Papp-Wallace, 2018). Data analysis was conducted using Graphpad Prism 7.

The decarbamylation rate was determined by incubation of Bla_{Mab} (1 µM) with relebactam (20 µM) for 1 hour at 25 °C. The reaction mixture was then subsequently diluted both 10,000 and 50,000 fold (1.8 nM and 0.36 nM relebactam with 90 pM and 18 pM Bla_{Mab} respectively), before addition of 100 µM nitrocefin. The reaction was monitored at 486 nm using a Multiskan Go plate reader (Thermo Scientific).

3.3.9. *In silico* modelling of the possible interaction of relebactam with Bla_{Mab}

The Bla_{Mab} X-ray crystal structure was obtained from the Protein Data Bank, accession code 4YFM. The A chain was submitted to the GHECOM pocket-finding server (GHECOM 1.0) (Kawabata, 2007) (Kawabata, 2010). The top six pockets identified were used to define the target site for protein-ligand docking experiments between the enzyme and relebactam using CACHe Worksystem Pro (version 7.5.0.85, Fujitsu Ltd). The amino acid residues lining these pockets are given in Fig. 3.7(c). Pocket 1 corresponded to the main (catalytic) site in the enzyme and for the purposes of the docking experiment was redefined as all amino acid residues within 8 Å of serine 71. Hydrogen atoms were added using the default settings in line with presumed protonation states for ionisable amino acid side-chains. The positions of the added hydrogen atoms were optimised by locking the coordinates of all the non-hydrogen atoms and subjecting the system to a molecular mechanics (MM2) geometry optimisation. Relebactam was docked four times into each pocket, using CACHe Worksystem Pro. The

amino acid side-chains in each pocket were allowed to be flexible as were all rotatable bonds in relebactam. The genetic algorithm settings for the docking protocol included population size 50, maximum generations 3000, crossover rate 0.8, mutation Rate 0.2 and convergence when the RMSD population fitness was less than 1. The best-scoring consensus complexes from each series of dockings were taken forward for molecular dynamics simulation. The required input files were prepared using the Antechamber module of the AMBER Tools package (Version 1435), implementing the ff14SB force field. The system was neutralised by addition of sodium ions and then solvated within a truncated octahedron of TIP3P water molecules extending 8 Å from the surface of the protein. Using the Amber 14 molecular dynamics package CUDA version (Gotz, 2012) (Salomon-Ferrer, 2013) (Le Grand, 2013), the system was energy-minimised for 2,000 cycles using a non-bonded cut-off of 12 Å and then heated under constant volume to 300 K over 25 ps under Langevin dynamics (time step = 1 fs). The heating was continued at 300 K for at least 200 ns under constant pressure also using Langevin dynamics (SHAKE on, time step = 2 fs) using the Particle–Mesh–Ewald (PME) method to treat the long range electrostatic interactions with a 12 Å non-bonded cut-off.

3.4. Results

3.4.1. Disk diffusions and broth microdilution assays

In this study, *M. abscessus* was made susceptible to amoxicillin by the addition of relebactam, a competitive β-lactamase inhibitor. It has also been shown that the MIC of the imipenem-relebactam combination can be further reduced with the addition of amoxicillin. When compared with a ceftazidime-avibactam combination, the amoxicillin-imipenem-relebactam combination is demonstrated to be superior. Furthermore, it was shown that meropenem can be enhanced with the addition of relebactam.

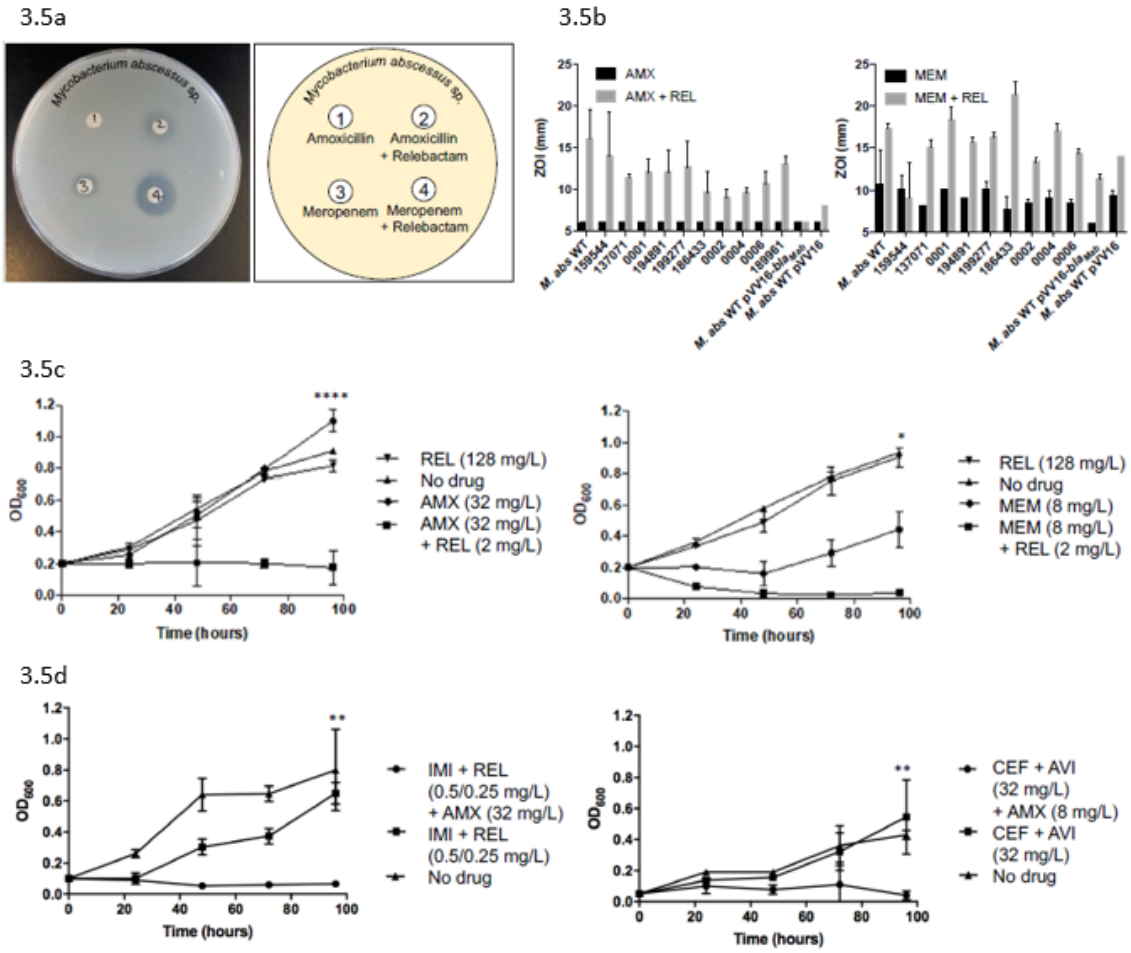


Figure 3.5a-d. *Relebactam makes M. abscessus susceptible to amoxicillin and increases susceptibility to meropenem and imipenem. This is indicated by a disk diffusion (3.5a, 3.5b), and DST using combinations of antibiotics and* β*-lactamase inhibitors (3.5c) (n=3)*

In a disk-diffusion experiment and corresponding plate map (Figure 3.5a), it is shown that the zone of inhibition (ZOI) on *M. abscessus* NCTC increased when relebactam is added to amoxicillin (2), compared with amoxicillin (1) which has no ZOI, demonstrating an increase in susceptibility. Meropenem (3) has a small ZOI when used on its own, however this is further enhanced with the addition of relebactam (4). Figure 3.5.b. shows the ZOI results for a panel of *M. abscessus* clinical isolates. It can be seen in the left chart that amoxicillin has no activity against any of the *M. abscessus* clinical isolates tested, and all 10 clinical isolates and the *M. abscessus* NCTC strain are made susceptible to amoxicillin with the addition of relebactam, shown by an increase in the diameter of

the ZOI. The *M. abscessus* pVV16-bla_{Mab} overexpressor strain remains resistant to amoxicillin with the addition of relebactam, and the control strain, *M. abscessus* pVV16 shows an increase in susceptibility to amoxicillin with the addition of relebactam. The right chart shows the ZOI diameters for 10 *M. abscessus* clinical isolates, *M. abscessus* NCTC strain, *M. abscessus* pVV16-bla_{Mab} overexpressor strain, and *M. abscessus* pVV16 strain when tested with meropenem and meropenem with relebactam. One hundred percent of the clinical isolates (10/10) had moderate susceptibility to meropenem, with an increased ZOI in 90% of the strains when meropenem was used in conjunction with relebactam. One isolate, 159544, showed a small decrease in the ZOI upon the addition of relebactam to meropenem, however this decrease is not statistically significant.

