A STUDY OF THE DIAGNOSIS, TREATMENT AND EPIDEMIOLOGY OF *MYCOBACTERIUM ABSCESSUS* IN PATIENTS WITH CYSTIC FIBROSIS

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A thesis submitted in partial fulfilment of the requirements of the University of Northumbria at Newcastle for the degree of Professional Doctorate

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and in collaboration with the Microbiology Department at the Freeman Hospital

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others. The work was done in collaboration with the Freeman Hospital, Newcastle upon Tyne, UK

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Faculty Ethics Committee / University Ethics Committee on 1st November 2013.

I declare that the Word Count of this Thesis is 44,296 words

Name: Clair L Preece

Signature:

Date:

Abstract

Members of the *Mycobacterium abscessus* complex (MABSC) are a highly antibiotic-resistant complex of organisms within the genus Mycobacterium, increasingly acknowledged as a significant cause of lung infection in patients with cystic fibrosis (CF) and associated with poor clinical outcomes. Current methods of isolation of MABSC are hindered by the fact that they grow at a slower rate in culture than other microorganisms with many patient samples having to be discarded due to the overgrowth of more rapidly growing species. Decontamination of samples has shown to have an adverse effect upon the viability of MABSC, therefore improvements in the isolation of MABSC are urgently required in order to offer the possibility of a more rapid and accurate diagnosis.

A novel medium (RGM) was developed for the isolation of MABSC. Commercially available pre-poured media were compared with RGM and challenged with isolates of rapidly growing mycobacteria and other species. In addition, in a multi-centre study sputum samples collected from patients with CF were inoculated onto RGM medium, BCSA and standard automated liquid culture method and assessed for growth. RGM demonstrated superior sensitivity over currently used methods without any requirement for decontamination and could easily be incorporated into any laboratory alongside routine culture for other CF pathogens.

Chromogenic and fluorogenic substrates were investigated for the possibility of differentiating between subspecies within the MABSC complex. However, the results established that these would not provide any additional benefit to RGM.

Possible environmental sources were explored in order to establish how patients with CF were acquiring MABSC. Although person-to-person transmission has been suggested, there are very few reports to substantiate this at present and many questions remain unanswered. In this study, MABSC was not isolated from any of the environments screened.

Finally, a selection of antimicrobials were investigated against MABSC with the purpose of ascertaining susceptibility and whether any may be used for a more successful treatment outcome. There were no clinically applicable results therefore further work is required in this area.

To conclude, RGM is a novel culture medium, which can be embedded alongside routine culture for other CF pathogens without any requirement for decontamination. This means that all respiratory samples submitted from patients with CF can be conveniently cultured for NTM, considerably improving the service offered to clinicians and patients. Furthermore, it is likely that formal AFB culture methods could be replaced by use of such a medium, potentially enabling substantial savings in terms of materials and labour time.

Dedication

I would like to dedicate this thesis not only to my children and grandchildren, Gary, Jamie, Emily, Heidi, Tyler, Alfie and Ivy, but also to my Mother Judith and Grandmother Jean. I am very proud to say I have undoubtedly inherited my Grandmothers brains and my Mothers free spirit, love of wine and sense of humour. Possibly a blessing they are both no longer with us, as I think the shock of me actually doing something worthwhile with my life at long last would have finished them off.

Acknowledgements

Undertaking this PhD has been a truly life-changing experience for me, predominantly in a positive way, and I have in truth enjoyed almost every moment. However, it has undeniably had its moments over the years, and I am not ashamed to admit that I am relieved it is finally over and I can have my life back again. That said, it would not have been possible to complete without the support and guidance that I have received from countless people.

I would like to begin by saying an enormous thank you to my supervisor Professor John Perry at the Freeman Hospital, Newcastle upon Tyne for providing me with the opportunity to undertake this PhD (… little did he know what he had let himself in for!). I am forever appreciative of his continued support, encouragement, leadership and above all else, his humour, which has kept me motivated throughout. Without his guidance and constant feedback, which I will admit on occasions may have been unwelcome, and whose maddening attention to detail finally drove me to learn how to reference correctly, this PhD would not have been achievable.

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I am especially grateful to Audrey Perry who has been an immense support throughout the past three years, offering advice, assistance and counselling. Audrey was also the perfect travel companion for the numerous conferences I attended throughout my PhD. I would like to thank my Freeman Hospital research colleagues Daniel Tierney, Arthur Codd, Emma Marrs and Katy Day for their continued support and for keeping a sense of humour when I lost mine, and to all the staff in the Microbiology Department at the Freeman Hospital for allowing me to work alongside them. Special thanks also go to Anne Barrett, Martin Appleby and all the staff in Newcastle Public Health England Regional Mycobacterial Reference

Laboratory, for their co-operation, advice and patience, and allowing me to work in their laboratory and collect data for my research.

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My heartfelt thanks and sincerest apologies go to Eddie, who has lovingly endured the most horrifying mood swings, tantrums and tears in the process of this thesis being written, but has continued to encourage, love and reassure me whilst never complaining. I could not begin to put into words what his persistent encouragement, support and above all else his sense of humour has meant to me. I know that he was happier and even more relieved than I was when this was finally complete. I will be forever grateful to him for sticking by me through what was unquestionably the hardest and most stressful time of my entire life.

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I would finally like to thank everyone else who has supported and encouraged me throughout this PhD; I am very appreciative to each and every one of you.

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4-MU	4-methylumbelliferone
7-AMC	7-amino-4-methylcoumarin
A475	4’-Diethylaminophenyl-9-methoxy-10-phenylacridan
A477/A480	9-Chloro-9-(4’-diethylamino)phenyl-9,10-dihydro-10-phenylacridine hydrochloride
A480a	4’-Diethylaminophenyl-9-chloroacridan
A503a	4’-Diethylaminophenyl-9-chloro-10-methylacridan
A503b	4’-Diethylaminophenyl-9-chloro-10-ethylacridan
A505	4’-Diethylaminophenyl-2-9-dichloro-10-phenylacridan
A520	4’-Diethylaminophenyl-9-chloro-10-phenyl-4”-chloroacridan
ABC	ATP binding cassette (transporters)
ABPA	Allergic bronchopulmonary aspergillosis
AFB	Acid-fast bacillus
AG	Arabinogalactan
AST	Antibiotic susceptibility testing
ATCC	American Type Culture Collection
ATP	Adenosine 5’-triphosphate
ATS	American Thoracic Society
BCC	*Burkholderia cepacia* complex
BCG	Bacillus Calmette-Guerin
BCSA	Burkholderia cepacia selective agar
BC-TSP	Benzalkonium chloride-trisodium phosphate
cAMP	Cyclic adenosine monophosphate
C390	9-Chloro-9-(4'-diethylamino)phenyl-9,10-dihydro-10-phenylacridine hydrochloride
CF	Cystic fibrosis

CFTR	CF transmembrane conductance regulator
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CPC-NaCl	Cetylpyridinium chloride-sodium chloride
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
H_2SO_4	Sulphuric acid
HRCT	High resolution computed tomography
IFN-ϒ	Interferon gamma
IND	Investigational New Drug Application
ITS	Internal transcribed spacer
KH_2PO_4	Monopotassium phosphate
LAI	Liposomal amikacin for inhalation
LM	Lipomannan
LAM	Lipoarabinomannan
LJ	Lowenstein–Jensen (medium)
MABSC	*Mycobacteria abscessus* complex
MAC	*Mycobacteria avium* complex
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MFS	Major facilitator superfamily (transporter)
MGIT	Mycobacterial growth indicator tube
MIC	Minimum inhibitory concentration
MLSA	Multilocus sequence analysis
MLST	Multilocus sequence typing
MmpL	Mycobacterial membrane protein large (transporter)

MST	Multispacer sequence typing
MTb	*Mycobacteria tuberculosis*
Mycobacterium AS	Mycobacteria additional species
Mycobacterium CM	Mycobacteria common bacteria
NALC-NaOH	*N*-acetyl-L-cysteine–2% sodium hydroxide
NALC-NaOH-OxA	*N*-acetyl-L-cysteine–2% sodium hydroxide 5% oxalic acid
NaOH	Sodium hydroxide
NCTC	National Collection of Type Cultures
NTM	Non-tuberculous mycobacteria
OADC	Oleic albumin dextrose catalase (growth supplement)
PANTA	Polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (antibiotic mix)
PCR	Polymerase chain reaction
PI	Phosphatidylinositol
PIMs	Phosphatidylinositol mannosides
QRDR	Quinolone resistance determining regions
RGM	Rapidly-growing mycobacteria (also RGM medium for rapidly-growing mycobacteria)
SGM	Slow-growing mycobacteria
TB	Tuberculosis
TNF-α	Tumour necrosis factor alpha
Trim-Sulf	Trimethoprim/sulfamethoxazole
TTD	Time-to-detection
VNTR	Variable number tandem repeat
WGS	Whole genome sequencing
XLD	Xylose lysine desoxycholate (Agar)

CHAPTER ONE

General Introduction

The genus Mycobacterium

The genus *Mycobacterium* is one of the earliest defined, with the name mycobacterium originating from the Greek prefix for fungus “myco”, initially designated to a group of organisms that grew as mould like pellicles on the surface of liquid media (Rastogi *et al.*, 2001).

Published in 1896 by Lehmann and Neumann in their Atlas of Bacteriology, the genus *Mycobacterium* at the time contained only two species, *Mycobacterium tuberculosis* and *Mycobacterium leprae*.

Taxonomically, mycobacteria belong to the genus *Mycobacterium*, a genus within the family of Mycobacteriaceae in the order of *Corynebacteriales*. The *Mycobacterium* genus to date comprises more than 170 diverse species (Euzeby, 2015), and with respect to pathogenicity three groups can be subdivided (Ng *et al.*, 2014); *M. tuberculosis* complex, *M. leprae* and non-tuberculous mycobacteria (NTM).

1.1 History of mycobacteria

M. tuberculosis was first discovered to be the causative agent of tuberculosis (TB) in 1882 by Robert Koch. On 24th March 1882, Koch declared to the Berlin Physiological Society that he had established the cause of TB (Koch, 1882). This was a principal event in the history of medicine, and a defining moment in the understanding of the deadly disease that had plagued mankind for centuries. Koch modified previously used staining techniques, and developed a solid culture medium rather than the liquid broths that were used at that time and was able to isolate, cultivate and observe the bacteria. He demonstrated that the disease was due to an external infectious agent by observing lesions characteristic of the disease, that were not present in normal tissues, then by culturing in *vitro*, outside the infected

animal, and reintroducing *in vivo* into a healthy animal subsequently leading to TB lesions (Cambau and Drancourt, 2014).

Only a few years later many other species of mycobacteria had been described (Migliori *et al.*, 2007), which at the time were all considered “atypical mycobacteria”. The pathogenicity of these “atypical” mycobacteria to humans was increasingly documented following a significant publication (Buhler and Pollak, 1953) where two cases of pulmonary disease comparable to TB and caused by what the authors termed the “yellow bacillus” after the bright yellow pigment observed upon exposure to light, were described. Today this species is known as *Mycobacterium kansasii*.

As these “atypical” mycobacteria currently make up the majority of species within the genus *Mycobacterium*, the term atypical is contentious, and they are today more frequently known as non-tuberculous mycobacteria.

1.2 Species of mycobacteria

1.2.1 *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex includes *M. tuberculosis, Mycobacterium africanum, Mycobacterium caprae, Mycobacterium bovis, Mycobacterium bovis* Bacille Calmette-Guerin (BCG), *Mycobacterium canettii, Mycobacterium microti, Mycobacterium mungi* and *Mycobacterium pinnipedii*.

With the exclusion of *M. bovis* BCG, which is a vaccine against TB prepared from a live attenuated strain of *M. bovis* (Orduna et al., 2011), these species are considered to cause TB in both humans and animals, and despite their genomic similarity, their epidemiology, pathogenicity and host continuum vary substantially.

1.2.2 Non-cultivable mycobacteria

Mycobacterium leprae, also known as Hansen`s bacillus, was the first acid-fast bacterium to be recognised as a source of a human disease in 1873 by Gerhard Armauer Hansen in the skin nodules of patients with leprosy.

Previously termed “the bacteriologist’s enigma” (Stewart-Tull, 1982) the causative organism of leprosy has to date obstinately eluded all attempts to culture it *in vitro* never having been successfully grown on artificial culture medium as it is only able to proliferate whilst acting as a intracellular parasite (Davis *et al.*, 2013; Kumar *et al.*, 2014). *Mycobacterium leprae* has the slowest doubling time of all known bacteria taking around fourteen days for cells to divide.

1.2.3 Non-tuberculous mycobacteria

NTM, also known as mycobacteria other than tuberculosis, or atypical mycobacteria, denote all species of mycobacteria that can cause human disease, other than TB and leprosy. NTM can be classified into two categories; slow-growing and rapid growing. This is based on interval to colony formation by subculture onto solid media, with the cut off distinguishing the two estimated at around seven days. To simplify organised clinical and taxonomical study, Runyon suggested the first classification system for NTM based on their colony morphology, growth rates and pigmentation (Runyon, 1959), (see Table 1-1), although this is now somewhat obsolete with the widespread use of molecular techniques. A more applicable method of categorising these organisms is based on the type of clinical disease they produce; pulmonary, cutaneous, lymphadenitis or disseminated disease (Koh et al., 2002).

1.2.3.1 Slow-growing non-tuberculous mycobacteria

This is a group of NTM that generally require more than seven-day's incubation to form visible colonies on culture media. The most common clinically relevant species are *Mycobacterium avium* complex (MAC), including but not limited to *M. avium* and *Mycobacterium intracellulare,* as well as other significant species such as *Mycobacterium kansasii, Mycobacterium xenopi, Mycobacterium malmoense, Mycobacterium simiae, Mycobacterium scrofulaceum, Mycobacterium marinum, Mycobacterium terrae, Mycobacterium gordonae* and *Mycobacterium szulgai*. *Mycobacterium ulcerans* is an environmental slow-growing NTM responsible for causing the Buruli ulcer, a chronic and often debilitating disease frequently seen in children and characterised by necrosis of subcutaneous tissue, affecting mainly the skin, but occasionally bones. The exact mode of transmission is unknown and there are currently no preventative measures that may be undertaken (Doig *et al.*, 2012).

Early determination of whether the causative organisms are rapidly-growing or slow-growing NTM is advantageous for selecting appropriate treatments; since antibiotic regimens can vastly differ, and those that are effective for slow-growing mycobacteria may not be effective for rapid growers (Kim *et al.*, 2013).

Table 1-1: Classification of non-tuberculous mycobacteria according to Runyon (Runyon, 1959)

Group	Characteristics	Important Species
I Photochromogens	Slow growth > 7 days Pigmentation after exposure to light	*Mycobacterium kansasii* *Mycobacterium szulgai* *Mycobacterium simiae*
II Scotochromogens	Slow growth > 7 days Pigmentation with or without light exposure	*Mycobacterium xenopi* *Mycobacterium gordonae* *Mycobacterium scrofulaceum*
III Non-Chromogens	Slow growth > 7 days No pigmentation	*Mycobacterium avium* *Mycobacterium intracellulare* *Mycobacterium malmoense*
IV Rapid Growers	Rapid growth < 7 days	*Mycobacterium fortuitum* *Mycobacterium abscessus* *Mycobacterium chelonae*

1.2.3.2 Rapidly-growing non-tuberculous mycobacteria

The group of NTM that are categorised as rapidly-growing will form visible colonies on culture media in less than seven days. As in the slow-growing mycobacteria, time to growth detection can vary between species with some rapid growers being detected in three to four days. These include but not limited to *Mycobacterium abscessus* complex (MABSC); (MABSC includes *M. abscessus* subsp. *abscessus, Mycobacterium abscessus* subsp. *bolletii* and *Mycobacterium abscessus* subsp. *massiliese*), *Mycobacterium chelonae, Mycobacterium fortuitum, Mycobacterium neoaurum, Mycobacterium smegmatis* and *Mycobacterium mucogenicum*.

1.3 The structure of the mycobacterial cell

Described as one of the most complex in nature, many of the distinctive characteristics of the mycobacterial cell are to be found in their intricate cell wall

(Figure 1-1), where a key finding in the past decade has been the remarkably low permeability of mycobacteria to nutrients and antimicrobial agents. This decelerates growth of mycobacteria, as well as making any disease instigated by pathogenic species very challenging to treat due to their inherent resistance to most antimicrobials and chemotherapeutic agents.

The cell wall of mycobacteria is extremely complex with its most prominent distinguishing feature being its high lipid content, said to account for around 60% of the cell wall weight, compared to 5% in other Gram-positive bacteria and 10% in Gram-negative bacteria (Jarlier and Nikaido, 1990), which could explain the tendency for NTM to grow in clumps. Its unusual structure makes it challenging for any host to impair the cell wall and while intact this offers substantial protection to the mycobacterium from any damage. The cell wall is composed of mycolic acids, complex waxes, and distinctive glycolipids, peptidoglycan (PG) and arabinogalactan (AG). Mycolic acids are a homologous succession of C_{60}-C_{90} exceptionally elongated side-chain alpha-alkyl and beta-hydroxy fatty acids joined to the muramic acid moiety of the peptidoglycan by phosphodiester bridges, and to arabinogalactan (D-arabinose and D-galactose) by esterified glycolipid linkages, signifying crucial components of the mycobacterial cell wall. They are imperative to mycobacterial growth, survival, and pathogenicity.

A major component of the mycobacterial cell wall is a macromolecule of peptidoglycan covalently-linked through a phosphodiester group to an arabinan-capped linear galactan. The arabinan cap is adapted with numerous mycolic acids. Aside from the mycolyl–arabinogalactan–peptidoglycan complex, the cell wall contains non-covalently associated lipids, glycolipids, glycophospholipids, glycopeptidolipids, sulfolipids, and sulfoglycolipids. Among the most abundant of these are a family of associated glycophospholipids containing mannose, termed

the phosphatidylinositol mannosides (PIMs), lipomannan (LM) and lipoarabinomannan (LAM). These molecules share a phosphatidylinositol (PI) anchor, and biochemical and genetic studies support a proposed biosynthesis pathway of PI → PIMs → LM → LAM (Khoo *et al.*, 1995; Schaeffer *et al.*, 1999; Kordulakova *et al.*, 2002).

PIMs are distinctive glycolipids found in abundance in the inner and outer membranes of all mycobacterium species. They are centred on a phosphatidylmyoinositol lipid anchor carrying one to six mannose residues and up to four acyl chains. Phosphatidylmyoinositol mannosides are considered to be crucial structural components of the cell envelope (Guerin *et al.*, 2009).

Glycolipids LAM and LM are key virulence factors in mycobacteria species. Besides their role in the cell wall structure, it is thought they function as toll-like receptors with immunoregulatory and anti-inflammatory effects (Fukuda *et al.*, 2013) allowing their survival by damaging host resistance and acquired immune responses. These mechanisms consist of the inhibition of T-cell proliferation and of macrophage microbicidal activity through reduced IFN-ϒ response (Knutson *et al.*, 1998).

Arabinogalactan (AG) is a major structural component of the cell wall. It is a highly branched polysaccharide providing a molecular framework facilitating the connection of peptidoglycan to the outer mycolic acid layer and a range of glycolsyltransferases are employed for its assembly. The biosynthesis of the arabinan domains of AG and LAM occurs via a combination of membrane bound arabinofuranosyltransferases (Alderwick *et al.*, 2011).

The role of the complex hydrophobic mycobacterial cell wall has been widely studied, and many of its properties including its characteristic acid-fastness, high lipid content, and the slow rate of growth are thought to play a large part in the poor

diffusion of hydrophilic molecules, including antibiotics (Jarlier and Nikaido, 1990). Genomic analysis has also revealed the presence of other prospective drug resistance elements such as predicted ß-lactamases, aminoglycoside phosphotransferases and aminoglycoside acetyltransferases (Ripoll *et al.*, 2009).

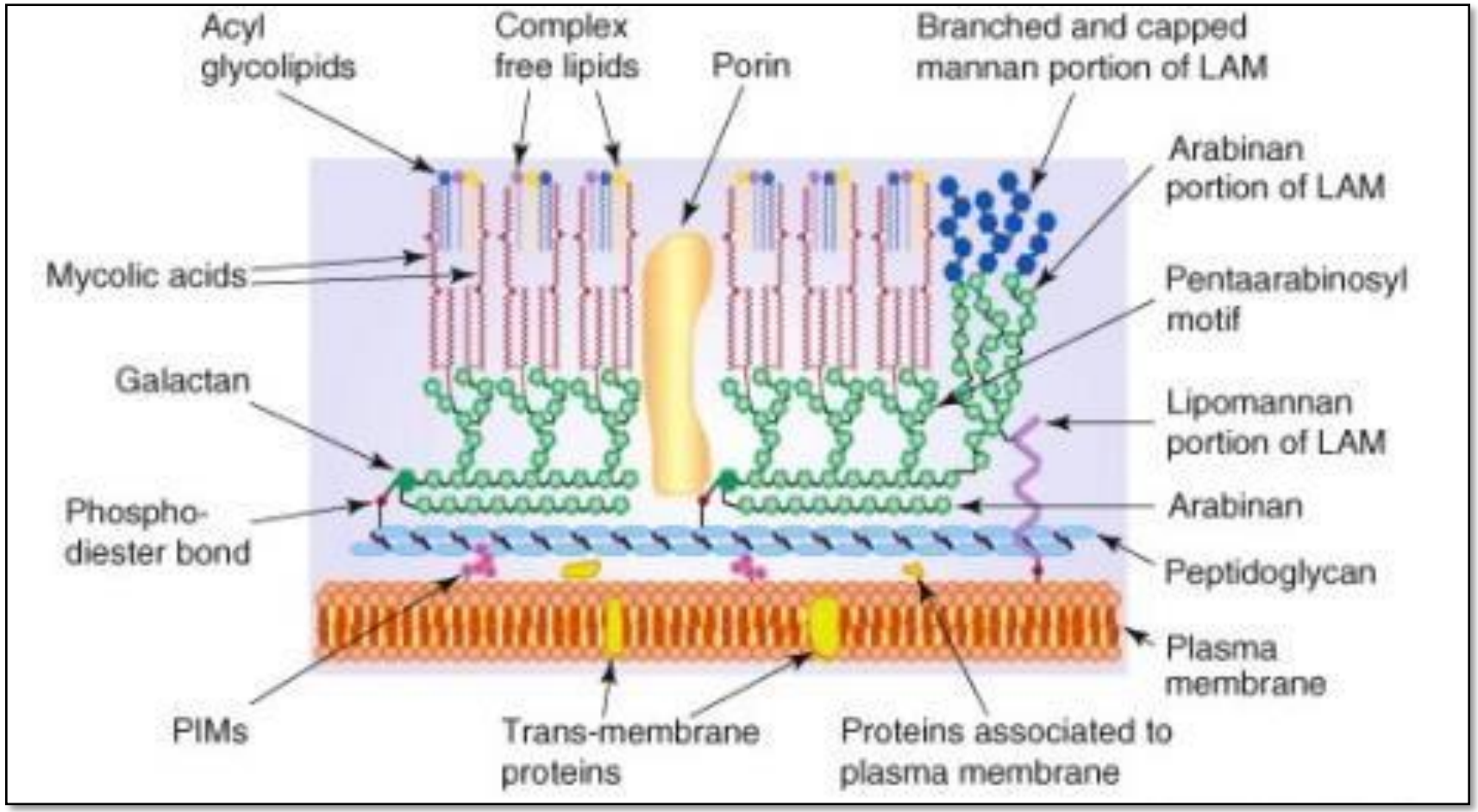


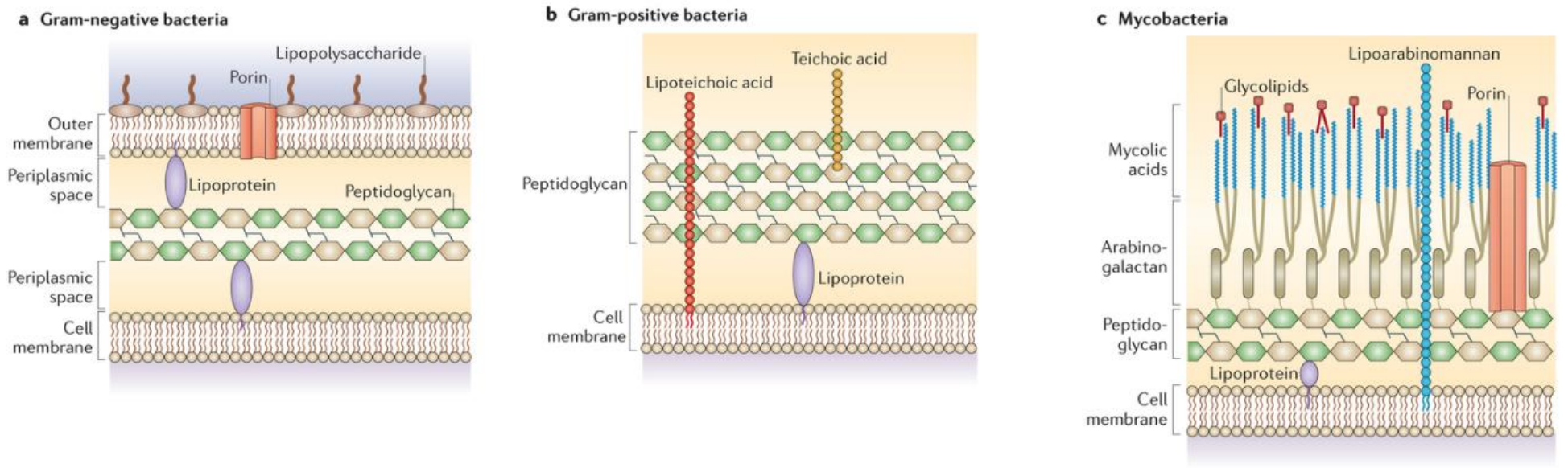
Figure 1-1: Structure of the cell wall of mycobacteria (Medjahed *et al.*, 2010)

Different species are characterised by variation in sugar substitutions in the glycolipids or peptidoglycolipids. Other significant cell wall components are trehalose dimycolate (cord factor), as it is thought to induce growth in serpentine cords on artificial medium, and mycobacterial sulfolipids, which are believed to play a role in virulence (Mendum *et al.*, 2015).

Mycobacteria are categorised as acid-fast organisms, signifying that they are resistant to decolourisation by acids during staining techniques and retain the carbol fuchsin dye when exposed to acid-ethanol.

The presence of porins protects the cell against harmful extracellular composites by allowing the passage of compounds and hydrophilic antibiotics where they can reach the cytoplasm and activate the expression of drug resistance genes (Trias *et al.*, 1992).

The permeability barrier of cell walls of mycobacteria was found to be between 10 – 100 fold lower than that of *Pseudomonas aeruginosa* with the chemical nature of the cell wall being unlike both Gram-negative and Gram-positive bacteria (Figure 1-2).



Gram negative **Gram positive** **Mycobacteria**

Figure 1-2: Typical bacterial cell walls showings the cell wall of Gram-negative bacteria (a) consists of a thin layer of peptidoglycan between the inner and outer lipid membranes. The outer membrane contains lipopolysaccharides and facilitates transport through channels such as porins. Gram-positive bacteria (b) have a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan and lipoteichoic acid, anchored to the cell membrane by diacylglycerol. Cell walls of mycobacteria (c) entail thin layers of peptidoglycan and arabinogalactan, and a thick layer of mycolic acids with glycolipids and porins also found in the cell walls, as well as lipoarabinomannan, which is anchored to the cell membrane by diacylglycerol (Brown *et al.*, 2015).

1.4 Pathogenicity of non-tuberculous mycobacteria and risk factors for infection.

NTM are ubiquitous environmental organisms found in soil, dust particles, biofilms and water. The virulence of NTM varies by species, but is usually low in immunocompetent individuals. As exposure to these organisms is widespread and disease is infrequent, it can be established that host defences are normally adequate to impede infection. The infective dose for NTM infection is unknown, however it follows that individuals that develop disease are thought to have abnormal susceptibility, intense exposure or immune defects that permit infection with NTM (Sexton and Harrison, 2008; Al-Anazi *et al.*, 2014).

Due to their profound immune suppression, individuals with HIV infection are frequently identified as having increased morbidity due to NTM (Corti and Palmero, 2008), and a CD4+ T cell count of less than 50 cells/μL is associated with increased risk of disseminated NTM disease (Mirsaeidi *et al.*, 2014). However the prevalence of NTM infections is also increasing in non-HIV patients, for example in patients on tumour necrosis factor α (TNF-α) pathway blockers (Winthrop *et al.*, 2009), individuals with bronchiectasis (Griffith and Aksamit, 2012) or chronic obstructive pulmonary disease (Huang *et al.*, 2012). Patients with genetic syndromes relating to mutations in the interleukin-12 or interferon γ pathways are also at risk for developing opportunistic infections such as those caused by NTM and the same applies to those with immunodeficiency syndromes (Sexton and Harrison, 2008). Additionally, female non-smokers aged between 50 and 80 with a lean body and some with characteristic features such as scoliosis, pectus excavatum, or mitral valve prolapse are more susceptible to pulmonary NTM compared to the rest of the population (Chan and Iseman, 2010). The body morphotype itself may be an

influential characteristic through features such as poor tracheobronchial secretion drainage or ineffective mucociliary clearance (Griffith *et al.*, 2007). Cystic fibrosis (CF) has also been progressively associated with an increased prevalence of NTM infection (Olivier *et al.*, 2003).

Typically the cause of morbidity and mortality is through slowly progressive and chronic lung disease, however NTM have the potential to infect any organ in the human body and have frequently been isolated from skin and soft tissue, bone, joint, septic arthritis and central nervous system disease. Disseminated disease generally occurs in patients who are immunocompromised, and lymphadenitis can occur in otherwise healthy young children (Mirsaeidi *et al.*, 2014).

The most common NTM species associated with pulmonary infection are members of MAC. *Mycobacterium kansasii*, another slow-growing NTM, is the second most common cause of pulmonary infection, followed by members of the rapidly-growing MABSC (Johnson and Odell, 2014).

1.5 Association of non-tuberculous mycobacteria with cystic fibrosis

Interest is now being paid to pulmonary disease in CF patients caused by NTM. Although the occurrence of NTM disease in the general population is around 1 in 100,000, there is a 10,000-fold greater prevalence within patients with CF. It is becoming progressively difficult to recognise and treat (Wentworth *et al.*, 2013) with overall prevalence of NTM in CF sputum varying between 6% to 13% (Olivier *et al.*, 2003; Martiniano *et al.*, 2016). The predominant species of NTM within the CF population in the UK are rapid growers within the MABSC (62% of NTM cases in adults, 68% of NTM cases in children) followed by slow-growing MAC (28% of NTM cases in adults, 27% of NTM cases in children) (Seddon *et al.*, 2013). Other species (*M. gordonae, M. kansasii, M. xenopi, M. fortuitum, M. simiae, M. malmoense, M.*

mucogenicum, M. perigrinum) together make up 8% of the total. In the US, major species are MAC 72% and MABSC 16% (Olivier *et al.*, 2003; Seddon *et al.*, 2013). This was consistent with similar studies completed in France (Roux *et al.*, 2009). A study in Israel had high rates of NTM with 22.6% isolation among patients with CF. Both MAC (14.3% of patients tested) and MABSC (31% of patients tested) were frequent; however the principal species was *M. simiae*, with 40.5% of all CF patients tested being infected with this species (Levy *et al.*, 2008). Intriguingly, of the two predominant species, MAC was rarely seen in patients younger than fifteen years, whereas MABSC was isolated from all ages (Pierre-Audigier *et al.*, 2005).

Before 1990, NTM were not predominantly linked to CF, with only 16 cases reported (Brown, 2010). Since then, NTM have been progressively isolated from the sputum of CF patients and are presenting increasingly complex diagnostic concerns. This could be for a variety of reasons, for example, the rising lifespan of CF patients, recent improvements in culture techniques and increased testing frequency, actively probing for NTM, transmission *via* contaminated water supplies, biofilms in showerheads (Feazel *et al.*, 2009), and hosts becoming more predisposed (Olivier *et al.*, 1996). Immune impairment due to chronic azithromycin therapy has recently been associated with an increase in the development of NTM infection. For long-term azithromycin therapy, it was demonstrated in primary macrophages that concentrations of azithromycin blocked autophagosome clearance by inhibiting lysosomal acidification, thus impairing autophagic and phagosomal degradation in mice infection models. As a consequence azithromycin treatment repressed intracellular killing of mycobacteria within macrophages consequently resulting in chronic infection with NTM (Renna *et al.*, 2011). Conflicting reports however state that patients with NTM on azithromycin treatment were not more likely to develop active NTM disease than those not taking azithromycin, and were also not less likely

to clear NTM upon treatment (Martiniano *et al.*, 2014). Recent data exists that NTM may also be spread by person to person transmission (Bryant *et al.*, 2013) however conflicting reports suggests there is no evidence for this (Harris *et al.*, 2015).

1.6 Cystic fibrosis

Cystic fibrosis is a complex genetic disease first described in 1938 by Dorothy Andersen, a pathologist at The Babies and Children's Hospital of Columbia University in New York (Andersen, 1938). However the gene responsible for CF was not identified and cloned until over half a century later in 1989 (Kerem *et al.*, 1989). CF is an autosomal-recessive disorder inherited in a typical Mendelian manner and triggered by molecular defects in the CF transmembrane conductance regulator (CFTR) gene (Cutting, 2015). The CFTR gene is located on the long arm of chromosome 7 at position q31.2, and encodes a 1480 residue multidomain protein for an ion channel responsible for the regulation and transportation of chloride (Gadsby *et al.*, 2006), bicarbonate (Chan *et al.*, 2006) and glutathione (Kogan *et al.*, 2003) across epithelial cell membranes.

The CFTR protein is a member of the ATP-binding cassette (ABC) transporter superfamily (Lewis *et al.*, 2004), controlled by cyclic adenosine monophosphate (cAMP), and is essential for the regulation of cell surface salt-water homeostasis, and the natural functioning of epithelia lining the intestinal tract and airways, as well as the salivary, sweat gland and pancreatic ducts. Defects in this gene, such as impaired protein folding, chloride channel gating or translation, subsequently result in a decreased permeability for chloride ions across the epithelial membrane. This in turn is responsible for the characteristic build-up of a thick sticky mucus accumulating in the airways, intestines and pancreas resulting in compromised mucociliary clearance, a predisposition to chronic infection, inflammation and

destruction or loss of tissues (Figure 1-3). This provides an ideal environment for harbouring frequent and often severe bacterial infections. The accumulative effect of chronic infection and inflammation over time will erode the function of the lungs, and this is frequently observed in patients with CF.

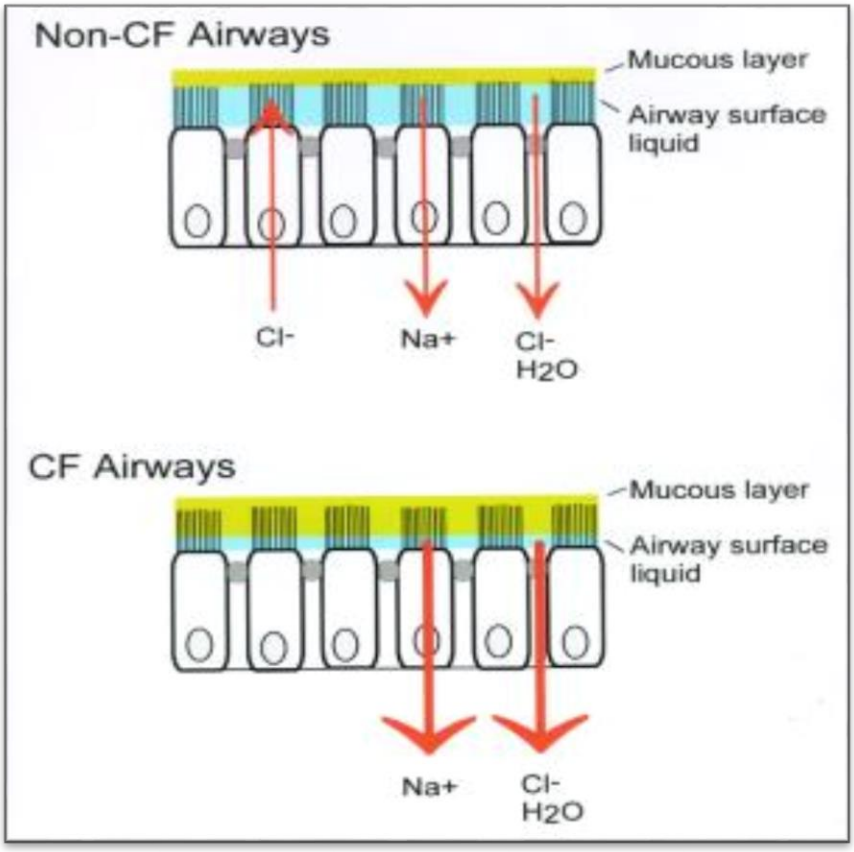


Figure 1-3: Transport in CF versus non-CF airways. In the CF airways due to defective or absent CFTR, mucociliary clearance is not able to occur (Kunzelmann, 2013)

1.6.1 Inheritance of cystic fibrosis

To date there are almost 2000 known CFTR defects (Cystic Fibrosis Mutation Database, 2011), and depending on their effect at the protein level, they are grouped into six classes (Kreindler, 2010; Boyle and De Boeck, 2013). Under normal circumstances, the CFTR gene is transcribed into mRNA, transferred to the

endoplasmic reticulum and in combination with tRNA will be translated into a fully functioning CFTR protein, which is subsequently folded, matured and transferred to the Golgi apparatus for post-translational modification and packaging into transport vesicles. Lastly, the channel is transported to the cell surface for final expression on the apical membrane of epithelial cells. Mutations in the *CFTR* gene can impact each step in protein synthesis: from gene transcription, through protein translation, folding and trafficking, to expression and gating of the channel on the cell surface (Tsui and Dorfman, 2013).

The most frequent mutation, although the frequency does vary among ethnic groups, occurring in around 70% of CF chromosomes is known as CFTRΔF508. This involves a deletion of phenylalanine at position 508 producing an abnormal CFTR protein (Villella *et al.*, 2013). As a result of this, the protein will be misfolded and subsequently retained within the endoplasmic reticulum failing to reach the cell membrane and function as a chloride channel in the apical membrane of epithelial cells.

As CF is a recessive disorder, two copies of the defective gene must be inherited, however patients can display severe or mild disease, depending on which clinical genotype they possess and whether it is homozygous or heterozygous. Many individuals possess only one copy of the defective gene and are unaware they are carriers of CF (see Figure 1-4). Reports suggest that in the UK one person in every 25 will carry a mutated CF gene, and in the US, one in 31 will unknowingly carry a mutated gene (Cystic Fibrosis Foundation, 2015; Cystic Fibrosis Trust, 2015). Prevalence of CF worldwide differs and is said to be underdiagnosed in Asia as existing evidence suggests it is rare. In the US the incidence is reported to be one in every 3500 births, and in Europe one in every 2000-3000 births (World Health Organization, 2013).

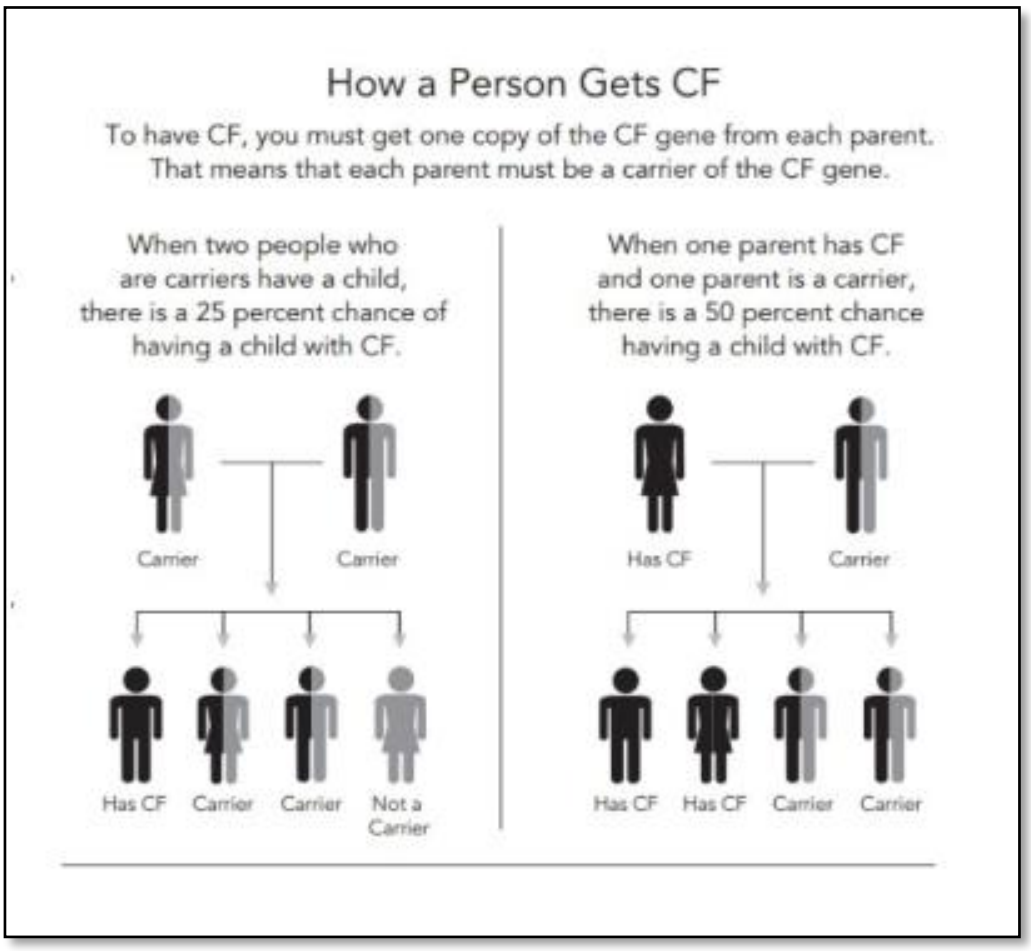


Figure 1-4: Ways in which an individual can be affected with cystic fibrosis (Cystic Fibrosis Foundation, 2015)

1.6.2 Symptoms of cystic fibrosis

The severity of symptoms can vary between individuals, and also fluctuates over time, however characteristic CF symptoms can manifest very early in life, some within days of birth and include salty tasting skin, meconium ileus, wheezing and failure to thrive (Farrell *et al.*, 2008). Patients may have recurrent or chronic lower respiratory tract infections, with approximately one third of infants aged three months being positive for *Staphylococcus aureus* infection rising to 47% by age 17 months alongside *Haemophilus influenzae* 15%, and *Pseudomonas aeruginosa*

13% (Metersky, 2012). Most patients with CF will be infected with *P. aeruginosa* by their late teens. This bacterium has the ability to form biofilms on damaged respiratory epithelium tissue, therefore establishing chronic infection and antibiotic resistance associated with early degeneration in lung function and a rise in mortality (Hurley *et al.*, 2014). Allergic bronchopulmonary aspergillosis (ABPA) is the most frequent fungal infection amongst CF patients (Smyth and Elborn, 2008). Other CF symptoms include stomach pain, nausea, weight loss, increased coughing, shortness of breath, pancreatitis, excess sputum, infertility and loss of appetite. Most patients with CF will die of respiratory failure with the majority of symptoms instigated by chronic and intermittent bacterial infections (FitzSimmons, 1994).

1.6.3 Lung disease in patients with cystic fibrosis

Patients with CF have a median survival of 40 years (Dodge *et al.*, 2006; Hardy *et al.*, 2015) and lung disease is the leading cause of morbidity and mortality. CF patients are predisposed to chronic pulmonary infections, with the lungs frequently colonised and/or infected with multiple species of bacteria concurrently due to defects within the transport of epithelial Na^+, Cl^-, $HCO3^-$ and unbalanced fluid secretions. It is these defects, which underlie the majority of clinical manifestations observed in CF.

CF patients enter a typically described “vicious cycle” of compromised mucociliary clearance and mucus retention, followed by infection and inflammation subsequently leading to tissue damage (Chmiel and Davis, 2003). This tissue damage, lack of fluid secretion and extreme electrolyte absorption, cause excess viscous and dense mucus to accumulate within the respiratory and gastrointestinal tracts, blocking the airways and preventing mucociliary clearance (Figure 1-5).

Consequently, this provides an ideal environment to promote bacterial growth whilst trapping and enabling inhaled pathogens to accumulate.

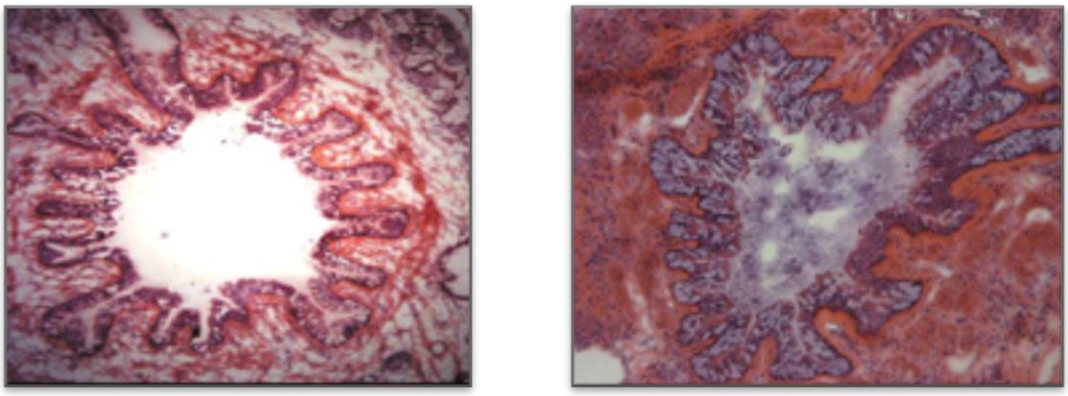


Figure 1-5: Normal airway with lumen free of secretions (left). In contrast, airway obstructed and distended by mucus secretions (right) (Quinton, 2010)

1.6.4 Common pathogens involved in lung disease of patients with cystic fibrosis

During childhood, the first bacterial pathogens to be detected are usually *S. aureus* and *H. influenza.* These pathogens are eventually superseded in adolescence and adulthood where the dominant pathogen is *P. aeruginosa*, and less frequently *Burkholderia cepacia* complex, *Achromobacter xylosoxidans, Aspergillus fumigatus* and NTM - in particular members of the MABSC (Coutinho *et al.*, 2008).

1.7 *Mycobacterium abscessus* complex

MABSC is a highly antibiotic-resistant complex of organisms within the *Mycobacterium* genus (Leung and Olivier, 2013) and increasingly acknowledged as a significant cause of lung infection in patients with CF. Infection with MABSC, predominantly *M. abscessus* subsp*. abscessus*, is associated with poor clinical outcome (Esther *et al.*, 2005) particularly following lung transplantation, with many CF centres eliminating patients infected with MABSC from undergoing transplant

(Gilljam *et al.*, 2010; Watkins and Lemonovich, 2012). Elevated resistance is attributed to a combination of the permeability barrier of the intricate multilayer cell membrane, drug export systems, enzymes that neutralize antibiotics in the cytoplasm and antibiotic targets with low affinity (Nessar *et al.*, 2012).

The genome of *M. abscessus* (CIP 104536T), as shown in Figure 1-6, consists of a 5,067,172 base pair circular chromosome including 4920 predicted coding sequences, an 81kb full length prophage, five insertion sequences and a 23kb mercury resistance plasmid (Ripoll *et al.*, 2009).

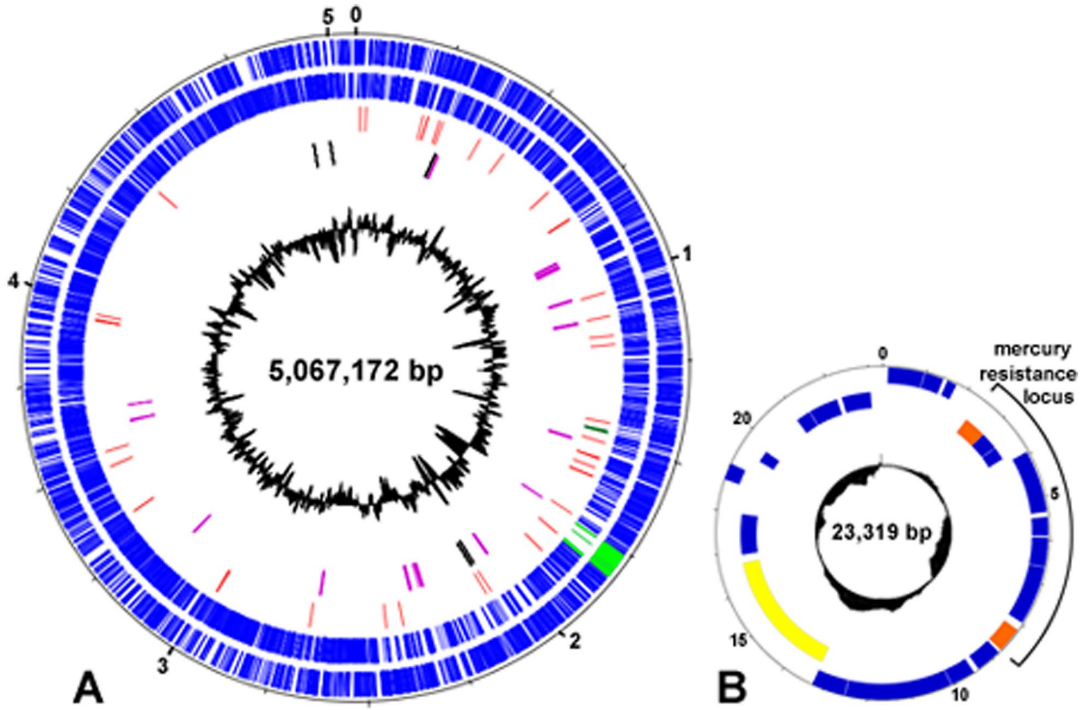


Figure 1-6: The *M. abscessus* CIP 104536T genome.

Figure 1-6 scale is in Mb with the outer blue circles displaying forward and reverse genes, light green phage genes and third circle tRNA genes (red) and rRNA operon (dark green). The fourth circle shows genes acquired from non-mycobacterial organisms, by horizontal gene transfer (purple) and insertion sequence elements (black). The inner black histogram shows the G + C content. The 23kb mercury resistance plasmid, (scale in kb) indicates the forward and reverse genes and the G+C content with the same code as the chromosome map. The plasmid carries a mercury resistance operon flanked by two genes encoding site-specific recombinases (MAB_p04c and MAB_p10, orange); it also encodes a relaxase/helicase that may function in conjugation or mobilization (MAB_p15c, yellow) (Ripoll *et al.*, 2009).

Members of the MABSC are rapidly-growing non-motile, Gram-positive, acid-fast rods of around 0.5 µm in width and 1 - 2.5 µm in length, and can grow on solid agar as either smooth non-cording biofilm-forming colonies, or rough cording, non-biofilm forming colonies as shown in Figure 1-7 (Roux *et al.*, 2016). The rough morphotype is associated with more severe and unrelenting infection and has been shown to persist for many years in an infected host. Glycopeptidolipids are accountable for the formation of smooth colonies and contribute to colonisation of NTMs in the environment through biofilm formation, whereas the loss of these glycopeptidolipids is associated with the formation of rough colonies and expedites survival in macrophages (Jonsson *et al.*, 2007). The rough colony types can evade internalisation by forming cords which compared to the size of most phagocytic cells are particularly large, therefore difficult for macrophages and neutrophils to surround and internalise (Bernut *et al.*, 2014). Strains are also reported to undergo frequent transition between smooth and rough morphotypes during the course of infection, however recent evidence suggests clearly distinct genetic lesions are responsible for the loss of production and transport of glycopeptidolipids making frequent switching between morphotypes questionable (Pawlik *et al.*, 2013).

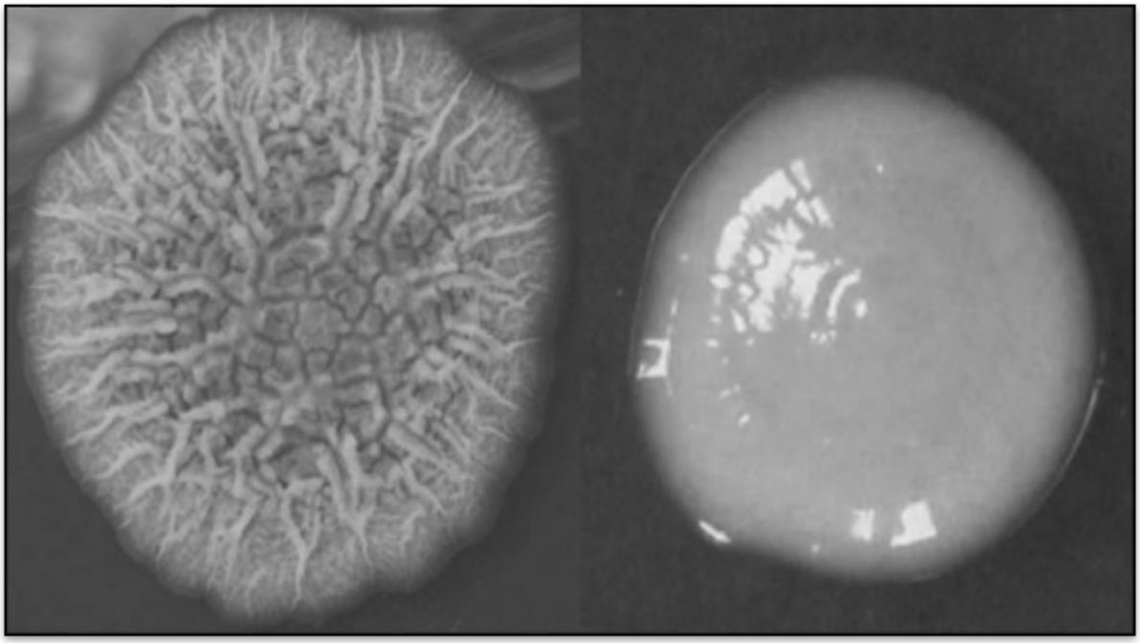


Figure 1-7: Growth characteristics of rough (left) and smooth (right*) M. abscessus* subsp*. abscessus* cultured on Middlebrook 7H11 agar. Image taken from (Ruger *et al.*, 2014)

Since *M. abscessus* was first described in 1953 by Moore and Frerichs after a case report of a human knee infection with deep subcutaneous abscess-like lesions (Moore and Frerichs, 1953), it has undergone numerous changes in its taxonomic status owing to indistinguishable results in discrimination. Prior to 1992, *M. abscessus* and *M. chelonae* were thought to be the same organism or subspecies within the *M. chelonae-abscessus* group (Kusunoki and Ezaki, 1992), and *M. abscessus* has also been grouped within the *M. fortuitum* complex (Kubica *et al.*, 1972).

A further two emerging species of mycobacteria closely related to *M. abscessus* have been discovered In the last decade; *M. abscessus* subsp*. massiliense* (Adekambi and Drancourt, 2004) and *M. abscessus* subsp*. bolletii* (Adekambi *et al.*, 2006). Collectively these three species are frequently referred to as the MABSC,

however, the taxonomy of these organisms, which cannot be distinguished by phenotypic techniques, has initiated some dispute in the literature.

All three species have identical 16S rRNA gene sequences; therefore, discrimination has proved challenging. Blauwendraat *et al* (2012) described a two-gene sequencing approach using PCR and the sequencing of housekeeping gene targets *hsp65* and *rpoB*, which claimed to accurately distinguish the three species (Blauwendraat *et al.*, 2012), however several others have reported findings that partial sequencing of *rpoB*, *hsp65*, and *secA* provided ambiguous results, suggesting members of the *M. abscessus* group undergo genetic exchange (Zelazny *et al.*, 2009). Reports have suggested that the three species are too strongly correlated to be considered as separate species (Macheras *et al.*, 2009), and Leao *et al.* combined *M. bolletii* and *M. massiliense* into one subspecies, named *M. abscessus* subspecies *bolletii* comb. nov and recognised a second subspecies named *M. abscessus* subspecies *abscessus* (Leao *et al*., 2011). Conflicting reports from higher resolution typing including whole genome sequencing recently supported the suggestion that the species should be divided into three subspecies; *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *bolletii* and *M. abscessus* subsp. *Massiliense* (Harris and Kenna, 2014; Nakanaga *et al.*, 2014; Tortoli *et al.*, 2016). Despite consensus not being reached to date, the three species are known to have differing antibiotic resistance phenotypes and genotypes (Kim *et al.*, 2010b), therefore strain typing is advantageous for patients with CF who have MABSC infection to receive the correct course of treatment. The use of comparative rRNA sequencing is frequently done to infer natural relationships between microorganisms (Woese and Fox, 1977). Figure 1-8 shows a phylogenetic tree of 29 mycobacterium species, clearly defining the separation of slow growers from rapid growers and also demonstrates the closeness of the three MABSC species.

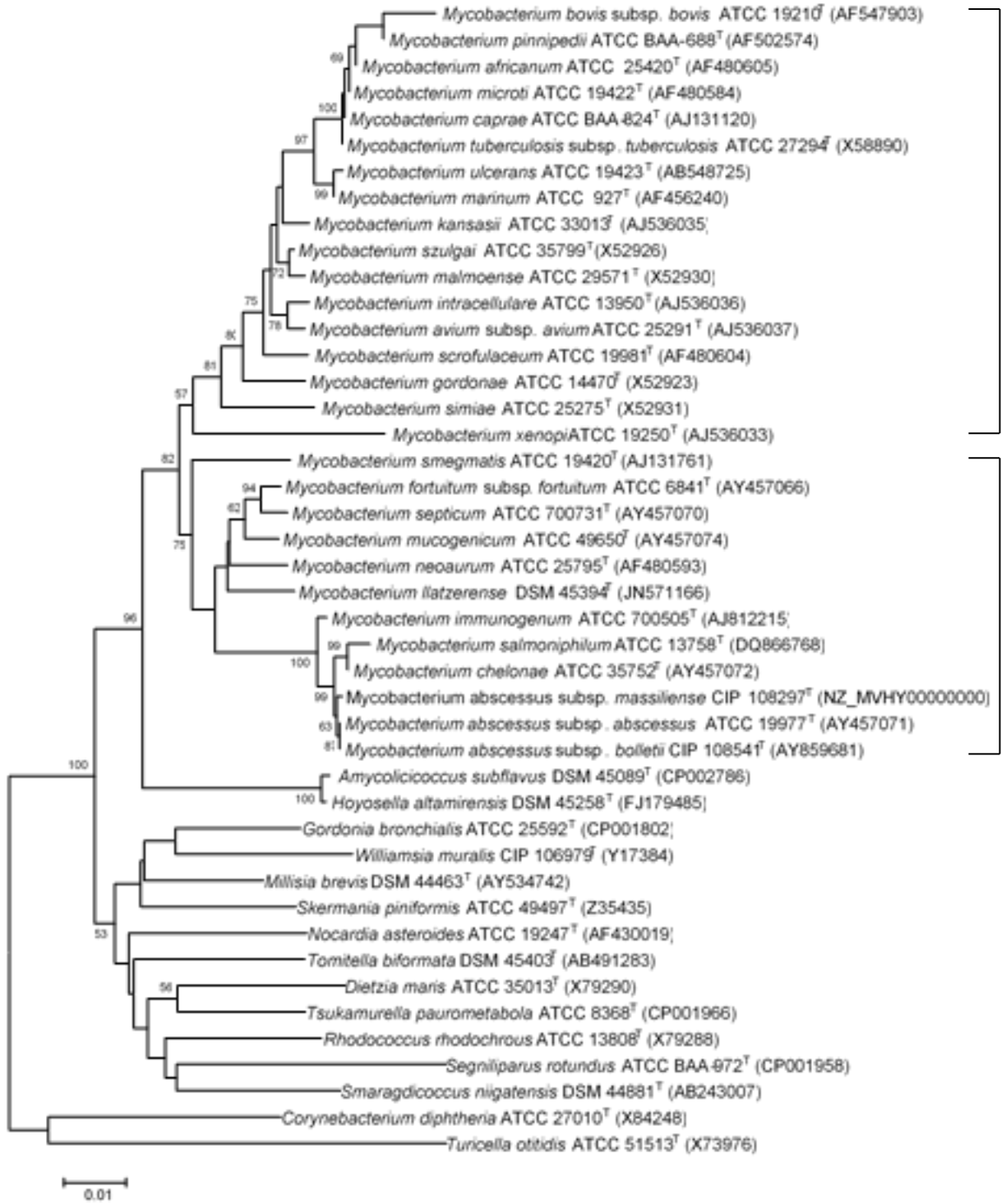


Figure 1-8: Neighbour-joining tree (Saitou and Nei, 1987) based on almost complete 16S rRNA gene sequences showing the positions of 29 members of the genus *Mycobacterium* discussed within this thesis, with representatives of the order *Corynebacteriales*. The numbers at the nodes indicate the levels of bootstrap support based on a neighbour-joining analysis of 1,000 resampled datasets; only values above 50 % are given. The scale bar indicates 0.01 substitutions per nucleotide position. Tree was produced using MEGA7 program (MEGA, 2017).

1.8 Host immune response to mycobacterial infection

Very little is known about the host response to NTM (Chan *et al.*, 2010) and despite the fact that they are ubiquitous in the environment only an small proportion of individuals exposed to NTM develop lung disease. Identifying these individuals with conditions that predispose them to NTM infection can provide an insight into what host immune factors are essential in protection against NTM.

As chronic respiratory infections due to NTM, particularly MABSC are increasingly seen in patients with CF this leads to the hypothesis that these patients may have an inherent susceptibility to NTM, or it could be that persistent bacterial infection is able to provide a suitable environment for colonisation by environmental mycobacteria.

CF is also known to cause a functional defect in antimicrobial peptides known as β-defensins found in epithelial cells. These protect against Gram-positive and Gram-negative bacteria, fungi and certain viruses by interacting with the membrane of invading microorganisms (Dalcin and Ulanova, 2013). The surface fluid of normal airways is low in salt, which favours the activity of defensins, however CF fluid has been described to be very high in salt due to dysfunction of CFTR protein that forms chloride ion channels. This high salt environment may inhibit defensin activity and compromise host respiratory defence, therefore patients with CF may have innate susceptibility to NTM infection (Chan *et al.*, 2010).

1.9 Diagnosing non-tuberculous mycobacteria in patients with cystic fibrosis

Identifying patients with NTM is challenging, with conventional culture and acid-fast microscopy continuing to be the mainstay of mycobacterial diagnostics. Current guidelines (Floto *et al.*, 2016) suggest that for any patient suspected of having NTM

lung disease, the following evaluation must be completed: (1) chest radiograph or, in the absence of cavitation, chest high resolution computed tomography (HRCT) (2) three or more sputum samples showing the presence of acid-fast bacilli (AFB) and (3) exclusion of other disorders, for example TB. All are equally significant and must be met. The following criteria have been established for symptomatic patients with radiographic opacities, nodular or cavitatary or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules (Floto *et al.*, 2016). The criteria best fit with MAC, MABSC and *M. kansasii*, as there are still too many ambiguities with regard to other NTM to ascertain whether these criteria are unanimously appropriate for all NTM respiratory pathogens.

Clinical:

(i) Pulmonary symptoms, nodular or cavitary opacities on chest radiograph or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
(ii) Appropriate exclusion of other diagnoses

Microbiological:

(i) At least two separate expectorated sputum samples with positive results OR
(ii) A positive culture result obtained from a bronchial lavage or wash OR
(iii) Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

Patients who do not meet the diagnostic criteria but are suspected of having NTM lung disease should be regularly monitored until a positive diagnosis is definitively documented or can be excluded (Griffith *et al.*, 2007).

1.9.1 Collection of samples from patients with cystic fibrosis

There are numerous methods that can be used for the collection of respiratory samples from patients with CF. The traditionally preferred method being the collection of sputum; however, in CF patients who do not expectorate, samples can be obtained preferably by cough swab, as this is less invasive than other methods. Cough swabs however can contain higher levels of oral flora and are less sensitive for detection of mycobacteria compared to culture of sputum. Bronchoalveolar lavage, cough plate, oropharyngeal culture, laryngeal or naso-pharyngeal aspirate can also be used. Current guidelines (Floto *et al.*, 2016) recommend against the use of oropharyngeal swabs as they are said to poorly reflect lung microbiota due to insufficient material for culture, and although culture of bronchoalveolar lavage gives an accurate representation of microorganisms present, it is a method that should be avoided in children as general anaesthesia is often required (Burns and Rolain, 2013). Transbronchial biopsies are to be circumvented, as there is a substantial risk of bleeding and pneumothorax. Updated evidence-based draft guidelines on the Management of Non-tuberculous Mycobacteria in individuals with Cystic Fibrosis have recently been published recommending that preference should be given to sputum, induced sputum, bronchial washings or bronchial lavage samples (Floto *et al.*, 2016).

1.9.2 Current detection methods of non-tuberculous mycobacteria in patients with cystic fibrosis

Although classed as “rapidly-growing” mycobacteria (RGM), the ‘rapidity’ of the growth rate is only relative to that of other mycobacteria, and they show slower growth when compared to most other CF pathogens (Esther *et al.*, 2011).

Conventionally, a time to detection cut-off of seven days has been used to differentiate rapidly-growing and slow-growing mycobacteria on solid media, however growth detection can fluctuate depending on the number of organisms in the sample and detection times become shorter as the mycobacterial burden rises. Other factors affecting this can include the type of medium used and the age of the sample. In comparison to RGM, *M. tuberculosis* has a TTD of around six to eight weeks. Liquid culture has a more rapid TTD, with a cut off between RGM and SGM of six days, however the rate of contamination of non-mycobacterial species is greater (Chihota *et al.*, 2010).

Overgrowth of cultures by a variety of other faster growing microorganisms, particularly *P. aeruginosa*, is commonly observed in patients with CF, (Bange *et al.*, 1999). Due to this overgrowth, laboratory detection and identification of NTM pose a major challenge, and mycobacterial infections can often be misdiagnosed, or concealed and completely overlooked in routine culture.

To decrease the commensal flora associated with the airways and digestive tract and consequently reduce overgrowth of cultures, samples are decontaminated prior to inoculating them on selective media (See Chapter 2 section 1.9.5 for information on current media used for isolation of NTM).

1.9.3 Decontamination steps performed upon receipt of a patient sample

Decontamination methods take advantage of the fact that mycobacteria demonstrate relative resistance to acids, bases and other antimicrobial agents, compared with non-acid-fast bacteria. *P. aeruginosa* characteristically survives routine sputum decontamination using *N*-acetyl-L-cysteine–2% sodium hydroxide (NALC-NaOH), therefore to ensure eradication, the most universally used laboratory

decontamination process is NALC-NaOH-OxA using 5% oxalic acid (De Bel *et al.*, 2013).

Following a study by Whitter *et al* (1997), it was shown that NALC-NaOH treatment followed by oxalic acid significantly reduced overgrowth by *P. aeruginosa*. This method was further evaluated and results confirmed that although this two-step NALC-NaOH-OxA process improved the yield of mycobacteria as well as reducing other microorganisms, it can also affect the viability of mycobacteria (Whittier *et al.*, 1997).

Bange *et al*., (1999) reported that treatment with oxalic acid may lead to false negatives being produced, particularly from specimens with low inocula of mycobacteria. Based on this, they reported that samples should be initially decontaminated with NALC-NaOH, and then inoculated for culture. Only those that still contained heavy growth of other species should then be subjected to a second round of decontamination with oxalic acid. This is the strategy currently recommended in the newly published guidelines produced by the US and European Cystic Fibrosis Societies (Floto *et al.*, 2016)

A comparison of four decontamination processes for stool samples from patients known to have *M. avium* was carried out by Yajko *et al*., (1993). These were NALC-NaOH, cetylpyridinium chloride-sodium chloride (CPC-NaCl), NALC-NaOH-OxA and benzalkonium chloride-trisodium phosphate (BC-TSP). Results demonstrated that a higher yield of colony forming units (CFU) of *M. avium* was obtained following oxalic acid treatment, whereas NALC-NaOH produced around half the number of CFU per millilitre when compared with treatment by oxalic acid. CPC-NaCl and BC-TSP had the lowest rates of survival of non-mycobacterial species, but also yielded the lowest number of CFU of *M. avium.* These results show that although the process of decontamination is essential for the recovery of mycobacteria using

traditional culture methods, a fine sense of balance must be sought as the process of decontamination can still influence the viability of mycobacteria, as well as reducing the growth of non-acid-fast bacteria (Yajko *et al.*, 1993).

The NALC-NaOH-OxA method was compared to a chlorhexidine-based method and it was found that although rates of growth of non-mycobacteria were higher with the chlorhexidine-based method, a higher yield of NTM was also recovered (Ferroni *et al.*, 2006). Although this study used sputum samples from patients with CF, it did not include clinical samples containing *M. abscessus*, a strain most frequently found in these patients so this may not provide an accurate representation. It was confirmed by De Bel *et al*. (2013) that the use of chlorhexidine did yield more mycobacteria than NALC-NaOH-Oxa, however if liquid culture is used as opposed to solid culture, the recovery of mycobacteria was consistent for both methods (De Bel *et al.*, 2013). A short study undertaken by the Health Protection Agency, Newcastle upon Tyne, UK (personal communication, March 15th 2013) over a 4-month period in 2012 exhibited the following rates of contamination of sputum samples in CF patients (See Table 1-2). The decontamination processes used in this study were 4% sodium hydroxide (NaOH) or 0.5N (N/2) sulphuric acid (H_2SO_4) and culture method used was BACTEC mycobacterial growth indicator tube (MGIT) 960 Mycobacterial Detection System with eight weeks incubation.

Current recommendations by the UK Cystic Fibrosis Trust are that either NALC-NaOH-OxA or chlorhexidine methods should be used in order to significantly reduce the viability of other microorganisms, particularly *P. aeruginosa* (The UK Cystic Fibrosis Trust Microbiology Laboratory Standards Working Group, 2010).

Table 1-2: Audit of respiratory samples from cystic fibrosis patients received by the Public Health England Laboratory, Newcastle upon Tyne showing contamination rates over a 4 month period in 2012

	SEPTEMBER	OCTOBER	NOVEMBER	DECEMBER
Number of samples received	39	41	49	33
Culture negative after 8 weeks incubation	27 (69.23%)	29 (70.73%)	29 (60%)	24 (72.7%)
Culture abandoned. Contamination after decontamination	10 (25.64%)	8 (19.51%)	16 (33%)	6 (18.2%)
Culture positive for mycobacteria	2 (5.13%)	4 (9.75%)	3 (6%)	3 (9.1%)

1.9.4 Staining of sputum smears for acid-fast bacilli (AFB)

Staining of sputum smears for AFB can be done, preferably using the fluorochrome method with auramine–phenol as opposed to staining by the Ziehl-Neelsen method as the former is reported to be more sensitive and can be advantageous in assessing the burden of organisms in the lungs (Cystic Fibrosis Trust, 2010).

The Ziehl-Neelsen stain is a low cost procedure but is burdensome and carries a higher risk to laboratory workers as it requires heat application during the carbol-fuchsin staining. Overheating may crack the slide, and also cause splashing of the stain. Slides are examined under oil immersion and acid-fast bacteria appear red/pink, and non-acid-fast bacteria as well as other organisms and cellular materials appear blue.

Fluorescent staining, utilises the same approach as Ziehl-Neelsen staining, but carbol fuchsin is replaced by a fluorescent dye (auramine-O, rhodamine). The advantage of the fluorescence method is that it is more sensitive and slides can be

observed at lower magnification therefore allowing the examination of a much larger area in a shorter time period (Annam *et al.*, 2009).

1.9.5 Current media used for isolation of non-tuberculous mycobacteria

The most frequently used solid-based culture media for the isolation of NTM are agar-based Middlebrook 7H10 and 7H11 media or egg-based Lowenstein–Jensen medium (Burns and Rolain, 2013). Culture on *Burkholderia cepacia* selective agar (BCSA) has been shown to be effective for isolation of NTM if incubation is prolonged from five to fourteen days and Esther *et al*., (2011) demonstrated that extended incubation of BCSA afforded an increased recovery rate of NTM from 0.7% to 2.8% using routine culture methods. However, not all NTM will grow on BCSA and overgrowth, particularly by fungi and Gram-negative bacteria, remains problematic (Esther *et al.*, 2011). Although some endorse this method, it is not specifically recommended in the CF guidelines for management of NTM (Floto *et al.*, 2016). *M. avium* complex cannot be recovered from BCSA, *Mycobacterium haemophilum* requires ferric ammonium citrate in order to grow and *Mycobacterium ulcerans* needs egg yolk supplement in the medium (Griffith *et al.*, 2007).

Other media that have been used for the isolation of mycobacteria include Kirchner medium (Sparham *et al.*, 1984), Stonebrink medium (Stonebrink *et al.*, 1969), Ogawa and modified Ogowa medium (Kalich *et al.*, 1976), however these are most suited to the culture of MTb, rather than NTM. It is recommended that samples should also be cultured in liquid broth medium as this can provide more rapid results as well as an increased yield of mycobacteria (Johnson and Odell, 2014).

The most commonly used liquid broth is the MGIT which contains modified Middlebrook 79H broth base, casein peptone, oleic albumin dextrose catalase (OADC) and PANTA antibiotic mixture comprising of polymyxin B, amphotericin B,

nalidixic acid, trimethoprim and azlocillin (Assawy *et al.*, 2014). A fluorescent compound embedded within the tube is sensitive to dissolved oxygen and when actively respiring bacteria consume the oxygen this allows the fluorescence to be visualised under ultra violet light and detected using sensitive instrumentation e.g. the BACTEC MGIT 960 Mycobacterial Detection System (Tortoli *et al.*, 1999).

BacT/ALERT 3D is an automated microbial detection system based on the colorimetric detection of CO_2 produced by growing microorganisms. The growth medium contains oleic acid, glycerol, bovine serum albumin, and amaranth in water, as well as amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin (Angeby *et al.*, 2003).

The use of solid media also has the added benefit that colony morphology can be observed, as well as growth rate, the option to perform antibiotic susceptibility tests and recognising if more than one species of NTM is present (Griffith *et al.*, 2007).

Detection of NTM is routinely achieved by culture of sputum onto both solid media, (e.g. Lowenstein–Jensen medium) and automated broth-based methods following decontamination of samples (Whittier *et al.*, 1993; Burns and Rolain, 2013).

1.9.6 Molecular detection methods for non-tuberculous mycobacteria

Although a typical diagnosis of NTM is achieved by direct analysis and culture, molecular based diagnostics are now becoming frequently used to diagnose disease, offering increased sensitivity and specificity (Lima *et al.*, 2013). Despite their availability they can however be costly, labour intensive, demand greater training requirements, and sample contamination could be responsible for producing false results. Sequencing of genes such as *rpoB*, 16S rRNA, and *hsp65* offer prompt and accurate identification although still have technical restrictions, and these

methods may not be readily available in many laboratories (Harris and Kenna, 2014).

Commercial line probe assays such as Genotype Mycobacterium CM (common mycobacteria) and Genotype Mycobacterium AS (additional species) (HAIN Lifescience, 2015), or INNO-LiPA MYCOBACTERIA v2 (Fujirebio, 2015) can accurately identify many of the frequently encountered NTM species. INNO-LiPA MYCOBACTERIA can identify sixteen different mycobacterial species targeting the 16S-23S rRNA internal transcribed spacer (ITS) region (Tortoli *et al.*, 2003). The GenoType Mycobacterium is centred on the reverse line probe hybridization assay, and utilises commercial DNA strip assays used for the detection and identification to the species level of mycobacteria isolated from positive liquid or solid cultures. It comprises two kits: the GenoType CM, for common mycobacteria and GenoType AS, for additional species assays. Genotype Mycobacterium CM (Figure 1-9) targets the 23S rRNA gene and is able to identify 24 NTM species and Genotype Mycobacterium AS (Figure 1-10), which also targets the 23S rRNA gene, can identify an additional 19 NTM species (Gitti *et al.*, 2006).

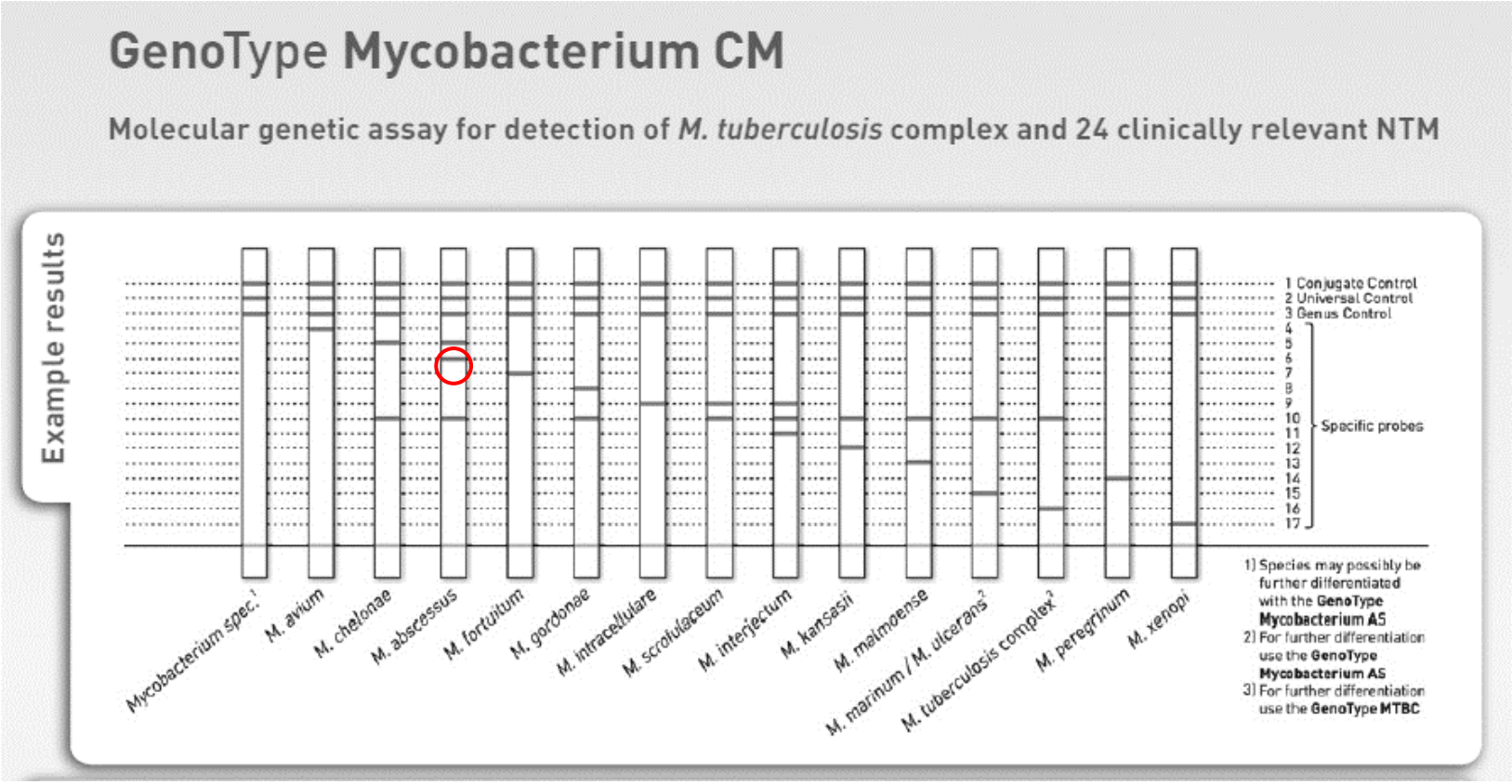


Figure 1-9: GenoType Mycobacterium CM permits the identification of the following mycobacterial species: *M. avium* subspecies, *M. chelonae*, *M. abscessus*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. scrofulaceum*, *M. interjectum*, *M. kansasii*, *M. malmoense*, *M. marinum/M. ulcerans*, *M. peregrinum*, *M. tuberculosis* complex, and *M. xenopi*.