Growth curves (Figure 3.5c, left) were also conducted with the *M. abscessus* NCTC strain in medium containing 128 μg/mL relebactam only, and 32 μg/mL amoxicillin with and without 2 μg/mL relebactam. No growth inhibition was observed with relebactam or amoxicillin alone, whereas full inhibition of growth was seen with 32 μg/mL amoxicillin and 2 μg/mL relebactam. Similarly, with Figure 3.5.c, right, relebactam alone has no inhibitory activity against *M. abscessus* NCTC. The addition of 8 μg/mL meropenem shows moderate but not complete inhibition, and with the addition of 2 μg/mL relebactam to 8 μg/mL meropenem, complete inhibition of growth can be observed. A t-test was used for end point analysis between samples with and without relebactam and the results were deemed to be statistically significant with p values of <0.0001 an 0.00152 for amoxicillin and meropenem respectively. Growth curves were conducted with *M. abscessus* NCTC in medium containing 0.5 μg/mL imipenem and 0.25 μg/mL relebactam, with and without amoxicillin at 32 μg/mL (Figure 3.5.d, left). The addition of 32 μg/mL amoxicillin to 0.5 μg/mL imipenem and 0.25 μg/mL relebactam significantly increases inhibition of growth (p=0.0028). Growth curves were also conducted with *M. abscessus* NCTC in medium containing 32 μg/mL ceftazidime and 32 μg/mL avibactam with and without 8 μg/mL amoxicillin (Figure 3.5d, right). Amoxicillin enhanced the inhibitory activity of ceftazidime and avibactam but to a lesser extent than imipenem and relebactam (p=0.0016)

In vitro DST was performed against 16 *M. abscessus* clinical isolates (including *M. abscessus* NCTC 13031), *M. abscessus* containing the Bla_{Mab} constitutive overexpression plasmid pVV16-bla_{Mab} and *M. abscessus* pVV16 with imipenem, amoxicillin and relebactam, either on their own, in conjunction with each other or as a triplicate combination (Table 3.1). All but one isolate (15/16, 93.75%) were resistant to amoxicillin with MICs of >128 µg/mL, with one isolate, 194891, having a susceptible MIC of 8 µg/mL, and all of the isolates had MICs of >32 µg/mL for relebactam, suggesting these drugs are not effective against *M. abscessus* clinical isolates within their acceptable clinical range. When combining amoxicillin with relebactam, 68.75% (11/16) of the isolates became susceptible to amoxicillin (range 4 – 128 µg/mL), requiring concentrations of relebactam ranging 0.5 - >32 µg/mL. *M. abscessus* pVV16-bla_{Mab} is resistant to amoxicillin alone (MIC > 128 µg/mL), and retains resistance with the addition of relebactam, whilst *M. abscessus* pVV16 is resistant to amoxicillin alone (MIC > 128 µg/mL), but becomes susceptible to amoxicillin with the addition of 2 µg/mL relebactam with an MIC of 32 µg/mL. MICs for imipenem ranged from 2-16 µg/mL, which are within the range for susceptibility/intermediate susceptibility according to the CLSI (CLSI, 2012). *M. abscessus* pVV16-bla_{Mab} had an imipenem MIC of 32 µg/mL. When combining imipenem with relebactam at concentrations of 1 -2 µg/mL, 50% (8/16) of the isolates saw a two-fold reduction in MIC of imipenem, with 6.25% (1/16) of the isolates showing a four-fold reduction in MIC of imipenem from 16 µg/mL to 4 µg/mL, with 2 µg/mL relebactam required to produce this effect. In 37.5% (6/16) of the isolates, the MIC of imipenem was unchanged by relebactam, and interestingly, 6.25% (1/16) saw a two-fold increase in imipenem MIC from 2 to 4 µg/mL with the addition of relebactam. The MIC of imipenem in *M. abscessus* pVV16-bla_{Mab} was reduced four-fold from 32 to 8 µg/mL with the addition of 4 µg/mL relebactam. The MIC of *M. abscessus* pVV16 to imipenem was unchanged with the addition of relebactam, remaining at 2 µg/mL.

When combined with amoxicillin and relebactam (concentration ranges 4 - 128 µg/mL and 0.25 – 2 µg/mL, respectively), the MICs of imipenem were reduced two-fold in 18.75% (3/16) of the isolates, four-fold in 62.5% (10/16) of the isolates, eight-fold in 12.5% (2/16) of the isolates and remained

unchanged in 6.25% (1/16) of the isolates. The MIC of imipenem in *M. abscessus* pVV16-bla_{Mab} saw a sixteen-fold decrease upon the addition of relebactam and amoxicillin, compared with imipenem alone. In this study it has been demonstrated that with the addition of relebactam, the MIC of imipenem can be reduced in *M. abscessus* clinical isolates from a range of 2 – 8 µg/mL to 2 – 4 µg/mL, and further reduced to a range of 0.5 – 4 µg/mL by adding both relebactam and amoxicillin.

The MICs for amoxicillin can be seen to be more variable, ranging from 4 – 128 µg/mL with the addition of relebactam. However, along with the ZOI method, it can be seen that amoxicillin has no activity on its own to 93.75% (15/16) of the clinical isolates and the addition of relebactam clearly creates susceptibility to amoxicillin within this cohort. The same occurs with the addition of relebactam and imipenem, where amoxicillin MIC ranges from 4 – 128 µg/mL, showing clear susceptibility.

Although 93.75% of *M. abscessus* clinical isolates are resistant to amoxicillin on its own, with MICs of > 128 µg/mL, the results in Table 3.1 show that amoxicillin susceptibility in *M. abscessus* isolates can be seen with the addition of the β-lactam inhibitor, relebactam. It is also well established that imipenem is effective against most strains of *M. abscessus,* but the results from Table 3.1 clearly show that the efficacy of imipenem can be enhanced with the addition of relebactam, and further enhanced with the addition of relebactam and amoxicillin.

Table 3.1. MICs of imipenem and/or amoxicillin in combination with relebactam against panel of 15 M. abscessus clinical isolates, M. abscessus NCTC 13031 strain, M. abscessus pVV16-bla$_{Mab}$ and M. abscessus pVV (n=3)

		MIC (µg/mL)									
Clinical isolate ID	**Subspecies**	**AMX**	**IMI**	**REL**	**AMX (+REL)**	**IMI (+REL)**	**REL (+AMX)**	**REL (+IMI)**	**AMX (+IMI +REL)**	**IMI (+AMX +REL)**	**REL (+AMX +IMI)**
NCTC	*M. abscessus*	> 128	2	> 32	64	2	2	1	32	0.5	0.25
DC088 A	*M. bolletii*	> 128	4	> 32	128	2	> 32	1	64	1	0.5
DC088 B	*M. abscessus*	> 128	4	> 32	32	4	> 32	2	32	1	0.5
DC088 C	*M. abscessus*	> 128	8	> 32	> 128	4	> 32	2	32	1	0.5
DC088 D	*M. massiliense*	> 128	4	> 32	> 128	4	> 32	2	4	2	1
DC088 E	*M. massiliense*	> 128	2	> 32	128	2	16	1	64	1	0.5
DC088 Ref	*M. abscessus*	> 128	4	> 32	> 128	2	> 32	1	64	1	0.5
211666	*M. abscessus*	> 128	4	> 32	128	2	8	1	64	1	0.5
137071	*M. abscessus*	> 128	2	> 32	128	4	16	2	16	2	1
199277	*M. abscessus*	> 128	4	> 32	64	4	16	2	128	1	0.5
194891	*M. abscessus*	8	4	> 32	4	2	0.5	1	64	1	0.5
159544	*M. abscessus*	> 128	4	> 32	128	2	1	1	8	1	0.5
186433	*M. abscessus*	> 128	2	> 32	128	4	32	2	128	1	0.5
186144	*M. massiliense*	> 128	8	> 32	128	4	4	2	64	1	0.5
186154	*M. massiliense*	> 128	16	> 32	> 128	4	> 32	2	8	4	2
147028	*M. abscessus*	> 128	4	> 32	> 128	4	> 32	2	16	1	0.5
M. abs pvv16-BlaMab	*M. abscessus*	> 128	32	> 32	128	8	16	4	>128	2	4
M. abs pvv16	*M. abscessus*	> 128	2	> 32	32	2	2	1	32	1	0.5

3.4.2. Biochemical analysis of relebactam inhibition of Bla_{Mab}

In order to validate the phenotypic evidence of inhibitory activity seen in Figure 3.5, biochemical analysis on the activity of relebactam on the *M. abscessus* β-lactamase, Bla_{Mab} was performed. The *Bla_{Mab}* gene was amplified by PCR, digested and then ligated into pET28a and sequenced, followed by transformation into chemically competent *E. coli* BL21. Subsequently, Bla_{Mab} was expressed, cells were harvested then the β-lactamase enzyme was purified by IMAC. A novel TLC-based assay was then devised to assess the β-lactamase activity of the purified Bla_{Mab} (Figure 3.6a). Avibactam was used as a control, based on previous evidence showing inhibitory activity against Bla_{Mab} by Dubee *et. al.* (Dubee, et al., 2015). Lane 1 contained protein purification buffer only, and lane 2 had the addition of enzyme (0.01 mg/mL). Following chromatography, two lower spots are observed in lanes 1 and 2, indicative of buffer. Penicillin V was added to lane 3 and gave a characteristic spot of high R_f value, demonstrating unhydrolysed penicillin resulted in a spot just below the solvent front. Lane 4 was identical to lane 3 with the addition of Bla_{Mab} protein. The hydrolysis of penicillin V to penicilloic acid by Bla_{Mab} resulted in a spot with reduced R_f value. The pre-incubation of enzyme with avibactam in lane 5 resulted in a loss of the lower penicilloic acid spot, demonstrating the inhibition of Bla_{Mab} activity. In lane 6, relebactam alone did not resolve on the TLC, but its pre-incubation with Bla_{Mab} before addition to the penicillin substrate in lane 7 resulted in total inhibition of hydrolysis as observed with avibactam (lane 5). Finally, the inhibition of Bla_{Mab} is further validated by the repeat of lane 4 and lane 7 conditions with heat-denatured Bla_{Mab} (lanes 8 and 9 respectively). This result confirms the direct inhibition of Bla_{Mab} by relebactam (*n=5*).