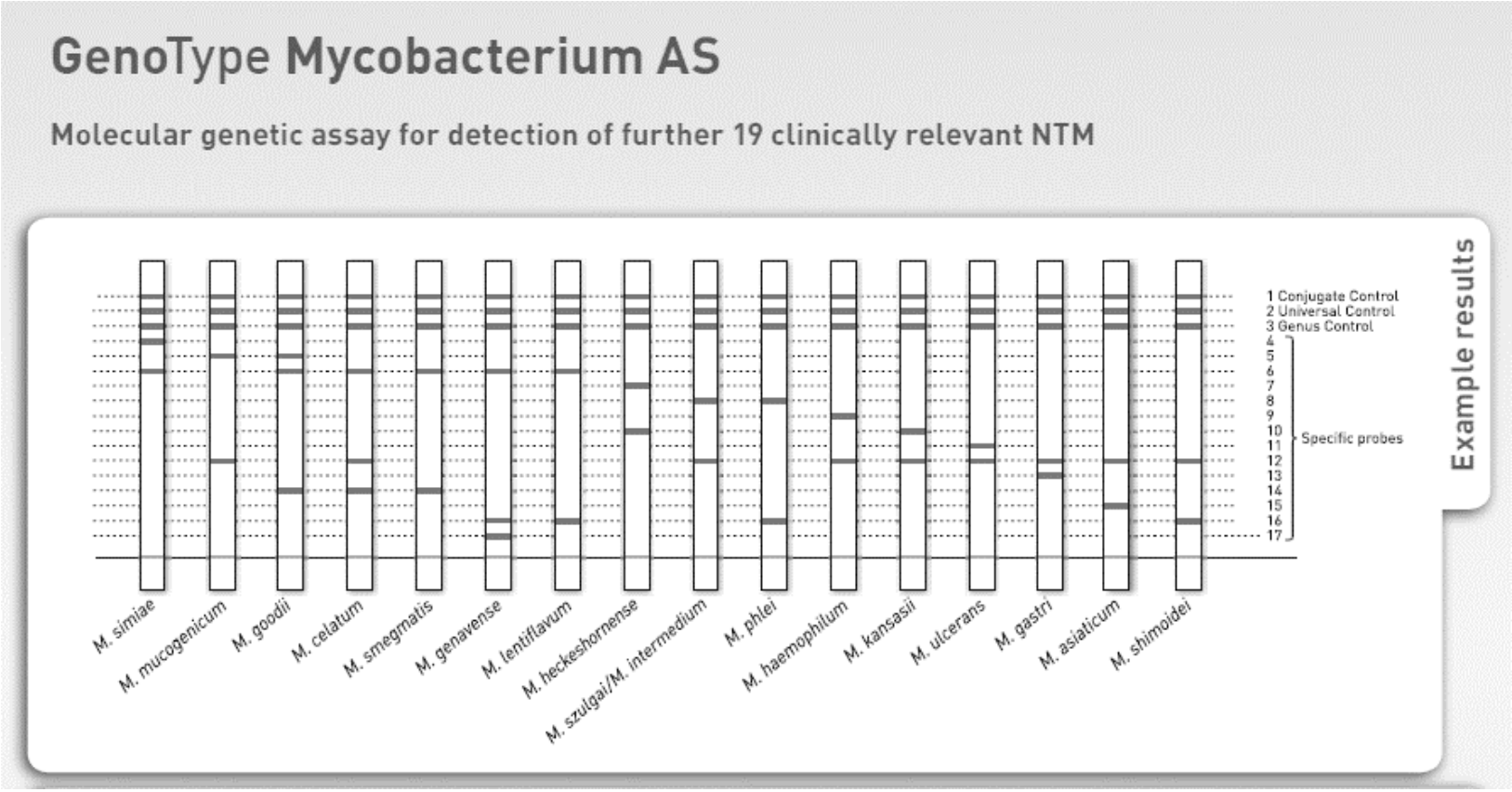


Figure 1-10: GenoType Mycobacterium AS provides probes for a series of additional NTM, namely, *M. simiae*, *M. mucogenicum*, *M. goodie*, *M. cellatum*, *M. smegmatis*, *M. genavense*, *M. lentiflavum*, *M. heckeshornense*, *M. szulgai*, *M. phlei*, *M. hemophilum*, *M. kansasii*, *M. ulcerans*, *M. gastri*, *M. asiaticum*, and *M. shimoidei*

The disadvantage of these kits is that the distinction between MABSC and *M. chelonae* can be questionable and is often a subjective judgement (Portaels *et al.*, 1996; Richter *et al.*, 2006; Arnold *et al.*, 2012). Genotype *Mycobacterium* CM kit has only one variance at position six between these two sub-species where *M. abscessus* has one extra band (as shown by circle on Figure 1-9), which on occasion can be very difficult to visualise, with often very faint and barely detectable bands subsequently giving a false identification of *M. chelonae*. Neither assay, CM or AM can discriminate between the three *M. abscessus* subspecies (Scarparo *et al.*, 2001). This lack of accuracy in identification is a cause for concern as it may influence patient management, in particular in making a judgement of whether to list patients for lung transplantation due to MABSC being linked to poorer clinical outcomes than those witnessed in patients infected with *M. chelonae*.

More accurate identification can be attained by performing PCR and sequencing of house-keeping gene targets such as *hsp65, sodA, rpoB* and *secA*, and whilst most isolates can be identified with the use of two target genes (Adekambi and Drancourt, 2004; Blauwendraat *et al.*, 2012), multilocus sequence analysis (MLSA) is required to differentiate between the three MABSC subspecies as often inconclusive results are attained where isolates have chimeric housekeeping gene sequences, likely as a result of recombination or horizontal gene transfer (Macheras *et al.*, 2011).

Many molecular methods for differentiation of MABSC isolates have been developed, including pulsed-field gel electrophoresis (Jonsson *et al.*, 2007), rep-PCR (Jamal *et al.*, 2014), multilocus/multispacer sequence typing (Macheras *et al.*, 2011) and variable number tandem repeat profiling (Harris *et al.*, 2012; Davidson *et al.*, 2013), however whole genome sequencing permits a much higher degree of resolution and improved typing than any other method (Kreutzfeldt *et al.*, 2013), and

can also provide valuable information about the transmission of MABSC (Bryant *et al.*, 2013). In many of these methods, the techniques used to lyse the mycobacterial cell wall can also fragment the genomic DNA therefore smearing can occur subsequently preventing identification.

To bypass the need for culture and afford a more rapid diagnosis, molecular methods can be used for the detection of NTM directly from clinical samples, however these methods also have limitations which can include difficulty in NTM cell lysis, and often the bacterial burden in NTM infection is of a low level in comparison to other CF pathogens (Caverly *et al.*, 2016).

In a recent study by Scoleri *et al.*, (2106), encouraging results were obtained from a quantitative PCR assay described to detect NTM directly from clinical samples based on the *hsp65* gene. However, restrictions to this are that DNA sequencing is not readily available or practical in many laboratories to identify PCR results and can be very costly and time consuming. The use of a single target can also give ambiguous results where chimeric housekeeping genes occur in some isolates (Harris and Kenna, 2014). In addition recently described NTM species where DNA sequence data is not yet available were unable to be identified (Scoleri *et al.*, 2016).

1.10 Treatment of non-tuberculous mycobacteria in patients with cystic fibrosis

The existence of NTM in the sputum of patients with CF may be indicative of transient contamination, colonisation or infection, which can pose a substantial diagnostic dilemma and not all patients will benefit from treatment for NTM. Although some patients with persistent NTM in their sputum have diminishing radiographic and clinical factors, this is not so for all patients and if treatment is required it should be customised according to the specific species of NTM isolated (Stout *et al.*, 2016).

The main threat posed by mycobacteria is predominantly due to their high resistance to antibiotics with *M. abscessus* subspecies *abscessus* recognised as one of the most resistant organisms to antibiotic therapies (Griffith, 2014). This resistance can be either natural or acquired, and where acquired, it is described as being via spontaneous mutation at targeted genes in the presence of antibiotics rather than through plasmid or transposon transmission (Nessar *et al.*, 2012).

Members of the MABSC are consistently resistant to typical anti-tuberculosis agents and due to varying *in vitro* drug susceptibilities, antibiotic susceptibility testing should be undertaken for all clinically significant isolates. NTM, in particular MABSC are notoriously difficult to treat, requiring a multi-drug regimen often extending up to twelve months and beyond (see Chapter 5) and treatment success is generally defined as having sustained culture conversion for at least twelve months (Griffith *et al.*, 2007).

1.10.1 Significance of a first positive non-tuberculous mycobacteria culture in patients with cystic fibrosis

Many patients will spontaneously clear their sputum after one single isolation of NTM, demonstrating that it is transiently present and has no clinical significance. Other patients may continue to have NTM-positive sputum samples, but require no treatment and do not progress to active NTM disease (Martiniano *et al.*, 2014).

1.11 *Mycobacterium abscessus* complex and lung transplantation

Lung disease caused by MABSC is often considered a relatively strong contraindication to lung transplantation with fatal post-transplant infection often being reported (Gilljam *et al.*, 2010). There are however more recent reports of positive transplantation outcomes for patients infected with MABSC with both positive short and long term success being documented (Qvist *et al.*, 2013)

In a study performed by the University of North Carolina, Chapel Hill, between 1990 and 2003 one hundred and forty patients with CF underwent lung transplantation and thirty-one were listed for transplantation. Of the patients referred, 19.7% were NTM positive. Following transplantation, 3.4% were identified as having NTM infection and those with infection prior to transplant were noted as having a higher risk of reoccurrence post-transplant (Chalermskulrat *et al.*, 2006).

Although severe infections can occur, the isolation of NTM prior to transplantation in patients with CF should not typically be an exclusion criterion for lung transplantation (Chalermskulrat *et al.*, 2006; Zaidi *et al.*, 2009). The most current NTM guidelines also recommend that in the presence of persistent MABSC infection, treatment should commence prior to transplant listing and this should not be an absolute contraindication to transplant approval (Floto *et al.*, 2016).

1.12 What does the future hold regarding *Mycobacterium abscessus* complex?

New treatment regimens are urgently required for the treatment of MABSC comparing and using a combination of longstanding and novel antimicrobials.

Furthermore, due to the complexity of the current techniques required in order to differentiate between the three MABSC species, a prompter and more economical identification method is essential.

1.13 Research aims and objectives

It is apparent from the literature reviewed that there are numerous areas of research that essentially require addressing with respect to mycobacteria, in particular MABSC. A reliable and convenient method of detection is vital in order to promptly diagnose NTM infection and fully understand the true prevalence in CF patients. Identification to the species level is fundamental in providing the correct treatments, as well as investigating new and improved treatment options for patients. Discovering means of possible transmission and whether they can be preventable or if they are entirely unavoidable is vital in preventing the spread of this infection. With all of these in mind, this research will focus on the following issues:

- Development and evaluation of a novel culture medium for rapidly-growing mycobacteria
- Methods of identification
- Environmental studies on transmission methods
- Exploration of potential antimicrobial treatments through *in vitro* susceptibility studies

CHAPTER TWO

Development of a novel culture medium for the isolation of rapidly-growing mycobacteria from the sputum of cystic fibrosis patients

Introduction

2.1 The origins of culture media

The origins of culture media date back to 1860 when Louis Pasteur published the first semi-synthetic medium, a nutrient broth, designed for the growth of bacteria and consisting of ammonium salts, yeast ash and sugar thus providing vitamins and trace elements as well as a nitrogen and carbon source (Pasteur, 1860). Developed further in 1872 by Ferdinand Cohn, a formula for what Cohn named a normal bacterial liquid, was published (Cohn, 1872). In 1881, Robert Koch described a medium containing aqueous meat extract, peptones and sodium chloride, the basic ingredients of many culture media still currently used (Koch, 1881).

The first solid culture medium was also developed by Robert Koch and published in 1881 using the cut surface of a boiled potato as this was a solid surface on which he could inoculate the bacteria (Koch, 1881). In the same year Frederick Loeffler published the formula for his nutrient broth, a meat extract peptone medium (Loeffler, 1881). Although this simple culture medium was used in the discovery of the anthrax pathogen *Bacillus anthracis*, potato slices were not deemed suitable for many microorganisms, as many could not thrive (Koch, 1884). Koch replaced the nutrient broth with silver salts in gelatin, obtaining this idea from photographic plates he made by coating glass plates with a solution of silver salts in gelatin, thus developing a solid culture medium superior to the sliced potato (Koch, 1887; Collard and Collard, 1976). Many bacteria however possessed enzymes that could digest the gelatin, and also on a warm day, the medium would liquefy, as the gelatin did not remain solid at 37^{o}C, the ideal temperature that most human pathogens required for growth. The main aims of Koch's work were that he wanted to be able to isolate

pure cultures of pathogenic bacteria in order to demonstrate a set of postulates that could be used to determine the cause of most infectious diseases (Koch, 1887).

Walter and Fanny Hesse, who were working in Koch's laboratory, suggested the use of agar as a replacement to gelatin, as Fanny had previously used this in the preparation of fruit jellies and puddings. Koch added this agar to the nutrient broths, and it proved to be far superior to the gelatin medium, as it remained solid at higher temperatures, produced a transparent medium, and was not often digested by bacterial enzymes (Koch, 1882).

Agar is a complex mix of polysaccharides acquired from species of the red algae agarophytes and comprising of two dominant polysaccharides. Agarose, which is responsible for the high strength gelling properties and is a (1-4) linked 3,6-anhydro-α-L-galactose alternating with (1-3) linked ß-D-galactose. The charged polymer agaropectin provides the viscous properties, and has the same repeating unit as agarose but with some of the 3,6-anhydro-L-galactose residues replaced with L-galactose sulphate residues, together with partial replacement of the D-galactose residues with pyruvic acid acetal 4,6-0-(1-carboxyethylidene)-D-galactose (Phillips and Williams, 2000).

Relatively unknown Italian pharmacist, Bartolomea Bizio, preceded Koch's work by many years, when in 1819 he discovered a chromogenic bacterium, which he named *Serratia marcescens*, growing as red spots on polenta. Bizio believed this to be a microorganism and to test his hypothesis placed some polenta in a saturated atmosphere at a constant temperature, and noted that within eight hours, red spots had appeared, and within twenty-four hours, the entire surface was discoloured. His findings were published in the Gazetta Privilegiata, however in the present day both Robert Koch and Louis Pasteur are principally credited for the discovery of culture media (Yu, 1979).

There are many different components used in culture media contributing to a variety of functions. Proteins, peptides and amino acids offer nutrients, energy is provided by carbohydrates, and components such as calcium, magnesium, iron, trace metals, phosphates and sulphates deliver essential metals and minerals that can be used as cofactors for certain enzymatic reactions, inorganic cellular cation and components of cytochromes and non-haem iron proteins (Morgan *et al.*, 1950). Buffering agents such as phosphates and acetates can be added, as well as pH indicators, for example phenol red, neutral red and bromothymol blue (Reznikov, 1972). Antibiotics and chemicals such as bile salts, selenite, tellurite and azide act as selective agents in culture media added at explicit concentrations to supress the growth of undesirable microorganisms in a polymicrobial sample. They must however be added at the correct concentration so as not to inhibit the desired organisms (Perlman, 1979). As bacteria can rapidly adapt it can be very challenging to obtain a completely specific selective medium, and the ultimate formulation is customarily a compromise which will supress most of the unwanted organisms whilst still allowing the growth of the majority of target organisms.

2.1.1 General purpose culture media

General purpose media are extensively used for the growth of a vast range of microorganisms. They do not contain any growth inhibitors and are generally rich in nutrients, therefore providing bacteria with carbohydrates, peptones, essential minerals, trace metals and other vital nutrients. These include nutrient agar, with or without the addition of blood and brain-heart infusion agar. The addition of blood enriches the medium and facilitates the growth of fastidious organisms such as *Haemophilus* spp. and *Neisseria gonorrhoeae*, and also supports the classification of certain microorganisms based upon their haemolytic reactions. When α-haemolysis is present, for example in *Streptococcus pneumoniae*, the agar beneath

the colony shows as a dark green colour. This is triggered by the bacterium's production of hydrogen peroxide subsequently oxidising haemoglobin to methemoglobin (Pericone *et al.*, 2000). β-haemolysis, also referred to as complete haemolysis, displays complete lysis of the red blood cells causing a yellow and transparent appearance beneath and around the colonies. Exotoxins, streptolysin O and streptolysin S produced by the bacteria, can be responsible for this, for example in *Streptococcus pyogenes*, however this species is not the only haemolytic bacteria. Staphylococci species can produce cytolytic toxins that can result in β-haemolysis as can *Enterococcus faecalis* and many more (Chopra *et al.*, 1991).

If an organism does not trigger haemolysis, this is known as γ-haemolysis, an example of this is in *Staphylococcus epidermidis*, and the agar remains unaltered (Pinheiro *et al.*, 2015).

2.1.2 Selective and differential culture media

Antimicrobials and chemicals can be included in culture media in order to inhibit the growth of unwanted microorganisms in a polymicrobial sample, permitting the growth of the desired pathogenic bacteria. There is a vast range of both synthetic and natural selective agents, depending on the selective requirements of the medium, with common selective agents including bile salts, selenite, tellurite, azide and many dyes and antibiotics (Bonev *et al.*, 2008).

Differential media employ biochemical tests in order to distinguish different species with the most frequent test being sugar utilisation. Lactose is metabolised to release gas and acid, and in the presence of a pH indicator dye, the acid released by the bacteria is detected as a colour change as the pH value decreases. Therefore, there is no colour change observed when bacteria are unable to utilise lactose. When an

acid is added to an indicator solution, the acid will donate protons to the water molecules. This increases the concentration of H_3O^+ ions in the solution. The H_3O^+ ions will subsequently donate protons to the indicator molecules causing the indicator to change colour.

Selective media will allow the growth of certain bacteria whilst inhibiting any unwanted bacteria. This can be achieved by adding certain ingredients, which can be utilised by particular species but not others. If certain species of bacteria are resistant to a specific antibiotic, this can be added to the medium to prevent the growth of other species and select only the desired bacteria. Eosin methylene blue (EMB) medium contains methylene blue which is toxic to Gram-positive bacteria, thereby only allowing the growth of Gram-negative species (Delost, 2015).

Sodium desoxycholate is a natural component in bile and used in many culture media. An example of such a medium is xylose lysine desoxycholate (XLD) agar which is both selective and differential and used for the isolation of *Salmonella* and *Shigella* species (Nye *et al.*, 2002). XLD works by utilising sodium desoxycholate as the selective agent thus making it inhibitory to commensal Gram-positive bacteria. Xylose incorporated into the medium as a differential agent is fermented by almost all enteric bacteria except for *Shigella* spp. and lysine enables *Salmonella* spp. to be differentiated from non-pathogenic bacteria. After the *Salmonellae* consume the supply of xylose, they decarboxylate the lysine via the enzyme lysine decarboxylase, thereby altering the pH to alkaline. Lactose and sucrose are added to the medium to produce excess acid by fermentation in order to prevent other lysine positive coliforms reverting the pH to alkaline. Degradation of xylose, lactose and sucrose to acid causes the phenol red indicator in the medium to change its colour from red to yellow. Bacteria that decarboxylate lysine can be identified by the appearance of a red colouration around the colonies due to an increase in the pH.

For further differentiation between *Salmonellae* and *Shigellae*, an H_2S indicator system, consisting of sodium thiosulfate and ferric ammonium citrate, is included in the medium for the identification of the hydrogen sulfide produced, resulting in the formation of black centred colonies. The non-pathogenic H_2S producers do not decarboxylate lysine hence the acid reaction produced by them prevents the blackening of the colonies which occurs only at neutral or alkaline pH (Taylor and Schelhart, 1971; Zajc-Satler and Gragas, 1977).

Other examples of selective media are modified charcoal cefoperazone desoxycholate agar (Chon *et al.*, 2012) for the detection of *Campylobacter* species, and modified buffered charcoal yeast extract agar (Feeley *et al.*, 1979) for the growth of *Legionella* species. Both of these contain charcoal as a key ingredient which absorbs bacterial toxins that would otherwise inhibit bacterial growth.

2.1.3 Chromogenic culture media

Chromogenic culture media employ one of more chromogenic enzyme substrates that are hydrolysed to release coloured dyes. Bacteria may therefore be identified with high specificity based on their possession (or lack of) of certain enzymes.

One of the first chromogenic media to be developed for use in clinical laboratories was Rambach agar (Kuhn *et al.*, 1994). This takes advantage of the fact that most *Salmonella* strains do not produce β-galactosidase. As the medium contains a chromogenic substrate for β-galactosidase, this enables the colonies of the most commonly encountered strains of Enterobacteriaceae, such as *Escherichia coli*, *Citrobacter* spp. and *Klebsiella* spp. to produce a blue coloration. The medium also contains neutral red and propylene glycol to allow differentiation of *Salmonella* from other species such as *Pseudomonas* spp. The propylene glycol is fermented by *Salmonella* to generate red-pink colonies (Heizmann, 1993).

Although these culture media are widely available to clinical microbiology laboratories for a wide range of pathogens, there are still many areas for possible improvement. An evident problem is their inability to detect pathogenic colonies beneath an overgrowth of other more rapidly-growing species, particularly where the pathogenic species is present in low numbers in a sample.

This chapter reports the development and evaluation of a novel agar-based culture medium (RGM medium) designed for the isolation of rapidly-growing mycobacteria from the sputa of patients with CF. RGM medium is based on Middlebrook agar and contains a combination of supplementary growth factors and selective agents. In a preliminary study, the medium was tested with pure cultures of mycobacteria and other bacteria and fungi. RGM medium was then compared to other brands of agar for the isolation of NTM, and then compared with BCSA for the isolation of NTM from 502 sputum samples from patients with CF. Further studies in seven centres were then undertaken, including a comparison of RGM medium to the gold standard automated liquid culture.

2.2 Aims and Objectives

Middlebrook and BCSA are both widely used solid culture media for the detection of NTM in patients with CF. However as demonstrated in the literature both of these methods are fraught with contamination caused by more rapidly-growing species found in CF sputa. Gold standard methods such as the MGIT also have contamination issues, and it may frequently be impossible to issue a result with respect to isolation of NTM. Such automated liquid culture methods are also time consuming and costly. It was therefore the aim of this study to develop a novel and highly selective agar-based medium for NTM based on the following objectives:

(i) To screen a variety of enrichment ingredients and antimicrobials to identify any which would be potentially useful for the optimal growth of NTM whilst inhibiting any other frequently growing bacterial species common in patients with CF.

(ii) To evaluate these to determine which would be the most effective in forming a novel agar-based medium for NTM.

(iii) To formulate a novel solid culture medium that will allow culture for NTM without the requirement for sample decontamination.

(iv) To evaluate the novel medium in different centres in comparison with standard methods.

2.3 Materials

2.3.1 Bacterial strains used in medium development and evaluation

A collection of 147 isolates of rapidly-growing mycobacteria previously isolated from CF sputum samples by standard methods was used for evaluation of all media including RGM. These included *M. abscessus* subsp. *abscessus* (*n* = 79), *M. chelonae* (*n* = 43), *M. abscessus* subsp. *massiliense* (*n* = 12), *M. abscessus* subsp. *bolletii* (*n* = 3), *M. fortuitum* (*n* = 3), *M. salmoniphilum* (*n* = 3), *M. llatzerense* (*n* = 2), *M. immunogenum* (*n* = 1) and *M. mucogenicum* (*n* = 1). Seventy-three of these isolates were obtained from the Microbiology Department, Freeman Hospital, Newcastle upon Tyne, UK and all were from distinct patients. Seventeen were kindly provided by St. Vincent's University Hospital, Dublin, Ireland and were from distinct patients. The remaining 57 were consecutive clinical isolates kindly supplied by Public Health England, Newcastle upon Tyne, UK. Due to being consecutive samples, (all CF samples sent to Public Health for testing) this does not reflect the number of patients, which is unknown for these isolates. The species and subspecies identity of all strains had been previously confirmed by sequencing of at least two of three housekeeping genes (*rpoB, hsp*65 and *sodA*) as described by (Blauwendraat *et al.*, 2012).

A collection of non-mycobacteria were selected to represent a variety of species frequently recovered from the sputa of patients with CF. Non-mycobacterial strains (*n* = 185) were obtained from national culture collections (*n* = 23) or from the culture collection of the Microbiology Department, Freeman Hospital, Newcastle upon Tyne (*n* = 162) and included an international *P. aeruginosa* reference panel (*n* = 43) (De Soyza *et al.*, 2013) and a *Burkholderia cepacia* complex experimental strain panel (*n* = 26) (Mahenthiralingam *et al.*, 2000; Coenye *et al.*, 2003; Vermis *et al.*, 2004) as

well as clinical isolates of both species. In total, the collection comprised: *P. aeruginosa* (*n* = 55), *B. cepacia* complex (*n* = 43), *S. aureus* (*n* = 28), Enterobacteriaceae (*n* = 11), *Achromobacter xylosoxidans* (*n* = 8), *Ralstonia mannitolilytica* (*n* = 7), *Stenotrophomonas maltophilia* (*n* = 4), *Streptococcus* spp. (*n* = 4), *Aspergillus* spp. (*n* = 3), *Bacillus subtilis* (*n* = 1), *Candida* spp. (*n* = 3), *Pandoraea* spp. (*n* = 3), *Acinetobacter* spp. (*n* = 2), *Enterococcus* spp. (*n* = 2), *Inquilinus limosus* (*n* = 2), *Scedosporium* spp. (*n* = 2), *Delftia acidovorans* (*n* = 1), *Elizabethkingia miricola* (*n* = 1), *Geosmithia argillacea* (*n* = 1), *Haemophilus influenzae* (*n* = 1), *Moraxella catarrhalis* (*n* = 1), *Neisseria flavescens* (*n* = 1) and *Ochrobactrum* sp. (*n* = 1).

A collection of fourteen isolates of slow-growing mycobacteria previously isolated by standard broth-based culture methods were used for evaluation of broth-based media (see section 2.4.18). These included *M. avium* (*n* = 6), *M. tuberculosis* complex (*n* = 6) and *M. intracellulare* (*n* = 2). All isolates were kindly provided and identified to species level by HAIN Genotyping by Public Health England, Newcastle upon Tyne, UK

All strains, origins and chapter/section referred to are shown in Appendix 1.

2.3.2 Growth media and general chemicals

Yeast extract, ready-prepared BCSA; (product Ref: 33631), bioMérieux cepacia agar (product Ref: 44347), BacT/ALERT MP (product ref 259797), MB/BacT Mycobacteria Antibiotic Supplement Kit including reconstitution fluid (product ref 259760) were purchased from bioMérieux, Basingstoke, UK. Blood agar was prepared from Columbia agar powder (Oxoid CM0331) (and supplemented with 5% defibrinated horse blood supplied by TCS Biosciences, Buckingham, UK), *Burkholderia cepacia* agar (product Ref: PO0938), proteose peptone, sputasol

liquid, Sabouraud dextrose agar (CM0041), Bacteriological agar nº. 1 (LP0011) and Mueller-Hinton agar (CM0337) were supplied from Oxoid Ltd, Basingstoke, UK. BD Cepacia medium (product Ref: 256180) and BD OFPBL (oxidation-fermentation-polymyxin-bacitracin-lactose) medium (product Ref: 254481) were purchased from BD Diagnostic Systems, Oxford, UK. Middlebrook 7H11 agar (Product Ref: PP4080) was obtained from E&O Laboratories, Bonnybridge, UK. Ammonium sulfate, calcium chloride, Tween-80 and sodium citrate were supplied by BDH, Poole, UK. Unless otherwise stated, all other ingredients were obtained from Sigma-Aldrich, Poole, UK.

2.3.3 Antimicrobials

Amphotericin B was supplied by Duchefa Biochemie BV, Haarlem, The Netherlands, colomycin (at 44.3% purity) was obtained from bioMérieux, Marcy l'Etoile, France, and C390 (9-Chloro-9-(4'-diethylamino)phenyl-9,10-dihydro-10-phenylacridine hydrochloride) was obtained from Biosynth, Staad, Switzerland. Derivatives of C390 were kindly synthesised and supplied by Dr Annette Johnson at Northumbria University, Newcastle upon Tyne, UK. All other antimicrobials were obtained from Sigma-Aldrich, Poole, UK.

2.3.4 Equipment

Sterilisation of media and equipment where appropriate was performed using a LTE Touchclave-R autoclave (LTE Scientific Ltd. Oldham, UK). All chemicals and media components were weighed on an A&D GR200 balance from A and D Weighing, California, USA, which is accurate to 0.1 mg. Sterile, disposable tips were used for the accurate measurement of small volumes of liquid using calibrated Gilson semi-automatic pipettes (P1000, P200 and P10) from Gilson Medical Electronics, Villiers-le-Bel, France. Larger volumes were dispensed using sterile disposable 3 ml

graduated pipettes from Alpha Laboratories, Hampshire, UK and sterile disposable 10 ml pipettes from L.I.P Limited, Shipley, UK. Antimicrobials were filter sterilised using 0.2 µm supor membrane filters from VWR International, Leicestershire, UK and Terumo two-part 20 ml syringe purchased from Medisupplies, Dorset, UK. Bacterial suspensions were prepared to known cell densities using a Densimat densitometer obtained from bioMérieux, Marcy l'Etoile, France. Glass beads (3mm) for vortexing rough isolates of mycobacteria were obtained from Sigma-Aldrich, Poole, UK. Sterile Petri dishes were obtained from Sarstedt AG & Co., Leicester, UK. All plates were incubated in a LEEC 30oC incubator obtained from Laboratory and Electrical Engineering Company, Nottingham, UK. The acidity or alkalinity of each medium was measured using a pH meter from Hanna Instruments Limited, Leighton Buzzard, UK to an accuracy of pH 0.1, and adjusted using sodium hydroxide or hydrochloric acid, both of which were purchased from BDH Chemicals Ltd., Poole, UK. Non-mycobacterial strains were inoculated using a Denley multipoint inoculator from Denley Instruments, Billinghurst, UK. Antimicrobials were injected into BacT/ALERT bottles using a Terumo 2 ml syringe and 0.8 x 40 mm needle also purchased from Medisupplies, Dorset, UK after being made sterile using Sterets swabs obtained from Molnlycke Healthcare, Bedfordshire, UK. The BacT/ALERT® 3D instrument used for the detection of mycobacteria was obtained from bioMérieux, Marcy l'Etoile, France. For identification of isolates matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Bruker, Coventry, UK) was used.

2.4 Methods

2.4.1 Evaluation of various ingredients for optimal growth of *Mycobacterium abscessus* complex

Various ingredients including nutrients and antimicrobials were tested initially using twenty isolates of Mycobacteria. The initial isolates were selected as VNTR profiling had been performed and identification could be certain (as shown in Table 2-1). This number was increased to include further isolates of Mycobacteria as well as a selection of non-mycobacterial isolates as shown in Appendix 1.

2.4.2 Mycobacterial Strains

Twenty isolates of MABSC were obtained as pure cultures from the Microbiology Department, Freeman Hospital, Newcastle upon Tyne and were stored at -20^{o}C in glycerol/skimmed milk. Frozen isolates were subcultured twice on Columbia agar with 5% horse blood prior to testing. The origin and species identity of these isolates is detailed in Table 2-1 and Appendix 1. Identity had previously been confirmed to subspecies level by *rpoB* and *hsp65* gene sequencing at Great Ormond Street Hospital, London, UK (Harris *et al.*, 2012).

Table 2-1: Species, origin and VNTR profile of mycobacteria used in medium testing

Species	Origin	VNTR Profile (variable-number tandem repeats)								
M. abscessus subsp. *abscessus*	Adult CF patient	2+	5	-	6	2	5	1+	2+	-
M. abscessus subsp. *abscessus*	Adult CF patient	1+	-	3	-	2	3+	4	2+	2
M. abscessus subsp. *abscessus*	Adult CF patient	2+	5+	3	2	4	4+	2+	2+	2
M. abscessus subsp. *abscessus*	Paediatric CF patient	3+	4+	3	2	4	3+	2	2+	2
M. abscessus subsp. *abscessus*	Paediatric CF patient	3+	4+	3	-	4	3+	2	2+	-
M. abscessus subsp. *abscessus*	Adult CF patient	-	5	3	2	7	3+	4	1	2
M. abscessus subsp. *abscessus*	Paediatric CF patient	1+	5	3	4	2	3+	4	2+	2
M. abscessus subsp. *abscessus*	Paediatric CF patient	2+	5	3	2	4	4+	1+	2+	3
M. abscessus subsp. *abscessus*	Adult CF patient	1+	3+	3	3	2	3+	4	-	2
M. abscessus subsp. *abscessus*	Paediatric CF patient	3+	5	2	3	2	5	1+	-	6
M. abscessus subsp. *abscessus*	Adult CF patient	1+	5	3	3	4	2	1+	2+	-
M. abscessus subsp. *bolletii*	Paediatric CF patient	1+	-	1	6	1	1+	1+	-	2
M. abscessus subsp. *bolletii*	Environmental	-	-	-	-	2	-	-	-	-
M. abscessus subsp. *massiliense*	Adult CF patient	1+	-	2	2	1	2	1+	1	2
M. abscessus subsp. *massiliense*	Adult CF patient	1+	5	2	4	2	3+	1+	2+	-
M. abscessus subsp. *massiliense*	Adult CF patient	2+	-	2	4+	2	2	1+	1	2
M. abscessus subsp. *massiliense*	Adult CF patient	-	5+	2	2	1	2	2+	2	-
M. abscessus subsp. *massiliense*	Paediatric CF patient	1+	-	2	3	2	3+	2+	2+	-
M. abscessus subsp. *massiliense*	Paediatric CF patient	-	-	2	2	1	3+	1+	1	2
M. abscessus complex (chimeric)*	Paediatric CF patient	1+	-	-	-	-	-	-	-	-

*This species was confirmed as *M. abscessus* subspecies *abscessus* by *hsp65* and *sodA* gene sequencing and *M. abscessus* subspecies *massiliense* by *rpoB* gene sequencing, suggesting a chimeric organism. VNTR typing failed as it would only amplify at one locus.

All experiments conducted in the development and evaluation of this medium were performed in duplicate unless otherwise stated.

2.4.3 Evaluation of basal culture media

Double-strength Middlebrook 7H9 broth base was prepared as follows: 1.0 g ammonium sulphate, 5.0 g disodium phosphate, 2.0 g monopotassium phosphate, 0.2 g sodium citrate, 0.1 g magnesium sulphate, 0.001 g calcium chloride, 0.002 g zinc sulphate, 0.002 g copper sulphate, 0.08 g ferric ammonium citrate, 1.0 g L-glutamic acid, 0.002 g pyridoxine, 0.001 g biotin and 8 ml glycerol were dissolved in 992 ml of deionised water and the pH adjusted to pH 6.6 $^{+}$/- 0.2 with 10M hydrochloric acid. The broth base was autoclaved at 116^{o}C for 10 minutes.

Each type of medium was prepared as shown in Table 2-2 to make a final volume of 100 ml with the OADC growth supplement omitted until after autoclaving.

OADC growth supplement was prepared using 10 g bovine albumin fraction, 4 g dextrose, 0.008 g catalase, 126 µl oleic acid and 200 ml deionised water. This was, filter sterilised once all components were dissolved.

Table 2-2: Ingredients of the various media used in initial basal medium evaluation

Media	Middlebrook Broth Base (2x strength) (ml)	Glycerol (ml)	Tween-80 (g)	Middlebrook OADC* Growth Supplement (ml)	Bacteriological Agar (g)	Columbia Agar (g)	Deionised Water (ml)
A	50.0	-	-	-	1.0	-	50.0
B	50.0	0.4	-	-	1.0	-	49.6
C	50.0	-	0.1	-	1.0	-	50.0
D	50.0	0.4	-	10.0	1.0	-	39.6
E	50.0	-	0.1	10.0	1.0	-	40.0
F	-	-	-	-	-	3.9	100.0
G	-	-	-	10.0	-	3.9	90.0

*OADC (oleic albumin dextrose catalase)

A further two media labelled H (ready prepared Columbia blood agar) and I (ready prepared bioMérieux cepacia agar) were also evaluated.

The twenty strains of mycobacteria were inoculated onto each of the nine media and time to detection (TTD) and appearance of resulting colonies was recorded after 24, 48, 72, 96 and 168 h.

The sensitivity value of a medium relates to the appearance and growth of target pathogens.

$$\text{Sensitivity (\%)} = \frac{\text{Number of target strains forming colonies}}{\text{Total number of target strains}} \times 100$$

2.4.4 Preparation of inocula and inoculation onto basal media

A series of suspensions containing 2 ml sterile saline (0.85%) and turbidity equivalent to a 0.5 McFarland standard (approx. 1.5×10^8 CFU/ml) were prepared for each isolate. For the rough colony types, where clumping occurred, vortexing with three sterile 3 mm glass beads for 10 minutes effectively dispersed all clumps. Each medium type was inoculated with a 1 µl aliquot of each isolate (i.e. approx. 1.5×10^5 CFU) and incubated at 30°C. For all subsequent experiments (unless stated otherwise) bacterial strains were retrieved from glycerol skimmed milk, subcultured, and suspensions were prepared as above.

2.4.5 Evaluation of various nutrients for the improved growth of mycobacteria

One litre of 10 x strength Middlebrook 7H9 broth base was made up using the same protocol as described previously adjusting the volumes accordingly.

Medium D from section 2.7.3 containing 10 ml aliquots of 10 x strength Middlebook broth base and 1% bacteriological agar had each of the following supplements added to separate 100 ml alliquots; 0.2 g activated charcoal (medium J), 5 ml 5% horse blood* (medium K), 0.4 g yeast extract (medium L), 1.5 g α-ketoglutarate (medium M), 0.1 g casein (medium N), 0.7 g sodium pyruvate (medium O), 1 g proteose peptone (medium P), 0.004 g amaranth (medium Q), 3.3 ml egg yolk emulison* (medium R), 0.005 g ribonucleic acid* (medium S), 0.01 g polyoxyethylene stearate* (medium T), 0.5 ml tyloxapol (medium U).

A further medium containing 0.4 g activated charcoal combined with 3.3 ml egg yolk emulsion* was prepared (medium V). Deionised water was added to each one to make up to 90 ml and autoclaved for 10 minutes at 116^{o}C.

A further 10 ml of OADC was added to each medium to take the total volume of each up to 100 ml.

*Supplements added to media K, R, S, T and the egg yolk emulsion from medium V were added after autoclaving, after the medium was cooled to 50°C.

A series of suspensions were prepared as in section 2.7.4 and each medium type was inoculated with a 1 µl aliquot of each isolate. Plates were inoculated as described previously using the twenty mycobacterial isolates as shown in Table 2-1, and incubated at 30^{o}C. Evaluation of all media after one week of incubation suggested that medium L supplemented with yeast extract demonstrated a noticeably improved and more rapid growth of mycobacteria in comparison to medium D.

2.4.6 Investigation of various antimicrobials as putative selective agents

Medium L was taken forward, with a selection of antimicrobials added in isolation in order to examine their inhibitory impact on frequently encountered bacteria and fungi

isolated from the sputum samples of patients with CF. The effect of the antimicrobials on the growth of mycobacteria was also evaluated. Antimicrobials evaluated were amphotericin B at 5, 10 and 20 mg/L, nalidixic acid at 16, 32 and 64 mg/L, vancomycin at 2.5, 5 and 10 mg/L, colomycin at 32, 64 and 128 mg/L, C390 at 32, 64 and 128 mg/L, fosfomycin at 400, 800 and 1600 mg/L and malachite green at 0.125, 0.25 and 0.5 mg/L.

Medium L was prepared as described previously and antimicrobials for each concentration were dissolved in 20 ml sterile deionised water at 10 x strength, placed in a water bath at 50^{o}C for five minutes to ensure they were fully dissolved then added to the medium, with the exception of amphotericin B. This was dissolved in 400 µl of N-methyl-2-pyrrolidone, a water-miscible organic solvent, placed in a water bath at 50^{o}C for five minutes to fully dissolve then added to 19.6 ml deionised water prior to adding to medium. A control with no antibiotics was included. OADC growth supplement was made up as previously and 20 ml added to each of the twenty two media so that final volume of each medium was 200 ml.

2.4.7 Non-mycobacterial strains and culture onto media containing antimicrobials

A selection of 88 non-mycobacterial species were selected to represent a broad variety of species commonly isolated from the sputum of patients with CF. The origin and species identity of these isolates is detailed in Appendix 1. Frozen bacterial isolates were subcultured on Columbia agar with 5% blood; yeasts and fungi were subcultured on Sabouraud medium prior to testing. Isolates 7037 through to 7079 are from an International *P. aeruginosa* reference panel collated by (De Soyza *et al.*, 2013) and chosen to cover frequently studied clones, transmissible strains,

sequential CF isolates, strains with particular virulence characteristics, and strains that represent serotype, genotype, or geographic diversity.

Isolates of mycobacteria were prepared and inoculated as previously in section 2.7.4. Non-mycobacterial isolates were suspended in 2 ml sterile saline (0.85%) and turbidity equivalent to 0.5 McFarland units (approx. 1.5×10^8 CFU/ml). Each medium type at all antimicrobial concentrations plus a control (medium L) were inoculated with 20 x 1 µl inocula per plate using a multipoint inoculator. Plates were incubated at 30^{o}C. Yeasts and fungi (see Appendix 1) were inoculated onto medium containing amphotericin B at all three concentrations.

2.4.8 Investigation of antimicrobial combinations in agar-based media

Medium W was identical to Medium L except it was supplemented with colomycin at 32 g/L, fosfomycin at 400 mg/L, amphotericin B at 5 mg/L and glucose-6-phosphate at 25 g/L. Medium X contained the same as medium W with the addition of C390 at 32 mg/L.

Glucose-6-phosphate was included in both media as research shows that with the addition of glucose-6-phosphate to the medium, fosfomycin MIC's are significantly lower for certain species, therefore susceptibility testing to fosfomycin is recommended with the addition of glucose-6-phosphate in the medium at a concentration of 25 mg/L (Barry and Fuchs, 1991).

2.4.9 Bacterial strains and culture onto media containing combined antimicrobials

Isolates of mycobacteria were used as in Table 2-1 plus an additional selection of 80 isolates of mycobacteria (Appendix 1) were included to examine the effect of the antibiotics on a larger selection of rapidly-growing mycobacteria. A further nine non-mycobacterial species frequently found in the sputum CF patients were also

included (see Appendix 1). All isolates were prepared and inoculated as previously in section 2.7.4

2.4.10 Plating efficiency studies performed on RGM medium

Plating efficiencies were undertaken to quantify the growth of mycobacteria on media L, W and X. Initial testing was performed on media W and X (hereafter known as RGM medium) to find the concentration which would yield approximately 100 CFU/ml. A series of suspensions containing 2 ml sterile saline (0.85%) and turbidity equivalent to a 0.5 McFarland standard unit was prepared for each isolate. Any rough colony types were vortexed as described previously in section 2.7.4 Isolates used in this study are shown in Appendix 1.

Each of the three media was inoculated with a 50 µl aliquot of each suspension using 1/1,000, 1/10,000 and 1/50,000 dilutions. Spread plates were prepared and incubated at 30°C for 96 h before colonies were counted and observed to see if the media also had any effect on the size of colonies.

Results showed that a 1/10,000 dilution provided the most countable number of colonies therefore ten isolates of mycobacteria were inoculated onto medium L, W and X (RGM) in triplicate as previously, using 50 µl of a 1/10,000 dilution. Plates were incubated for 96 h at 30^{o}C

2.4.11 Statistical analysis

Any difference in performance of media was investigated for statistical significance using McNemar's test with the continuity correction applied. Statistical significance was assigned to a probability (*P*) value of ≤ 0.05. Two-sample t-tests were performed to analyse any differences between control medium and RGM.

2.4.12 Evaluation of the stability of RGM medium

Twenty-four plates of RGM medium were prepared and stored at 4ºC. These were inoculated as described above on a weekly basis over a period of 12 weeks with four isolates of mycobacteria (*M. abscessus* subsp. *abscessus, M. abscessus* subsp. *bolletii, M. abscessus* subsp. *massiliense* and *M. chelonae*), seven isolates of fungi and yeast (*A. fumigatus, A. terreus, C. albicans, C. glabrata, G. argillacea, S. apiospermum* and *S. prolificans*) and six other isolates (*P. aeruginosa* (*n* = 4), *B. multivorans* and *E. cloacae*) (See Appendix 1). The six non-mycobacteria were carefully selected as indicators of deterioration of each of the selective agents in the medium. Media were incubated at 30°C and examined daily for up to 14 days.

2.4.13 Investigation of various derivatives of C390 as putative selective agents

C390 and the following C390 derivatives as seen in Figure 2-1 to Figure 2-7 below were used in the initial MIC testing using a range of 1 – 128 mg/L.

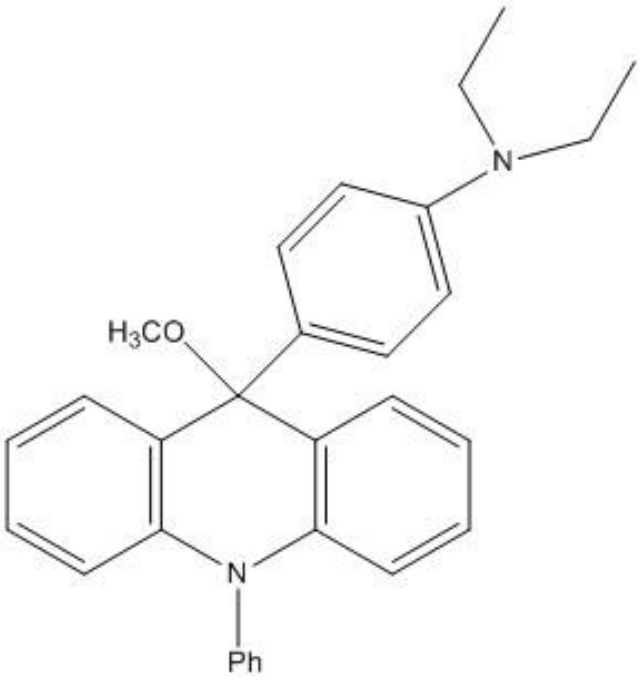


Figure 2-1: A475: 4'-Diethylaminophenyl-9-methoxy-10-phenylacridan

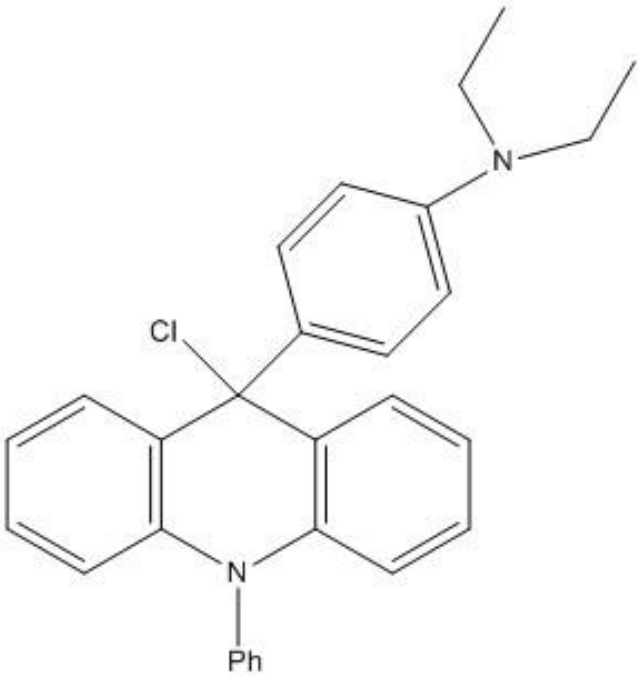


Figure 2-2: A477/A488/C390: 4'-Diethylaminophenyl-9-chloro-10-phenylacridan

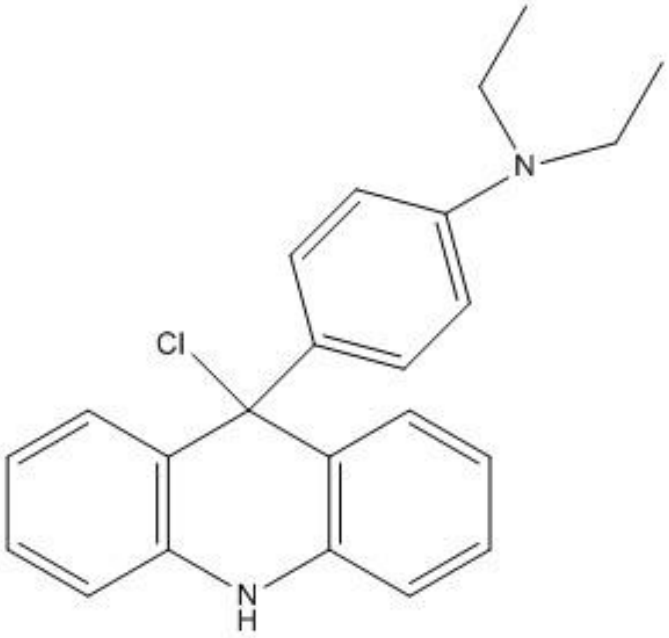


Figure 2-3: A480a: 4'-Diethylaminophenyl-9-chloroacridan

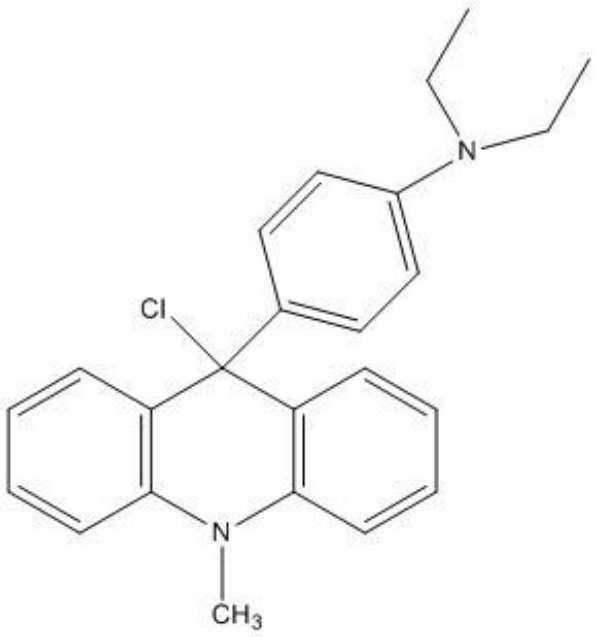


Figure 2-4: A503a: 4’-Diethylaminophenyl-9-chloro-10-methylacridan

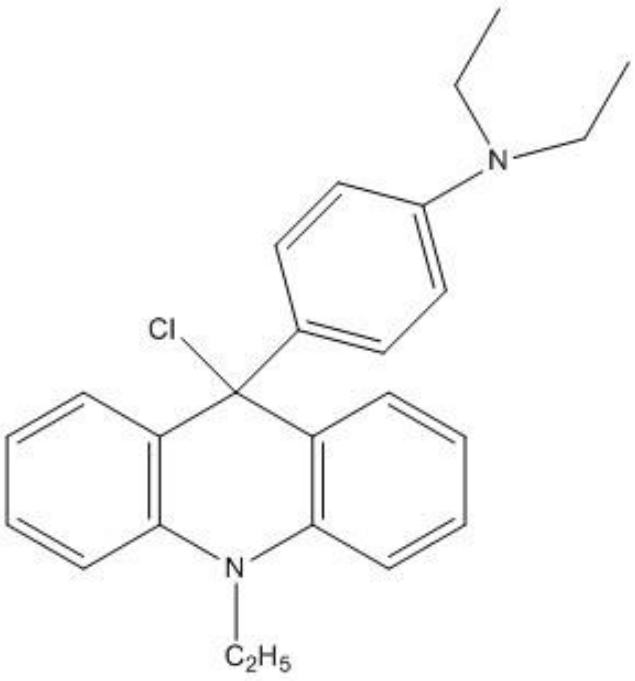


Figure 2-5: A503b: 4’-Diethylaminophenyl-9-chloro-10-ethylacridan

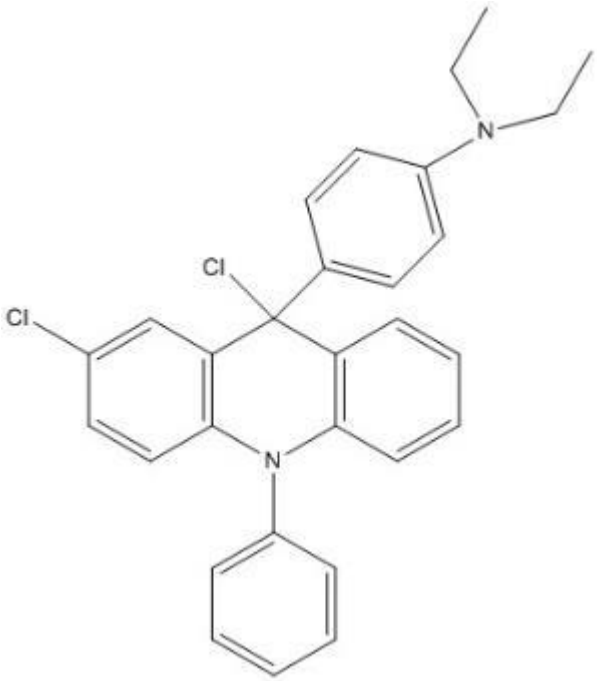


Figure 2-6: A505: 4'-Diethylaminophenyl-2-9-dichloro-10-phenylacridan

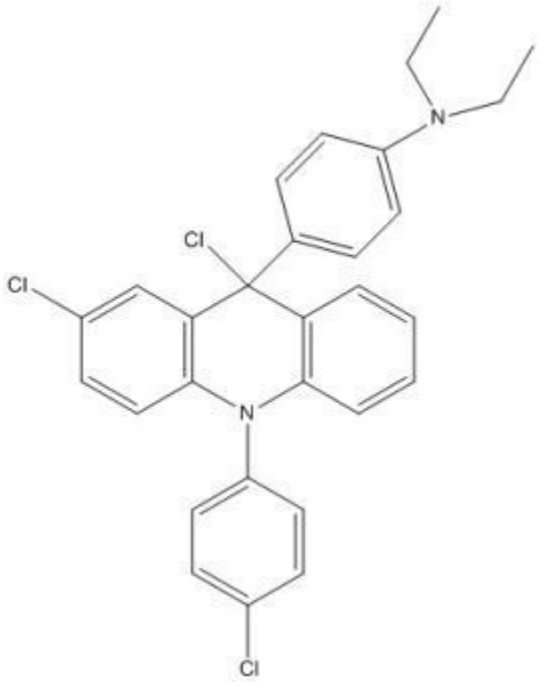


Figure 2-7: A520: 4'-Diethylaminophenyl-9-chloro-10-phenyl-4"-chloroacridan

2.4.14 Preparation of bacterial strains and inoculation of isolates onto media containing C390 and various C390 derivatives to evaluate the effectiveness in RGM medium

Mueller Hinton agar was made up according to manufacturer's instructions and autoclaved at 116^{o}C for 10 minutes and quantities of 5.12 mg were measured out for C390 and each derivative of C390. C390, A477, A488, A503a and A503b were dissolved in 2 ml of sterile distilled water; A475 was dissolved in 200 µl 1M HCl and added to 1.8 ml sterile distilled water. A480a, A505 and A520 were dissolved in 200 µl of N-methyl-2-pyrrolidone then added to 1.8 ml sterile distilled water. The dissolved components underwent serial dilutions in sterile distilled water to achieve each desired concentration ranging from 2560 mg/L down to 20 mg/L and 1 ml of each was added to 19 ml agar giving a total volume of 20 ml for each compound at each concentration which ranged from 128 mg/L to 1 mg/L. Plates were poured into sterile Petri dishes and allowed to set and dry.

A series of suspensions containing 2 ml sterile saline (0.85%) and turbidity equivalent to a 0.5 McFarland standard were prepared for each isolate (Appendix 1). Each medium type at each concentration plus Mueller Hinton control plates were inoculated using a multipoint inoculator with a 1 µl aliquot of each isolate (i.e. approx. 1.5 x 10^5 CFU/ml), incubated at 30^{o}C and results recorded after 24, 96, 168 and 240 hours

2.4.15 Preparation of bacterial strains and inoculation of isolates onto various media to evaluate the effectiveness of RGM medium against currently available media

Strains, which consisted of 147 mycobacteria and 185 non-mycobacteria (Appendix 1) were prepared as in section 2.7.4 and sub-cultured onto Columbia agar with 5%

horse blood prior to testing. A 1 µl aliquot of each suspension of mycobacteria was inoculated onto each medium type and the inoculum was spread using a loop. Filamentous fungi were inoculated in the same way. Suspensions of all other isolates were inoculated onto media using a multipoint inoculator to deliver inocula of approximately 1 µl per spot (i.e. approximately 1.5×10^5 CFU/spot). All plates were incubated at 30 °C and growth was recorded after four, seven days and ten days of incubation. The eleven media types evaluated included RGM medium using C390 at 32 mg/L, 64 mg/L, 128 mg/L, A477 at 32 mg/L in place of C390 (one of the C390 derivatives described in section 2.4.12), RGM medium minus OADC supplement, Middlebrook 7H11 selective (PP4080), Oxoid *B. cepacia* (PO0938), BD cepacia (256180), BD OFPBL (254481), bioMérieux cepacia (44347) and bioMérieux BCSA (33631). To demonstrate the viability of isolates, Columbia blood agar for bacterial isolates and Sabouraud agar for fungal isolates were used as controls.

2.4.16 Inoculation of sputum samples onto RGM medium and bioMérieux cepacia agar

Consecutive sputum samples (*n* = 502) obtained from 210 adults and children with CF were processed between February and September 2014. The age range of patients was < 1 year to 77 years. All samples were routine samples submitted to the Microbiology Department, Freeman Hospital, UK for culture. No additional samples were taken for the purposes of this study (See Appendix 4 for details of ethical approval). Sputum samples received from patients with CF were digested (1:1) with sputasol and 10 µL aliquots cultured onto whole plates of both RGM medium and bioMérieux cepacia agar. The inoculum was spread using a loop to obtain isolated colonies. Both media were incubated for 10 days at 30°C and growth was recorded after four, seven and ten days. All isolates recovered were identified

by MALDI-TOF MS. For any suspected mycobacteria, full protein extractions were performed as recommended by Saleeb *et al*. (2011) and species and subspecies identification was further confirmed at a reference laboratory as previously described (Saleeb *et al.*, 2011; Blauwendraat *et al.*, 2012).

2.4.17 Investigation of the use of RGM medium for the isolation of mycobacteria from the sputum of patients with non-cystic fibrosis bronchiectasis

Consecutive sputum samples (*n* = 400) obtained from 310 adults with bronchiectasis or patients undergoing transplant assessment (*n* = 67) from 60 patients were processed between May and November 2015. All samples were routine samples submitted to the Microbiology Department, Freeman Hospital, UK for culture. No additional samples were taken for the purposes of this study. Sputum samples received were digested (1:1) with sputasol and 10 µL aliquots cultured onto plates of RGM medium. The inoculum was spread using a loop to obtain isolated colonies. The medium was incubated at 30°C and all growth was recorded after ten days. Identification of colonies was performed as in section 2.4.16.

2.4.18 Adaptation and evaluation of RGM medium for recovery of mycobacteria including *Mycobacterium tuberculosis* complex in a broth-based culture medium

A bottle of 15 ml MB/BacT reconstitution fluid was transferred into a sterile universal and the following antimicrobials were weighed out to give final concentrations of; colomycin at 32 mg/L, fosfomycin at 400 mg/L, amphotericin B at 5 mg/L, C390 at 32 mg/L and glucose-6-phosphate at 25 mg/L. In the following order, colomycin, glucose-6-phosphate and fosfomycin, were added and allowed to dissolve. C390 and amphotericin B were each dissolved in 100 µl N-methyl-2-pyrrolidone then

added to the reconstitution fluid, the bottle briefly vortexed and the solution filter sterilised. The top of each BacT/ALERT bottle was cleaned with a sterile disposable wipe and 0.5 ml of reconstitution fluid containing antimicrobials was injected into each bottle using a 2 ml syringe and 0.8 x 40 mm needle. Bottles (designated Bottle B) were placed in the refrigerator at 4^{o}C until required.

RGM medium was prepared as described in Appendix 2 and an aliquot of 15 ml was poured into sterile universals, tilted and allowed to set.

2.4.18.1 Health and safety

Good laboratory practice was followed at all times. The processing of these samples was carried out inside a Class I Safety Cabinet in a category 3 containment suite. Disposable gloves were worn at all times, and any spillage was mopped up using tristel activated chlorine dioxide.

2.4.18.2 Preparation of clinical samples from cystic fibrosis patients

Each sample was digested and liquefied using an excess of dithiothreitol and vortexed at full speed until completely homogenous. The sample was centrifuged at 3000g for 18 minutes and supernatant poured off to leave a pellet. The pellet was subsequently split into two equal aliquots and two different decontamination procedures were performed.

2.4.18.3 Decontamination procedure for Gram-positive organisms

A volume of 7 ml of sodium hydroxide (NaOH) (4% w/v) was added to one of the aliquots and left for 25 minutes during which the sample was vortexed at full speed every five minutes. At the end of the treatment time the sample was neutralised with 14 ml of neutralising buffer solution (1M anhydrous monopotassium phosphate (KH_2PO_4) + 0.2% Phenol red; final pH 6.8), and re-concentrated by centrifugation at

3000g for 18 minutes. The sample was then re-constituted to 2 ml and using a safety needle and syringe one BacT/ALERT culture bottle (ref 259797) with the addition of MB/BacT Mycobacteria Antibiotic Supplement Kit including reconstitution fluid (ref 259760), designated Bottle A (see Appendix 3) was inoculated with 0.5 ml of neutralised specimen, one BacT/ALERT culture bottle with prototype antibiotic mix, designated Bottle B (see Appendix 3) was inoculated with 0.5 ml of neutralised specimen and an aliquot also stored at –20ºC.

Using sterile disposable graduated pipettes, the surface of a neutral pyruvate Lowenstein Jensen (LJ) slope and an RGM medium slope were each inoculated with 0.2 ml of specimen. These were rotated to allow the specimen to inoculate the entire surface; the caps were tightly fitted and incubated at 30°C for eight weeks. This was repeated with a further neutral pyruvate LJ slope and an RGM medium slope and incubated at 37°C for eight weeks for LJ and three weeks for RGM medium. BacT/ALERT bottles were logged onto the system and incubated for 28 days.

2.4.18.4 Decontamination procedure for Gram-negative organisms

An equal volume of N/2 sulfuric acid (H_2SO_4) was added to the second aliquot (as described in section 2.4.18.2) and treated for 60 minutes. At the end of 60 minutes, the sample was topped up with sterile distilled water to reduce the action of the acid, centrifuged at 3000g for 18 minutes and the supernatant discarded. A volume of 2 ml of neutralising buffer solution at pH 6.8 (as previously) was added and 0.5 ml inoculated into both bottles A and B as previously described, and incubated for 28 days. As in the decontamination procedure for Gram-positive organisms, the surface of two neutral pyruvate LJ slopes and two RGM medium slopes were each

inoculated with 0.2 ml of specimen and incubated for eight weeks for LJ and three weeks for RGM medium at both 30°C and 37°C

In addition to this, two RGM medium slopes were inoculated with a 0.2 ml volume of untreated specimen. One was incubated at 30^{o}C and the other at 37^{o}C for three weeks. Fourteen control strains (Appendix 1) were inoculated into both bottle A and bottle B and incubated for 28 days.

2.4.18.5 Processing of clinical samples from non-cystic fibrosis patients

Non-CF patient samples that were pre-selected due to their AFB-positive status on microscopy were treated with the same procedure as above for Gram-positive organisms and 0.5 ml inoculated into a BacT/ALERT culture bottle with a further 0.5 ml inoculated into one BacT/ALERT culture bottle with prototype antibiotic mix, logged and incubated for 28 days. The surface of two neutral pyruvate LJ slopes were each inoculated with 0.2 ml of specimen and incubated for eight weeks at both 30°C and 37°C. RGM medium slopes were not included in non-CF patient samples as their purpose was solely to investigate the detection of RGM in CF patients.

2.4.19 Multi-centre study to examine the effectiveness and convenience of RGM medium

In order to obtain a more accurate representation of the selectivity and ease of use for RGM medium a multi-centre study performed in eight laboratories in six geographical locations was completed, with 2679 RGM plates being prepared and distributed in total.

RGM plates were prepared as in Appendix 2 and sent to the eight laboratories for analysis using sputum samples of CF patients. These were Aarhus University Hospital, Aarhus, Denmark (100), Centre de Biologie et Pathologie Est, Bron, France (50), Centre Hospitalier Lyon Sud, France (50), Kings College Hospital,

London, UK (200), Klinikum der Johann Wolfgang Goethe-Universität, Frankfurt, Germany (280), Papworth Hospital, Cambridge, UK (930), Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands (200) and UNC Healthcare, Chapel Hill, North Carolina, USA (869).

2.5 Results

2.5.1 Evaluation of basal media for optimal growth of *Mycobacterium abscessus* complex

From the data obtained, the sensitivity values of each of the nine media described in section 2.4.3 was calculated (Table 2-3).

Table 2-3: Sensitivity values of basal media.

	Medium	Sensitivity %				
		24 h	**48 h**	**72 h**	**96 h**	**168 h**
A	Middlebrook 7H9	0	0	0	0	0
B	Middlebrook with glycerol	0	0	0	25	95
C	Middlebrook with Tween-80	0	0	0	10	100
D	Middlebrook with glycerol and OADC	0	95	100	100	100
E	Middlebrook with Tween-80 and OADC	0	95	100	100	100
F	Columbia Agar	0	0	75	80	100
G	Columbia with OADC	0	70	100	100	100
H	Columbia with 5% horse blood	0	25	100	100	100
I	bioMérieux cepacia agar (44347)	0	20	90	95	95

One strain of *M. abscessus* subspecies *abscessus*, (isolate 1050) failed to grow on BCSA, and only a trace of isolate 1034, also *M. abscessus* subspecies *abscessus*, was visible on medium B after 168 h.

Figure 2-8 shows left (medium A) Middlebrook 7H9, centre (medium B) with the addition of glycerol, and right (medium D) with the addition of glycerol and Middlebrook OADC growth supplement. Substantially improved growth of mycobacteria on the medium containing glycerol and Middlebrook OADC growth supplement is clearly visible. Although medium D and medium E had identical sensitivity values throughout, growth of mycobacteria on medium D was enhanced

in comparison to medium E. Results also demonstrate the value of the addition of OADC supplement as shown in Figure 2-8.

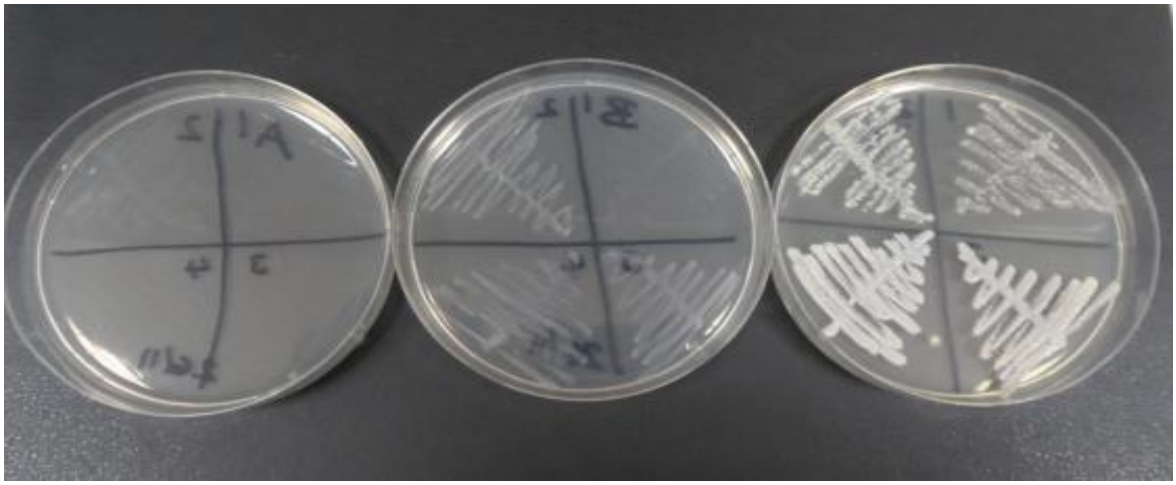


Figure 2-8: Growth of mycobacteria with and without OADC growth supplement after 168 h incubation

2.5.2 Evaluation of enrichment ingredients in basal media

After analysis of growth on all media after 168 h, medium D supported the strongest growth and shortest TTD and was therefore selected as the basal medium to which further supplements were evaluated to promote mycobacterial growth and further enhance the medium. The twenty strains of mycobacteria were inoculated onto each of the thirteen media and TTD in days and appearance of resulting colonies was recorded after 24, 48, 72, 96 and 168 h. Results suggested that medium D, with the addition of yeast extract (medium L), gave noticeably improved and more rapid growth of mycobacteria in comparison to medium D alone. (See Figure 2-9 below). After only 24 h incubation, a trace of growth was visible for 65% of strains (13/20) as opposed to 10% (2/20) on medium D, demonstrating that yeast extract was a useful ingredient for the enhanced growth of mycobacteria.

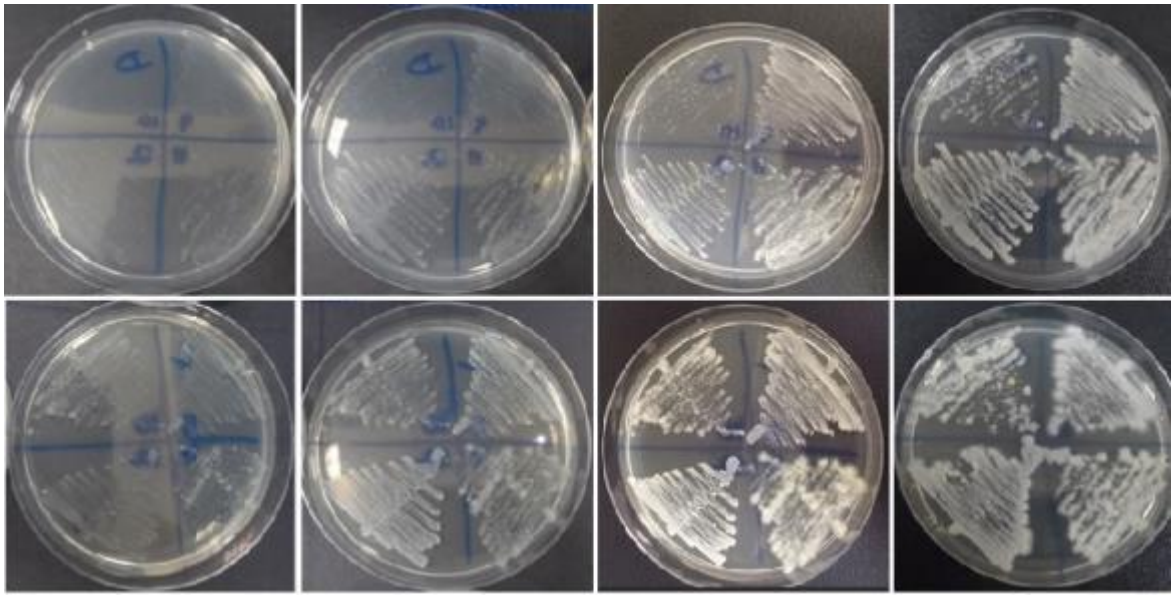


Figure 2-9: Comparison of the growth of mycobacteria on medium D, without yeast extract (top) and medium L (bottom) containing yeast extract at 48, 72, 96 and 168 h

2.5.3 Investigation of various antimicrobials for the inhibition of non-mycobacterial strains frequently encountered in sputum samples from patients with cystic fibrosis

Results as shown in Table 2-4 revealed that all non-mycobacterial isolates tested (excluding fungi) were inhibited by a combination of 32 mg/L colomycin, 400 mg/L fosfomycin, 5 mg/L amphotericin B and 128 mg/L C390 with the exception of *P. apista* and *P. pnomenusa*. However, growth of these two species was substantially reduced. Thirty-five percent of the mycobacteria tested were inhibited by the higher concentration of C390, but no inhibition was observed using C390 at 32 mg/L. At the lower concentration of 32 mg/L C390, *Burkholderia* and *Pandoraea* species were able to grow, although both displayed significantly reduced growth and for the majority of isolates of these species just a trace of growth was visible.

Isolate 1050, *M. abscessus* subspecies *abscessus* was susceptible to C390 and vancomycin at all concentrations. Malachite green was inhibitory to 35% of mycobacteria at the lowest concentration of 0.125 g/L. Vancomycin caused a reduction in the growth of 30% of mycobacteria and only inhibited 2% of *Pseudomonas* spp. and 35% of other species at the highest concentration.

Nalidixic acid had no effect on the growth of mycobacteria, however only inhibited 42% of non-mycobacterial species at the highest concentration (64 mg/L). Amphotericin B had no inhibitory effects on mycobacteria, but did not inhibit *Aspergillis terreus*, *Scedosporium apiospermum* and *Scedosporium prolificans* at any concentration, and *Geosmithia argillacea* was only inhibited at 20 mg/L. Colomycin inhibited 92% of *Pseudomonas* spp. at the lowest concentration, and 33% of other non-mycobacterial species, with no significant effect upon the growth of mycobacteria. Fosfomycin had no inhibitory effects on the mycobacteria at all concentrations tested, and inhibition of *Pseudomonas* spp was 81%, 86% and 93% and other non-mycobacteria species were inhibited at a rate of 65%, 73% and 73% at concentrations 400 mg/L, 800 mg/L and 1600 mg/L respectively.

Table 2-4: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30ºC using various antimicrobials in agar-based media

Ref No.	MABSC Subspecies	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
1034	*M. abscessus*	+++	+++	++	+++	+++	+++	++	++	+	++
1042	*M. abscessus*	+++	++	+++	+++	+++	+++	++	++	++	+
1044	*M. abscessus*	+++	+++	+++	+++	+++	+++	+++	+++	+++	++
1045	*M. abscessus*	++	++	++	++	++	++	++	++	++	++
1047	*M. abscessus*	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
1050	*M. abscessus*	++	++	++	++	++	++	++	NG	NG	NG
1051	*M. abscessus*	++	++	++	++	++	++	++	++	++	+
1052	*M. abscessus*	++	++	+++	+++	+++	++	++	++	++	+
1053	*M. abscessus*	++	++	++	++	++	++	++	+	+	+
1054	*M. abscessus*	++	++	++	++	++	++	++	+	+	+
1055	*M. abscessus*	+++	+++	+++	+++	+++	++	+++	+++	++	++
1000	MABSC - (chimeric)	++	++	++	++	++	+++	++	++	+	+
3016	*M. bolletii*	+++	++	++	++	++	++	++	++	++	+
3017	*M. bolletii*	+++	++	+	++	++	++	++	+	++	+
3015	*M. massiliense*	+++	+++	++	++	+++	+++	+++	+++	+++	+++
3010	*M. massiliense*	+++	+	++	++	++	++	++	+	+	+/-
3011	*M. massiliense*	+++	++	+++	+++	+++	+++	+++	++	++	++
3012	*M. massiliense*	++	++	++	++	++	++	++	+	++	+
3013	*M. massiliense*	++	++	+	++	++	++	++	+	++	++
3014	*M. massiliense*	++	+++	++	++	++	++	++	++	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	MABSC Subspecies	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
1034	*M. abscessus*	++	++	+	+++	+++	++	+++	+++	+++	++	+++	+++
1042	*M. abscessus*	+++	+++	++	+++	+++	++	+++	+++	+++	++	+++	+++
1044	*M. abscessus*	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
1045	*M. abscessus*	++	++	+++	++	++	++	+++	++	++	+	++	++
1047	*M. abscessus*	+++	++	++	+++	+++	+++	+++	+++	+++	++	+++	+++
1050	*M. abscessus*	++	+	+	NG	NG	NG	++	++	++	NG	++	++
1051	*M. abscessus*	++	++	++	++	++	++	++	++	++	+	++	++
1052	*M. abscessus*	++	++	++	++	+++	+++	++	++	++	NG	++	++
1053	*M. abscessus*	++	++	++	++	++	++	++	++	++	+	++	++
1054	*M. abscessus*	++	++	++	++	+++	+++	++	++	++	+	++	++
1055	*M. abscessus*	+++	++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
1000	MABSC - (chimeric)	++	++	++	++	++	+++	++	++	++	+	++	++
3016	*M. bolletii*	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	++	++
3017	*M. bolletti*	+	++	+++	+++	++	++	+++	++	+++	NG	+	++
3015	*M. massiliense*	+++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++
3010	*M. massiliense*	+	++	++	++	+	++	++	++	+++	NG	+	++
3011	*M. massiliense*	++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	+++
3012	*M. massiliense*	++	++	++	+++	+++	+++	+++	++	++	+	++	++
3013	*M. massiliense*	++	++	++	++	++	++	++	++	++	+	++	++
3014	*M. massiliense*	++	+	+	++	++	+++	+++	+++	++	+	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
	Gram-negatives										
8001	*A. xylosoxidans*	+	+	+	+	+	+	+	+	+	+
8003	*Acinetobacter* sp.	+	+/-	+/-	+/-	+/-	+/-	+/-	NG	NG	NG
8004	*B. multivorans*	+++	+++	++	+++	+++	+/-	+	++	++	+++
8006	*B. cenocepacia* IIIA	+	+	+	+	+	+	+	+	+	+
8007	*B. cepacia* (G1)	+++	+++	+++	+++	+++	+	+/-	++	++	+++
8008	*B. contaminans*	+	+	+	+	+	+	+	+	+	+
8032	*C. freundii*	+	-	-	-	NG	NG	NG	+	+	+
9007	*E. aerogenes*	+++	-	-	-	+++	NG	NG	+++	+++	+++
8038	*E. cloacae*	+++	-	-	-	+++	NG	NG	+++	+++	+++
8036	*E. coli*	+	-	-	-	++	NG	NG	+	+	+
9010	*H. influenzae*	NG	-	-	-	NG	NG	NG	NG	NG	NG
9009	*M. catarrhalis*	NG	-	-	-	NG	NG	NG	NG	NG	NG
9008	*N. flavescens*	+/-	-	-	-	+/-	+/-	NG	+/-	+/-	+/-
8009	*P. aeruginosa*	+++	+++	++	++	++	+	+/-	++	++	++
8010	*P. aeruginosa*	+	++	+	+	+	+	NG	+	+/-	+/-
8014	*P. aeruginosa*	+	+	+	+	+	+/-	NG	+	+/-	+/-
8015	*P. aeruginosa*	+	+	+	+	+	+	NG	+	+	+
8016	*P. aeruginosa*	+	+	+	+	+	+	NG	+	+	+
8018	*P. aeruginosa*	+	+	+	+	+	+	+/-	+	+	+
8019	*P. aeruginosa*	+	+	+	+	+/-	+	NG	+	+/-	+/-

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
7037	*P. aeruginosa*	+	+	+	+	+	+	+/-	+	+	+
7038	*P. aeruginosa*	+/-	+/-	+/-	+/-	+/-	+/-	NG	+	+/-	+/-
7039	*P. aeruginosa*	+	+	+	+	+	+	+/-	+	+	+
7040	*P. aeruginosa*	+	+	+	+	+	+	NG	Tr	+	+
7041	*P. aeruginosa*	++	++	++	+++	+	++	NG	++	+	+
7042	*P. aeruginosa*	+++	++	++	++	+	++	NG	++	++	++
7043	*P. aeruginosa*	+++	++	++	++	+	+	NG	+	++	++
7044	*P. aeruginosa*	++	++	++	++	+	+	+	+	+	+
7045	*P. aeruginosa*	++	++	++	++	+	NG	NG	+	+	++
7046	*P. aeruginosa*	+	++	++	++	+	NG	+	+	+	++
7047	*P. aeruginosa*	+	++	++	++	+	NG	+	++	+	++
7048	*P. aeruginosa*	++	++	++	++	+	+	+	++	++	+++
7049	*P. aeruginosa*	+	++	+	+	+/-	+	+	++	+	+
7050	*P. aeruginosa*	+	++	+	+	+	+	Tr	++	+	+
7051	*P. aeruginosa*	+	+	+	+	+	+	Tr	++	+	+
7052	*P. aeruginosa*	++	++	++	++	+	++	Tr	++	++	++
7053	*P. aeruginosa*	++	++	++	++	+	+	NG	++	++	++
7054	*P. aeruginosa*	++	++	++	++	+	+	Tr	++	++	+++
7055	*P. aeruginosa*	++	++	++	++	+	+	Tr	++	++	++
7056	*P. aeruginosa*	++	++	++	++	+	+	+	++	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
7057	*P. aeruginosa*	+	+	+	+	++	NG	NG	+	++	++
7058	*P. aeruginosa*	+	+	+	+	+	NG	+	+/-	+/-	+/-
7059	*P. aeruginosa*	+	+	+	+	+/-	NG	+/-	+	+/-	+/-
7060	*P. aeruginosa*	+/-	+/-	+/-	+/-	+/-	Tr	NG	+	+/-	Tr
7061	*P. aeruginosa*	++	++	++	++	+	+	++	+	+	+
7062	*P. aeruginosa*	++	+++	++	++	++	+	+	++	+	+
7063	*P. aeruginosa*	++	++	++	+	+	NG	NG	++	+	+
7064	*P. aeruginosa*	+	+	+	++	+	+	+	+	+	+
7065	*P. aeruginosa*	+	+	+	++	+	+	+	+	+	+
7066	*P. aeruginosa*	+	+	+	+	+	+	+/-	+	+	+
7067	*P. aeruginosa*	+	++	+	+	++	+	+	+	+	+
7068	*P. aeruginosa*	++	++	+	+	++	+	+	++	+	+
7069	*P. aeruginosa*	++	++	+	+	++	+	+	+	++	++
7070	*P. aeruginosa*	++	++	+	+	+	+	+	+	+	+
7071	*P. aeruginosa*	++	++	+	+	+	+	+	+	+	+
7072	*P. aeruginosa*	++	++	+	+	+	+	+	++	+	+
7073	*P. aeruginosa*	++	++	+	+	++	+	+	++	++	++
7074	*P. aeruginosa*	++	++	++	+	++	+	+	++	+	+
7075	*P. aeruginosa*	++	+	+	+	+	+	+/-	++	+	+
7076	*P. aeruginosa*	++	+	+	+	+	+	+/-	++	++	++
7077	*P. aeruginosa*	+	++	++	++	+	+	+	++	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
7078	*P. aeruginosa*	+	+	+	+	+	+	+	++	++	++
7079	*P. aeruginosa*	+	++	++	++	++	++	++	++	++	+++
8020	*P. apista*	+	+	+	+	+	+	+	+	+	+
8021	*P. pnomenusa*	+	+	+	+	+	+	+	+	+	+
8031	*P. rettgeri*	+	-	-	-	NG	NG	NG	+	+/-	+
8037	*R. planticola*	++	-	-	-	NG	NG	NG	++	++	++
8022	*S. maltophilia*	+	+	+	+	+	+/-	Tr	+	+	+
8023	*S. maltophilia*	+	+	+	+	+	+/-	+/-	+	+	+
8039	*S. marcescens*	++	-	-	-	+	NG	NG	++	++	++
	Gram-positives										
9006	*B. subtilis*	+	-	-	-	NG	NG	NG	NG	NG	NG
9003	*E. faecalis*	+/-	-	-	-	+/-	+/-	+/-	NG	NG	NG
9005	*E. faecium*	+/-	-	-	-	+/-	+/-	+/-	NG	NG	NG
9001	*S. aureus*	+	-	-	-	+	NG	NG	NG	NG	NG
9002	*S. aureus* (MRSA)	+	-	-	-	+	+	+	NG	NG	NG
9004	*S. epidermidis*	+	-	-	-	+	Tr	NG	NG	NG	NG
8035	*S. gordinii*	Tr	-	-	-	Tr	Tr	+/-	NG	NG	NG
8040	*S. pneumoniae*	Tr	-	-	-	Tr	Tr	Tr	NG	NG	NG
8033	*S. pyogenes*	+/-	-	-	-	+/-	+/-	+/-	NG	NG	NG
8034	*S. salivarius*	+	-	-	-	+	+	+	NG	NG	NG

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30ºC using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Control	Amphotericin B (mg/L)			Nalidixic Acid (mg/L)			Vancomycin (mg/L)		
			5	10	20	16	32	64	2.5	5	10
	Fungi and Yeast										
9013	*A. fumigatus**	+++	+/-	NG	NG	-	-	-	-	-	-
9014	*A. fumigatus***	+++	+/-	NG	NG	-	-	-	-	-	-
9015	*A. terreus*	+++	+++	+++	+++	-	-	-	-	-	-
9001	*C. albicans*	+++	NG	NG	NG	-	-	-	-	-	-
9012	*C. glabrata*	+++	NG	NG	NG	-	-	-	-	-	-
9018	*G. argillacea*	+++	Tr	+/-	NG	-	-	-	-	-	-
9016	*S. apiospermum*	++	+++	+++	+++	-	-	-	-	-	-
9017	*S. prolificans*	++	+++	+++	+++	-	-	-	-	-	-