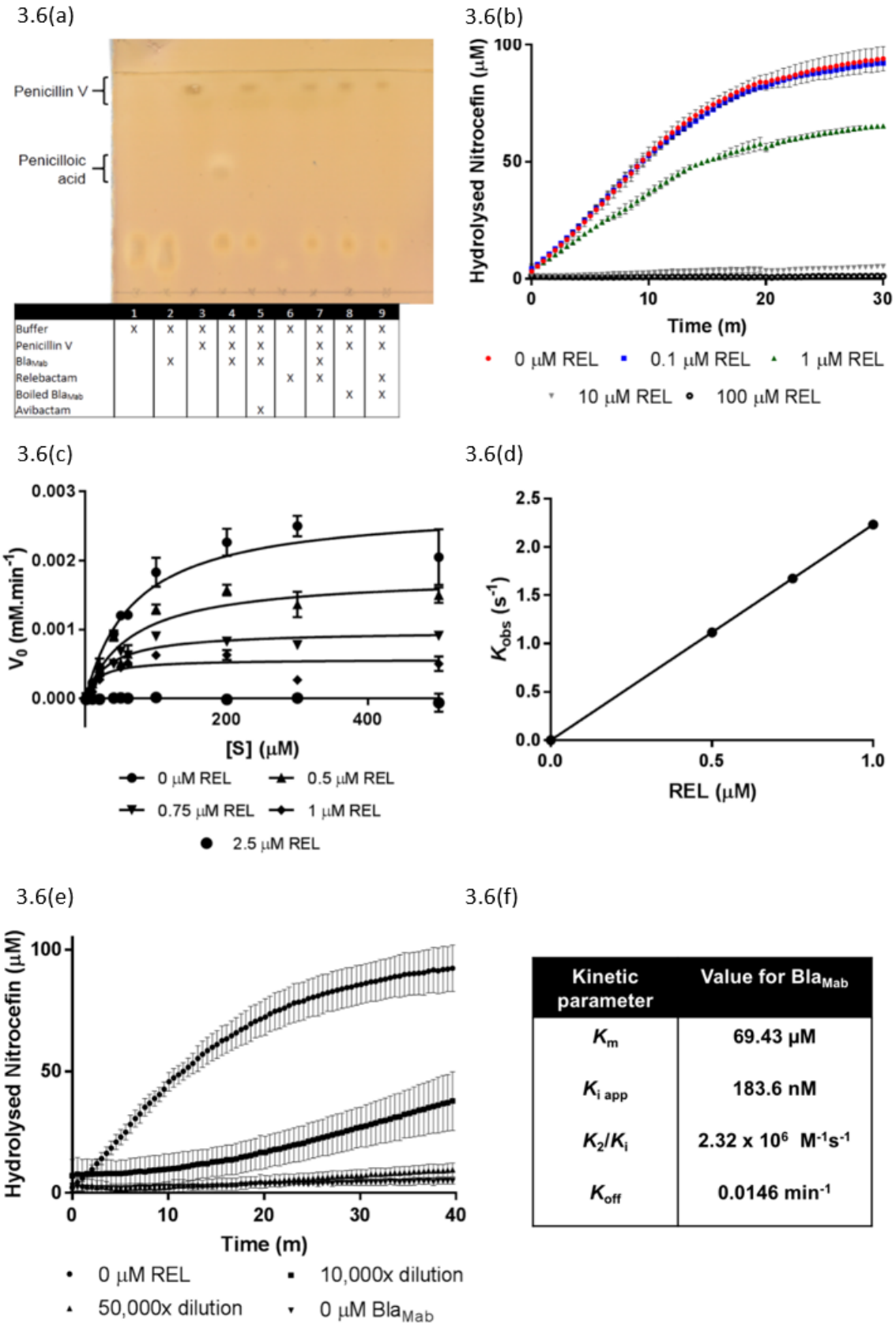
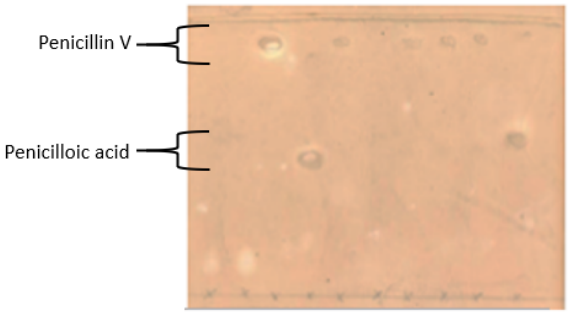


***Figure 3.6a-f. Biochemical analysis of relebactam inhibition of* M. abscessus β-*lactamase, Bla*$_{Mab.}$**

We investigated the kinetic parameters of the Michaelis-Menten kinetics, the apparent K_i ($K_{i\ app}$), the second-order carbamylation rate constant (k_2/K_i) and the decarbamylation rate constant (k_{off}) of relebactam inhibition of soluble recombinant Bla_{Mab} using a commercially available β-lactamase substrate, nitrocefin, as a reporter substrate. Nitrocefin is selectively hydrolysed by β-lactamases, resulting in an increase in absorbance which can be monitored at 486 nm. By pre-incubating Bla_{Mab} enzyme with a dose response of relebactam (from 0-100 μM), before initiation of the absorbance assay with the addition of nitrocefin substrate, partial inhibition at 1 μM (0.348 μg/mL) was observed and a complete loss of activity at 10 μM (3.48 μg/mL), confirming the direct inhibitory activity of relebactam on Bla_{Mab} (Fig 3.6b).

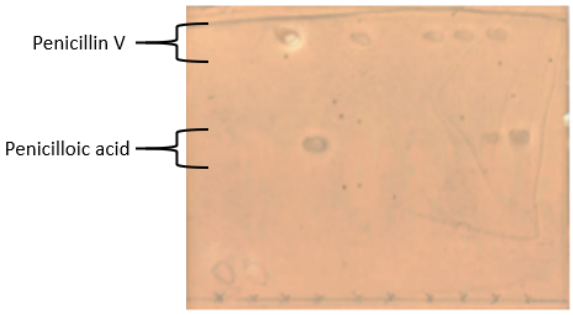
The initial velocities (v_i) of nitrocefin turnover were recorded for a range of nitrocefin concentrations (1-500 μM) over a range of relebactam concentrations (0-2.5 μM). These results were plotted as classical Michaelis-Menten curves (v_i vs [S]) (Figure 3.6c), resulting in Michaelis constants (K_m) between 69.43-16.96 μM, before initial velocities were abolished at 2.5 μM relebactam. However, a K_i value was unable to be derived using this data. In response, the reciprocal initial velocities against relebactam concentrations were plotted as a linear equation and derived $K_{i\ app}$ observed from the Y intercept/slope, which was then normalised for the use of nitrocefin (Figure 3.6f). The $K_{i\ app}$ value obtained of 183.6 nM is indicative of the high inhibitory potency of relebactam against Bla_{Mab}. The carbamylation rate constant (k_2/K_i) of 2.32 x 10^6 $M^{-1}s^{-1}$ is similar to the observed rate for avibactam inhibition of Bla_{Mab} (4.9 x 10^5 $M^{-1}s^{-1}$) [20]. The rate of decarbamylation (k_{off}) of Bla_{Mab} for relebactam was 0.0146 min^{-1}, which is again similar to the rate previously obtained for that of avibactam (0.047 min^{-1}) [19]. Kinetics analysis shows that, like avibactam, relebactam is a potent, competitive and reversible inhibitor of Bla_{Mab}, displaying a reasonably rapid “on” rate and slow “off” rate, with only half the enzyme recovering activity after 40 minutes in the absence of relebactam (Figure 3.6e) (Dubee, et al., 2015).