*3 colonies on Day 7 at 5mg/L **4 colonies on day 7 at 5mg/L

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
	Gram-negatives												
8001	*A. xylosoxidans*	+	+	+	+/-	NG	NG	+	+	+	+	+	+
8003	*Acinetobacter* sp.	+	+/-	+/-	NG	NG	NG	NG	NG	NG	+	+/-	+/-
8004	*B. multivorans*	++	+	+	NG	NG	NG	++	++	++	++	++	+++
8006	*B. cenocepacia* IIIA	+	+	+/-	Tr	Tr	NG	+	+	+/-	+	+	+
8007	*B. cepacia* (G1)	+++	++	+	Tr	Tr	NG	+	+	+	++	++	++
8008	*B. contaminans*	+	+	+	Tr	NG	NG	+	+	+	+	+	+
8032	*C. freundii*	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	+	+
9007	*E. aerogenes*	NG	NG	NG	NG	NG	NG	++	NG	NG	++	++	++
8038	*E. cloacae*	++	++	+	NG	NG	NG	+++	+++	+	+++	+++	+++
8036	*E. coli*	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	+	+
9010	*H. influenzae*	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
9009	*M. catarrhalis*	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
9008	*N. flavescens*	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG	+/-	+/-
8009	*P. aeruginosa*	NG	NG	NG	+	+	NG	NG	NG	NG	++	++	++
8010	*P. aeruginosa*	+	+/-	Tr	NG	NG	NG	+/-	NG	NG	+/-	+	+
8014	*P. aeruginosa*	+/-	+/-	+/-	+/-	NG	NG	NG	NG	NG	+	+	+
8015	*P. aeruginosa*	Tr	Tr	Tr	NG	NG	NG	+/-	Tr	Tr	+/-	+	+
8016	*P. aeruginosa*	+/-	+/-	+/-	+	+	NG	NG	NG	NG	+	+	+
8018	*P. aeruginosa*	NG	NG	NG	+	+/-	NG	+	+	+	+	+	+

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
8019	*P. aeruginosa*	+/-	Tr+	Tr	+/-	+/-	Tr	NG	NG	NG	+/-	+	+
7037	*P. aeruginosa*	NG	NG	NG	+	+	NG	+/-	+/-	+/-	+	+	+
7038	*P. aeruginosa*	NG	NG	NG	+/-	Tr	NG	+/-	+/-	Tr	+/-	+/-	+/-
7039	*P. aeruginosa*	NG	NG	NG	+	+	+/-	+	+	+/-	+	+	+
7040	*P. aeruginosa*	NG	NG	NG	+	+	+	+	+/-	NG	+	+	+
7041	*P. aeruginosa*	NG	NG	NG	+	+	+	+/-	NG	NG	+	+	+
7042	*P. aeruginosa*	NG	NG	NG	+	++	+	NG	NG	NG	+	+	++
7043	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	++
7044	*P. aeruginosa*	NG	NG	NG	++	+	NG	NG	NG	NG	++	++	++
7045	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	++	++	++
7046	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	++	++	++
7047	*P. aeruginosa*	NG	NG	NG	+	+	Tr	NG	NG	NG	++	++	++
7048	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	+++
7049	*P. aeruginosa*	NG	NG	NG	+	+/-	NG	Tr	NG	NG	+	+	+
7050	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	+
7051	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	+
7052	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	++
7053	*P. aeruginosa*	NG	NG	NG	++	+	+	Tr	NG	NG	++	++	++
7054	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	++	++	++
7055	*P. aeruginosa*	NG	NG	NG	+	+	NG	+	+	+	++	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
7056	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	++
7057	*P. aeruginosa*	NG	NG	NG	+	+	NG	Tr	NG	NG	+	+	++
7058	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	+	+	+
7059	*P. aeruginosa*	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	+	+
7060	*P. aeruginosa*	NG	NG	NG	NG	NG	NG	Tr	NG	NG	Tr	+/-	+/-
7061	*P. aeruginosa*	NG	NG	NG	++	+++	+++	NG	NG	NG	+	++	++
7062	*P. aeruginosa*	NG	NG	NG	+	++	+	NG	NG	NG	++	++	++
7063	*P. aeruginosa*	NG	NG	NG	+	+	+	+/-	NG	NG	+	+	+
7064	*P. aeruginosa*	NG	NG	NG	+	+	+	+	+/-	NG	+	+	+
7065	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	+
7066	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	+	+	+
7067	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	+
7068	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	++
7069	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	++
7070	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	++	++
7071	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	++
7072	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	+	+	++
7073	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	+++
7074	*P. aeruginosa*	NG	NG	NG	+	+	NG	NG	NG	NG	+	++	++
7075	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	+	++	++

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
7076	*P. aeruginosa*	NG	NG	NG	++	+	+/-	NG	NG	NG	+	++	++
7077	*P. aeruginosa*	NG	NG	NG	++	+	+	NG	NG	NG	++	++	++
7078	*P. aeruginosa*	NG	NG	NG	+	+	+	NG	NG	NG	++	++	+++
7079	*P. aeruginosa*	NG	NG	NG	+	+	+/-	NG	NG	NG	++	++	++
8020	*P. apista*	+	+	+	+	+	Tr	+	+	+/-	+	+	+
8021	*P. pnomenusa*	+	+	+	+/-	+/-	Tr	+	+	+/-	+	+	+
8031	*P. rettgeri*	+	+	+	NG	NG	NG	NG	NG	NG	+	+	+
8037	*R. planticola*	NG	NG	NG	NG	NG	NG	+++	+	+	+	++	++
8022	*S. maltophilia*	+	+	+	Tr	NG	NG	NG	NG	NG	+	+	+
8023	*S. maltophilia*	+	+	+	NG	NG	NG	NG	NG	NG	+	+	+
8039	*S. marcescens*	+	+	+	NG	NG	NG	NG	NG	NG	+	+	+
	Gram-positives												
9006	*B. subtilis*	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	+	++
9003	*E. faecalis*	+/-	+	+	NG	NG	NG	NG	NG	NG	+/-	+/-	+/-
9005	*E. faecium*	+/-	+/-	+/-	NG	NG	NG	NG	NG	NG	+/-	+/-	+/-
9001	*S. aureus*	NG	NG	NG	NG	NG	NG	NG	NG	NG	+	+	NG
9002	*S. aureus* (MRSA)	+	+	+	NG	NG	NG	NG	NG	NG	+	+	+
9004	*S. epidermidis*	+	NG	NG	NG	NG	NG	NG	NG	NG	+	+	+
8035	*S. gordinii*	+/-	+/-	+/-	NG	NG	NG	NG	NG	NG	Tr	Tr	+/-
8040	*S. pneumoniae*	NG	NG	NG	NG	NG	NG	NG	NG	NG	Tr	Tr	Tr

Table 2-4 continued: Investigation of the growth of NTM and inhibition of non-NTM isolates after seven days of incubation at 30°C using various antimicrobials in agar-based media

Ref No.	Non-mycobacteria Species	Colomycin (mg/L)			C390 (mg/L)			Fosfomycin (mg/L)			Malachite Green (mg/L)		
		32	64	128	32	64	128	400	800	1600	0.125	0.25	0.5
8033	*S. pyogenes*	+/-	+/-	NG	NG	NG	NG	NG	NG	NG	+/-	+/-	+/-
8034	*S. salivarius*	+	+	+	NG	NG	NG	NG	NG	NG	+	+	+
	Fungi and Yeast												
9013	*A. fumigatus**	-	-	-	-	-	-	-	-	-	-	-	-
9014	*A. fumigatus***	-	-	-	-	-	-	-	-	-	-	-	-
9015	*A. terreus*	-	-	-	-	-	-	-	-	-	-	-	-
9001	*C. albicans*	-	-	-	-	-	-	-	-	-	-	-	-
9012	*C. glabrata*	-	-	-	-	-	-	-	-	-	-	-	-
9018	*G. argillacea*	-	-	-	-	-	-	-	-	-	-	-	-
9016	*S. apiospermum*	-	-	-	-	-	-	-	-	-	-	-	-
9017	*S. prolificans*	-	-	-	-	-	-	-	-	-	-	-	-

2.5.4 Investigation of combined antimicrobials for the inhibition of non-mycobacterial strains present in cystic fibrosis sputum

Out of 100 isolates of mycobacteria, one was excluded due to contamination with *C. albicans.* Another isolate, *M. intracellulare* did not grow on any of the media formulations within seven days, however this was not unexpected as *M. intracellulare* is known to be a slow-growing species, and usually has a TTD of 28 days on LJ medium (Wasilauskas and Morrell, 1994). After 168 h incubation both medium L and medium W had a sensitivity value of 99.0% (98/99), and medium X (RGM), 98.0% (97/99), however these values would rise to 100% and 99.0% if *M. intracellulare* was discounted.

Inhibition of 93.8% and 85.6% of all non-mycobacterial species tested was demonstrated by RGM, and medium W respectively. Six species; *Achromobacter* sp, *B. cepacia, P. apista, P. pnomenusa, B. contaminans* and *B. stabilis*, remained uninhibited on RGM medium although growth was significantly reduced. Fungi that were uninhibited by amphotericin B, and able to grow on media L and W were inhibited on RGM medium when combined with C390 as shown in Figure 2-10 with *A. terreus*. This was also the case for *Scedosporium apiospermum, Scedosporium prolificans*, and *Geosmithia argillacea.* All isolates grew well on control media.

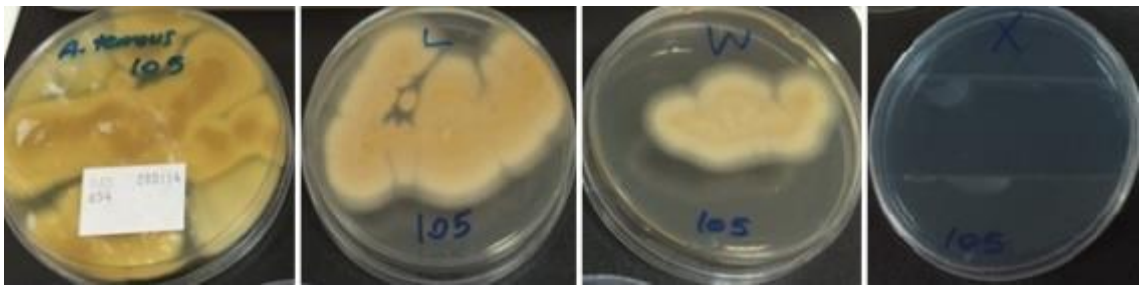


Figure 2-10: Growth of *Aspergillus terreus* on (left to right) Sabouraud control medium, Medium L, Medium W and RGM

Figure 2-10 shows the growth of *Aspergillus terreus* on Sabouraud control medium, medium L, the basal medium containing no antimicrobials showing slightly reduced growth, medium W, with only a small amount of growth and total inhibition on RGM medium, containing C390.

2.5.5 Evaluation of plating efficiency studies on RGM medium

On average, RGM medium was 84.0% as effective for the growth of mycobacteria as medium L. Two-sample t - tests gave a *P* value for medium L (control) and RGM medium as P=0.052. By conventional criteria, this difference is considered to be not statistically significant, indicating that there is no substantial difference between the two media for the growth of mycobacteria. Growth of colonies established that on RGM medium, colonies were marginally smaller than on medium L, but with regard to the numbers of colonies, the difference was minimal (Figure 2-11)

Following the addition of various antimicrobials into the medium, mycobacterial cell counts reduced from 225.3 ± 33.3 to 189.3 ± 28.8 (p >0.05); a decline of 36.0 ± 4.6. The probability of obtaining a difference of 36.0 between the mean colony counts, given that the media does not affect the counts, is 0.052, or 5.2%.

Figure 2-11: Plating efficiencies showing colonies on medium L (left) and RGM medium (right)

2.5.6 Evaluation of the stability of RGM medium

There was no reduction in the quality of growth of the four species of *Mycobacterium* after storage of RGM media at 4°C for 12 weeks and the medium maintained complete inhibition of the six non-mycobacteria and seven fungal and yeast isolates tested (See Appendix 1).

2.5.7 Evaluation of various C390 derivatives in RGM medium

Results from the testing of C390 derivatives shown in Table 2-5 and Figure 2-12 to Figure 2-14 showed that the MIC's of A477 were almost equivalent to C390 with the only significant difference being for *I. limosus*. A488 also performed well, but with slightly higher MIC's than A477. However, all other compounds tested were not able to provide sufficient inhibition of the Gram-negative species, displaying increased MICs for all isolates tested.

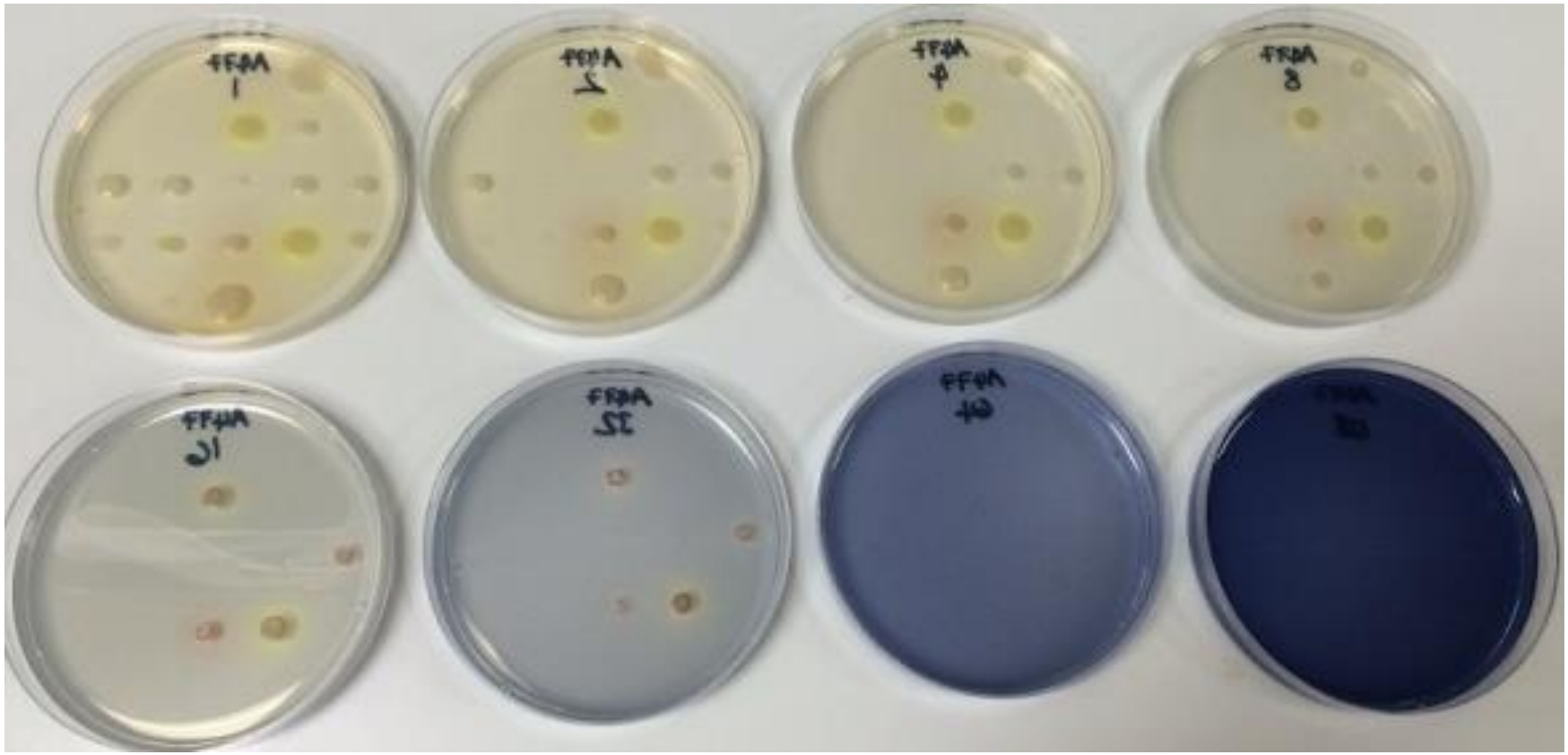


Figure 2-12: A477 at concentrations of 1 – 128 mg/L for twenty selected Gram-negative species after four days of incubation

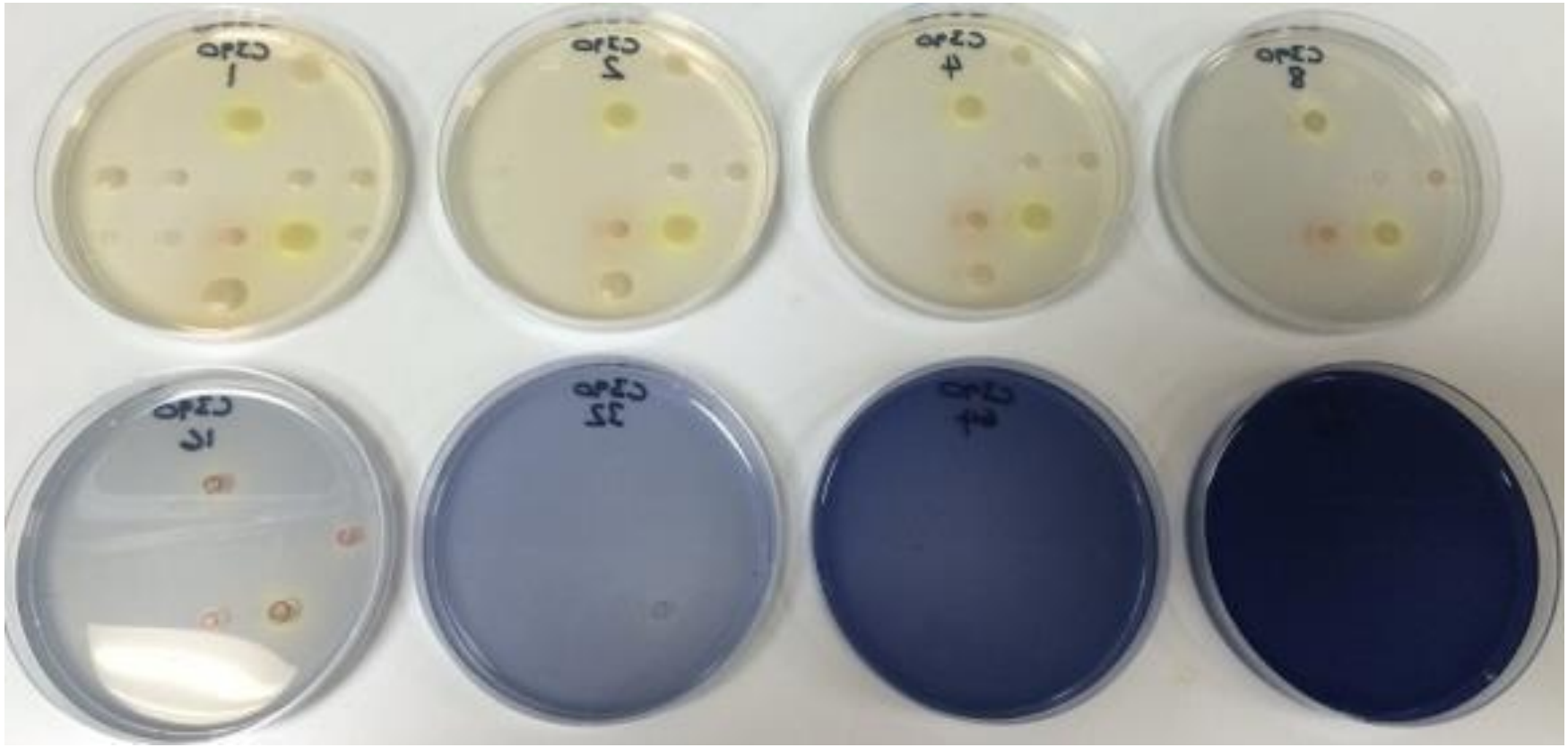


Figure 2-13: C390 at concentrations of 1 – 128 mg/L for twenty selected Gram-negative species after four days of incubation

Table 2-5: MIC's (mg/L) of a selection of Gram-negative bacteria for C390 and derivatives of C390 after ten days at 30ºC

Species	Ref	C390	A477	A475	A480a	A488	A503a	A503b	A505	A520
A. baumannii	NCTC 12156	4	4	4	128	8	128	>128	>128	>128
A. xylosoxidans	8001	32	64	>128	128	64	>128	>128	>128	>128
B. cepacia	NCTC 1222	8	8	>128	128	4	>128	>128	>128	>128
B. contaminans	8008	8	8	>128	128	8	>128	>128	>128	>128
B. multivorans	8004	8	8	>128	128	16	>128	>128	>128	>128
B. vietnamiensis	7032	4	4	4	128	2	64	>128	64	>128
C. albicans	ATCC 90029	≤1	≤1	≤1	>128	2	64	128	>128	>128
E. cloacae	NCTC 11936	16	16	>128	>128	32	>128	>128	>128	>128
E. coli	NCTC 10418	≤1	≤1	≤1	128	≤1	16	32	64	>128
E. faecalis	NCTC 775	≤1	≤1	2	>128	≤1	64	64	32	>128
I. limosus	7007	2	16	16	128	64	>128	>128	>128	>128
R. planticola	NCTC 9528	≤1	2	≤1	128	2	64	32	>128	>128
P. apista	8020	64	64	>128	128	128	>128	>128	>128	>128
P. aeruginosa	NCTC 10662	64	64	128	128	128	>128	>128	>128	>128
P. aeruginosa	7044	64	64	128	128	128	>128	>128	>128	>128
P. aeruginosa	8009	64	64	128	128	128	>128	>128	>128	>128
P. pnomenusa	8021	32	64	>128	128	64	>128	>128	>128	>128
S. aureus	NCTC 6571	≤1	≤1	≤1	128	≤1	16	32	8	32
S. maltophilia	NCTC 10257	≤1	≤1	≤1	128	≤1	16	32	32	>128
S. marcescens	7020	8	8	>128	>128	16	>128	>128	>128	>128

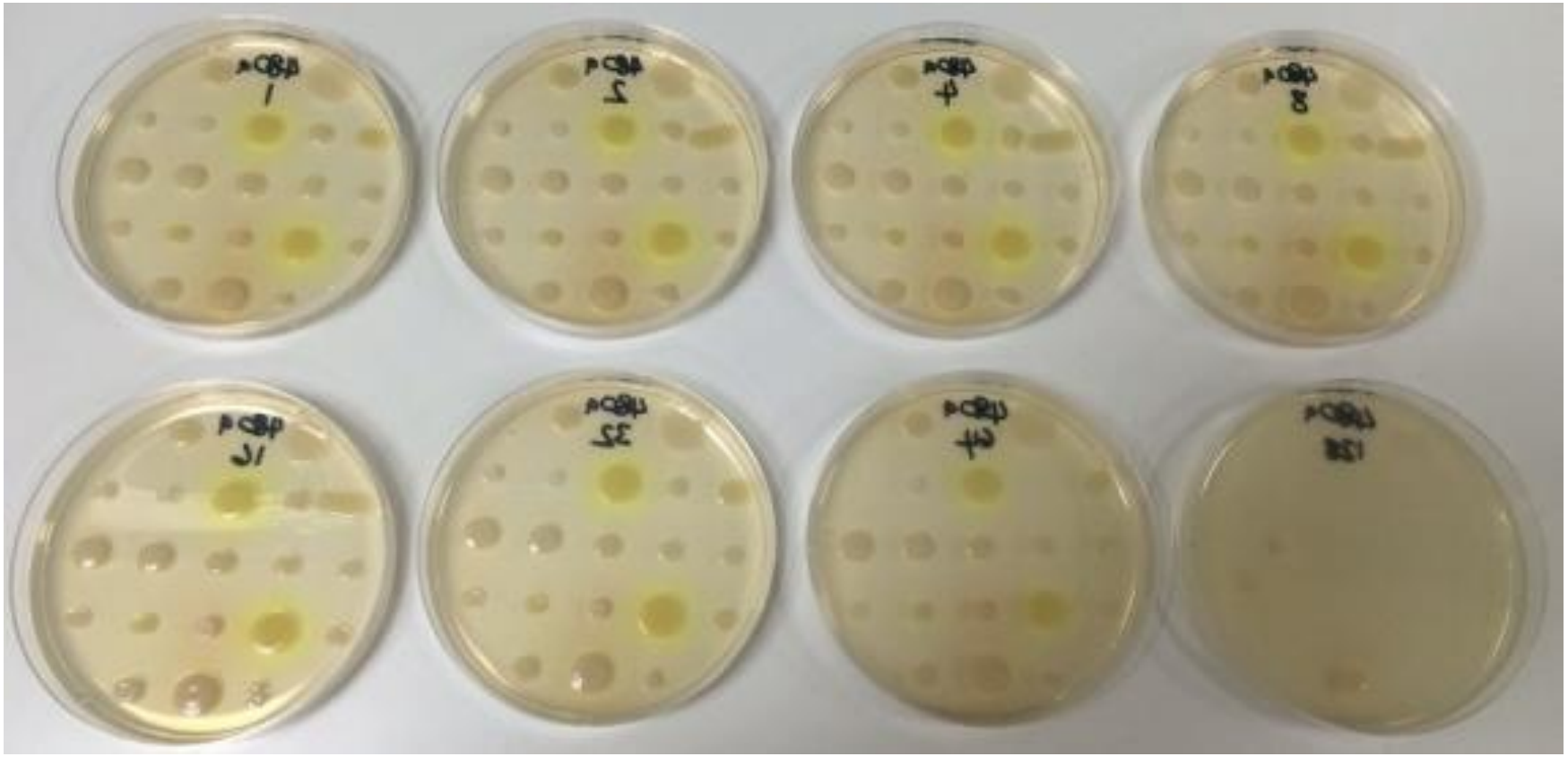


Figure 2-14: 480a at concentrations of 1 – 128 mg/L for twenty selected Gram-negative species after four days of incubation

These results demonstrated that A477, being locally synthesised by Dr Annette Johnson at Northumbria University, could be a more cost effective substitution for C390, working equally as well as a crucial ingredient in RGM medium. This could potentially be prepared in bulk for future large-scale studies.

2.5.8 Evaluation of selective agars in comparison to RGM for the growth of mycobacteria

Clear differences were revealed between the five different brands of BCSA in terms of their ability to support the growth of mycobacteria (Figure 2-15 below). For example, on Cepacia selective agar (bioMérieux) 95.9% of mycobacteria generated growth within four days of incubation compared with only 40.1% of isolates on Oxoid *B. cepacia* agar as shown in Table 2-6.

After ten days of incubation, ten isolates had failed to grow on Oxoid *B. cepacia* agar including MABSC (*n* = 4), *M. chelonae* (*n* = 3), *M*. *llatzerense* (*n* = 2) and *M. mucogenicum* (*n* = 1). All isolates were recovered on Cepacia selective agar (bioMérieux) whereas other brands of BCSA failed to support the growth of between four and eight isolates. All isolates were recovered on Middlebrook 7H11 selective agar and RGM medium with C390 at 32 mg/L. Two isolates (*M. abscessus* and *M. mucogenicum*) were inhibited on RGM medium with C390 at 64 mg/L, 128 mg/L and C390 at 32 mg/L with no OADC, and one isolate (*M. abscessus*) failed to grow on RGM medium using A477. Figure 2-15 shows the growth at day four of four isolates of *M. abscessus* subspecies *abscessus*.

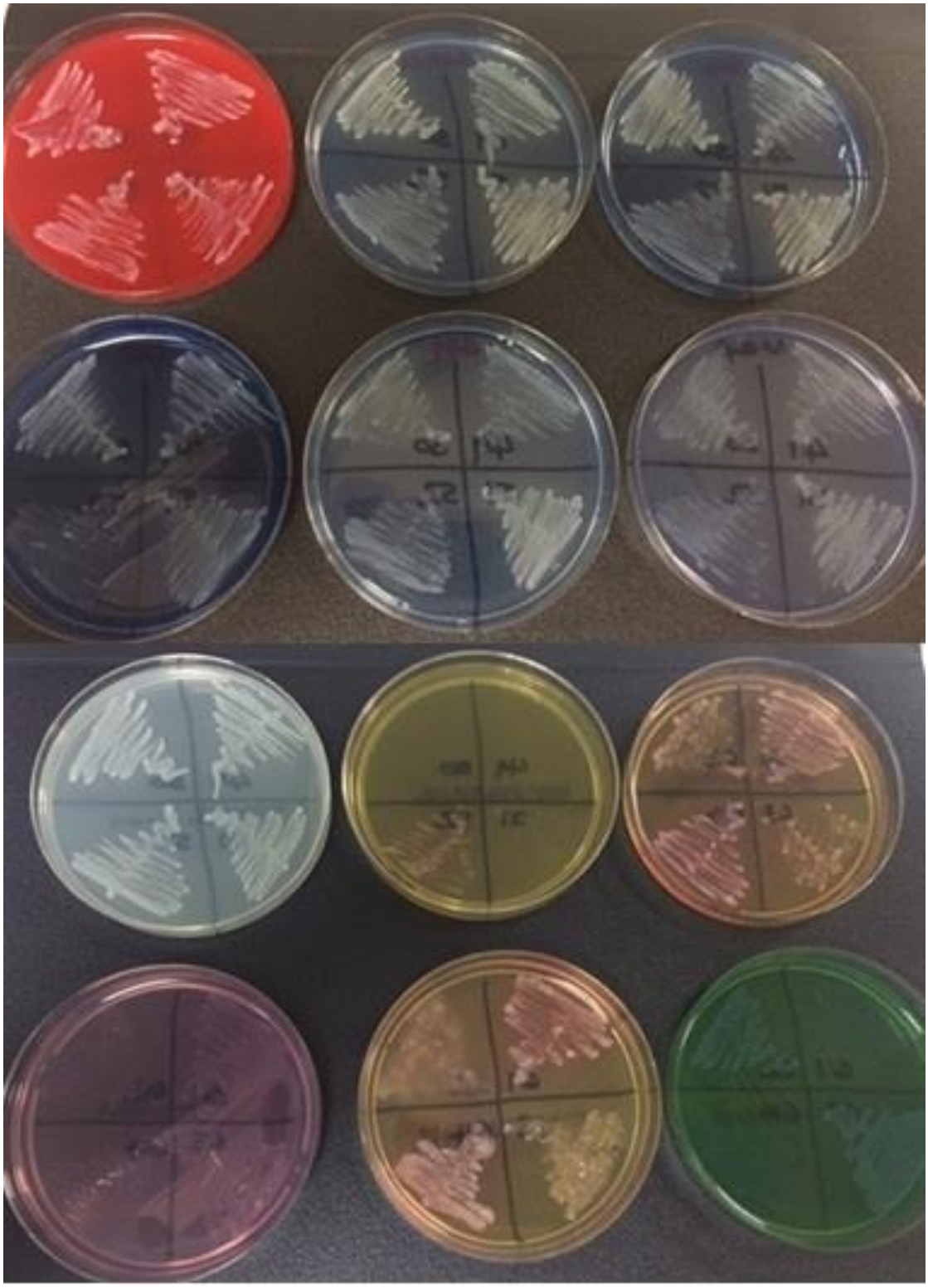


Figure 2-15: Growth at day four of four isolates of *M. abscessus* subspecies *abscessus* on various media. Columbia blood agar, RMG with 32 mg/L, C390 and RGM with 64 mg/L C390, (top row). RGM with C390 at 128 mg/L, RGM with A477 at 32 mg/L and RGM with 32 mg/L C390 minus OADC supplement (second row). Middlebrook, Oxoid cepacia, bioMérieux cepacia (third row), and bioMérieux BCSA, BD cepacia and BD OFPBL (bottom row)

Table 2-6: Percentage of mycobacteria recovered on various selective agars at 30ºC

		BCSA	Cepacia	B. cep	Cepacia	OFPBL	M'brook	RGM	RGM	RGM	RGM	RGM	Blood/Sab
	n	bioMérieux	bioMérieux	Oxoid	BD	BD	7H11	C390 32mg/L	C390 64mg/L	C390 128mg/L	A477 32mg/L	No OADC*	Controls
		33631	**44347**	**PO0938**	**256180**	**254481**	**PP4080**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**
MABSC	94												
Day 4		92.6	96.8	57.4	96.8	93.6	98.9	98.9	98.9	97.9	97.9	98.9	100
Day 7		98.9	98.9	91.5	98.9	98.9	98.9	98.9	98.9	98.9	98.9	98.9	100
Day 10		98.9	100	95.7	100	98.9	100	100	98.9	98.9	98.9	98.9	100
M. chelonae	43												
Day 4		97.7	100	9.3	95.3	100	100	100	100	100	100	100	100
Day 7		100	100	69.8	95.3	100	100	100	100	100	100	100	100
Day 10		100	100	93	97.7	100	100	100	100	100	100	100	100
Other NTM	10												
Day 4		10	70	10	70	40	70	90	30	30	30	60	100
Day 7		30	80	60	70	40	70	90	90	60	90	70	100
Day 10		30	100	70	70	70	100	100	90	90	100	90	100
Total NTM	147												
Day 4		88.4	95.9	40.1	94.6	91.8	97.3	98.6	94.6	93.9	93.9	96.6	100
Day 7		94.6	98	83	95.9	95.2	97.3	98.6	98.6	96.6	98.6	97.3	100
Day 10		94.6	100	93.2	97.3	97.3	100	100	98.6	98.6	99.3	98.6	100

*OADC: oleic acid, bovine serum albumin, dextrose, catalase

2.5.9 Evaluation of selective agars including RGM for the inhibition of non-mycobacteria

Table 2-7 and Figure 2-16 shown below provide an insight into the selectivity of the eleven selective media with 185 non-mycobacteria. Effective inhibition of *P. aeruginosa*, an essential characteristic of such media, was demonstrated on all five brands of BCSA. Inhibition of other species was more inconsistent however, and of 28 isolates of *S. aureus* (mainly methicillin-resistant strains (27/28)), 21 (75.0%) were able to grow on BD OFPBL medium whereas only three isolates were able to grow on Oxoid *B. cepacia* agar and bioMérieux BCSA. All brands of media for isolation of *B. cepacia* complex showed a poor ability to inhibit the growth of fungi and yeasts, particularly *Aspergillus* spp. Overall, bioMérieux BCSA displayed the greatest selectivity and BD OFPBL exhibited the weakest selectivity among the five brands tested.

Although Middlebrook selective medium is designed specifically for the isolation of mycobacteria from clinical samples, the growth of other non-mycobacterial species occurred frequently with 74 out of 186 (39.8%) isolates able to grow. Overall, it demonstrated inferior selectivity in comparison to the two most selective media for *B. cepacia* complex, although it was able to inhibit the growth of *Aspergillus fumigatus*. The various types of RGM medium were by far the most selective of all of the agars tested, with between 89.9% and 98.4% of non-mycobacteria inhibited including all fungi and Gram-positive bacteria. Species shown in Figure 2-16 include *Achromobacter* spp (*n* = 2), *Acinetobacter* (*n* = 1), BCC (*n* = 2), *P. aeruginosa* (*n* = 11), *C. freundii* (*n* = 1), *M. morganii* (*n* = 1) and *S. maltophilia* (*n* = 2).

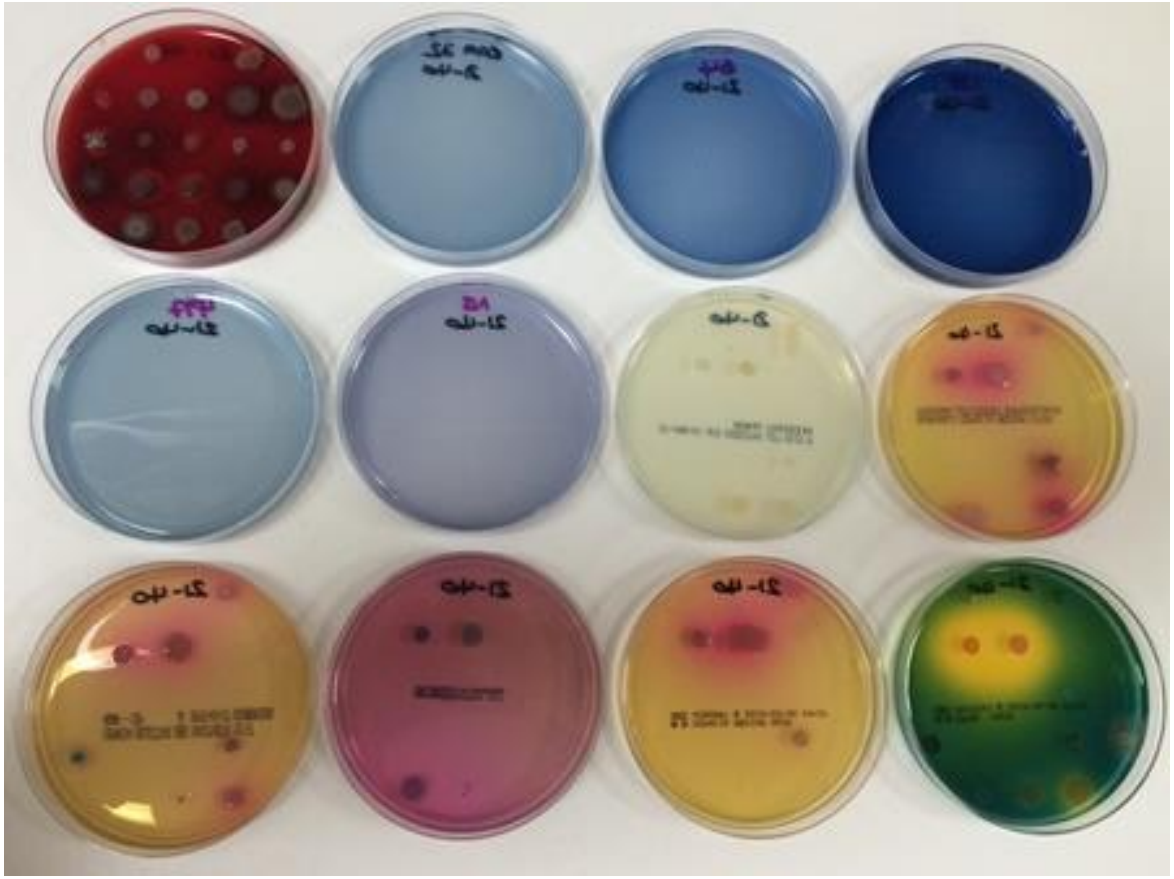


Figure 2-16: Growth of Gram-negative bacteria on various selective agars. Top row Columbia blood agar, RGM 32 mg/L C390, RGM 64 mg/L C390 and RGM 128 mg/L C390. Middle row RGM with A477 at 32 mg/L and RGM with 32 mg/L C390 minus OADC supplement, Middlebrook, Oxoid cepacia and bottom row bioMérieux cepacia and (bottom row) bioMérieux BCSA, BD cepacia and BD OFPBL

Table 2-7: Number of non-mycobacterial isolates recovered on various selective agars after 10 days at 30ºC

	n	**BCSA** bioMérieux **33631**	**Cepacia** bioMérieux **44347**	**B. cep** Oxoid **PO0938**	**Cepacia** BD **256180**	**OFPBL** BD **254481**	**M'brook** 7H11 **PP4080**	**RGM** C390 32mg/L **N/A**	**RGM** C390 64mg/L **N/A**	**RGM** C390 128mg/L **N/A**	**RGM** A477 32mg/L **N/A**	**RGM** No OADC* **N/A**
Gram Negatives	**141**	**56**	**61**	**56**	**60**	**73**	**64**	**18**	**6**	**3**	**19**	**11**
Enterobacteriaceae	11	2	0	2	2	6	1	0	0	0	0	0
A. xylosoxidans	8	3	3	3	5	8	3	2	0	0	2	0
Acinetobacter sp.	2	2	0	0	0	0	0	0	0	0	0	0
B. cepacia complex	43	37	40	36	37	41	39	12	4	2	13	8
D. acidovorans	1	1	0	0	0	1	0	0	0	0	0	0
E. miricola	1	1	1	1	0	1	1	0	0	0	0	0
H. influenzae	1	0	0	0	0	0	0	0	0	0	0	0
I. limosus	2	0	2	0	2	0	2	1	0	0	1	1
M. catarrhalis	1	0	0	0	0	0	0	0	0	0	0	0
N. flavescens	1	1	1	1	1	1	1	1	0	0	1	0
Ochrobactrum sp.	1	0	1	1	1	1	1	0	0	0	0	0
P. aeruginosa	55	0	2	1	2	2	2	0	0	0	0	0
Pandoraea spp.	3	3	3	3	3	3	3	2	2	1	2	2
R. mannitolilytica	7	6	6	6	6	5	7	0	0	0	0	0
S. maltophilia	4	0	1	1	0	3	3	0	0	0	0	0
Gram positives	**35**	**3**	**11**	**3**	**14**	**21**	**7**	**0**	**0**	**0**	**0**	**0**
B. subtilis	1	0	0	0	0	0	0	0	0	0	0	0
Enterococcus spp.	2	0	0	0	0	0	0	0	0	0	0	0
S. aureus	28	3	11	3	14	21	7	0	0	0	0	0
Streptococcus spp.	4	0	0	0	0	0	0	0	0	0	0	0
Yeast and Fungi	**9**	**5**	**8**	**9**	**8**	**8**	**3**	**0**	**0**	**0**	**0**	**0**
A. fumigatus	2	2	2	2	2	2	0	0	0	0	0	0
A. terreus	1	1	1	1	1	1	1	0	0	0	0	0
Candida spp.	3	2	3	3	3	3	1	0	0	0	0	0
G. argillacea	1	0	0	1	0	0	0	0	0	0	0	0
S. apiospermum	1	0	1	1	1	1	1	0	0	0	0	0
S. prolificans	1	0	1	1	1	1	0	0	0	0	0	0
Total	**185**	**64**	**80**	**68**	**82**	**102**	**74**	**18**	**6**	**3**	**19**	**11**
Total excluding *B. cepacia* complex	**142**	**27**	**40**	**32**	**45**	**61**	**35**	**6**	**2**	**1**	**6**	**3**

2.5.10 Evaluation of the effectiveness of RGM medium versus bioMérieux cepacia agar using clinical samples

Results with sputum samples showed that out of 502 samples tested from 210 distinct patients, 55 samples from 33 distinct patients yielded NTM giving an overall prevalence of 15.7%. Twenty-one of the 210 patients were colonised with MABSC (prevalence: 10%). The mycobacteria isolated from 55 samples comprised *M. abscessus* subsp. *abscessus* (56.4%), *M. abscessus* subsp. *massiliense* (20%), *M. chelonae* (10.9%) *M. avium* (3.6%), *M. llatzerense* (3.6%), *M. salmoniphilum* (3.6%) and *M. mucogenicum* (1.8%). Table 2-8 shows the numbers of each species recovered by the two media, with the calculation of sensitivity shown for comparative purposes only and assuming that all mycobacteria were recovered by a combination of the two methods. Evidently, this cannot be proven and furthermore might be considered highly improbable for species such as *M. avium* and other slower growing NTM. RGM medium enabled the detection of NTM from 54 of 55 positive samples whereas BCSA recovered NTM from 17 of 55 positive samples (sensitivity: 98% *vs*. 31%; $P \leq 0.0001$).

Table 2-8: Mycobacteria recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BSCA) and RGM medium

Species or subspecies	Total (either medium)	BCSA		RGM	
	n	*n*	Sensitivity (%)	*n*	Sensitivity (%)
M. abscessus subspecies *abscessus*	**31**	13	42	31	100
M. abscessus subspecies *massiliense*	**11**	3	33	11	100
M. chelonae	**6**	1	17	5	83
M. avium	**2**	0	0	2	100
M. llatzerense	**2**	0	0	2	100
M. salmoniphilum	**2**	0	0	2	100
M. mucogenicum	**1**	0	0	1	100
Total mycobacteria	**55**	**17**	**31**	**54**	**98**

For patients who had NTM found in their sputum (*n* = 33), 23 were detected using RGM medium only, one was detected using BCSA only (*M. chelonae*) and nine were detected using both media ($P \leq 0.0001$). After four days of incubation, 59% of mycobacteria were recovered on RGM medium in comparison to 35% of mycobacteria isolated on BCSA. No further isolates of NTM were isolated after seven days of incubation using BCSA.

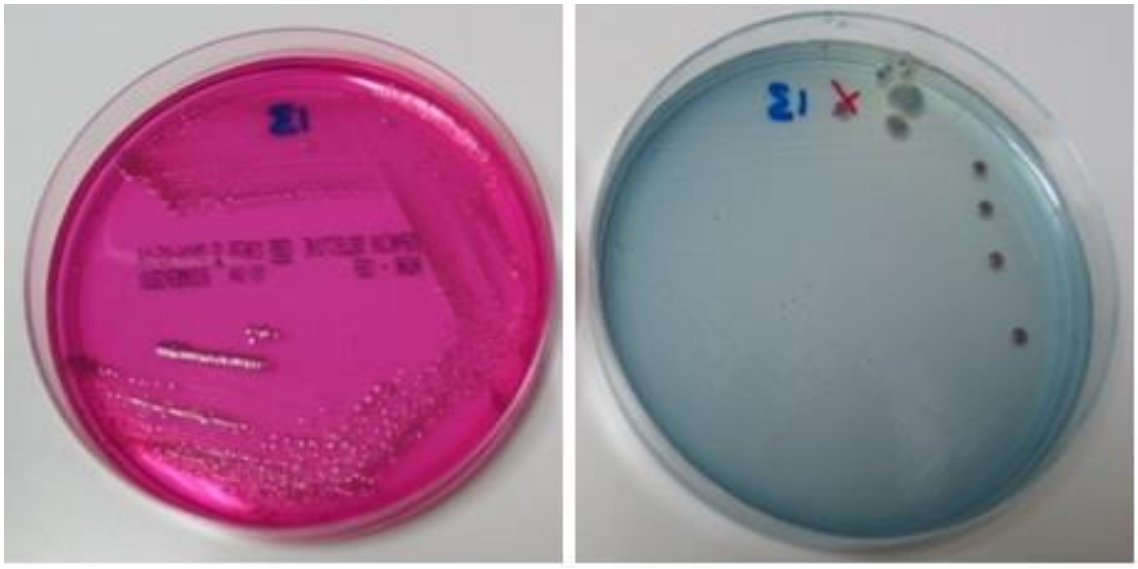


Figure 2-17: Patient sample at day 4 on BCSA (left) and RGM (right) demonstrating significantly reduced growth of *B. multivorans* on RGM

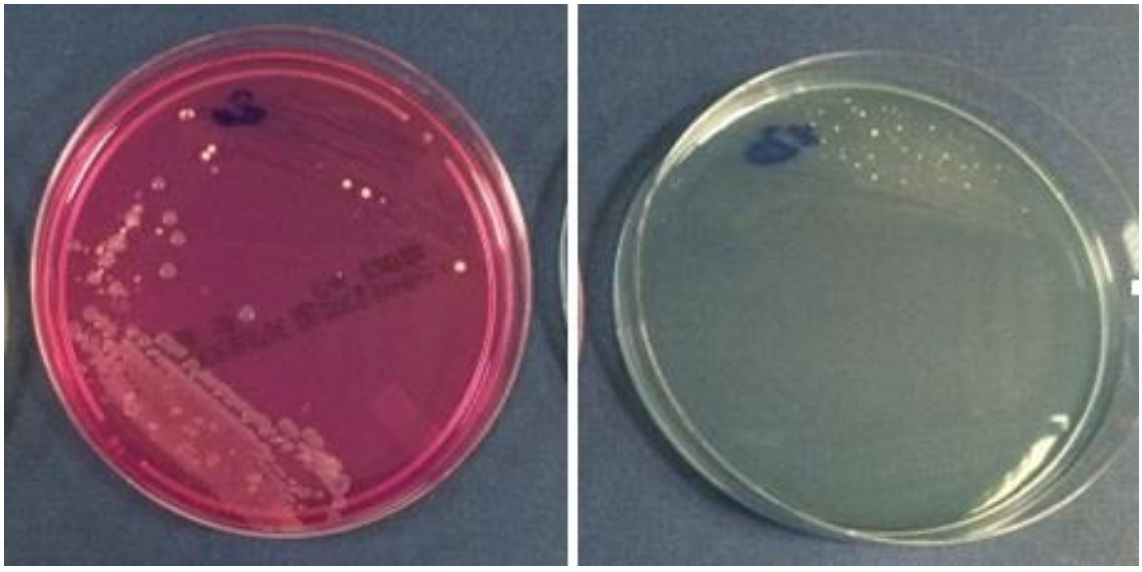


Figure 2-18: Patient sample at day 4 on BCSA (left) and RGM (right) showing growth of *C. parapsilosis* and *S. maltophilia* on BCSA, but only *M. abscessus* subspecies *abscessus* on RGM

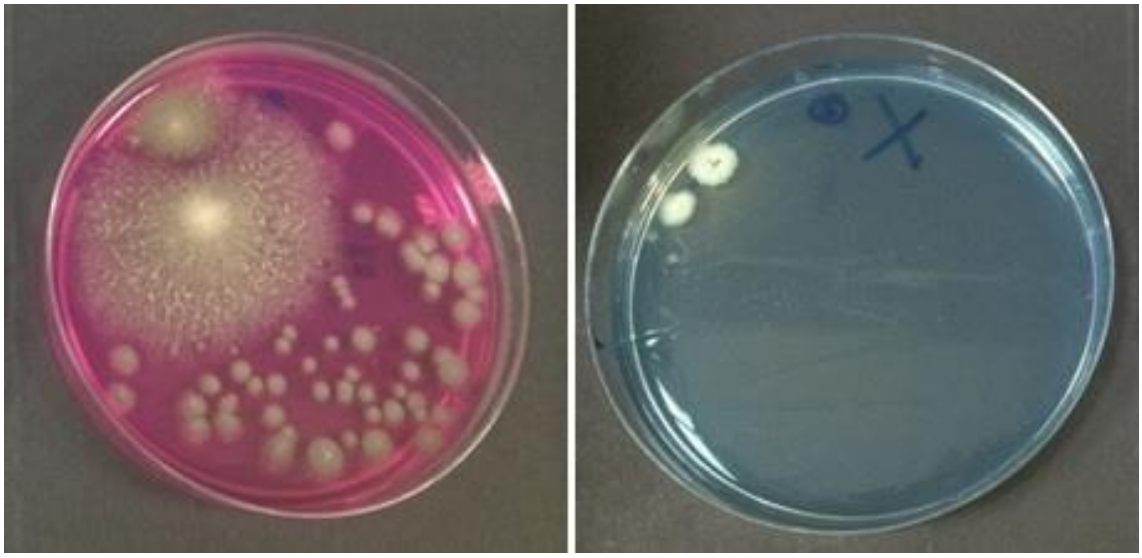


Figure 2-19: Patient sample at day 4 on BCSA (left) and RGM (right) showing *A. fumigatus* on BCSA and two rough colonies of *M. abscessus* subspecies *abscessus* on RGM

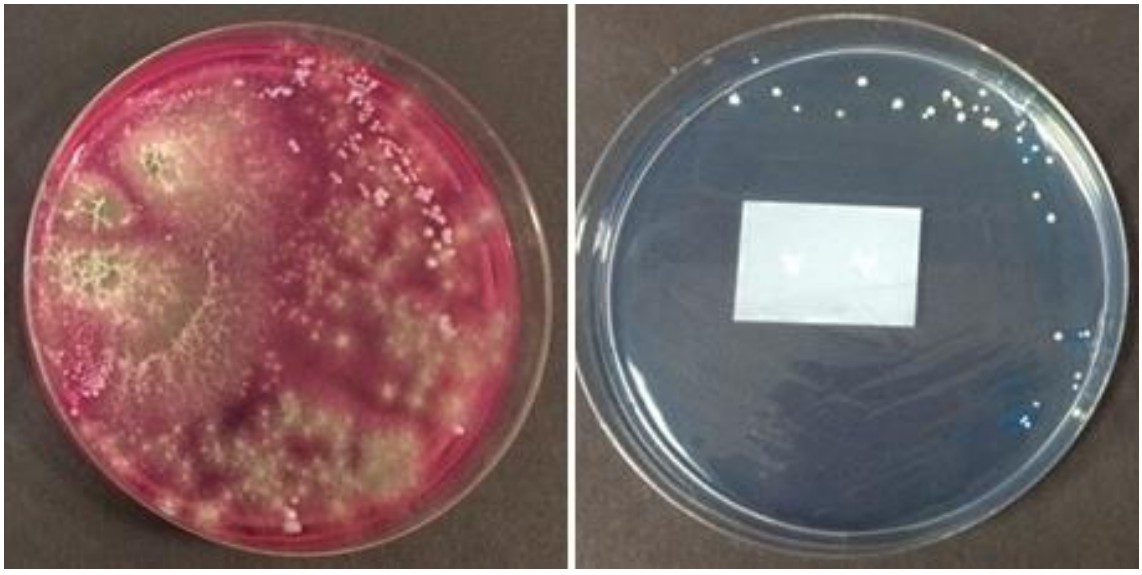


Figure 2-20: Patient sample at day 4 on BCSA (left) and RGM (right) showing growth of *A. fumigatus, C. albicans* and *C. lusitaniae* on BCSA, and only pure colonies of *M. abscessus* subspecies *massiliense* on RGM

Table 2-9: Other species recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BSCA) and RGM medium

	Number of isolates (*n*)	
	BCSA	**RGM**
Fungi and Yeasts	**226**	**0**
Arthrographis kalrae	2	0
Aspergillus fumigatus	69	0
Aspergillus terreus	6	0
Yeasts (Grams stain)	121	0
Exophiala dermatitidis	23	0
Scedosporium apiospermum	5	0
Gram-negative bacteria	**136**	**46**
Pseudomonas spp.	32	2
Burkholderia cepacia complex	30	18
Stenotrophomonas maltophilia	24	0
Achromobacter spp.	21	18
Enterobacteriaceae	14	2
Inquilinus limosus	4	2
Ochrobactrum spp.	4	0
Pandoraea spp.	3	3
Acinetobacter lwoffii	1	0
Methylobacterium radiotolerans	1	0
Rhizobium radiobacter	1	0
Sphingomonas sp.	1	0
Delftia acidovorans	0	1
Gram-positive bacteria	**57**	**0**
Staphylococcus spp.	29	0
Enterococcus spp.	14	0
Streptococcus spp.	8	0
Granulicatella adiacens	2	0
Lactobacillus paracasei	2	0
Micrococcus luteus	1	0
Nocardia cyriacigeorgica	1	0
Total non-mycobacteria	**419**	**46**

A remarkable feature of RGM medium was its ability to inhibit the growth of fungi and Gram-positive bacteria, and during ten days of incubation, no isolates of either of these groups were recovered from 502 sputum samples (Table 2-9). In addition, although both of these selective media are designed to inhibit the growth of

Pseudomonas, RGM medium was much more effective with only two isolates recovered, compared with 32 isolates isolated on BCSA. Some isolates of Gram-negative bacteria, particularly *B. cepacia* complex and *Achromobacter* spp., could not be wholly inhibited on RGM medium but growth was somewhat restricted. Additionally, mycobacteria produced bright white colonies on RGM medium (Figure 2-21) whereas Gram-negative species that were able to grow generated blue, or occasionally pink, colonies. The high selectivity of RGM (Figure 2-17 to Figure 2-20) medium enabled the recovery of NTM in pure culture from 52 samples whereas BCSA enabled the recovery of a pure culture of NTM from only two samples.

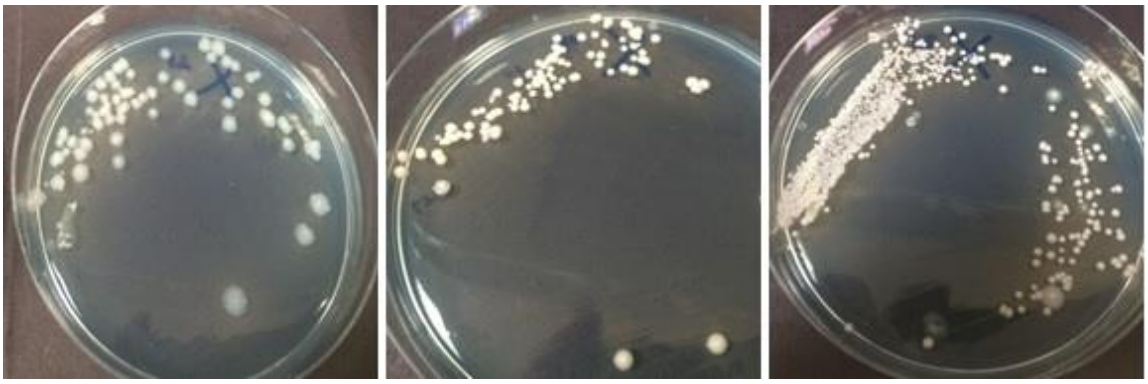


Figure 2-21: RGM medium demonstrating the visual appearance of both smooth and rough colonies of *M. abscessus* subspecies *abscessus*

Figure 2-21 demonstrates the growth on RGM medium of rough *M. abscessus* subspecies *abscessus* colonies (left), smooth *M. abscessus* subspecies *abscessus* colonies (centre), and a combination of both rough and smooth colonies (right), which upon typing have the same VNTR profile.

2.5.11 Investigation of RGM medium for sputum samples of control group patients with non-cystic fibrosis bronchiectasis and cystic fibrosis transplant assessment patients

Out of 310 bronchiectasis patients, NTM was recovered from 4.5% (*n* = 14) of patients with 1.3% of patients (*n* = 4) harbouring *M. abscessus* subsp*. abscessus*. Furthermore 6.7% (*n* = 4) of transplant assessment patients were positive for NTM, with 1.7% (1 patient) having both *M. abscessus* subsp. *abscessus* and *M. neoaurum* (See Table 2-).

Of the 400 bronchiectasis samples, 385 were negative for NTM (96.3%), as were 61/67 (91.0%) transplant assessment samples. As a result of this evaluation, one bronchiectasis patient was reviewed, and after undergoing genetic testing was diagnosed with CF for the first time at the age of 71.

Table 2-10: Number of NTM-positive samples from bronchiectasis and transplant assessment samples

Species	Positives (*n*)	Patient (*n*)
Bronchiectasis		
M. abscessus subsp. *abscessus*	5	4
M. chelonae	7	7
M. mucogenicum	3	3
Transplant Assessment		
M. abscessus subsp. *abscessus*	2	1*
M. chelonae	1	1
M. neoaurum	1	1*
M. species	2	2

*Patient co-infected with both *M. abscessus* subsp*. abscessus and M. neoaurum*

2.5.12 Evaluation of RGM medium for optimal growth of *Mycobacterium tuberculosis* complex and other slow-growing mycobacteria in broth-based culture medium

Although results were comparable in terms of isolation of slow-growing mycobacteria, the TTD was not as rapid using broth-based RGM as shown in Table 2-11 below.

Table 2-11: Results of 44 AFB positive bottles inoculated with non-CF samples

	Total positive samples	Bottle A positive samples	Mean Time to Detection (days)	Bottle B positive samples	Mean Time to Detection (days)
M. tuberculosis	17	17	10.8	16	13.9
M. avium	14	11	9.7	12	10.5
M. intracellulare	2	2	6.2	2	7.9
M. malmoense	2	2	15.7	2	18.6

In treated samples from CF patients, MABSC was detected in more of the RGM (bottle B) based bottles (6/56) than in the traditional (bottle A) MBacT bottles (3/56). A significant improvement was apparent in the rates of contamination of non-mycobacterial species being considerably reduced as well as the TTD of MABSC in these samples (Table 2-12).

Table 2-12: Recovery of mycobacteria from 56 pairs of MBacT bottles inoculated with treated samples from patients with CF

	Positive samples		Time to detection (days)		Contaminated bottles	
	MABSC	*M. avium*	*M. abscessus*	*M. avium*	Number	Rate (%)
Bottle A (soda treated sample)	3	0	9.0	-	20/56	36
Bottle A (acid treated sample)	2	0	5.0	-	13/56	23
Total Bottle A	3	0	-	-	-	29
Bottle B (soda treated sample)	2	1	5.2	21.0	4/56	7
Bottle B (acid treated sample)	6	0	4.2	-	3/56	5
Total Bottle B	6	1	-	-	-	6
TOTAL	6	1				

This was also evident across the slopes, with LJ slopes (Table 2-13) showing a dramatically higher rate overall of non-NTM contaminating species at both temperatures and after all decontamination treatments. The highest rate of NTM recovered was on the untreated 30ºC RGM slopes at 13.9%. Although these also showed a contamination rate of 17.6%, it was very easy to visualise the NTM on the slope in comparison to the non-NTM species due to the colour difference.

Table 2-13: Percentage contamination of samples from patients both with CF and non-CF and percentage of NTM isolated using RGM and LJ slopes at 21 days incubation at 30ºC and 37ºC

	% Contamination	% NTM isolated
Soda Treated Samples 30ºC		
RGM	2.8	4.6
LJ	28.7	1.9
Soda Treated Samples 37ºC		
RGM	1.9	1.9
LJ	25.9	1.9
Acid Treated Samples 30ºC		
RGM	2.8	8.3
LJ	18.5	3.7
Acid Treated Samples 37ºC		
RGM	5.6	1.9
LJ	14.8	3.7
Untreated Samples 30ºC		
RGM	17.6	13.9
Untreated Samples 37ºC		
RGM	4.6	7.4

It was impractical to perform a direct comparison of RGM medium with standard AFB culture as specimens were submitted for standard AFB culture infrequently. For example, for the 33 patients who were found to harbour mycobacteria in this study (i.e. using RGM plus BCSA as described in section 2.4.10), specimens were only submitted for standard AFB culture for ten of these patients (30%) during the 8-month trial period and only six of these yielded mycobacteria (18%). Equally, for the 177 patients that were found not to harbour mycobacteria, only 22 patients (12%) had specimens referred for standard AFB culture during the 8-month trial period. Of these 22 patients, mycobacteria was only recovered from one patient (*M. abscessus* subsp. *abscessus*).

In a 15-month period where RGM medium was used routinely at the Freeman Hospital, Newcastle upon Tyne, 4408 respiratory samples collected from 625

patients, both adult and paediatric, with CF were cultured onto RGM medium and incubated up to ten days (Table 2-14). AFB culture was continued for those patients who spontaneously produced sputum. Upon analysis of these patients, only 213 (34%) actually had AFB cultures requested, and 73 (15.5%) were contaminated and abandoned therefore no report could be issued with regard to the isolation of mycobacteria. NTM was only detected in 21 patients, in comparison to 56 patients with the routine use of RGM medium.

RGM medium was able to recover NTM from cough swabs as well as sputum samples, and although the yield was lower (1.9%), NTM was detected for the first time by cough swab culture in 15 patients.

Table 2-14: Comparison of RGM medium and formal AFB culture for the recovery of NTM from respiratory samples from patients with CF over a 15-month period

	Total		**Sputum**	**Other**	**Patients tested using RGM medium (10 day incubation)**	**Patients tested using formal AFB culture[a]**
	4408	2443	1557	408	625	213
Total mycobacteria	195	46	133	16	56	21[b]
M. abscessus complex	168	40	114	14	34	13
M. chelonae	10	2	8	0	9	3
M. fortuitum	2	0	2	0	1	0
M. avium complex	0	0	0	0	0	6
Other mycobacterial species	15	4	9	2	12	0

[a] 469 samples cultured from 213 patients using culture on LJ medium plus automated liquid culture

[b] Two different *Mycobacterium* spp. Isolated from one patient sample

Studies have also been completed comparing RGM medium (minus decontamination of samples) against the gold standard MGIT method using decontaminated samples with one study in London, UK showing a ten-day incubation period of RGM was equivalent to MGIT for the recovery of NTM (*P* = 1.00). Another evaluation performed in Chapel Hill, USA demonstrated that with an incubation period of 28 days, the sensitivity of RGM medium for the isolation of mycobacteria was significantly higher than combined MGIT/LJ with a *P* value of (*P* = 0.001) for total mycobacteria including *M. avium*, and (*P* = 0.046) for MABSC only.

2.5.13 Summary of studies in other centres using RGM medium for the isolation of NTM from patients with cystic fibrosis

An evaluation similar to the one described in section 2.4.16 was performed in Frankfurt, Germany, and showed that RGM was significantly more sensitive than BCSA (*P* = 0.023) (Preece *et al.*, 2016). A further study in Chapel Hill. USA demonstrated that from 869 samples incubated for up to 28 days also provided a significantly higher sensitivity for RGM medium (*P* = <0.0001) (Plongla *et al.*, 2016). Both of these studies compared RGM against a brand of *B. cepacia* medium (Table 2-15).

Table 2-15: Summary of studies using RGM medium for the isolation of NTM from respiratory samples from patients with CF

Study Location	No. of specimens / No. of patients	Prevalence of NTM (%)	Method used	Decontamination	Sensitivity (5)	*P*
Newcastle upon Tyne, UK	502 / 210	15.7%	RGM 10 days incubation	None	98.0	
						≤0.0001
			BCSA 10 days incubation	None	32.0	
Frankfurt, Germany	224 / 133	9.0%	RGM 10 days incubation	None	100	
						0.023
			BCSA 10 days incubation	None	41	
Chapel Hill, USA	869 / 493		RGM 28 days incubation	None	96.9	
						<0.0001
			BCSA 28 days incubation	None	34.7	
London, UK	187 / 187	15%	RGM 10 days incubation	None	82	
						1.00
			MGIT 28 days incubation	3% oxalic acid	86	
Chapel Hill, USA	212 / 172	24.1%	RGM 28 days incubation	None	93.2	
						0.0001
			MGIT / LJ medium 6 weeks incubation	NALC-NaOH then 5% oxalic acid	47.7	
Aarhus, Denmark	97 / 86	Sample size too small, only 1 MABSC isolated during test period	RGM 14 days incubation	None	Only 1 MABSC isolated during test period, on both RGM and BCSA	
			BCSA 14 days incubation	None		N/A
Cambridge, UK	564 / environmental samples	-	RGM 10 days incubation	None	100.0	
						0.04
			PANTA* 10 days incubation	None	14.0	

* PANTA solid agar plates made in house at Papworth Hospital, Cambridge, UK and contains Middlebrook 7H11 Agar (Difco Mycobacteria 7H11 Agar - BD) with PANTA Antimicrobial Supplement (Becton, Dickinson and Company) at final antibiotic concentrations as follows; Polymyxin B 30units/ml, Amphotericin 3µg/ml, Nalidixic Acid 12µg/ml, Trimethoprim 3µg/ml and Azlocillin 3µg/ml.
Studies performed in Nijmegen, The Netherlands; Bron, France and Lyon, France were too small and an insignificant number of MABSC isolates were isolated to validate any substantial results.

2.6 Discussion

Annual screening for NTM is recommended in patients who spontaneously produce sputum, with the suggested methodology from the CF Foundation and the European Cystic Fibrosis Society comprising of decontamination of sputum samples using N-acetyl-L-cysteine (0.5%)-NaOH (2%), or upon a sample remaining contaminated, it should be further treated with either 5% oxalic acid or 1% chlorohexidine (Floto *et al.*, 2016). Staining of sputum smears for acid-fast bacilli and culture on both solid and liquid media for a minimum of six weeks is also recommended. The use of oropharyngeal swabs should be avoided, due to insufficient material for culture (Saiman *et al.*, 2014). Transbronchial biopsies are to be circumvented if possible as although they can reveal NTM in culture or microscopy and exhibit granulomatous inflammation there is a substantial risk of bleeding and pneumothorax occurring. Preference should be given to sputum, induced sputum, bronchial washings or bronchial lavage samples. Updated evidence-based draft guidelines on the Management of Non-tuberculous Mycobacteria in individuals with Cystic Fibrosis have recently been published (Floto *et al.*, 2016).

The limitations of these methods are highlighted in these guidelines and include a significant reduction in the viability of mycobacteria due to the decontamination process, or only limited elimination of non-mycobacteria requiring further rounds of decontamination (De Bel *et al.*, 2013). The method is both costly and labour intensive. It is suggested that the currently used and most sensitive method following decontamination is an automated growth detection system such as MGIT, with simultaneous culture on solid media to possibly increase the diagnostic yield. The potential use of BSCA is highlighted in these guidelines, but not overtly recommended.

There is currently no explicit culture medium for the sole isolation of rapidly-growing mycobacteria, as in the case of *B. cepacia* complex despite the fact that the prevalence of rapidly-growing NTM may be considerably higher, as illustrated by this study where 54 isolates of mycobacteria were recovered on RGM medium (Table 2-8) compared with 30 isolates of *B. cepacia* complex recovered using BCSA (Table 2-9). One reason for this may be the lack of a convenient, rapid and effective method for isolation of rapidly-growing NTM. RGM medium provides a simple and convenient technique that can be implemented for the culture of all routinely submitted sputum samples from patients with CF. Such methodical screening will ensure that diagnosis of a significant infection with NTM is not unreasonably hindered. For example, a positive sputum culture for MABSC is more likely to indicate the presence of NTM-mediated lung damage rather than asymptomatic colonization (Seddon *et al.*, 2013; Floto and Haworth, 2015) and multiple positive cultures despite treatment is associated with a poor outcome (Esther *et al.*, 2010; Qvist *et al.*, 2015). Additionally, routine screening of all submitted sputum samples from CF patients may assist in prompt identification of risk factors leading to acquisition, subsequent infection and transmission of NTM (Bryant *et al.*, 2013). Any screening method may lead to the detection of transient colonisation with mycobacteria that may have no clinical significance. This can be problematic, as patients may be assumed to be infected and may be unnecessarily segregated from other patients. This problem can be alleviated largely by prompt species identification of the isolate (Blauwendraat *et al.*, 2012; Harris and Kenna, 2014) to assess likely pathogenicity and, wherever possible, prompt submission of further sputum samples.

As media for isolation of *B. cepacia* complex have been suggested for isolation of mycobacteria, this prompted an investigation of different commercial brands of

Cepacia selective media in order to compare their ability to support the growth of mycobacteria and their selectivity against other bacteria associated with sputum samples from CF patients. Cepacia selective agar (bioMérieux; 44347) was at least as effective for culture of pure strains of mycobacteria as any other Cepacia selective agar (Table 2-7). It was less selective than some other agars and much of this could be attributed to lack of inhibition of methicillin-resistant *S. aureus*. Cepacia selective agar was less selective than bioMérieux BSCA but more selective than BD OFPBL.

In 1985, Gilligan *et al.* were the first to report the design of a selective culture medium for *B. cepacia*, PC medium, for use with sputum samples from patients with CF (Gilligan *et al.*, 1985). This medium comprised of polymyxin B, ticarcillin, crystal violet and bile salts as selective agents, many of which are frequently used in commercial brands. At around the same time, Welch *et al.* evaluated the use of OFPBL medium, exploiting the use of polymyxin B and bacitracin as selective agents (Welch *et al.*, 1987). In addition, over a decade later, Henry *et al.* described *B. cepacia* selective agar (BCSA) that was shown to have superior selectivity when compared to PC medium and OFPBL medium. In this medium, polymyxin B and crystal violet were retained as selective agents with the addition of gentamicin and vancomycin (Henry *et al.*, 1997). In a large trial with 656 clinical samples, Henry *et al.* concluded that BCSA was superior to both OFPBL and PC medium for supporting the growth of *B. cepacia* and suppressing the growth of other flora (Henry *et al.*, 1999). In this study the high selectivity of BCSA is confirmed, demonstrating much more selectivity than OFPBL, however, six isolates of BCC were inhibited using BCSA.

The growth rate of mycobacteria is slower than most if not all of the other bacterial and fungal isolates frequently recovered from sputum samples from patients with

CF. For this reason, high selectivity of media is vital in order to inhibit or at least restrict the growth of non-mycobacteria so that the mycobacteria do not go undetected due to overgrowth by other species. Although BCSA was the most selective of the agars designed for recovery of BCC, it was much less selective than RGM medium. If BCC is excluded (as BCSA is designed to grow this), 24 non-mycobacteria were able to grow on BCSA compared with only six on RGM medium. A particular flaw of selective agars for BCC is their failure to inhibit fungi, particularly *Aspergillus* species. On extended incubation of these media, the growth of *Aspergillus* can consume the entire culture plate, thus compromising the likelihood of isolating mycobacteria. This is particularly problematic with sputum samples from CF patients where concomitant isolation of *Aspergillus* sp. has been frequently associated with mycobacteria infection (Esther *et al.*, 2010; Verregghen *et al.*, 2012)

Middlebrook 7H11 selective agar, designed specifically for the isolation of mycobacteria, was superior at inhibiting fungi due to the inclusion of amphotericin. However, species such as *Aspergillus terreus* and *Scedosporium apiospermum* persisted and largely the selectivity of Middlebrook 7H11 selective agar was inferior to that of bioMérieux BCSA and Oxoid *B. cepacia* agar. In contrast, no yeasts or fungi were able to grow on RGM medium. From this analysis, it can be concluded that RGM medium offers a superior option to any of the other selective agars for isolation of rapidly-growing mycobacteria from the sputum of patients with CF.

Under existing guidelines (Floto *et al.*, 2016) NTM may be undetected for up to a year, and occasionally longer if successive conventional cultures are compromised by frequent contamination.

In conclusion, it is proposed that routine systematic use of RGM medium could expedite a greater understanding of the true prevalence and clinical significance of

rapidly-growing mycobacteria in patients with CF. The superior sensitivity of RGM medium over recognised AFB culture strongly supports the proposal that decontamination of samples will have an adverse effect upon the viability of mycobacteria, as previously described in section 1.9.3. The use of this medium can easily be incorporated into any laboratory alongside routine culture for other CF pathogens without any requirement for the laborious decontamination of samples and potentially facilitate a considerable saving in both labour time and materials cost of replacing formal AFB culture. NTM were detected in over twice as many patients by performing routine investigation with RGM medium and it is probable that this would have been additionally increased with prolonged incubation of up to 28 days.

Burkholderia cepacia complex is routinely screened for in patients with CF, yet the results of the studies completed in Table 2-9 confirm that NTM was isolated at a much greater rate than *B. cepacia* complex so it would seem irrational that screening for NTM is only recommended annually.

This method however will not preclude the need to perform annual screening using conventional mycobacterial culture methods, as slow-growing mycobacteria such as *M. avium* may not be consistently detected. However, the existing evidence validates the significant potential of such a highly selective medium for the isolation of NTM from patients with CF.

CHAPTER THREE

An evaluation of fluorogenic and chromogenic enzyme substrates as prospective identification tests for the *Mycobacterium abscessus* complex and for potential differentiation of subspecies.

Introduction

3.1 Current methods of identification of *Mycobacterium abscessus* complex

MABSC and *M. chelonae* are closely related species that cannot always be differentiated by clinical laboratories, and prior to 1992, they were thought to be the same organism or subspecies within the *M. chelonae-abscessus* group (Kusunoki and Ezaki, 1992). This is particularly problematic as they cause infections requiring diverse treatment regimens, with *M. chelonae* not notably as clinically significant if isolated from a patient sample. MABSC however is increasingly acknowledged as a substantial cause of lung infection in cystic fibrosis (CF) patients (Esther *et al.*, 2005), particularly *M. abscessus* subsp. *abscessus*, with many CF centres rejecting patients colonised with MABSC for lung transplant (Watkins and Lemonovich, 2012).

Accurate identification can be accomplished by PCR and sequencing of housekeeping gene targets, as previously discussed in section 1.9.6, however many reports in the literature describe ambiguous sequencing results from isolates that have chimeric house-keeping gene sequences. This is possibly as a result of horizontal gene transfer and recombination, which further highlights the difficulties in accurate identification and differentiation of these three subspecies (Zelazny *et al.*, 2009; Macheras *et al.*, 2014b).

3.2 Direct observation of non-tuberculous mycobacteria in culture medium

Mycobacteria are grouped according to speed of growth as either rapidly-growing mycobacteria or slow-growing mycobacteria. The formation of visible colonies on culture media in less than seven days constitutes “rapid” growth, (e.g. as shown by

M. abscessus), whilst those necessitating more than seven days are known as slow-growing, (e.g. *M. avium*). In general mycobacteria form either rough or smooth white/cream coloured colonies, although many species can, like *M. kansasii*, produce pigment generating bright yellow colonies (Lima and Magalhaes, 2014).

Many rapidly-growing species can grow on MacConkey agar without crystal violet, whereas *M. tuberculosis* and *M. bovis* do not have this capability, and *M. abscessus* is noted as having the ability to grow in media with 5% sodium chloride. LJ medium supplemented with a concentration of 250 mg of hydroxylamine can distinguish between NTM and other species of mycobacteria for example *M. bovis* as NTM do not have the capacity to grow in the presence of hydroxylamine. Rapidly-growing mycobacteria excluding *M. chelonae* can grow on Sauton picric medium and Sauton agar with 0.2% picric acid (Garcia-Agudo *et al.*, 2011).

3.3 Biochemical tests used in identification of mycobacteria

Biochemical tests, such as citrate utilisation or tolerance to sodium chloride, are simple to perform, require minimal equipment, and in general are able to correctly differentiate between the more common mycobacterial species. Nonetheless, they are laborious and due to lengthy incubation times present a delay in final identification. Other methods based on mycolic acid analysis, for example high performance liquid chromatography and gas liquid chromatography, are challenging and costly. Experience in interpretation is essential, and can be limited by subjectivity and low specificity. Biochemically unreactive or inert organisms can be difficult to identify and phenotypic misidentification can occur due to species homogeneity, variability, and the increasing recovery of novel species (Springer *et al.*, 1996; Tortoli *et al.*, 2001).

At present, there are almost 200 currently established species, a number that continues to rise, and in general, biochemical algorithms will only differentiate around twenty species. This therefore makes this approach too complex, resulting in a characteristic preference towards the more familiar species (Kent and Kubica, 1985). Despite these challenges, phenotypic methods are still used in some laboratories to identify NTM regardless of their recognised limitations.

3.4 Genotypic methods for the identification of *Mycobacterium abscessus* complex

The use of nucleic acid probes is a prompt and extensively used procedure for identification, however as such a small range of mycobacterial species are covered, there are well documented concerns regarding specificity and sensitivity (Lim *et al.*, 1991; Bull and Shanson, 1992; Viljanen and Olkkonen, 1993).

The earliest commercially available method was the Gen-Probe AccuProbe (Gen-Probe Inc.), and more recently, the INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), Geno-Type MTBC and Genotype Mycobacterium (Hain Lifesciences, Nehren, Germany). INNO-LiPA Mycobacteria v2 is still quite antiquated in that it integrates *M. abscessus* and *M. chelonae* into *M. chelonae* complex (Garcia-Agudo *et al.*, 2011), and as discussed in section 1.9.6, none of these kits are not able to identify individual members of the MABSC (Reisner *et al.*, 1994).

3.5 Molecular typing of *Mycobacterium abscessus* complex isolates

Differentiation of MABSC isolates has been performed using various molecular techniques; including variable number tandem repeat (VNTR) analysis, whole genome sequencing, multilocus sequence typing (MLST) and multispacer sequence typing (MST), pulsed field gel electrophoresis and rep-PCR.

3.5.1 Variable number tandem repeat

VNTR is a rapid and convenient PCR-based technique targeting variation in tandem DNA repeats at specified loci, generally between nine and fifteen, with a numerical profile created that is based upon the number of repeats at each locus. This method has been successfully used for the differentiation of *M. tuberculosis* strains for many years (Mazars *et al.*, 2001), but is not as well established for MABSC and there are varying reviews of its success and accuracy in being able to separate *M. abscessus* complex isolates. A two-tandem repeat locus was demonstrated by Choi *et al.* (2011), which was reported to be able to split the three members of MABSC in eighty-five clinical isolates that were all initially identified as *M. abscessus* subsp*. abscessus* by PCR-restriction fragment length polymorphism analysis of *rpoB.* Using this method, if there is one band on the gel an identification of *M. abscessus* subsp*. massiliense* is given. If two amplification bands are displayed, they were either *M. abscessus* subsp*. abscessus* (>393 bp) or *M. abscessus* subsp*. bolletii* (393 bp), and if there was a single band of >393 bp in length, the result was interpreted as *M. abscessus* subsp*. abscessus* (Choi *et al.*, 2011). Wong *et al*. described a VNTR assay for *M. abscessus* complex declaring complete reproducibility in 38 clinical isolates (Wong *et al.*, 2012) previously all identified as *M. abscessus* by PCR and HAIN Genotyping (Telenti *et al.*, 1993). A further study in 2012 by Harris *et al*. however performed a comparison of VNTR with the Diversilab rep-PCR method and demonstrated that the patients were infected with isolates which shared a limited number of VNTR profiles, suggesting the possibility of cross contamination having occurred, or a possible mutual environmental source of infection (Harris *et al.*, 2012).

3.5.2 Rep-PCR for the identification of mycobacteria

Rep-PCR uses PCR primers that will amplify repetitive sequences in the genome in order to give a profile (Healy *et al.*, 2005). A relatively basic commercial kit from Diversilab, (bioMerieux) can be used in routine diagnostics, however reports are unclear as to whether this method provides representation of same strain isolates, or if they are in fact just highly related strains when identical rep-PCR profiles are obtained (Harris *et al.*, 2012).

3.5.3 Multilocus sequence typing and multispacer sequence typing

MLSA is a previously described technique for the identification of *M. abscessus* isolates to subspecies level (Macheras *et al.*, 2011). Multilocus sequence typing (MLST) is an extension of the MLSA method however, a greater number of house-keeping gene targets are sequenced for MLST, usually around eight. Although reproducible and not requiring substantial amounts of DNA, the sequences of the house-keeping targets used are well conserved in *M. abscessus* complex, therefore MLST does not provide discrimination of the three subspecies (Macheras *et al.*, 2014a). Multispacer sequence typing (MST) is a PCR and sequence based technique that targets intergenic spacers, which are more variable than the housekeeping genes in MLST therefore this technique may offer improved discrimination. A recent study described the development of this MST scheme for identification of *M. abscessus* complex and is able to discriminate between the three subspecies when combined with phylogenetic analyses (Sassi *et al.*, 2013).

3.5.4 Whole genome sequencing

Whole genome sequencing (WGS) provides the most comprehensive technique in order to analyse the entire genome. It provides rapid and accurate microbial genome sequence information, which is crucial for detecting mutations, discovering

significant deletions or insertions, and ascertaining other genetic changes among microbial strains. It can also provide an insight into whether cross contamination is an issue as conflicting information has frequently been described (Bryant *et al.*, 2013; Davidson *et al.*, 2013; Harris *et al.*, 2015).The high degree of resolution provided by WGS allows for much finer discrimination of strains than any of the other current or previous methodologies.