3.7(a)



	1	2	3	4	5	6	7	8	9	10
Buffer	X	X		X	X		X	X	X	X
Pencillin V			X	X	X		X	X	X	X
Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Relebactam 0s						X				X
Relebactam 10s							X			
Relebactam 30s								X		
Relebactam 60s									X	

3.7(b)



	1	2	3	4	5	6	7	8	9	10
Buffer	X	X		X	X		X	X	X	X
Pencillin V			X	X	X		X	X	X	X
Bla_{Mab}		X		X	X		X	X	X	X
Avibactam					X					
Rel 200 µg/mL						X	X			
Rel 20 µg/mL								X		
Rel 2 µg/mL									X	
Rel 0.2 µg/mL										X

Figure 3.7a-b. Thin Layer Chromatography (TLC) assay exhibiting the activity of Bla_{Mab} in the turnover of penicillin V (high R_f value) to penicilloic acid (lower R_f value).

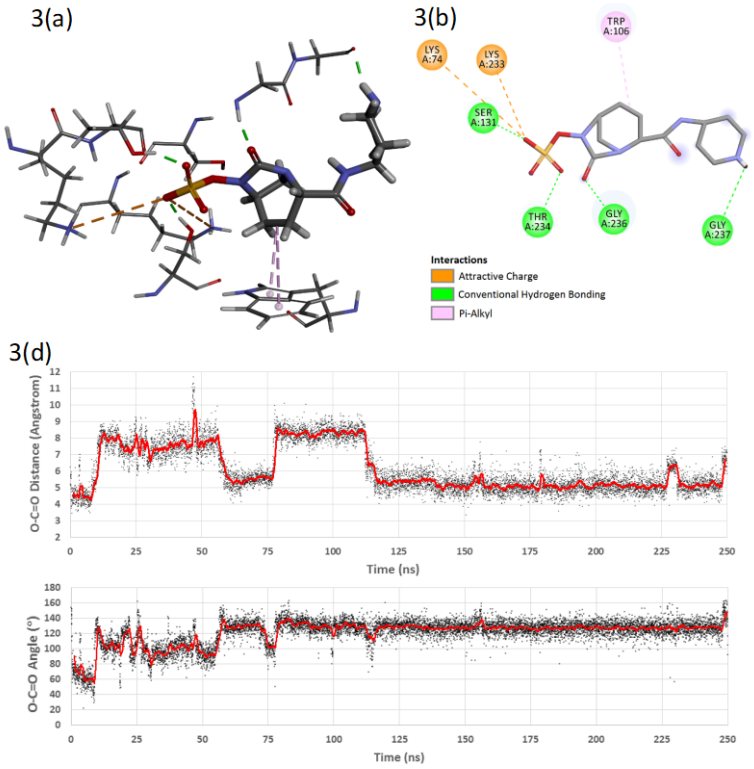
Our TLC-based β-lactamase assay enabled us to further explore the parameters of the inhibitory activity of relebactam by varying the time of pre-incubation of relebactam with Bla_{Mab} (Fig 3.7a) and the minimum inhibitory concentration required to abrogate catalytic turnover of the penicillin V substrate to the penicilloic acid substrate (Fig 3.7b).

We found that penicillin V turnover was rapid and that only addition of relebactam at the same time as the substrate demonstrated turnover of penicillin V, suggesting competitive, reversible inhibition of Bla_{Mab} by relebactam (Fig 3.7a). The dose response of relebactam demonstrated total inhibition down to 20 µg/mL, and activity of Bla_{Mab} was maintained below a relebactam concentration of 2 µg/mL (Fig 3.7b). This suggested a minimal concentration of relebactam required to inhibit Bla_{Mab} in the assay is within the range of 20 to 2 µg/mL. This corresponds to a less than or equal to 100 fold stoichiometric

excess of relebactam required to completely inhibit Bla_{Mab} (0.5 µM BlaMab to 57.5 µM relebactam corresponding to 20 µg/mL).

3.4.3 *In Silico* modelling

In order to further investigate the mechanism of relebactam inhibition of Bla_{Mab}, molecular docking simulations were conducted *in silico*. From this, 6 potential binding sites were identified (Figure 3.8c). For pockets 2-6 the ligand was weakly-held and generally exited the pocket after a few tens of nanoseconds. For pocket 1 (corresponding to the main active site) the ligand reoriented itself relative to the docked conformation and thereafter remained relatively stable within the pocket. The binding interactions for the stable pose are shown in Figure 3.7a and 3.7b and include several polar interactions with the sulphonamide moiety and hydrophobic interactions between the relebactam central piperidine ring and tryptophan 106. In addition, after the initial reorientation, the relebactam carbonyl carbon remained in the vicinity of the hydroxyl oxygen of the catalytically-active serine 71 as can be seen after 120 ns in the distance plot given in Figure 3.8(d). The average distance in this period was approximately 5.5 Å and there were many close approaches. Furthermore, the corresponding O-C=O angle (Figure 3.8d) in the same period was roughly 130 ° and as such it is reasonable to assume that it would be possible for the serine hydroxyl to attack the relebactam carbonyl and effect a ring-opening. A further molecular dynamics simulation of the enzyme in a periodic box of explicit water was undertaken but this time in the presence of ten copies of unbound relebactam. Over the course of a 200 ns simulation, one ligand found its way into the main active site (pocket 1) after 130 ns and remained stable therein. The other nine copies of the ligand found no place to reside in the enzyme.



Pocket 1	Pocket 2	Pocket 3	Pocket 4	Pocket 5	Pocket 6
LEU68	ARG45	MET69	PHE76	THR207	ARG95
MET69	GLU65	CYS70	ILE77	ASP208	TYR98
CYS70	ARG66	SER71	VAL78	TRP209	ALA107
SER71	PHE67	THR72	SER79	LEU210	PRO108
TYR72	LEU68	VAL73	ALA80	LYS211	ILE109
VAL73	MET69	LYS74	ILE81	ARG212	THR110
LYS74	CYS70	THR75	LEU82	ASN213	SER111
THR75	ARG165	PHE76	ARG83	THR214	GLN112
PHE76	LEU170	ILE77	ARG84	THR215	HIS113
ILE77	ASN171	VAL78	ARG85	GLY216	GLY117
TRP106	ILE172	ALA80	LEU86	ASP127	MET118
THR127	PRO173	THR127	SER87	GLN218	THR119
LEU128	ASP174	ALA136	GLU88	SER219	VAL120
TYR130	GLY175	ASN137	LEU91	ILE220	SER121
SER131	ASP176	LEU139	GLN142	ARG221	GLU122
ASP132	LEU177	ILE140	LEU143	ALA222	LEU123
ASN133	ASP178	THR141	GLY145	ALA223	CYS124
ALA136	THR179	LEU143	GLY145	VAL224	ASP125
GLU167	SER180	GLY144	LYS147	PRO225	ALA126
VAL168	GLY237	GLY145	GLU148	ALA226	THR127
LEU170	PHE238	PRO146	THR149	GLY227	LEU128
ASN171	LYS239	LYS147	GLU150	TRP228	ARG129
ASP178	GLY240	GLU148	LYS151	THR229	TYR130
LEU210	GLU241	THR149	PHE152	VAL230	ASP132
THR215	THR242	GLU150	VAL153	ALA231	GLY135
LYS233	ASN243	LYS151	ARG154	ASP232	TRP209
THR234	LEU261	VAL153	SER155	LYS233	ARG212
GLY235	THR262	THR161	LEU156	THR234	
GLY236	VAL263	ARG162	GLY197	ASP244	
GLY237	PRO264	MET163	LEU198	ILE245	
ASN243		ASP164		ALA246	
ILE234		ARG165		VAL247	
		THR166		ILE248	
		GLU167		TRP249	
		GLN169		PRO250	
		LEU170		PRO251	
		ASN171		ILE256	
		LEU177		MET258	
		ASP178		ILE276	
		THR179		ALA277	
				THR380	
				ARG281	
				LEU284	
				ALA289	

Figure 3.8a-d. **In silico *modelling of the possible interaction of relebactam with the* M. abscessus β-*lactamase,* Bla_{Mab}.**

3D and 2D protein-ligand interaction diagrams for relebactam in the main (catalytic) active site after molecular dynamics simulation (Figures 3.8a and 3.8b). Amino acid residues featured in the top six potential binding pockets identified for Bla_{Mab}. (Figure 3.8c) Pocket 1 corresponded to the main (catalytic site) in the enzyme and for the purposes of the docking experiment was redefined as all amino acid residues within 8 Å of serine 71. Time-courses of the serine 71 hydroxyl oxygen – relebactam carbonyl carbon distance and the corresponding O-C=O angle. (Figure 3.8d) The actual values are plotted as black points and a 50-frame moving average is over-plotted in red.