3.6 Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

As an alternative to chromatographic, biochemical or molecular methods, mass spectral analysis has become progressively more widespread for the identification of microorganisms (Lau *et al.*, 2014). MALDI-TOF MS can be used for accurate and speedy identification of innumerable microorganisms. This technique is based upon the detection of highly abundant proteins in a mass range between 2 and 20 kDa by computing their mass (*m*) to charge (*z*), *m*/*z* values generating a characteristic spectrum for each microorganism. This can then be used for comparison with stored reference spectra and in so doing providing identification of the isolate (Panda *et al.*, 2013).

3.6.1 Principles of MALDI-TOF MS

MALDI-TOF MS measures a unique molecular fingerprint, specifically the proteins found in all microorganisms (Singhal *et al.*, 2015). The distinguishing patterns of these extremely abundant proteins can consistently and accurately identify a particular microorganism by matching its individual pattern with an extensive database to determine the identity to species level. This system is highly accurate with a rapid turnaround time and high throughput (96 samples per run) and is cost effective. Its ease of use and versatility does not require extensive training for

laboratory workers (Marvin *et al.*, 2003). Limitations can however include the need for isolated pure colonies, a fresh culture, and on occasions an inability to differentiate between closely related organisms and repeat analysis may be required (Dhiman *et al.*, 2011). Once the inoculum is dried, the matrix is added. Once this is dried the prepared target plate is placed into the ionization chamber where each sample is irradiated with momentary pulses of energy from an ultraviolet nitrogen laser (337 nm). This process desorbs individual sample and matrix molecules from the target plate into the gas phase, with the majority of energy absorbed by the matrix, which becomes ionized with a single positive charge. This positive charge is consequently transferred from the matrix to native sample proteins through their random collision in the gas phase. The ionized proteins are funnelled through a positively charged, electrostatic field that accelerates the molecules into the time of flight (TOF) mass analyser. The TOF chamber is an empty, pressurized tube that allows ions to travel down a field-free region toward the ion detector. The speed at which individual ions move through the TOF chamber is dependent on their mass-to-charge ratio and ions are ultimately separated based on their difference in mass. Heavier ions will travel through the mass analyser at a slower velocity than lighter ions and as they emerge, they collide with the ion detector, which measures their charge and time to impact. Based on standards of known mass, the time to impact for each unknown analyte is converted into a mass-to-charge ratio, which is illustrated on a mass spectrum. As each spectral profile is attained, the software automatically generates a MALDI-TOF spectrum that is matched against a reference database in order to provide identification alongside a score value. Using a biostatistical algorithm the peak list is compared to reference peaks of organisms in the reference database and a log (score) value is given between 0.00 and 3.00. The higher the log (score) value, the higher the degree of similarity to a given

organism, with values of ≥2.00 indicating a high probability of correct identification at the suggested species level (Bruker, 2015).

3.6.2 The use of MALDI-TOF in the identification of *Mycobacterium abscessus* complex

Although MALDI-TOF MS has been shown to be a highly accurate method for identifying mycobacterial isolates to species level, this technique has not been shown to be able to successfully differentiate between the three subspecies of MABSC (Saleeb *et al.*, 2011; Balada-Llasat *et al.*, 2013). A report by Teng *et al*. (2013) suggested that this was achievable, however this report also claimed that *M. abscessus* subsp*. bolletii* was currently the correct taxonomic name for *M. abscessus* subsp*. massiliense* and only a limited number of isolates (*n* = 58) were tested. Other reports suggest that although *M. abscessus* subsp*. massiliense* can be differentiated from *M. abscessus* subsp*. abscessus* and *M. abscessus* subsp*. bolletii*, the latter too are too closely related to be differentiated by MALDI-TOF MS (Fangous *et al.*, 2014; Suzuki *et al.*, 2015; Luo *et al.*, 2016).

3.7 The use of chromogenic and fluorogenic substrates in culture media

Enzymes are present in all microorganisms in vast numbers with quantities and varieties produced differing between species and often also between strains. All species of bacteria possess a unique enzyme profile comprising of a number of enzymes required for growth, nutrition and replication and this differentiation permits an appropriate means of identification (Bascomb and Manafi, 1998).

The biochemical activity within bacteria is known as metabolism, and the majority of these biochemical reactions do not transpire spontaneously, but are catalyzed by enzymes accelerating the chemical reaction without undergoing any permanent

modification themselves (Chubukov *et al.*, 2014). Without enzymes, these reactions would be at a rate that was too slow. Many enzymes necessitate the presence of other compounds, known as cofactors in order to exert their catalytic activity and one property of enzymes that makes them so significant as a diagnostic tool is the specificity they exhibit. Certain enzymes require a particular type of bond (linkage specificity) or functional group (group specificity), whereas others will only catalyze one reaction (absolute specificity), or only act upon a particular steric or optical isomer (stereochemical specificity) (Rago *et al.*, 2015).

Several fluorogenic and chromogenic reactions have been used for detection of bacteria. Fluorogenic methods exploit synthetic substances, such as those based on 4-methylumbelliferone (4-MU) or 7-amino-4-methylcoumarin (7-AMC) that are hydrolysed by bacterial enzymes to release powerful fluorophores. This will produce an increase in fluorescence attributable to enzymatic activity (Dyer, 1970), a fluctuation in fluorescence or absorbance of a pH indicator (Goodwin and Kavanagh, 1950), or a change of intensity of fluorescence as a result of adsorbance of fluorescent dye within the bacterial cell (Ramsey *et al.*, 1980).

Hydrolysis of esculin to glucose and esculetin (6,7 dihydroxycoumarin) and detection of esculetin by its reaction with iron was first described in bacteria by Meulen in 1907 (Ter Meulen 1907, cited in Harrison and van der Leck 1909). This reaction was then incorporated into esculin medium to examine water for the presence of coliforms by Harrison and van der Leck in 1909 (Harrison and Van Der Leck, 1909).

3.8 Enzyme substrates

Enzymatic substrates are fundamental tools in biochemistry and are extensively used in microbiology to study metabolic pathways, observe metabolism and to

detect, count and identify microorganisms. Rapid detection and identification of microorganisms is particularly important in microbiology and both chromogenic and fluorogenic substrates have proved to be a powerful aid, utilising specific enzymatic activities of particular microorganisms, either in conjunction with or as an alternative to traditional methods (Maddocks and Greenan, 1975; Manafi and Kneifel, 1991). By incorporation of these substrates into primary selective media, colony counts and detection can be achieved directly on the isolation plate. The introduction of many of these media and identification tests has led to enhanced accuracy and more rapid detection of target organisms, frequently decreasing the need for isolation of pure cultures and supplementary confirmatory tests (Gee *et al.*, 1999).

Glycosidases catalyse the hydrolysis of glycosidic linkages, and in so doing degrade oligosaccharides and glycoconjugates. These effective and very specific catalysts are crucial in biological processes therefore a comprehensive knowledge of their function is instrumental in regulating disease (Falk *et al.*, 1990). Peptidases are enzymes that catalyse the cleavage of proteins into both shorter fragments, as well as into their component amino acids with two major groups being endopeptidases, targeting sites within the proteins and exopeptidases, cleaving terminal ends of proteins (van Roosmalen *et al.*, 2004). Esterases catalyse the hydrolysis of an ester bond into its acid and alcohol. They catalyse three types of reaction; esterification, interesterification and transesterification (Xiang *et al.*, 2007). As they do not require cofactors, this makes them attractive biocatalysts. Chromogenic substrates allow the rapid and simple identification of microorganisms in clinical samples, and when exploited in a culture medium can decrease the need for time consuming and labour intensive subcultures and biochemical tests (Perry and Freydiere, 2007).

Developed in 1908 by Wohlgemuth, the first enzyme assay for the detection of amylase activity was using starch and iodine (Wohlgemuth, 1908). Maltose

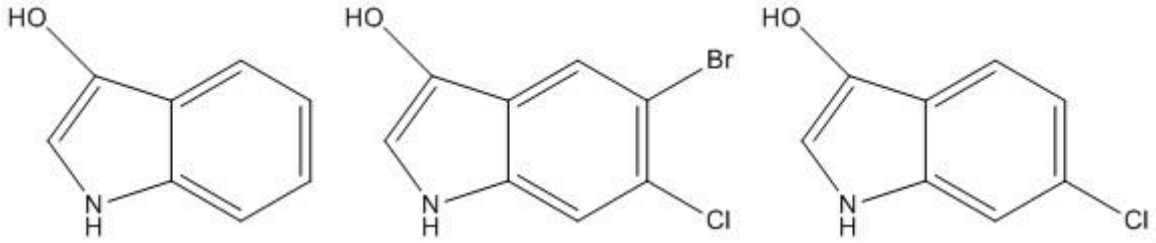
disaccharides are produced when amylase catalyses the hydrolysis of starch and a colour change of the iodine from dark brown to yellow occurs.

Chromogenic substrates are molecules designed to imitate metabolic substrates and are colourless until they are cleaved by the target enzyme (Perry and Freydiere, 2007). Once cleaved the released chromogen is usually insoluble and brightly coloured. Colonies that possess the enzyme can then be simply differentiated from those that do not possess the enzyme. The addition of chromogenic substrates into a selective culture medium can allow differentiation and identification of groups of bacteria.

The first commercially available culture medium that utilised a synthetic chromogenic substrate was Rambach agar, which uses a chromogenic substrate for β-galactosidase known as X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Composed of propylene glycol, peptone, yeast extract, sodium deoxycholate, neutral red and Xgal, upon incubation at 37^{o}C for 24 hours, *Salmonella* spp will ferment the propylene glycol to generate acid causing precipitation of the neutral red and consequently producing colonies with a characteristic red colour (Rambach, 1990).

The chromogenic substrate (X-gal) will facilitate the most common Enterobacteriaceae to produce blue or violet colonies whereas most species that fail to produce ß-galactosidase (e.g. *Proteus* spp.) remain colourless. The inhibition of Gram-positive microorganisms in the sample is attributed to the sodium deoxycholate in the medium. There is a disadvantage to this medium in that it does not detect *Salmonella typhi, Salmonella paratyphi* or some rare strains such as *Salmonella wassenaar* and *Salmonella moscow*. Additionally, some *Salmonella* strains are able to produce β-galactosidase, for example *Salmonella arizonae*, therefore show as blue/violet colonies on the medium (Manafi, 1996).

The most widely used chromogenic substrates are those based on indoxyl or its halogenated derivatives such as 5-bromo-6-chloro-indoxyl (forms a magenta dye) or 6-chloro-indoxyl (forms a rose pink dye) (See Figure 3-1 below) and their popularity is due to their heat resistance, water solubility and their predisposition to form strongly coloured indigo-based precipitates upon enzymatic action (Perry and Freydiere, 2007).



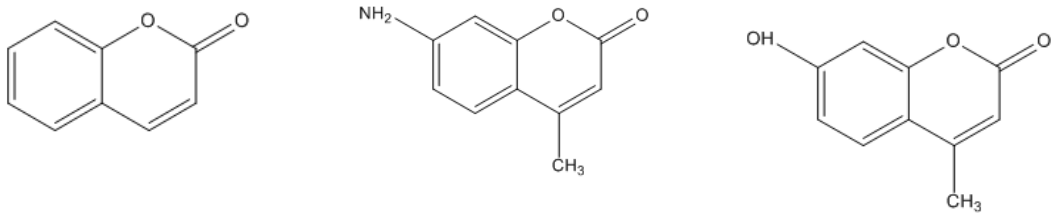
(i) (ii) (iii)

Figure 3-1: Structure of indoxyl (i) and it halogenated derivatives (ii) 5-bromo-6-chloro-indoxyl and (iii) 6-chloro-indoxyl

The fluorogenic enzyme substrates used for bacterial detection largely consist of derivatives of coumarin, such as 4-methylumbelliferone (4-MU or 7-hydroxy-4-methylcoumarin) or 7-amino-4-methylcoumarin (7-AMC), (Chilvers *et al.*, 2001), the structures of which are shown in Figure 3-2. This is due to the accessibility of an extensive range of substrates with diverse metabolic moieties, their non-carcinogenicity, simplicity of visual detection of the products of enzyme activity with UV light sources, and availability of appropriate tools for measurement of fluorescence (Manafi *et al.*, 1991).

The use of fluorogenic substrates for bacterial enzyme detection was first described by Dyer (1970) followed by Dahlen and Linde (1973) who employed an agar plate containing the enzyme substrate, 4-methylumbelliferyl-β-D-glucuronide, in order to

detect β-glucuronidase activity in microorganisms (Dahlen and Linde, 1973). Maddocks and Greenan (1975) subsequently described a ten minute spot test using 4-MU derivatives outlining a simplistic and rapid alternative to standard biochemical tests and Fujiwara and Tsuru (1978) were the first to use 7-AMC derivatives for the measurement of bacterial peptidases (Fujiwara and Tsuru, 1978).



(i) (ii) (iii)

Figure 3-2: Structures of fluorescent core molecules (i) coumarin (ii) 7-amino-4-methylcoumarin (iii) 4-methylumbelliferone

Substrates based on such fluorophores are non-fluorescent when conjugated to a metabolically active moiety, for example glucose, and upon hydrolysis the aglycone is cleaved from glucose, freeing up the electrons at the hydroxyl group and altering the molecule so that it yields a bright blue fluorescent light under UV light at an excitation wavelength of 365 nm and emission wavelength of 445 nm (Haughland, 1996).

These substrates are more suitable when used in liquid assays, as when used in solid agar based media, the fluorescent product will diffuse away from the bacteria which expresses the target enzyme making it difficult to differentiate between a negative and positive colony in a mixed culture (Manafi *et al.*, 1991).

As discussed, there are an abundant number of commercially available kits based upon enzyme function, providing a rapid and accurate result within a very short space of time (Bascomb and Manafi, 1998) as well as numerous chromogenic and fluorogenic media readily obtainable (Manafi *et al.*, 1991), but currently there is nothing available for the rapid identification of mycobacteria therefore enzymic activity cannot be accurately or promptly estimated.

3.9 Aims and objectives

To screen a collection of fluorogenic and chromogenic enzyme substrates (structures are shown in Appendix 4) to characterise the enzymatic profiles of mycobacteria and selected Gram-negative strains and to determine whether mycobacteria can be differentiated from Gram-negative bacteria in order to exploit the use of these substrates within RGM medium.

To determine if different species within the *Mycobacterium abscessus* complex (*M. abscessus* subsp. *abscessus, M. abscessus* subsp*. bolletii* and *M. abscessus* subsp. *massiliense*) can be distinguished from each other, and also from lesser pathogenic mycobacterial species such as *M. chelonae.*

3.10 Materials

3.10.1 Bacterial strains used in the evaluation of fluorogenic substrates

A collection of 45 isolates were used; these included two control strains from the National Collection of Type Cultures: *E. coli* NCTC 10418 and *S. aureus* NCTC 6571, 15 Gram-negative isolates; *A. xylosoxidans* (*n* = 3), *B. multivorans* (*n* = 3), *B. cenocepacia* (*n* = 2), *B. stabilis* (*n* = 1), *B. vietnamiensis* (*n* = 1), *D. acidovorans* (*n* = 1), *I. limosus* (*n* = 1), *P. aeruginosa* (*n* = 1), *P. norimbergenesis* (*n* = 1), *S. marcescens* (*n* = 1), and 28 isolates of mycobacteria (*M. abscessus* subsp*. abscessus* (*n* = 11), *M. abscessus* subsp*. massiliense* (*n* = 8), *M. chelonae* (*n* = 6) and *M. abscessus* subsp*. bolletii* (*n* = 3). Further details in Appendix 1.

3.10.2 Bacterial strains used in the evaluation of chromogenic substrates

A collection of 20 mycobacteria isolates were used: *M. abscessus* subsp*. abscessus* (*n* = 12), *M. abscessus* subsp*. massiliense* (*n* = 5) and *M. abscessus* subsp*. bolletii* (*n* = 3) and 31 non-mycobacterial isolates for the evaluation of magenta octanoate; *Burkholderia cepacia* complex (*n* = 10), *A. xylosoxidans* (*n* = 4), *P. aeruginosa* (*n* = 4), *Pandoraea* spp. (*n* = 3), *S. maltophilia* (*n* = 2), *S. marcescens* (*n* = 2), *Acinetobacter* sp. (*n* = 1), *D. acidovorans* (*n* = 1), *E. miricola* (*n* = 1), *I. limosus* (*n* = 2) and *Ochrobactrum* sp. (*n* = 1).

For the evaluation of L-ala-ala-ala-4(4’-aminostyryl)-N-methylquinolinium dichloride a collection of 12 mycobacteria isolates were used: *M. abscessus* subsp*. abscessus* (*n* = 6), *M. abscessus* subsp*. massiliense* (*n* = 4) and *M. abscessus* subsp*. bolletii* (*n* = 2). Further details in Appendix 1.

A further collection of 11 mycobacteria isolates were used for all other chromogenic substrates: *M. abscessus* subsp*. abscessus* (*n* = 6), *M. abscessus* subsp*. massiliense* (*n* = 2), *M. chelonae* (*n* = 2) and *M. abscessus* subsp*. bolletii* (*n* = 1).

All isolates used unless stated otherwise were supplied by the Freeman Hospital Microbiology Department and were isolated from the sputa of patients with CF. Further details can be found in Appendix 1.

3.10.3 Growth media and general chemicals

RGM broth was prepared in house using Middlebrook broth base and glycerol as described in Appendix 2 with the addition of yeast extract obtained from bioMérieux, Marcy l'Etoile, France. OADC supplement was prepared as described previously in section 2.4.3. No antimicrobials or agar were added to the broth. Methyl red was purchased from Sigma-Aldrich, Poole, UK.

3.10.4 Fluorogenic enzyme substrates

4-Methylumbelliferyl lignocerate, 4-Methylumbelliferyl nonanoate, 4-Methylumbelliferyl riboside, 4-Methylumbelliferyl α-D-glucopyranoside, 4-Methylumbelliferyl α-D-glucuronide, 4-Methylumbelliferyl α-D-mannopyranoside, 4-Methylumbelliferyl α-L-idopyranoside, 4-Methylumbelliferyl α-L-iduronide, 4-Methylumbelliferyl β-D-glucopyranoside and 4-Methylumbelliferyl β-D-glucuronide, 4-Methylumbelliferyl β-D-ribofuranoside were all obtained from Glycosynth, Warrington, UK. 4-Methylumbelliferyl acetate, 4-Methylumbelliferyl 7-β-D-xyloside, 4-Methylumbelliferyl acetamido-4,6-0-benzylidene-2-deoxy-β-D-glucopyranoside, 4-Methylumbelliferyl butyrate, 4-Methylumbelliferyl heptanoate, 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide, 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, 4-Methylumbelliferyl palmitate, 4-Methylumbelliferyl p-guanidinobenzoate hydochlorate, 4-Methylumbelliferyl phosphate, 4-

Methylumbelliferyl propionate, 4-Methylumbelliferyl stearate, 4-Methylumbelliferyl sulfate, 4-Methylumbelliferyl α-D-galactoside, 4-Methylumbelliferyl α-L-arabinopyranoside, 4-Methylumbelliferyl β-D-cellobioside, 4-Methylumbelliferyl β-D-fucoside, 4-Methylumbelliferyl β-D-galactoside, 4-Methylumbelliferyl β-D-glucoside, 4-Methylumbelliferyl β-D-mannopyranoside, 4-Methylumbelliferyl β-D-N,N'-diacetylchitobioside and 4-Methylumbelliferyl β-D-N,N'-triacetylchitotriose were purchased from Sigma-Aldrich, Poole, UK. 4-Methylumbelliferyl laurate was obtained from Koch-Light Laboratories Limited, Suffolk, UK. Ac-Met-7-amino-4-methylcoumarin (AMC), Boc-Leu-Gly-Arg-AMC acetate salt, H-Arg-AMC.2HCl, H-Arg-AMC.2HCl, H-Asn-AMC.TFA, H-Asp(AMC)-OH, H-Gln-AMC, H-Glu-AMC, H-Gly-AMC.Hbr, H-Gly-pro-AMC.HBr, H-His-AMC, H-Ile-AMC.TFA, H-Leu-AMC.HCl, H-Lys-AMC.acetate, H-Met-AMC acetate salt, H-Orn-AMC.2HCl, H-Phe-AMC.TFA, H-Pro-AMC hydrobromide salt (Prolyl), H-Thr-AMC, H-Try-AMC, H-Val-AMC, H-β-Ala-AMC.TFA, Pyr-AMC, L-alanine-AMC trifluoroacetate salt and Z-Arg-AMC.HCl were purchased from Bachem, Saffron Walden, UK. All structures are shown in Appendix 4.

3.10.5 Chromogenic enzyme substrates

5-bromo-6-chloro-3-indolyl-ß-D-galactopyranoside, 6-chloro-3-indolyl-ß-D-glucuronide, 6-chloro-3-indolyl-ß-D-glucopyranoside and magenta octanoate were purchased from Glycosynth, Warrington, UK. Alizarin-2-α-D-galactopyranoside was obtained from Inalco, Italy. Alizarin-2-ß-D-galactopyranoside, Alizarin-2-ß-D-glucopyranoside, 3,4-cyclohenenoesculetin-ß-D-galactopyranoside, 3,4-cyclohenenoesculetin-ß-D-glucopyranoside and L-ala-ala-ala-4(4'-aminostyryl)-N-methylquinolinium dichloride were kindly supplied by Professor Arthur James, University of Northumbria, UK.

3.10.6 Equipment

In addition to equipment used in section 2.3.4, a Proline Plus 30-300µl, 8-Channel multi pipette from Alpha laboratories, Hampshire, UK was used for dispensing substrates into the microtitre trays. Flat-bottomed, 96 well microtitre trays used in the evaluation of enzyme substrates were purchased from Sarstedt AG & Co., Numbrecht, Germany. The Synergy HT multi-detection microplate reader, obtained from BioTek Instruments, Bedfordshire, UK (see section 3.10.6.1) was used to measure the change in absorbance in enzyme assays containing chromogenic and fluorogenic substrates.

3.10.6.1 The Synergy HT multi-detection microplate reader

The Synergy HT multi-detection microplate reader is a microplate reader capable of measuring absorbance, fluorescence and luminescence. It utilises a unique dual optics design and has both monochromator/xenon flash systems with a silicone diode detector for absorbance, which allows the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm in 1 nm increments. It also has a tungsten halogen lamp with blocking interference filters, excitation and emission for wavelength selection and photomultiplier detection for fluorescence (BioTek, 2017).

3.11 Methods

3.11.1 Culture medium used in the evaluation of chromogenic and fluorogenic substrates

One litre of Middlebrook 10 x strength broth base described in Appendix 2 containing 40 ml glycerol and 4 g yeast extract was prepared, autoclaved for 10 minutes at 121^{o}C, cooled and stored at 4^{o}C. As the OADC supplement cannot be autoclaved, 100 ml was prepared as previously and described in Appendix 2, filter sterilised and added to the broth as required at a concentration of 10 ml per 100 ml broth base.

3.11.2 Preparation of bacterial strains

All Gram-negative strains were subcultured onto blood agar, and mycobacteria onto previously described RGM medium and incubated at 30^{o}C for 72 hours. A bacterial suspension was prepared by suspending a loopful of fresh culture in 2 ml broth base in a sterile bijou bottle. The cell density of all 45 strains was measured with a densitometer and adjusted to a turbidity equivalent to 1.0 McFarland units (approx. 3.0 x 10^{8} colony forming units (cfu)/ml) using additional broth base where required.

3.11.3 Evaluation of fluorogenic substrates for both mycobacteria and non-mycobacterial isolates

Fluorogenic substrates were prepared at 2.96 mM and initially dissolved in 200 µl N-methyl-2-pyrrolidone and made up to 5.5 ml with broth base. Esterase substrates had an additional 300 µl tween 20 added to prevent them precipitating out of solution and all were made up to a final volume of 5.5 ml with broth base. Substrates which did not go into solution readily at room temperature were heated until fully dissolved and clear in colour.

A 50 µl volume of each isolate suspension was loaded into the wells of a microtitre tray in duplicate, with their position recorded, and an equal volume of the substrate solution added. In addition, three negative control organism-free wells were prepared, and two controls containing *E. coli* (NCTC 10418) and *S. aureus* (NCTC 6571). Each substrate was tested in duplicate. Microtitre trays were read to obtain an initial "time zero" measurement of fluorescence with a fluorescence microtitre plate reader using a 365 nm excitation wavelength and a 440 nm emission wavelength. Trays were incubated at 30^{o}C for 72 hours after which the fluorescence readings were repeated. Average readings after 72 hours incubation are shown in Appendix 5.

3.11.4 Initial evaluation of chromogenic substrates for mycobacterial isolates

Magenta octanoate was evaluated for activity in NTM and L-ala-ala-ala-4(4'-aminostyryl)-N-methylquinolinium dichloride was evaluated for activity in both NTM and a selection of non-mycobacteria, which included a number of common Gram-negative CF pathogens. Columbia agar was prepared in 200 ml volumes and magenta octanoate was added at a concentration of 0.45 g/L. This was dissolved in N-methyl-2-pyrrolidone (4.5 ml/L) and tween 20 (7.9 ml/L) then added to the agar once cooled to 50^{o}C. Twenty isolates of NTM were inoculated onto the medium and incubated at 30^{o}C for four days. L-ala-ala-ala-4(4'-aminostyryl)-N-methylquinolinium dichloride was added at a concentration of 0.1 g/L dissolved in N-methyl-2-pyrrolidone (1 ml/L) and added to the agar once cooled to 50^{o}C. Twelve isolates of NTM and 28 non-mycobacterial isolates were inoculated onto the medium and incubated at 30^{o}C for four days.

Eight further chromogenic substrates were evaluated to consider if there were any benefits to including these in RGM medium. RGM agar was prepared in 100 ml volumes as previously described and the following concentrations of each substrate added; 5-bromo-6-chloro-3-indolyl-ß-D-galactopyranoside 0.08g/L, 6-chloro-3-indolyl-ß-D-glucopyranoside 0.2g/L, 6-chloro-3-indolyl-ß-D-glucuronide 0.2g/L, alizarin-2-α-D-galactopyranoside 0.05g/L, alizarin-2-ß-D-galactopyranoside 0.05g/L, alizarin-2-ß-D-glucopyranoside 0.05g/L, 3,4-cyclohexenoesculetin-ß-D-galactopyranoside 0.3g/L and 3,4-cyclohexenoesculetin-ß-D-glucopyranoside 0.3g/L. All substrates were dissolved in N-methyl-2-pyrrolidone (1 ml/L) and added to the agar once cooled to 50^{o}C. Eleven isolates of NTM were used in total, with five inoculated onto each media and incubated at 30^{o}C for four days. Control plates containing no substrates were inoculated for all isolates.

3.12 Results

3.12.1 Activity of fluorogenic substrates in both non-mycobacteria and mycobacterial isolates

The results for the activity detected with fluorogenic substrates are shown in Table 3-1. Mycobacteria were showed most activity with 4-Methylumbelliferyl laurate, 4-Methylumbelliferyl palmitate, 4-Methylumbelliferyl stearate, 4-Methylumbelliferyl β-D-galactoside, 4-Methylumbelliferyl β-D-glucoside, H-Leu-AMC.HCl, H-Met-AMC acetate salt, H-His-AMC, H-Arg-AMC.2HCl, H-Try-AMC, H-β-Ala-Amc.TFA, L-alanine-AMC trifluoroacetate salt, Boc-Leu-Gly-Arg-AMC acetate salt and 4-Methylumbelliferyl phosphate. For all of these however the majority of non-mycobacteria also demonstrated activity, therefore discriminating between NTM and other species would still be problematic. Methyl Red 291 was highly sensitive in 23/26 (88.5%) of NTM, with only three isolates of *M. chelonae* showing no activity. Inactivity for all of the non-mycobacteria tested was observed, except for the two control organisms tested, *S. aureus* and *E. coli*. No activity was detected with six of the substrates for any of the species tested; 4-Methylumbelliferyl propionate, 4-Methylumbelliferyl *p*-guanidinobenzoate hydochlorate hydrate, 4-Methylumbelliferyl lignocerate, 4-Methylumbelliferyl 7-β-D-xyloside, 4-Methylumbelliferyl α-D-glucopyranoside and SR266, and two showed 100% sensitivity for all isolates; H-Leu-AMC.HCl and L-alanine-AMC trifluoroacetate salt. None of the fluorogenic substrates tested was able to successfully differentiate between *M. chelonae* or between subspecies of the *M. abscessus* complex.

Table 3-1: Percentage of isolates possessing enzyme activity with a range of fluorogenic substrates

	(*n*)	4MA	4MPr	4MB	4MH	4MN	4MLa	4MPgh	4MPa	4MSt	4MLi	4Mara	4Mxyl	4Mribs	4Mribf
A. xylosoxidans	3	0	0	0	0	0	100	0	0	0	0	0	0	33.3	33.3
B. cenocepacia	2	0	0	0	50	0	50	0	50	0	0	0	0	100	100
B. multivorans	3	0	0	0	0	0	100	0	66.7	66.7	0	0	0	0	0
B. stabilis	1	0	0	0	100	0	100	0	100	100	0	0	0	100	100
B. vietnamiensis	1	0	0	0	0	0	0	0	0	0	0	0	0	100	0
D. acidovorans	1	0	0	0	0	0	0	0	100	0	0	0	0	0	0
I. limosus	1	0	0	0	0	0	0	0	0	0	0	0	0	100	100
P. norimbergenis	1	0	0	0	0	0	0	0	0	0	0	0	0	100	0
P. aeruginosa	1	100	0	0	0	0	100	0	100	100	0	0	0	100	100
S. marcescens	1	0	0	100	100	0	100	0	100	100	0	100	0	100	100
*M. abscessus**	12	0	0	0	8.3	8.3	100	0	100	100	0	33.3	0	75	25
*M. bolletii***	2	0	0	0	0	0	100	0	100	100	0	0	0	0	0
*M. massiliense****	8	0	0	0	0	0	100	0	100	100	0	33.3	0	66.7	16.7
M. chelonae	6	0	0	0	25	12.5	100	0	100	100	0	62.5	0	87.5	25
S. aureus	1	0	0	0	0	0	0	0	0	0	0	100	0	100	100
E. coli	1	0	0	0	0	0	0	0	100	0	0	0	0	100	100

	(*n*)	4Mfuc	4Mglupα	4Mglupβ	4Mmanα	4Mgalα	4Mgalβ	4Mgluβ	4MIdo	4Manβ	4Mcurβ	4MIdu	4MNgal	4MNglu	4Mcel
A. xylosoxidans	3	0	0	0	0	0	100	0	0	0	0	0	0	0	0
B. cenocepacia	2	0	0	50	0	0	50	50	0	0	0	0	0	0	50
B. multivorans	3	0	0	0	0	0	33.3	0	0	0	0	0	0	0	0
B. stabilis	1	0	0	0	0	0	100	0	0	0	0	0	0	0	0
B. vietnamiensis	1	0	0	100	0	0	100	0	0	0	0	0	0	0	0
D. acidovorans	1	0	0	0	0	0	100	0	0	0	100	0	0	0	100
I. limosus	1	0	0	100	100	0	100	100	0	0	0	0	0	0	0
P. norimbergenis	1	0	0	0	0	0	100	0	0	0	0	0	0	0	0
P. aeruginosa	1	0	0	0	0	0	100	0	0	0	0	0	0	0	0
S. marcescens	1	0	0	100	100	100	100	100	0	100	100	100	100	0	100
*M. abscessus**	12	33.3	0	100	91.7	83.3	100	100	33.3	83.3	83.3	58.3	0	8.3	66.7
*M. bolletii***	2	0	0	100	100	50	100	100	0	50	100	50	0	0	0
*M. massiliense****	8	0	0	83.3	100	0	100	100	0	66.7	83.3	16.7	0	0	16.7
M. chelonae	6	25	0	100	100	75	100	100	62.5	75	100	62.5	37.5	50	75
S. aureus	1	100	0	0	0	0	100	0	0	0	100	0	0	0	0
E. coli	1	0	0	0	0	0	100	0	0	0	0	0	0	0	0

**M. abscessus* subsp. *abscessus*, ***M. abscessus* subsp. *bolletii*, ****M. abscessus* subsp. *massiliense*

Key : 4MA : 4-Methylumbelliferyl acetate, 4MPr : 4-Methylumbelliferyl propionate, 4MB: 4-Methylumbelliferyl butyrate, 4MH: 4-Methylumbelliferyl heptanoate, 4MN : 4-Methylumbelliferyl nonanoate, 4MLa : 4-Methylumbelliferyl laurate, 4MPgh : 4-Methylumbelliferyl p-guanidinobenzoate hydochlorate hydrate, 4MPa : 4-Methylumbelliferyl palmitate, 4MSt : 4-Methylumbelliferyl stearate, 4MLi : 4-Methylumbelliferyl lignocerate, 4Mara : 4-Methylumbelliferyl α-L-arabinopyranoside, 4Mxyl : 4-Methylumbelliferyl 7-β-D-xyloside, 4Mribs : 4-Methylumbelliferyl riboside, 4Mribf : 4-Methylumbelliferyl β-D-ribofuranoside, 4Mfuc : 4-Methylumbelliferyl β-D-fucoside, 4Mglupα : 4-Methylumbelliferyl α-D-glucopyranoside, 4Mglupβ : 4-Methylumbelliferyl β-D-glucopyranoside, 4Manα : 4-Methylumbelliferyl α-D-mannopyranoside, 4Mgalα : 4-Methylumbelliferyl α-D-galactoside, 4Mgalβ : 4-Methylumbelliferyl β-D-galactoside, 4Mgluβ : 4-Methylumbelliferyl β-D-glucoside, 4MIdo : 4-Methylumbelliferyl α-L-idopyranoside, 4Manβ : 4-Methylumbelliferyl β-D-mannopyranoside, 4Mcurβ : 4-Methylumbelliferyl β-D-glucuronide, 4Midu : 4-Methylumbelliferyl α-L-iduronide, 4MNgal : 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide, 4MNglu : 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, 4Mcel : 4-Methylumbelliferyl β-D-cellobioside,

Table 3-1 continued: Percentage of isolates possessing enzyme activity with a range of fluorogenic substrates

	(*n*)	Pro	Val	Thr	Ile	Leu	Orn	Gln	MetH	His	Phe	ArgH	Try	MetA	Ala
A. xylosoxidans	3	100	0.0	0	0.0	100	0	100	100	33.3	100	100	100	0	0
B. cenocepacia	2	50	0.0	0	0.0	100	0	100	100	50	100	50	100	0	100
B. multivorans	3	100	0.0	0	0.0	100	0	33.3	33.3	0	100	33.3	100	0	100
B. stabilis	1	100	0.0	0	100	100	0	100	100	100	100	100	100	0	100
B. vietnamiensis	1	100	100	100	100	100	100	100	100	100	100	100	100	0	0
D. acidovorans	1	0	0.0	0	0	100	0	100	100	0	100	100	100	0	0
I. limosus	1	100	100	100	100	100	0	100	100	100	100	100	100	0	100
P. norimbergenis	1	100	0.0	0	0	100	0	100	100	0	100	100	100	0	0
P. aeruginosa	1	0	0.0	0	0	100	0	100	0	0	100	100	100	0	100
S. marcescens	1	100	100	100	100	100	100	100	100	100	100	100	100	0	100
*M. abscessus**	12	91.7	91.7	91.7	91.7	100	100	100	100	100	100	100	100	33.3	100
*M. bolletii***	2	50	50	50	100	100	50	50	100	100	100	100	100	0	100
*M. massiliense****	8	100	100	83.3	100	100	83.3	100	100	100	10	100	100	0	100
M. chelonae	6	100	100	100	100	100	100	100	100	100	100	100	100	62.5	100
S. aureus	1	0	0	0	0	100	0	100	100	100	100	100	100	0	0
E. coli	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0

	(*n*)	AAMC	GlyP	Boc	4MPh	4MSu	SR123	SR124	S521	SR266	MR291	7N3CA
A. xylosoxidans	3	100	0	0	100	0	0	0	0	0	0	100
B. cenocepacia	2	100	0	50	100	0	0	0	0	0	0	0
B. multivorans	3	100	0	0	100	0	0	0	0	0	0	66.7
B. stabilis	1	100	0	0	100	0	0	0	0	0	0	0
B. vietnamiensis	1	100	100	0	100	0	0	0	0	0	0	0
D. acidovorans	1	100	0	0	100	100	0	0	0	0	0	100
I. limosus	1	100	0	100	100	0	0	0	0	100	0	0
P. norimbergenis	1	100	0	0	100	0	0	0	0	0	0	0
P. aeruginosa	1	100	0	100	0	0	0	0	0	100	0	0
S. marcescens	1	100	100	100	100	0	0	0	0	100	0	100
*M. abscessus**	12	100	0	100	100	33.3	0	8.3	0	91.7	100	16.7
*M. bolletii***	2	100	0	100	100	0	0	0	0	100	100	0
*M. massiliense****	8	100	0	100	100	66.7	16.7	16.7	0	100	100	0
M. chelonae	6	100	12.5	100	100	50	0	0	0	62.5	50	0
S. aureus	1	0	0	100	100	0	0	0	0	100	100	100
E. coli	1	100	0	0	100	0	0	0	0	100	100	100

**M. abscessus* subsp. *abscessus, **M. abscessus* subsp. *bolletii, ***M. abscessus* subsp. *massiliense*

Key : Pro : H-Pro-AMC hydrobromide salt (Prolyl), Val : H-Val-AMC, Thr : H-Thr-AMC, Ile : H-Ile-AMC.TFA, Leu : H-Leu-AMC.HCl, Orn : H-Orn-AMC.2HCl, Gln : H-Gln-AMC, MetH : H-Met-AMC acetate salt, His : H-His-AMC, Phe : H-Phe-AMC.TFA, ArgH : H-Arg-AMC.2HCl, Try : H-Try-AMC, MetA : Ac-Met-AMC, Ala : H-β-Ala-Amc.TFA, AAMC : L-alanine AMC trifluoroacetate salt, GlyP : H-Gly-pro-AMC.HBr, Boc : Boc-Leu-Gly-Arg-AMC acetate salt, 4MPh : 4-Methylumbelliferyl phosphate, 4MSu : 4-Methylumbelliferyl sulfate, SR123 : , SR124 : , S521 : , SR266 : , MR291 : Methyl Red 291, 7N3CA : 7-nitrocoumarin-3-carboxylic acid.

None of the fluorogenic substrates specifically stood out with a profile that would indicate they would be of any benefit if they were included in RGM medium for the differentiation of NTM and other non-NTM species.

3.12.2 Activity of chromogenic substrates in non-tuberculous mycobacteria

All isolates of mycobacteria demonstrated a colour change with magenta octanoate and grew as purple colonies except one isolate of *M. abscessus* subsp*. bolletii*, which failed to grow. No colour change was observed on all control plates (see Table 3-2 and Figure 3-3) below. This indicated that production of a C8-esterase was a consistent feature of the mycobacteria that were tested that were able to grow in the presence of this substrate.

Table 3-2: Appearance and growth of NTM isolates with magenta octanoate

MABSC Subspecies	Isolate Number	Growth After 4 Days	Colour of Colonies
M. abscessus complex (chimeric)	1000	+	purple
M. abscessus subsp. *abscessus*	1034	+	purple
M. abscessus subsp. *abscessus*	1042	+	purple
M. abscessus subsp. *abscessus*	1044	++	purple
M. abscessus subsp. *abscessus*	1045	+++	purple
M. abscessus subsp. *abscessus*	1047	+++	purple
M. abscessus subsp. *abscessus*	1050	+	purple
M. abscessus subsp. *abscessus*	1051	+++	purple
M. abscessus subsp. *abscessus*	1052	+	purple
M. abscessus subsp. *abscessus*	1053	+++	purple
M. abscessus subsp. *abscessus*	1054	+++	purple
M. abscessus subsp. *abscessus*	1055	+	purple
M. abscessus subsp. *bolletii*	3015	+++	purple
M. abscessus subsp. *bolletii*	3016	No Growth	N/A
M. abscessus subsp. *bolletii*	3017	+++	purple
M. abscessus subsp. *massiliense*	3010	+++	purple
M. abscessus subsp. *massiliense*	3011	++	purple
M. abscessus subsp. *massiliense*	3012	+++	purple
M. abscessus subsp. *massiliense*	3014	+++	purple
M. abscessus subsp. *massiliense*	3015	+	purple

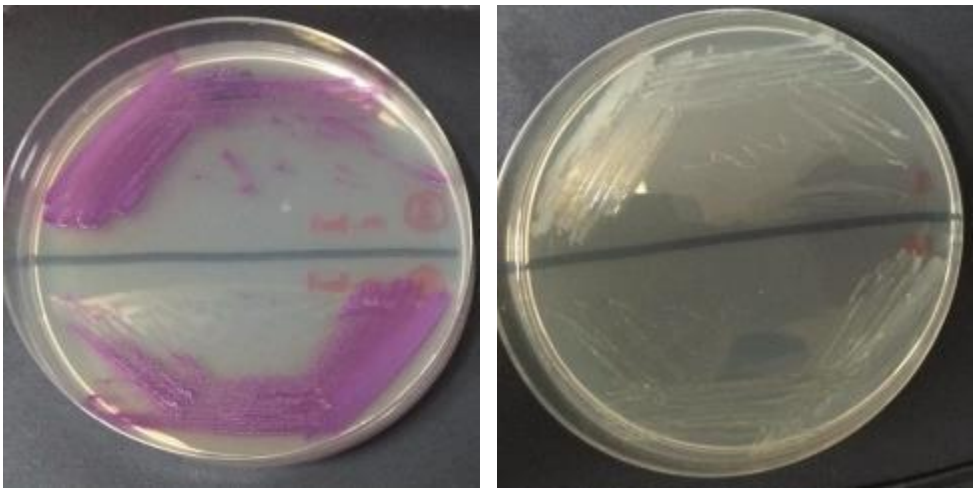


Figure 3-3: Isolates 3017 *M. abscessus* subsp. *bolletii* and 1054 *M. abscessus* subsp. *abscessus* shown with magenta octanoate (left) added and control plate without magenta octanoate (right)

Results for L-ala-ala-ala-4(4’-aminostyryl)-N-methylquinolinium dichloride are shown below in Table 3-3 and Figure 3-4 and Figure 3-5. No colour change was observed on all control plates.