3.5. Discussion

In this study, the inhibitory activity of relebactam, a non-β-lactam β-lactamase inhibitor against the *M. abscessus* β-lactamase, Bla_{Mab} has been demonstrated, resulting in increased susceptibility of *M. abscessus* clinical isolates to certain β-lactam antibiotics. It has been shown that the inhibition of Bla_{Mab} by relebactam, whilst not able to inhibit *M. abscessus* growth on its own, can re-establish the

usefulness of a wide range of β-lactam antibiotics that are otherwise not active against the organism. Therefore, the therapeutic potential of relebactam as part of a drug combination that includes one or more β-lactam antibiotic has been established, and moreover is able to enhance the inhibitory capabilities of imipenem, a drug already widely used to treat *M. abscessus* infections. Relebactam has been recently approved by the FDA for use in conjunction with imipenem/cilastatin, therefore the results of this study represent a significant step forward in treatment options available for *M. abscessus* infections. When used in a triplicate combination, the concentrations required of relebactam, imipenem, and amoxicillin to inhibit *M. abscessus* are significantly reduced, therefore reducing the risk and severity of adverse drug reactions, that are unfortunately all too common in *M. abscessus* treatment regimes. Imipenem in particular is associated with high levels of adverse drug reactions, therefore demonstrating the capability of adding amoxicillin and relebactam in reducing the MIC of imipenem has significant potential in easing the therapeutic burden placed on patients receiving treatment for *M. abscessus* infection. This is particularly important considering the treatment regimen consists of multiple antibiotics each with its own range of severe and debilitating adverse effects. This effect is not as pronounced with a ceftazidime-avibactam combination, in which relatively higher concentrations are required to produce the same effect as the triplicate combination demonstrated in this study.

This study has also demonstrated a novel TLC-based assay to assess β-lactamase inhibition, validated using the widely used nitrocefin assay. The results of both the kinetics analysis and the *in silico* binding studies demonstrate the mechanism of inhibition of Bla_{Mab} by relebactam as competitive reversible, displaying a reasonably rapid “on” rate and slow “off” rate, indicative of the high potency of the inhibitor against Bla_{Mab}.

Chapter 4: Drug susceptibility screening using an *in vitro* model of hypoxic non-replicating persistent *M. abscessus.*

4.1. Aims and objectives

The drug discovery pipeline for *M. abscessus* currently takes a 'two-dimensional' approach; lacking a comprehensive assessment of drug efficacy in models of different physiological states, instead focussing only on *M. abscessus* growth inhibition in high nutrient, aerobic models. This chapter aims to take a *MTb*-like approach, for which drug efficacy is measured in various physiological states. Using both an aerobic and an anaerobic model of *M. abscessus*, this chapter with assess the effect of latency on drug susceptibilities of *M. abscessus.* The results in this chapter can be used to inform the development of a robust, multi-model approach to *M. abscessus* drug discovery.

4.2. Introduction

The drug discovery pipeline of *MTb* involves several assays that assess drug activity against *MTb* in a range of physiological states, such as actively replicating cells and cells that are in a state of non-replicating persistence (NRP). This ensures that novel compounds undergoing testing against *MTb* can be shown to be effective against bacteria in all physiological states (Mdluli, 2015). No such pipeline exists for NTM, where only classical aerobic models are used, in which bacteria are rapidly growing (Wu, 2018). This does not encompass the varied and complex environments encountered during NTM infection. Pathogenic mycobacteria such as *M. tuberculosis* are able to evade the immune response and survive within the harsh environment of a granuloma in a metabolically altered NRP state (Betts, 2002). The reduction of available oxygen and nutrients, and an increase in nitric oxide are largely responsible for this shift in physiology, allowing the mycobacteria to enter the NRP state (Wipperman, 2014) (Matin, 1989). When in the NRP state, *Mycobacterium* spp. enter a state of anaerobiosis, surviving hypoxic conditions and severe nutrient deprivation and nitric oxide assault. Changes seen at the protein level result in the slowdown of transcription apparatus, energy metabolism, lipid biosynthesis and cell division (Betts, 2002). *Mycobacteria* spp. are also able to scavenge cholesterol

from macrophage cell membranes found within the granuloma, allowing for a reduced metabolome in the NRP state (Wipperman, 2014). Considering that NTM are environmental bacteria, where they will likely encounter nutrient deprivation, in contrast to the relatively rapid growth observed in traditional nutrient-rich laboratory assays (Matin, 1989) (Rittershaus, 2013), it stands to reason that pathogenic NTM may be predisposed to survive the harsh environments of the host. Of course, the environment the bacteria encounter in the host will differ to the outside environment, but common pressures mean that the micro-organism is already adapted to nutrient starvation and oxygen deprivation (Rittershaus, 2013).

Previous work has established that oxygen deficiency and nutrient starvation induce the non-pathogenic NTM, *M. smegmatis,* to enter a state of dormancy very similar to what is seen in *M. tuberculosis* pathology (Dick, 1998) (Wu, 2016), with persister cells shown to not synthesize DNA, as well as displaying synchronised cell division upon reactivation in aerobic medium, suggesting that hypoxic conditions cause the arrest of cells at a specific stage of the cell cycle (Dick, 1998). Furthermore, the transcription factor DosR, that regulates dormancy in *M. tuberculosis,* is shown to be conserved across all NTM including *M. abscessus* (Gerasimova, 2011). Mirand-Casoluengo *et al.* showed that the DosR transcription factor is upregulated following nitric oxide-induced hypoxia (Miranda-CasoLuengo, 2016).

Unlike what is seen in *M. tuberculosis,* mucus plays a large role in the pathogenesis of NTM infection. The excessive accumulation of mucus in the CF airway is likely to provide NTM with a unique environment, where oxygen and nutrient levels may vary (Wu, 2018). Studies have suggested that the unique composition of CF sputum slows the growth of *M. abscessus*, inducing a 'low energy' transcriptional response (Miranda-CasoLuengo, 2016). Due to the dual physiology of *M. abscessus*, showing either rough or smooth colonies, this further complicates *in vitro* testing. It is believed that the smooth variants of *M. abscessus* establish infection within the host, due to the tendency of these variants to form robust biofilms within the airways of the host (Howard, et al., 2006), and the rough

variants are associated with chronic *M. abscessus* infection, causing inflammation (Davidson, 2011), promoting tissue invasion, and generally being more virulent than their smooth counterparts (Howard, et al., 2006). *M. abscessus* biofilm studies have fallen short of recreating the complex environment of an infectious biofilm; thus, the effect biofilms have on the susceptibilities of *M. abscessus* to drug regimens remains to be seen (Hunt-Serracin, 2019).

Whilst the majority of research into NRP states in *Mycobacteria* spp. focuses on *M. tuberculosis,* there is growing evidence that *M. abscessus* may also reside within micro-environments in the host that include oxygen starvation and nutrient deprivation (Yam, 2020). It is believed that, with a similar physiology to *M. tuberculosis*, NTM infection may be characterised by necrotising granulomas with minimal blood vessel penetration and a hypoxic central environment (Wu, 2018).

The growing evidence of the ability of NTM, including *M. abscessus*, to persist within harsh, hypoxic, and nutrient deprived states may point to why *M. abscessus* is so recalcitrant to standard drug regimens, and go some way to explaining why *in vitro* drug susceptibilities are not always consistent with treatment outcomes. Knowing that *M. abscessus* physiology shares many traits with that of *M. tuberculosis* (Griffith, 2007)*,* it can be tempting to hypothesise that the NRP state is the sole reason that *in vitro* testing under aerobic conditions and high nutrient media often fails to correlate with *in vivo* results. However, this is unlikely to be the whole picture. *M. abscessus* likely encounters a wide range of oxygen and nutrient levels within the host (Berube, 2018), and so it is important that *in vitro* testing covers these conditions as much as possible to provide a more accurate representation of true susceptibilities. It stands to reason that if *M. abscessus* was to exclusively reside in nutrient and oxygen starved environments in the host, causing cell replication to slow significantly, that agents that inhibit cell replication (such as the β-lactam antibiotics cefoxitin and imipenem) would have little effect on *M. abscessus in vivo.* It is understood, however, that this is not the case, in fact imipenem and cefoxitin are two of the most consistently active agents against *M. abscessus* infection, often correlating with treatment success (Story-Roller, 2018) (Griffith, 2007) (Jeon, 2009).

In this study, a novel NRP model was employed to further elucidate the effect of the NRP state of *M. abscessus* on its drug susceptibilities. Alongside the conventional aerobic MIC testing performed in rich media, it has been shown that *M. abscessus* is not just able to survive harsh hypoxic conditions, but that when in the NRP state, the susceptibilities of *M. abscessus* to some of the frontline drugs recommended by the ATS and the BTS are significantly altered.

4.3. Materials and methods

4.3.1. Hypoxic-induced rich media NRP assay

To produce NRP *M. abscessus* cultures, approximately 10 µL of *M. abscessus* glycerol stock stored at -80 °C was inoculated into vented culture flasks containing 10 mL of Middlebrook 7H9 broth supplemented with ADC. These were incubated for 5 days at 37 °C with 5% CO_2 or until cultures reached a mid-logarithmic phase of growth (OD_{600} of 0.6) The cultures were diluted to an OD_{600} of 0.3 in sterilised glass tubes to a total volume of 3 mL using Middlebrook 7H9 broth. Then 4.5 µL of methylene blue was added to each tube to achieve a final concentration of 1.5 µg/mL. With the lids loose, the tubes were placed into an anaerobic cabinet in order to allow slow degassing of the cultures to take place, which will be indicated by the decolourisation of the methylene blue. This colour change indicated the cultures had reached NRP stage I and were ready for drug susceptibility testing (DST).

4.3.2. Drug susceptibility testing on NRP *M. abscessus*

Once the cultures had reached NRP state I, cultures were dosed with either amikacin, clofazimine, clarithromycin, tigecycline, linezolid, moxifloxacin, or dapsone at concentrations of 100, 50, 25, and 12.5 µg/mL, in triplicate. Culture tube lids were then tightened and sealed with paraffin wax heated to >70 °C, and subsequently placed in a shaking incubator set to 100-120 RPM. The OD_{600} was measured every 24 hours for 420 hours. This assay was performed in triplicate.

4.3.3. Drug susceptibility testing on aerobic models of *M. abscessus*

For the DST on *M. abscessus* growing in a nutrient rich, aerobic environment, *M. abscessus* NCTC 13031 was grown in Middlebrook 7H9 broth supplemented with 10% ADC and 5% glycerol in a shaking incubator at 37 °C until an OD_{600} of~1.0 was reached. The OD600 of the culture was then normalised to 0.1 for experimentation. In a two-fold dilution starting at 128 µg/mL, *M. abscessus* cultures were dosed with amikacin, clofazimine, clarithromycin, tigecycline, linezolid, and moxifloxacin in a 96 well plate, in triplicate. Plates were then placed in an aerobic incubator at 37 °C for 5 days, with the OD_{600} read every 24 hours. After the end of the 5 days, the final OD_{600} of the cultures was determined. This assay was performed in triplicate.

4.4. Results

When grown in traditional aerobic media, the drugs amikacin, clarithromycin, linezolid and moxifloxacin displayed MICs against *M. abscessus* with a range of 8 – 64 µg/mL (Table 4.1). With the exception of amikacin, all of the drugs tested were within the resistant range of predetermined breakpoints for *M. abscessus,* they did however have MICs within the ranges tested in this study. When grown in hypoxic media, all drugs tested see an increase in MIC, with clarithromycin showing an almost three-fold increase. Amikacin MIC goes from 16 µg/mL to 25 µg/mL, a small increase that nonetheless pushes the MIC from the susceptible to intermediate range. The only drugs that did not show a significant difference in MIC were linezolid and clofazimine, for which both assays the MICs were well into the resistant range.

Table 4.1. *MIC of amikacin, clofazimine, clarithromycin, linezolid, and moxifloxacin against M. abscessus grown in aerobic conditions and hypoxic rich media (n=3).*

Antibiotic	Aerobic (7H9) MIC (μg/mL)	Hypoxic MIC (μg/mL)
Amikacin	16	25
Clofazimine	>4	>100
Clarithromycin	32	>100
Linezolid	64	100
Moxifloxacin	8	12.5

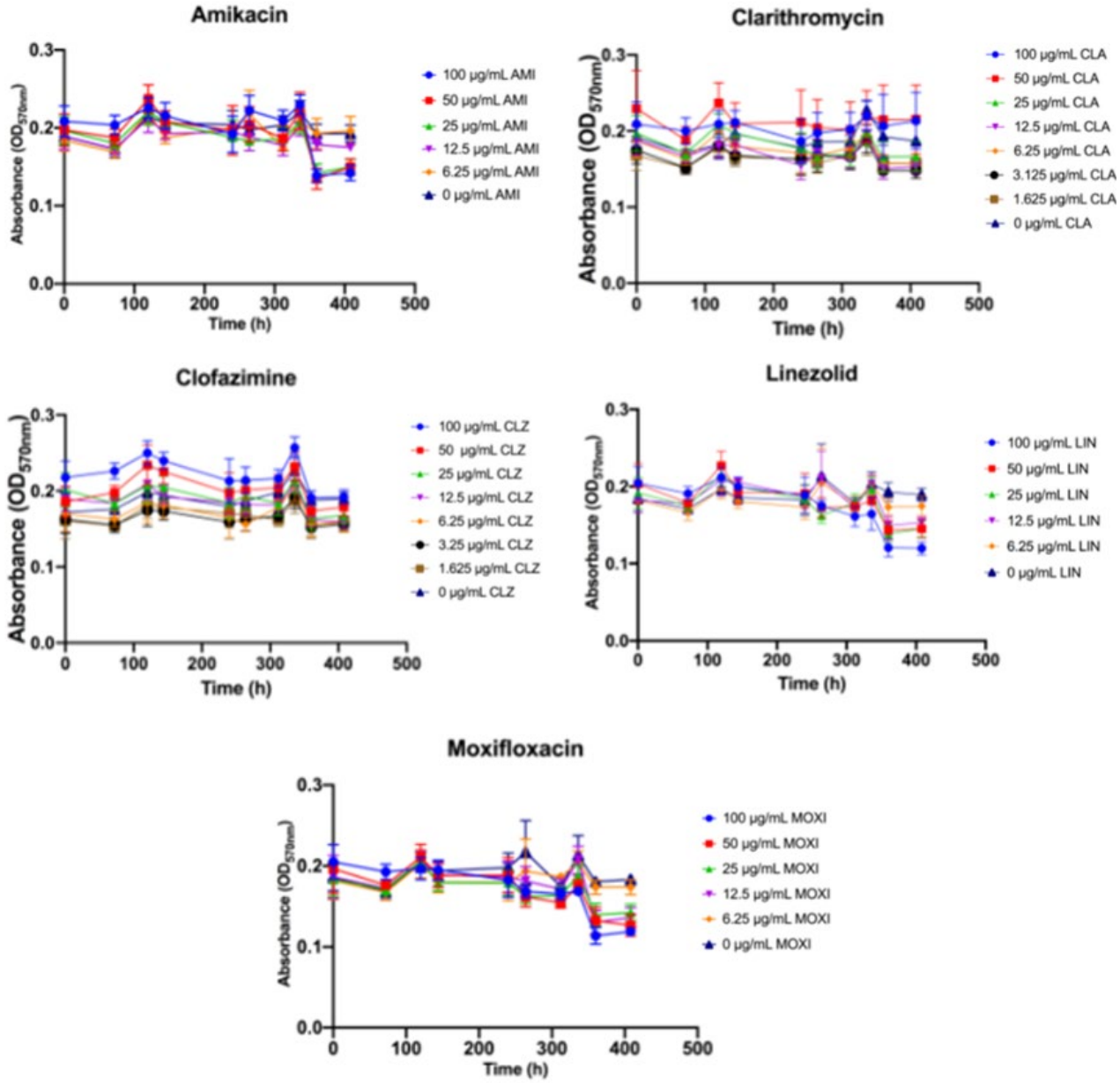


Figure 4.1. Growth curves over 420 hours of* M. abscessus *in rich media (7H9) under anaerobic conditions with amikacin, clarithromycin, clofazimine, linezolid, moxifloxacin, and dapsone, at concentrations of 100, 50, 25, 12.5, 6.25 and 0 µg/mL.

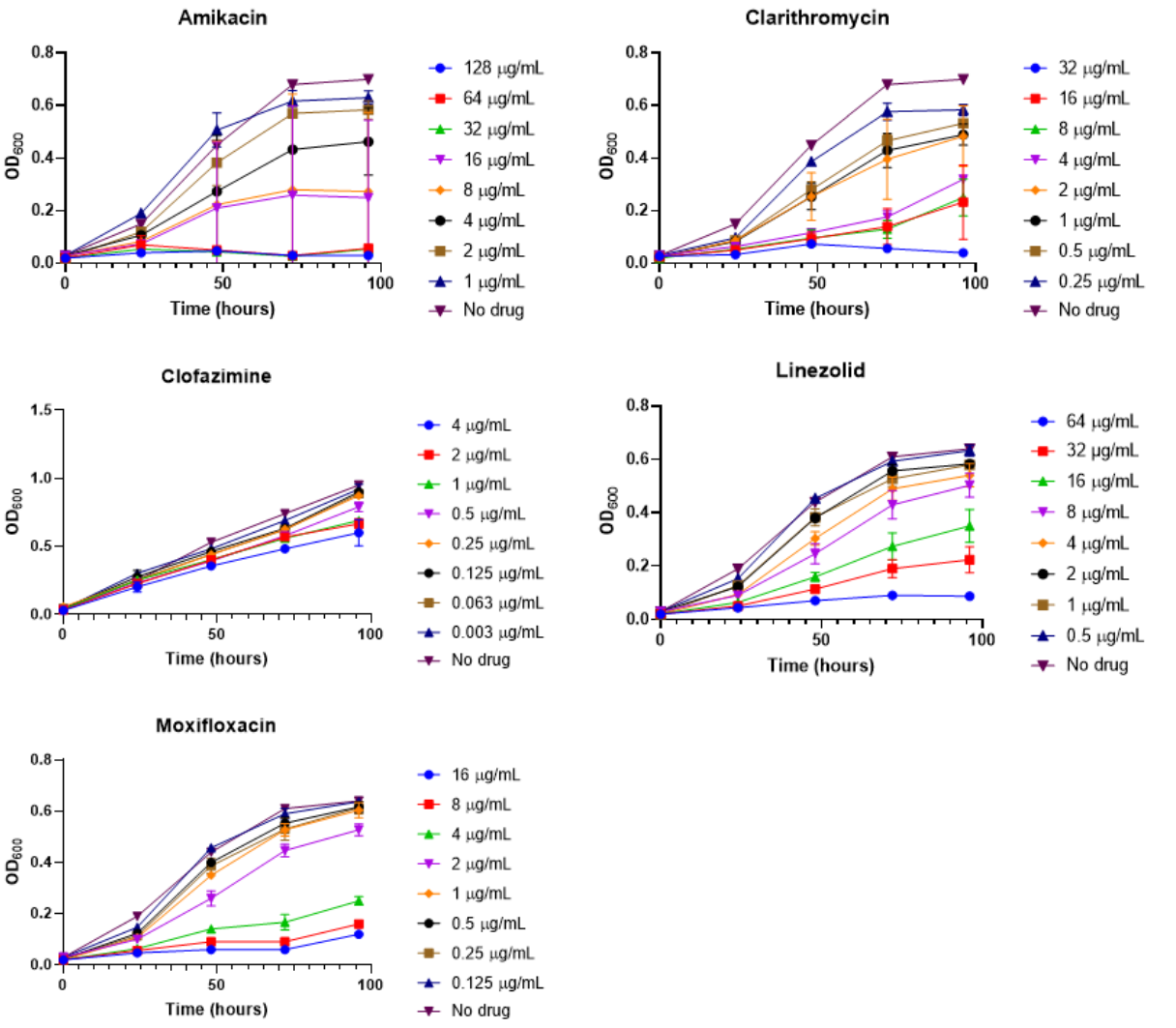


Figure 4.2. Growth curves of* M. abscessus *over 96 hours in rich media (7H9) in aerobic conditions with the addition of amikacin, clarithromycin, clofazimine, linezolid, moxifloxacin, and dapsone, at differing concentrations.

As seen in Table 4.1, under aerobic conditions, amikacin has an MIC of 16 µg/mL, which is within the range of susceptibility (≤16 µg/mL). Using the traditional hypoxia model in rich media, the MIC is increased to 25 µg/mL, somewhere in between an intermediate and susceptible MIC. Similarly, with moxifloxacin, under aerobic conditions, it has an MIC of 8 µg/mL. Whilst this is not within the range of susceptibility (≤1 µg/mL), it is relatively low, and when grown in hypoxic media, the MIC increases to 12.5 µg/mL.

Clarithromycin, when tested against *M. abscessus* in aerobic conditions to 5 days, shows an MIC of 32 µg/mL, which is within the resistant range (≥8 µg/mL), but nonetheless displays some level of susceptibility. When grown in hypoxic rich media the MIC jumps to over 100 µg/mL, an almost three-fold increase, showing total resistance.

For linezolid, it is likely that the levels of hypoxia do not affect susceptibility. In both assays, the MIC was within the resistant range (≥ 32 µg/mL), showing MICs of 64 and 100 µg/mL for aerobic nutrient rich media and hypoxic nutrient rich media respectively (Figure 4.1). As the hypoxic assay used concentrations of 100, 50, 25, and 12.5 µg/mL, and the aerobic assay used concentrations of 128, 64, 32, and 16 µg/mL, it is likely that the MIC remains constant in each assay, perhaps being negligibly higher under hypoxic conditions. Similarly for clofazimine, aerobic testing was performed up to 4 µg/mL, as a resistant phenotype is ≥ 1 µg/mL. When testing under hypoxic conditions, the MIC was >100 µg/mL. Therefore, under all assays, *M. abscessus* shows extremely high levels of resistance, however it is likely that the MIC is indeed increased under hypoxic conditions however the exact increase was beyond the scope of this study.

4.5. Discussion

In one of the more extensively studied *Mycobacteria* spp., *M. tuberculosis,* so called "persister assays" are employed alongside the standard aerobic, nutrient-rich MIC testing, allowing for a more comprehensive picture of susceptibilities (Gibson, 2018). Whilst there is yet to be a global consensus reached on exactly which model best represents the clinical phenotype of *M. tuberculosis*, it may be worth considering adopting a multi-model *in vitro* approach to *M. abscessus* drug discovery.

M. abscessus displays multiple phenotypes *in vivo,* with smooth and rough variants playing different roles in *M. abscessus* pathogenesis, including the formation of biofilms by infecting smooth variants and the virulent and invasive nature of rough variants. Furthermore, mucus, particularly CF mucus, provides an environment with constantly changing oxygen and nutrient levels, and growing evidence

supports the existence of necrotising granulomas within the infected lung, allowing for bacteria to persist in a slow-growing state that results in redundancy of many antimicrobial targets.

In this study it is shown that when *M. abscessus* cultures are grown in standard hypoxic nutrient rich media, almost all of the antibiotics tested display a significant increase in MIC compared with nutrient rich aerobic media. This suggests that using only standard aerobic MIC testing may not sufficiently predict antibiotic susceptibilities in the complex and varied environment of the host.

Kolpen *et al.* showed in 2020 that under aerobic conditions, amikacin enhances the killing of *M. abscessus* by 1 to 6 log units compared with anaerobic conditions (Kolpen, 2020). In a nutrient-starved model of NRP *M. abscessus,* it was shown that amikacin retains activity (MIC 16 µg/mL), and this study demonstrated retained activity using the nutrient-rich hypoxic model.

In a model of nutrient starved NRP *M. abscessus*, it has been shown that moxifloxacin MIC is higher than 200 µg/mL (Berube, 2018), suggesting that it is nutrient starvation, rather than just hypoxic conditions, that may drive moxifloxacin resistance in NRP *M. abscessus,* as the results show an MIC of 12.5 µg/mL under nutrient rich hypoxic conditions.

Clarithromycin was inactivated when tested against *M. abscessus* under hypoxic rich media. Despite being a protein synthesis inhibitor like amikacin, which retained activity under hypoxic rich media, its resistance may be in part due to the known presence of the inducible *erm*(41) gene conferring macrolide resistance (Nash, 2009).

Linezolid demonstrated poor activity against *M. abscessus* in both models of MIC testing in this study. In contrast to these findings, Yam *et al* showed in 2020 that linezolid displays an MIC of 1.4 µg/mL in aerobic nutrient rich media, whereas this study revealed an MIC of 64 µg/mL, constituting resistance. The study performed by Yam *et al.* used *M. abscessus* Bamboo strain, isolated from a patient in Taiwan, whereas this study utilised the more commonly used NCTC 13031 strain, perhaps explaining this inconsistency in MIC results. Furthermore, Yam *et al.* demonstrated that the MBC_{90} of linezolid was in

fact >100 μM, and again, along with this study, oxygen deprivation resulted in the loss of activity against *M. abscessus* Bamboo (Yam, 2020). Linezolid works by inhibiting protein synthesis and is established as a bacteriostatic antimicrobial (Ament, 2002). Considering that *M. abscessus* has been shown to enter the NRP state under nutrient and oxygen deprivation, this explains why linezolid loses activity against non-replicating cells, further cementing the need for bactericidal agents against *M. abscessus* infections.

In the nutrient rich aerobic model used in this study, tigecycline displayed good activity against *M. abscessus,* with an MIC of 1 μg/mL, with the hypoxic condition resulting in loss of activity. Kolpen *et al,* using a model of biofilm formation, illustrated that tigecycline loses its activity, and upon disaggregation, regains activity. In this study it was demonstrated that tigecycline is inactive against *M. abscessus* when in the NRP state. Tigecycline is a bacteriostatic antibiotic, so does not have activity against non-replicating bacteria, however Kolpen *et al.* demonstrated that activity can be regained if biofilms can be disaggregated. In the clinic, tigecycline is an effective antimicrobial used to treat *M. abscessus,* with tigecycline being associated with treatment success in up to 66% of patients (Wallace, et al., 2014). This suggests that actively replicating *M. abscessus* within the host can still be targeted with tigecycline, and provides further evidence of heterogenic states of oxygenation and nutrient deprivation within the host.

Clofazimine displayed resistance in both models used in this study, but MICs were considerably higher (>100 μg/mL) for NRP *M. abscessus* than actively replicating cells (>4 μg/mL). Clofazimine works by binding to the guanine bases of bacterial DNA, resulting in the inhibition of bacterial replication. In 2020, Yam *et al.* demonstrated differing MICs for nutrient-starved aerobic non-replicating *M. abscessus* (>100 μM) and nutrient-rich anaerobic non-replicating *M. abscessus* (31 μM), suggesting that levels of oxygenation may drive clofazimine resistance in NRP *M. abscessus.* In this study, the hypoxic model of NRP *M. abscessus* showed resistance with an MIC of >100 μg/mL. It is difficult to surmise the true effect of oxygen starvation on the activity of clofazimine against *M. abscessus*

considering these results contrast with the findings of Yam *et al*. However, considering clofazimine inhibits replicating cells, it is indeed likely that the NRP state that can be present in an infected host will affect the efficacy of this drug.

In this study it has been shown that antibiotic susceptibility is significantly altered by differing levels of oxygen and nutrients. The unique and complex pathogenesis of *M. abscessus* infection necessitates the use of multiple *in vitro* models specific to *M. abscessus* pathology in drug discovery pipelines.

Chapter 5: General Discussion

Global incidence of infections by the multi-drug resistant pathogen, *M. abscessus* is quickly becoming a significant healthcare concern. *M. abscessus* primarily affects individuals with chronic structural lung disorders, such as CF and bronchiectasis, and those who are immunocompromised, causing severe disease burden (Esther, et al., 2010) (Griffith, et al., 1993) (Sermet-Gaudelus, et al., 2003) (Radhakrishnan, et al., 2009). As the treatment and management of such disorders such as CF improves and longevity of those living with these disorders increases, this will provide the opportunistic *M. abscessus* with increasing numbers of potential hosts, meaning that incidence is likely to rise further. Furthermore, mounting evidence of patient – patient transmission of *M. abscessus* (Bryant, et al., 2013) (Grogono, et al., 2017) clearly highlights a need to understand this pathogen and discover effective treatments. Currently, the ATS and the BTS recommend a treatment regimen lasting from 6 to 18 months, depending on infection type (Griffith, 2007) (Haworth, 2017), and the regimen for *M. abscessus* infection involves a cocktail of antibiotics that are often associated with adverse drug reactions, and unfortunately despite this, treatment failure rates remain unacceptably high (Sfeir, 2018) (Huang, 2010). High treatment failure rates are often attributed to the extreme drug tolerance displayed by *M. abscessus* (Nessar, 2012), as well as inconsistencies between *in vitro* susceptibility and *in vivo* efficacy (Griffith, 2007). Both of these attributes necessitate the need for not only new, effective and safe treatments, but an *M. abscessus* drug discovery pipeline that is able to close the gap between *in vitro* susceptibility results and *in vivo* treatment response.

The *M. abscessus* complex consists of three subspecies; *M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii,* and *M. abscessus* subsp. *massiliense.* This work has identified that drug susceptibilities vary between the three subspecies, and the results correlate with the clinical observation that *M. abscessus* subsp. *massiliense* is associated with higher levels of treatment success. This is likely due to an *erm*(41) gene that confers inducible macrolide resistance in isolates of *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *bolletii,* that is not present in isolates of *M. abscessus* subsp. *massiliense* (Nash, 2009) (Kim, 2010). Chapter 2 of this thesis set out to investigate the prevalence of

each subspecies within a cohort of *M. abscessus* clinical isolates and identify a correlation between subspecies and DSPs. Using a previously described PCR-based typing method (Shallom, 2013), it was found that within the cohort of *M. abscessus* clinical isolates, 66.7% were *M. abscessus* subsp. *abscessus,* 29.2% were *M. abscessus* subsp. *massiliense,* and 4.2% were *M. abscessus* subsp. *bolletii.* The prevalence of each subspecies was found to be in line with similar studies performed globally (Roux, et al., 2009) (O'Driscoll, 2016) (Jeong, 2017) (Tan, 2018) (Bryant, 2013) (Kim, 2008) (van Ingen, 2009), and due to the low prevalence of *M. abscessus* subsp. *bolletii,* it is difficult to identify a link between DSPs and subspecies, apart from *erm(41)*-induced macrolide resistance. A large-scale global study would be particularly useful for identifying a potential link between subspecies and DSPs, particularly with regard to *M. abscessus* subsp. *bolletii.* Genomic typing of *M. abscessus* subspecies represents a significant challenge for clinical microbiology laboratories. This study built upon previous work to use a simple PCR-based typing assay (Shallom, 2013) that could be easily translated into clinical practice. It was found that this PCR-based assay was able to accurately subspeciate the *M. abscessus* clinical isolates within the cohort, representing further evidence that this method could be used clinically to predict treatment outcomes, as well as epidemiologically to track global transmission of the disease. Overall, this study found a high level of resistance to all drugs tested against the cohort of clinical isolates, with only three drugs, imipenem, cefoxitin, and amikacin displaying efficacy against 50% or more of the isolates. There is a clear need for effective drugs to treat *M. abscessus* infection and perhaps a rethink of the treatment guidelines to better match the DSPs of clinical isolates.

Imipenem, a carbapenem β-lactam antibiotic used to treat *M. abscessus* infection, has been found to be one of the more effective drugs to treat *M. abscessus,* both by the studies in chapter 2 and elsewhere in the literature (Kaushik, et al., 2015) (Lefebvre, 2016). Despite its relative superior efficacy, imipenem is impeded by the presence of an endogenous β-lactamase in *M. abscessus,* Bla_{Mab} (Soroka, et al., 2014). Highly efficient at hydrolysing β-lactam antibiotics, Bla_{Mab} is also resistant to the classical β-lactam β-lactamase inhibitors on the market today, such as clavulanic acid, tazobactam, and sulbactam (Soroka, et al., 2014). Avibactam, a non-β-lactam β-lactamase inhibitor, developed for use

alongside ceftazidime, was shown to be able to reduce the MICs of imipenem (Lefebvre, 2016) and amoxicillin (Dubee, et al., 2015), however its approval for use alongside ceftazidime, a drug not used against *M. abscessus*, limits its potential in a clinical setting. It does, however, open up the opportunity to explore other non- β-lactam β-lactamase inhibitors against *M. abscessus.* Relebactam, another non-β-lactam β-lactamase inhibitor, was approved for use alongside imipenem/cilastin, with early studies suggesting that relebactam can reduce the MIC of imipenem against *M. abscessus.* The study in chapter 3 aimed to probe the usefulness of relebactam/imipenem against a panel of clinical isolates, and identify potential drug combinations that can further reduce the MIC of imipenem, which is associated with severe adverse drug reactions and is often considered a 'last resort' antibiotic. In this study we showed for the first time, that the addition of amoxicillin, a β-lactam drug with no efficacy on its own against *M. abscessus,* can significantly reduce the MICs of both imipenem and relebactam against all clinical isolates tested. This is important from a clinical standpoint, if you can reduce the antibiotic burden placed on patients during their months-long treatment with the addition of a safe, widely available drug, like amoxicillin, it not only reduces adverse drug reactions and therefore increases compliance, but it has the clear potential to provide a new, rapidly implementable treatment option for patients for whom all other therapy has failed.

The *M. tuberculosis* drug discovery pipeline has long included models of both replicating and non-replicating bacteria in order for researchers to identify potential drug targets and compounds that can inhibit growth within all the subpopulations of *MTb* that are found within the host (Mdluli, 2015). The presence of persister cells within a hypoxic granuloma is a hallmark of *MTb* infection that has largely driven this multi-faceted approach to TB drug discovery. The NTM drug discovery pipeline, however, relies largely on classical aerobic assays, in which bacteria are rapidly multiplying in aerobic media (Wu, 2018). This does not encompass the different environments encountered by the pathogen when infecting the host, and suggests that the basic aerobic assays used to determine DSPs may only be showing a partial picture. In chapter 4, a classic aerobic assay in which *M. abscessus* cells are rapidly dividing, was employed alongside an anaerobic assay (typically used in TB drug discovery), in which

non-replicating persistence was induced in *M. abscessus* prior to antibiotic challenge. It was shown that oxygen deprivation significantly alters the antibiotic susceptibility of various antibiotics tested, resulting in MICs increasing across the board. The results of this study clearly show the value of exploring more physiologically relevant means of drug discovery, and employing both aerobic and anaerobic assays when investigating novel compounds against *M. abscessus*.

The studies shown in this thesis represent an expansion of our understanding of *M. abscessus* drug susceptibilities and subspecies prevalence. This knowledge can be used to inform NTM research and cement the notion that novel drugs are desperately needed to treat this infection. It has also shown, for the first time, that a triplicate combination of imipenem, relebactam, and amoxicillin have great potential in improving treatment outcomes of *M. abscessus,* furthermore, this combination can be clinically implemented immediately as all of the drugs are currently available and do not need to undergo the lengthy regulatory processes that novel drugs will have to go through. Lastly, we have shown that non-replicating persistent *M. abscessus* sees phenotypic changes to its drug susceptibility profile, therefore we propose a TB-like pipeline be immediately implemented into the NTM antibiotic drug discovery pipeline.

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