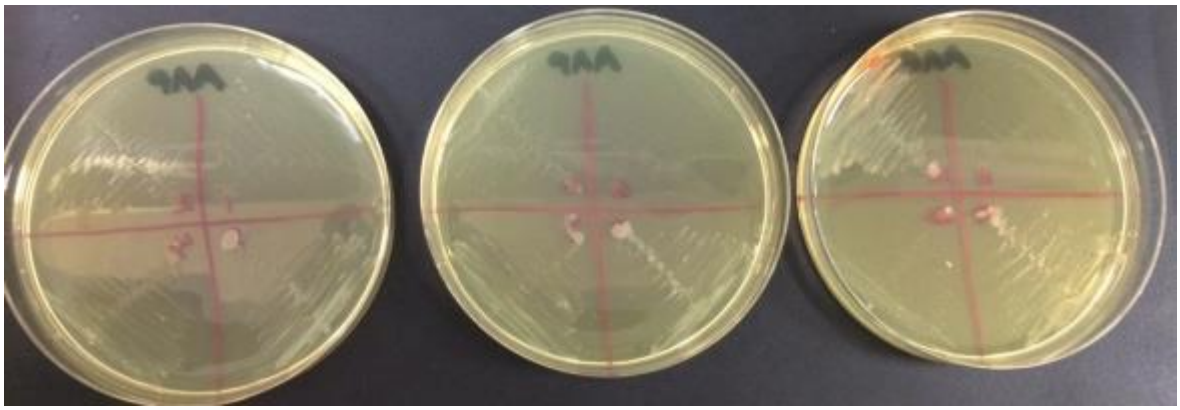


Figure 3-4: Non-tuberculous mycobacteria isolates with L-ala-ala-ala-4(4’-aminostyryl)-N-methylquinolinium dichloride included in the agar

Table 3-3: Appearance and growth of NTM isolates with L-ala-ala-ala-4(4'-aminostyryl)-N-methylquinolinium dichloride

Species	Reference	Growth After 4 Days	Colour of colonies
Non-tuberculous mycobacteria			
M. abscessus complex	1000	+	white
M. abscessus subsp. *abscessus*	1034	+/-	white
M. abscessus subsp. *abscessus*	1042	+/-	white
M. abscessus subsp. *abscessus*	1044	+/-	white
M. abscessus subsp. *abscessus*	1054	++	white
M. abscessus subsp. *abscessus*	1055	++	white
M. abscessus subsp. *bolletii*	3015	++	white
M. abscessus subsp. *bolletii*	3016	+	white
M. abscessus subsp. *massiliense*	3010	+/-	white
M. abscessus subsp. *massiliense*	3011	+	white
M. abscessus subsp. *massiliense*	3012	+	white
M. abscessus subsp. *massiliense*	3013	+	White
Non-mycobacterial isolates			
A. xylosoxidans	7010	++	pink
A. xylosoxidans	7015	++	pink edges
A. xylosoxidans	7027	+++	pale pink
A. xylosoxidans	7037	++	pink
Acinetobacter sp.	8003	++	pink
B. cenocepacia	7009	++	pink
B. cenocepacia	7012	+	red
B. cepacia	8007	+++	pink
B. contaminans	8008	++	red
B. multivorans	7017	++	red
B. multivorans	7022	++	red
B. multivorans	7024	++	red
B. multivorans	7036	+	pink
B. stabilis	7026	++	red
B. vietnamiensis	7032	+	pink
D. acidovorans	7008	+++	red
E. miricola	8025	+++	red
I. limosus	7007	++	red
I. limosus	7011	++	no colour
Ochrobactrum sp.	7031	++	pale pink
P. aeruginosa	7004	+++	pink
P. aeruginosa	8017	+	pink/red
P. aeruginosa	8011	++	pale pink
P. aeruginosa	8012	+++	pink
P. apista	8020	+	pink
P. norimbergenis	7018	+	pink
P. pnomenusa	8021	+	pink
S. marcesens	7019	+++	red
S. marcesens	7020	+++	red
S. maltophilia	8022	+++	red
S. maltophilia	8023	+++	red

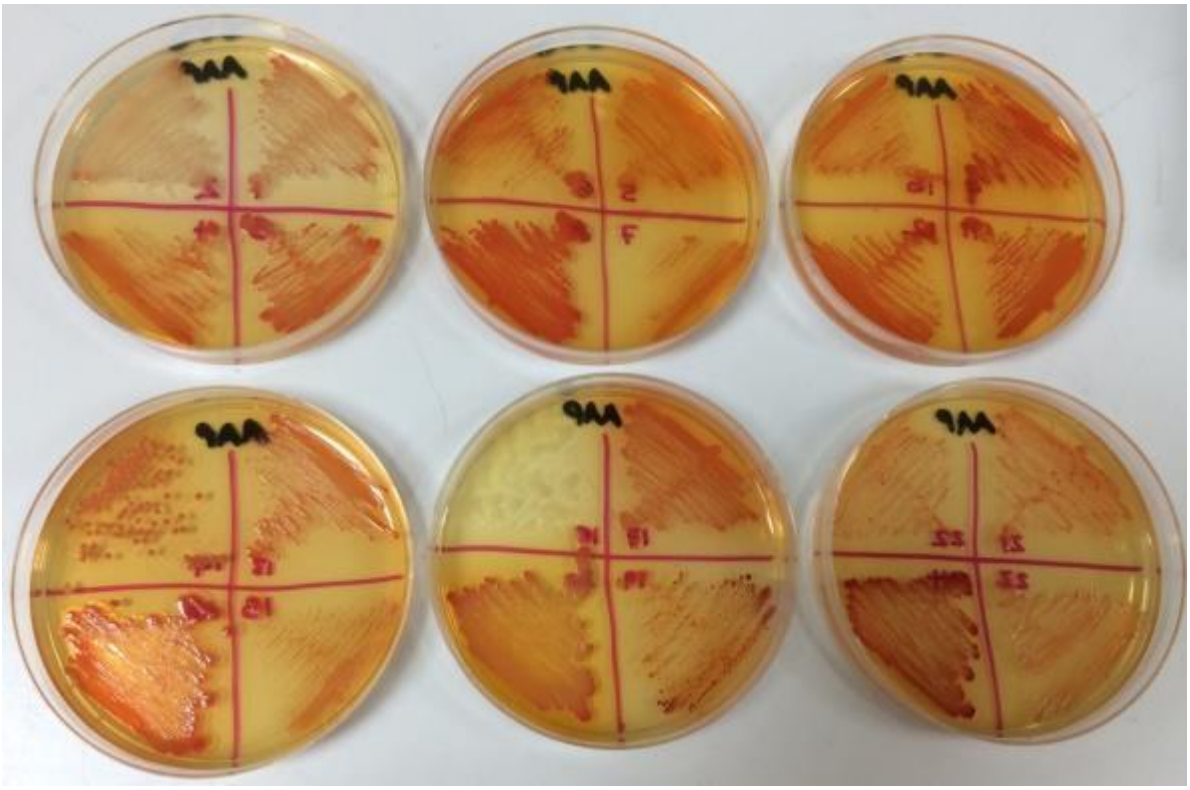


Figure 3-5: Showing growth and colouration of Gram-negative isolates as follows: *A. xylosoxidans* x4 (top left), *Acinetobacter* spp, *B. cenocepacia* x2, *B. cepacia* (top centre), *B. contaminans, B. multivorans* x3 (top right), *B. multivorans*, *B. stabilis, B. vietnamiensis, D. acidovorans* (bottom left), *E. miricola, I. limosus x2, P. aeruginosa* (bottom centre) and *P. apista, P. norimbergenis, P. pnomenusa, S. marcescens* (bottom right) with L-ala-ala-ala-4(4'-aminostyryl)-N-methylquinolinium dichloride encompassed in the agar

Five of the eleven NTM strains were inoculated onto each RGM (minus antibiotics) with chromogenic substrates added and results are shown below in Table 3-4 and Figure 3-6. No colour change of NTM was observed on any control plates.

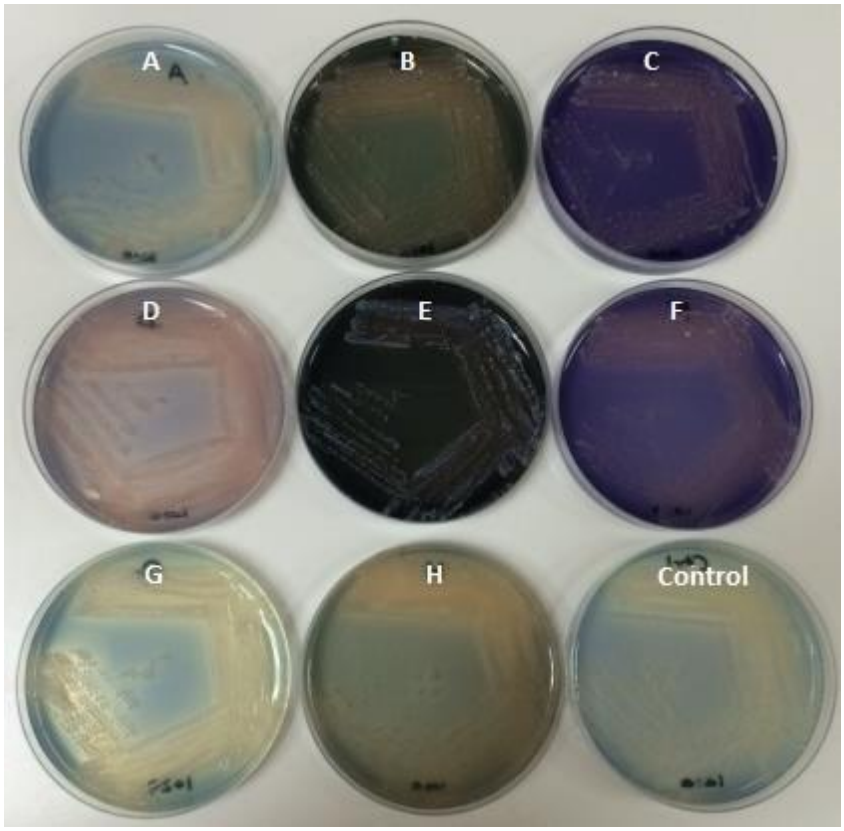


Figure 3-6: The appearance of isolates 1010 *M. abscessus* subsp*. abscessus* and 1029 *M. abscessus* subsp*. abscessus* using the substrates A – H as shown in Table 3-4 and control plate containing no chromogenic substrates

Table 3-4: The appearance of NTM inoculated onto RGM medium with the addition of various chromogenic substrates

ID	Species	A	B	C	D	E	F	G	H
2002	*M. chelonae*								pale orange/white
2014	*M. chelonae*								pale orange/white
1010	*M. abscessus* subsp. *abscessus*	white	white	white	pale pink/white	white	white		pale orange/white
1013	*M. abscessus* subsp. *abscessus*	white	white	white					
1026	*M. abscessus* subsp. *abscessus*	white	white	white					
1027	*M. abscessus* subsp. *abscessus*								pale orange/white
1029	*M. abscessus* subsp. *abscessus*				pale pink/white	white	white	white	
1032	*M. abscessus* subsp. *abscessus*				pale pink/white	white	white	white	
3002	*M. abscessus* subsp. *massiliense*	white	white	white	pale pink/white	white	white	white	
3007	*M. abscessus* subsp. *massiliense*				pale pink/white	white/blue	white	white/blue	pale orange/white/ blue
3016	*M. abscessus* subsp. *bolletii*	white	white	white				white	

Key: **A**; 5-bromo-6-chloro-3-indolyl-ß-D-galactopyranoside, **B**; 3,4-cyclohenenoesculetin-ß-D-galactopyranoside, **C**; alizarin-2-ß-D-galactopyranoside, **D**; 6-chloro-3-indolyl-ß-D-glucopyranoside, **E**; 3,4-cyclohenenoesculetin-ß-D-glucopyranoside, **F**; alizarin-2-ß-D-glucopyranoside, **G**; 6-chloro-3-indolyl-ß-D-glucuronide, **H**; alizarin-2-α-D-galactopyranoside. Good (++) growth of all species was observed after 4 days incubation at 30°C.

3.13 Discussion

Rapid species-level identification of mycobacteria is of high importance in patients with CF in order for accurate and prompt treatment to be provided in patients deemed to require therapy. Identification of mycobacteria to species level by conventional biochemical tests have lengthy turnaround times subsequently leading to delays in diagnosis, as well as giving ambiguous and unreproducible results. The phenotype of many mycobacterial species can also be exceptionally variable.

The aim of this study was to evaluate a range of chromogenic and fluorogenic substrates containing coumarin derivatives 4-methylumbelliferone (4-MU) or 7-amino-4-methylcoumarin (7-AMC) to look at the presence or absence and quantities of a range of mycobacterial glycosidases, peptidases and esterases for their capability to discriminate between *M. abscessus* complex and the less pathogenic *M. chelonae*, as well as the potential to differentiate between the three members of the *M. abscessus* complex, (*M. abscessus*, *M. massiliense* and *M. bolletii*) and additionally to ascertain whether mycobacteria can be differentiated from Gram-negative bacteria recurrently found in the sputum of patients with CF, and occasionally recovered on RGM medium.

If such a substrate was available, incorporation into a primary isolation medium such as RGM could greatly increase the appeal of the medium allowing for easy enumeration and detection directly on the isolation plate, subsequently providing a rapid and effective means of identifying pathogenic NTM in a CF sputum sample, which could potentially harbour a diverse range of bacterial species. This will also eliminate the need for subculture or further biochemical tests.

The use of umbelliferone derivatives were reported to be a sensitive technique in the detection of mycobacterial group-specific hydrolases such as lipases, phosphatases or glucosidases, and it has been advocated they may be appropriate for investigating the glycosidase activity of slow-growing mycobacteria, (Grange and Clark, 1977; Grange, 1978; Slosarek, 1980). *Mycobacterium nonchromogenicum, Mycobacterium szulgai* and *Mycobacterium marinum* exhibited characteristic enzyme activities which distinguished them from the other species, with *M. marinum* being unique amongst mycobacteria for its very obvious α-L-fucosidase activity (Grange and McIntyre, 1979).

The methods employed in this study would not prove to be technically difficult or time consuming, and substrates could be incorporated into the medium, however the results established that the use of these would not provide any additional benefit to RGM.

As mycobacteria appear on RGM as bright white colonies, either smooth or rough, and other species that are able to grow generally appear blue or pink, mycobacteria are already quite distinguishable on the medium, however to differentiate *M. abscessus* complex from less pathogenic *M. chelonae* or *M. mucogenicum* will still require further testing and sequencing.

CHAPTER FOUR

Epidemiology, possible sources, transmission and prevention of rapidly-growing mycobacteria from the environment

Introduction

4.1 The epidemiology of non-tuberculous mycobacterial infections

Non-tuberculous mycobacteria (NTM) are opportunistic pathogens whose sources include water, dust and soil. They are not contaminants, but somewhat typical inhabitants of these environments. By occupying the same surroundings as humans, this allows frequent exposure to occur on a daily basis from these sources via inhalation and/or ingestion (Cook, 2010; Falkinham *et al.*, 2015).

NTM represent a substantial threat emerging in patients with CF, with until recently, limited reports presenting conflicting results of global prevalence.

Estimates of the prevalence of NTM in the CF population have ranged from 1.3% in 1984 (Smith *et al.*, 1984) to 32.7% in a US study in 2005 (Rodman *et al.*, 2005). The largest published studies to date looked at 986 (Olivier *et al.*, 2003), 1216 (Esther *et al.*, 2010) and 1582 (Roux *et al.*, 2009) individuals with CF, and reported positive NTM cultures of 13.0%, 13.7% and 6.6% respectively.

The predisposing factors for acquisition of NTM are still largely unknown though recently a large US study reported possible risk factors for NTM using CF patient registry data from 2003 to 2011 (Binder *et al.*, 2013). Limitations of this study were that not all incidences of NTM were reported, and it may also include patients that were only transiently colonised and not experiencing true NTM disease. No such study has been completed in Europe. There are a wide range of infections caused by rapidly-growing mycobacteria (RGM), but in recent years, members of the MABSC have emerged as major pathogens in patients suffering from CF.

The most fundamental difference between *M. tuberculosis, M. leprae* and non-tuberculous mycobacterial disease is that person-to-person transmission seems to be extremely rare in the latter, with limited reports of this occurring, and disease

almost certainly arising due to NTM being extensively dispersed in the environment. Defining the epidemiology of NTM is particularly challenging as unlike *M. tuberculosis*, the documentation of NTM infection is not compulsory or enforced and therefore does not necessitate reporting to Public Health authorities. This is due to the fact it is not considered a major public health concern, as there is very little evidence to substantiate human-to-human transmission. In a recent study, whole-genome sequencing of *M. abscessus* in CF patients indicated human-to-human transmission can occur, however this study has yet to be replicated (Bryant *et al.*, 2013).

An accurate understanding of the true epidemiology of NTM disease is still lacking. It is also arduous to precisely quantify the true prevalence of NTM as a positive culture could indicate transient colonization and does not always indicate genuine clinical infection.

An increase in NTM infection could in part be due to improved diagnostic techniques as well as recognition of the significance of NTM, however current evidence does show a genuine increase globally (Griffith *et al.*, 2007; Weiss and Glassroth, 2012; Wentworth *et al.*, 2013) and in a number of studies an escalation of cases can be witnessed where no variation in intensity of monitoring or modifications in culture techniques have occurred. Other explanations for the increase have been suggested, for example, a rise in environmental exposure due to home water heaters and shower aerosols (Falkinham, 2011; Thomson *et al.*, 2013a; Feazel *et al.*, 2009) and an upsurge in the use of antibiotics, including chronic use of medications impairing host immunity (Renna *et al.*, 2011; Catherinot *et al.*, 2013a).

4.2 The global incidence of non-tuberculous mycobacterial disease

Evidence suggests that the increase in the prevalence of NTM over the past few decades will continue to rise with multifactorial causes. The increasing age of the population, along with an escalation in the occurrence of diseases such as diabetes mellitus or chronic obstructive pulmonary disease, as well as a rise in the use of immunosuppressant medication would seem to play a major role in the increase of NTM (Winthrop *et al.*, 2009). Another factor is NTM being abundant in the environment, in water and water supply systems, aerosols, decaying vegetation, soil and biofilms, swimming pools and hot tubs (Fjallbrant *et al.*, 2013), therefore exposure to NTM would appear to occur on a daily basis (September *et al.*, 2004; Falkinham, 2009). Increased awareness by physicians and enhanced culture techniques may also play a substantial role, leading to increased rates of detection and more prompt and accurate identification.

A vast collaborative study by NTM-NET collected species identification data for 20,182 patients, from 62 laboratories in 30 countries across six continents (Hoefsloot *et al.*, 2013). Each hospital or reference laboratory was invited to provide data from patients where NTM was isolated from pulmonary samples, provided that number exceeded 30, within the year 2008, as well as providing the species and method of identification. One isolate per species per patient qualified for analysis. Results showed that 91.3% (18,418) of all isolates were identified to species or complex level, and the remaining 1,764 could not be identified beyond *Mycobacterium* species (other than *M. tuberculosis*). The six most frequent species recovered, and accounting for 80% of all isolates, were *M. avium* complex (MAC; 47%), *M. gordonae* (11%), *M. xenopi* (8%), *M. fortuitum* complex (7%), *M. kansasii* (4%) and MABSC (3%).

Out of the rapidly-growing mycobacteria, MABSC and *M. fortuitum* were the most frequently isolated, however significant geographical variances were observed with the highest number of rapid growers encountered in East Asia making up 27% of all NTM isolates in comparison to North America (17.9%), South America (16%) and Europe (14%). It was also noted that within Asia, significant differences occurred with 6.6% of all isolates being rapid growers compared to 50% in Taiwan and 28.7% in South Korea.

In Europe *M. avium* was the most frequently isolated NTM, accounting for 44% of all species isolated in Northern Europe and 31% in Southern Europe. This is consistent with other reports (Olivier *et al.*, 2003; Floto *et al.*, 2016), although in many European centres MABSC has overtaken all other species as the most commonly isolated RGM (Sermet-Gaudelus *et al.*, 2003), (Roux *et al.*, 2009; Esther *et al.*, 2010; Qvist *et al.*, 2015).

In individuals with CF, acquisition of NTM is largely associated with age, and prevalence would seem to increase after patients reach adulthood. MABSC however is isolated at all ages, whereas MAC does not seem to be recovered until patients are considerably older (Olivier *et al.*, 2003; Pierre-Audigier *et al.*, 2005; Catherinot *et al.*, 2013b).

4.3 Environments providing favourable conditions for the transmission of non-tuberculous mycobacteria

NTM are reported to be common in all natural environments, and transmission is thought to occur via ingestion, inoculation or inhalation (Gangadharam and Jenkins, 1998). Rapidly growing mycobacteria withstand a much more extensive range of temperature, salinity, oxygen tension and pH than other pathogenic bacteria, with a higher tolerance to standard disinfectants such as chlorine (Le Dantec *et al.*, 2002).

Their capacity for biofilm formation and ability to grow at low carbon levels also enables their survival in both natural and manmade environments, including water systems (Falkinham, 1996).

The original emphasis of NTM environmental investigations centred on natural waters and soils (Falkinham *et al.*, 1980; Brooks *et al.*, 1984). However, with the outbreak of AIDS and HIV in the early 1980's reports of NTM infections were increasing and led to the deduction that NTM were widely distributed within the environment (Greene *et al.*, 1982).

NTM are able to colonise drinking water distribution systems due to their cell surface hydrophobicity preventing flushing from the system and their ability to form biofilms (Bendinger *et al.*, 1993; Torvinen *et al.*, 2004). In untreated water systems, NTM are poor competitors for nutrients due to their slow growth rates, however as the typical disinfection process kills off any competitors for nutrients, they are able to thrive (Taylor *et al.*, 2000). NTM are also somewhat heat-resistant and have been recovered from household plumbing where temperatures have been set to up to 55^{o}C, and even higher for some NTM species such as *M. xenopi* (Falkinham, 2011; Schulze-Robbecke and Buchholtz, 1992). Low oxygen concentrations due to reduced or discontinuous water flow does not impede NTM growth, as they are able to acclimatise and endure low concentrations of oxygen (Dick *et al.*, 1998; Lewis and Falkinham, 2015). However, in areas of high levels of oxygen, quantities of NTM were lower (Kirschner *et al.*, 1992).

High numbers of NTM are found in soil, where due to their hydrophobicity they can adhere to soil particles and be aerosolised as dusts (De Groote *et al.*, 2006). In a study by Narang *et al*., 26 isolates comprising of seven species of NTM, including three *M. abscessus,* were reported to have been isolated from water and soil samples in India where samples were obtained from household and work areas of

NTM positive patients (Narang *et al.*, 2009). The methods used for identification of species in this report encompassed phenotypic testing including growth at varying temperatures on different media and several biochemical tests as well as PCR restriction analysis of *hsp65* genes using *Hea*III and *Bst*E2 restriction enzymes (Telenti *et al.*, 1993). However, none of the environmental isolates correlated with any of the patient isolates.

NTM have also been associated with metal removal fluids (Wu *et al.*, 2015), and it has been suggested that hypersensitivity pneumonitis in exposed workers may be connected to NTM present in metal removal fluid aerosols. However as these fluids are diluted with water in order to form an emulsion, it is highly probable that the water is in fact providing the source of the NTM (Kapoor and Yadav, 2012; Wu *et al.*, 2015).

The most commonly reported cause of NTM-associated hypersensitivity pneumonitis is *via* aerosolisation of contaminated hot-tub and swimming pool water, with numerous reports describing patients who developed NTM hypersensitivity pneumonitis from these sources following frequent use (Embil *et al.*, 1997; Rickman *et al.*, 2002; Glazer *et al.*, 2007).

There have been very few accounts of MABSC isolated from any of the above sources, with the majority of rapidly growing NTM documented being *M. chelonae*, *M. fortuitum*, *M. gordonae*, *M. mucogenicum*, and *M. kansasii* (Falkinham, 2009). Due to many taxonomic changes over the years, particularly relating to MABSC and *M. chelonae*, which is frequently isolated from the environment, caution should be applied in interpreting many of these reports describing the isolation of MABSC from the environment, as they were typically reported prior to these being designated as separate species in 1992 when *M. abscessus* was still thought to be a subspecies of *M. chelonae* (Kusunoki and Ezaki, 1992). In more recent publications, the

presence of MABSC is seldom reported, however in a recent case *M. abscessus* was said to be isolated from drinking water in Brisbane (Thomson *et al.*, 2013b), and from skin infections detected after the 2004 tsunami in South-East Asia (Appelgren *et al.*, 2008). In South Africa, *M. abscessus* was reported to have been isolated from drinking water distribution centres (September *et al.*, 2004), and in ready to eat vegetables in Mexico (Cerna-Cortes *et al.*, 2015).

4.4 Reducing exposure to non-tuberculous mycobacteria

Recommendations to avoid NTM exposure for those who are susceptible, for example patients with CF, include disinfection of showerheads by submerging in undiluted bleach for thirty minutes, or by replacing the showerhead with one that produces streams rather than a fine mist as this type generates lower numbers of droplets containing NTM (Aksamit *et al.*, 2014).

Raising the temperature of water heaters to 55^{o}C has been shown to reduce NTM, and as the highest number are recovered in the sediment at the bottom of the heater, periodically draining and refilling can greatly reduce numbers of NTM (Johnson and Odell, 2014).

It is recommended by National Jewish Health (National Jewish Health, 2015) that water is boiled for ten minutes at 100^{o}C for drinking and cooking, as well as avoiding water from built in refrigerator taps. Care should be taken to avoid if possible granular activated carbon water filters, as the pores of these filters are not small enough to prevent the passage of bacteria, and NTM can attach and grow on the carbon bound organics and metals due to their resistance to disinfectants. Filters that have pore sizes less than 0.2 micrometres can prevent NTM; however, these are costly and can congest easily requiring frequent changing. Humidifiers should be avoided as they can generate aerosols with high numbers of NTM, and the

substitution of piped water for well water can be of benefit, as this harbours significantly less NTM. Commercial potting soil containing peat and the dust generated from this is rich in NTM, therefore moistening garden soils can reduce dust inhalation (Huitt, 2015).

4.5 Non-tuberculous mycobacteria biofilm formation and its significance.

The formation of biofilms leads to an increase in resistance to antimicrobials (Hoiby *et al.*, 2010), and there have been several reports of biofilm development by NTM. In one study by El Helou *et al*, *M. abscessus* subsp*. abscessus* was observed to have a more rapid biofilm growth, which could be a factor in the pathogenicity of this species (El Helou *et al.*, 2013). Growth conditions can influence the time for biofilm formation, and tap water was shown to promote biofilm perhaps due to its content of nutrients for mycobacteria (Howard *et al.*, 2006; Esteban *et al.*, 2008).

In a report by Steed and Falkinham, *M. avium* cells grown in biofilms have been reported to be more resistant to chlorine in comparison to those grown in suspension in the same medium (Steed and Falkinham, 2006). Other elements may also contribute to biofilm formation in mycobacteria, such as the long-chain mycolic acids in their cell envelopes. Glycopeptidolipids coat the cell wall and have been suggested to be important for attaching the mycobacterial biofilm to surfaces (Recht and Kolter, 2001). In a study of showerheads in the US, the presence of pink pigmented *Methylobacterium* was associated with the absence of NTM and it was discovered that biofilms of methylobacteria inhibit the adherence of *M. avium* to stainless steel (Feazel *et al.*, 2009).

The major factor of NTM ecology, epidemiology and physiology is the existence of their lipid rich outer membrane with long chain mycolic acids contributing to their

hydrophobicity and impermeablility (see section 1.3 in Chapter 1). These characteristics make possible a high resistance to antimicrobials and disinfectants, and favour attachment to surfaces. Their slow rates of growth can also contribute to their persistence by allowing time for adaptation to any changes in their environment.

4.6 Aims and objectives

In order to further understand potential environmental sources of NTM, in particular *M. abscessus* complex, a large study was performed on sampling taken from areas within the local environment, in homes and from animals and various food items. Samples were also obtained from the local CF adult and paediatric outpatient centres and inpatient wards.

RGM medium (as described in Chapter 2) was used for the culture of all samples as it was thought to be the most relevant and effective for isolating NTM in comparison to other media commercially available where contamination by non-NTM could be anticipated.

4.7 Materials

Water samples were collected in sterile containers purchased from BDH, Poole, UK and filtered using Gelman Sciences vacuum control box model 4205 and filter funnel manifold 3-place polyurethane 629-4205 with GN-6 metricel 0.45 µm 47 mm grid sterile membrane disc filters purchased from Gelman Sciences, Portsmouth, UK. Sterile polywipes MW 729A were purchased from Medical Wire and Equipment, Wiltshire, UK. Sterilisation of media and equipment where appropriate was performed using a LTE Touchclave-R autoclave (LTE Scientific Ltd. Oldham, UK). Ingredients for RGM medium were purchased as described in Chapter 2. All plates were incubated in a LEEC 30^{o}C incubator obtained from Laboratory and Electrical Engineering Company, Nottingham, UK. All isolates were identified using MALDI biotyper (Bruker, Coventry, UK). Stringent wash solution, hybridisation buffer, conjugate, substrate and rinse solutions used in the HAIN hybridisation procedure (Section 4.8.5.3.2) were all purchased from HAIN Lifescience, Nehren, Germany.

4.8 Methods

4.8.1 Culture medium

RGM medium was used for the processing of all samples and prepared as described in Appendix 2. RGM broth was made up as in section 3.10.3 in Chapter 3 and a 10 ml aliquot added to sterile 20 ml plastic universals.

4.8.2 Environmental samples

Samples were collected from various items and locations (shown in Table 4-1). New sterile disposable gloves were worn for each sample taken, so as not to introduce any contamination. A 10 x 5 cm sterile polywipe premoistened in phosphate buffer was used to thoroughly wipe down each area, and stored in an individual sterile sealed bag to transfer to the laboratory. Polywipes were aseptically placed onto RGM medium and spread over the whole plate to maximise transfer of any material. Plates were incubated at 30^{o}C for ten days.

Table 4-1: Environmental areas tested for non-tuberculous mycobacteria

No.	Environmental area sampled
1	Washing up sponge
2	Floor Dust
3	Kitchen bench
4	House phone
5	Toilet seat
6	Sink draining board
7	Sofa (leather)
8	Hallway (door handles, surfaces, floor)
9	Bathroom (basin, showerhead)
10	Kitchen (basin, shelves, taps)
11	Living room (table, sofa, mantelpiece, handle, light switch)
12	Chicken egg
13	Can of diet coca cola lid
14	Horse manure
15	Countryside soil
16	Bus seats

Table 4-1 continued: Environmental areas tested for non-tuberculous mycobacteria

No.	Environmental area sampled
17	Cash machine
18	Computer desk
19	Window sill dust
20	Laptop keyboard
21	House plant
22	Grass
23	Cigarette ash
24	Lab Coat Microbiology Research Dept.
25	Medical Student Shirt
26	Medical Student ID Badge
27	Cigarette (Lambert and Butler) - Interior
28	Cigarette (Lambert and Butler) - Filter
29	Paediatric Outpatients Green Area Reception Desk (Not cleaned)
30	Paediatric Outpatients Red Area Children's Desk (Not cleaned)
31	C1 Consultants Desk
32	C1 Sink
33	C1 Children's Desk & Chair
34	C1 BP Cuff
35	C1 Pillow
36	C1 Couch
37	C1 Couch Blue Roll
38	C2 Consultants Desk
39	C2 Sink
40	C2 Children's Desk & Chair
41	C2 BP Cuff
42	C2 Couch
43	C2 Pillow
44	C2 Stethoscope
45	C3 Consultants Desk
46	C3 Sink
47	C3 Children's Desk & Chair
48	C3 BP Cuff
49	C3 Couch
50	C3 Pillow
51	C3 Children's Toys
52	C4 Consultants Desk
53	C4 Sink
54	C4 Children's Desk & Chair
55	C4 BP Cuff
56	C4 Couch
57	C4 Pillow
58	C4 Couch Blue Roll
59	C4 Stethoscope
60	C5 Consultants Desk

Table 4-1 continued: Environmental areas tested for non-tuberculous mycobacteria

No.	Environmental area sampled
61	C5 Sink
62	C5 Children’s desk & chair
63	C5 BP Cuff
64	C5 Couch
65	C5 Pillow
66	C5 Children's Toys
67	Treatment Room Desk
68	Treatment Room Couch
69	Treatment Room Sink
70	Treatment Room Pillow
71	Treatment Room Cuff
72	Treatment Room Height Machine
73	Treatment Room Weight Machine
74	Treatment Room Nurse's Trolley
75	Treatment Room Children's Toys
76	Treatment Room Child's Wheelchair
77	Adult C1 Patient chair
78	Adult C1 Bed
79	Adult C1 Desk
80	Adult C1 Sink
81	Adult C2 Patient chair
82	Adult C2 Bed
83	Adult C2 Desk
84	Adult C2 Sink
85	Adult C3 Patient chair
86	Adult C3 Bed
87	Adult C3 Desk
88	Adult C3 Sink
89	Adult C4 Patient chair
90	Adult C4 Bed
91	Adult C4 Desk
92	Adult C4 Sink
93	Adult C5 Patient chair
94	Adult C5 Bed
95	Adult C5 Desk
96	Adult C5 Sink
97	Adult C6 Patient chair
98	Adult C6 Bed
99	Adult C6 Desk
100	Adult C6 Sink
101	Adult C7 Patient chair
102	Adult C7 Bed
103	Adult C7 Desk
104	Adult C7 Sink
105	Adult C8 Patient chair

Table 4-1 continued: Environmental areas tested for non-tuberculous mycobacteria

No.	Environmental area sampled
106	Adult C8 Bed
107	Adult C8 Desk
108	Adult C8 Sink
109	Adult C8 Observation machine
110	Adult C9 Patient chair
111	Adult C9 Bed
112	Adult C9 Desk
113	Adult C9 Sink
114	Lung function room 1 sink
115	Lung function room 1 spirometer tubes
116	Lung function room 1 desk
117	Lung function room 1 chair
118	Lung function room 2 sink
119	Lung function room 2 spirometer 1
120	Lung function room 2 spirometer 2
121	Lung function room 2 spirometer 3
122	Lung function room 2 HD pft chair/glass
123	Ward 52 C6 room sink
124	Ward 52 C6 showerhead
125	Ward 52 C7 room sink
126	Ward 52 C7 WC sink
127	Ward 52 C7 armchair
128	Ward 52 C7 showerhead
129	Ward 52 C8 WC sink
130	Ward 52 C8 room sink
131	Ward 52 C8 room armchair
132	Ward 52 C8 room bathroom door handles
133	Ward 52 C8 room showerhead
134	Ward 52 C10 room sink
135	Ward 52 C10 WC sink
136	Ward 52 C10 showerhead
137	Ward 52 C10 O_2 wall mounts
138	Ward 52 C10 armchair
139	Ward 52 C10 window sill
140	Ward 52 C10 patient table
141	Ward 52 C10 bed
142	Ward 52 C11 WC sink
143	Ward 52 C11 room sink
144	Ward 52 C11 showerhead
145	Ward 52 C11 armchair
146	Ward 52 Corridor observation machine
147	Ward 52 Corridor patient chair
148	Flower Petal

Table 4-1 continued: Environmental areas tested for non-tuberculous mycobacteria

No.	Environmental area sampled
149	Flower Stem
150	Flower Soil
151	Flower Root

4.8.3 Water Samples

Sampling was completed on hospital water from showers, sink units and drinking fountains from around the CF wards, waiting areas, consultant and patient treatment rooms at the Royal Victoria Infirmary, Newcastle upon Tyne, UK. Samples were also taken from local homes, local rivers and ponds and various bottled waters, juices and carbonated fruit drinks (as shown in Table 4-2). From each area, 500 ml volumes were collected and stored in sterile bottles. GN-6 metricel membrane disc filters, diameter 47 mm, pore size 0.45 µm were used and 250 ml was filtered using the membrane filtration apparatus. Membrane filters were then aseptically placed onto RGM medium and plates were incubated at 30^{o}C for ten days.

Table 4-2: Water samples tested for non-tuberculous mycobacteria

No.	Water sampling area
1	House 1 bathroom hot
2	House 1 bathroom cold
3	House 1 Kitchen hot
4	House 1 kitchen cold
5	House 1 toilet
6	House 1 outdoor tap
7	House 2 bathroom hot
8	House 3 bathroom cold
9	House 2 kitchen cold
10	House 2 outdoor tap
11	House 2 shower hot
12	Pathology Dept. Freeman Hospital Male Bathroom Hot
13	Pathology Dept. Freeman Hospital Male Bathroom Cold
14	Pathology Dept. Freeman Hospital Female Bathroom Hot
15	Pathology Dept. Freeman Hospital Female Bathroom Cold

Table 4-2 continued: Water samples tested for non-tuberculous mycobacteria

No.	Water sampling area
16	Freeman Hospital Outpatient Drinking Water
17	Pathology Dept. Freeman Hospital Kitchen Hot
18	Pathology Dept. Freeman Hospital Kitchen Cold
19	Ward 52 CF Unit C6 Shower
20	Ward 52 CF Unit C6 WC Sink
21	Ward 52 CF Unit C6 Room Sink
22	Ward 52 CF Unit C8 Shower
23	Ward 52 CF Unit C8 WC Sink
24	Ward 52 CF Unit C8 Room Sink
25	Ward 52 CF Unit C10 Shower
26	Ward 52 CF Unit C10 WC Sink
27	Ward 52 CF Unit C10 Room Sink
28	Ward 52 CF Unit C7 Shower
29	Ward 52 CF Unit C11 Shower
30	Adult CF Clinic Waiting Rm Drinking Fountain
31	Adult CF Clinic C1 sink
32	Adult CF Clinic C5 sink
33	Adult CF Clinic C6 sink
34	Adult CF Clinic C7 sink
35	Adult CF Clinic C8 sink
36	Adult CF Clinic C9 sink
37	Paediatric C1 sink
38	Paediatric C2 sink
39	Paediatric C3 sink
40	Paediatric C4 sink
41	Paediatric C5 sink
42	Paediatric treatment room sink
43	Paediatric drinking water outpatients main waiting area
44	Paediatric drinking water green (area 1)
45	Paediatric drinking water red (area 2)
46	Evian bottled water
47	Eden Falls bottled water
48	Asda Smart Price bottled water
49	Highland Spring bottled water
50	Buxton bottled water
51	Ouseburn River
52	Paddy Freeman Pond, High Heaton
53	Coca-Cola
54	Fanta Zero
55	Tango
56	Sun Magic Orange Juice
57	Sun Magic Pineapple Juice
58	Sun Magic Apple Juice
59	Volvic Touch of Fruit Cherry

Table 4-2 continued: Water samples tested for non-tuberculous mycobacteria

No.	Water sampling area
60	Volvic Touch of Fruit Lemon/Lime
61	Ribena
62	Robinsons Fruit Shoot Orange
63	Robinsons Fruit Shoot Apple/Blackcurrant
64	Pepsi cola

4.8.4 Food Samples

Various unsystematic food items from local supermarkets and outdoor markets, both pre-packed and loose (see Table 4-3) were collected. Each sample was aseptically diced, mashed and transferred into 10 ml sterile RGM broth and incubated at 30^{o}C. After ten days' incubation, 10 µl aliquots were taken from each broth and inoculated onto RGM medium and incubated for a further ten days at 30^{o}C.

Table 4-3: All foods tested for non-tuberculous mycobacteria

No	Food samples tested
1	Brussel Sprouts
2	Salad Cress
3	Salad Cress Soil
4	Cheese
5	Spring Onion - White
6	Spring Onion - Green
7	Strawberry
8	Raspberry
9	Sweet and Crunchy prepacked salad
10	Coleslaw prepacked mix
11	Iceberg Lettuce
12	Blackberry
13	Sliced Red Onions
14	Grape - Red
15	Grape - Green
16	Mushrooms
17	Savoy Cabbage
18	Grower's Selection - Red Onion
19	Grower's Selection - Courgette
20	Grower's Selection - Red Pepper

Table 4-3 continued: All foods tested for non-tuberculous mycobacteria

No	Food samples tested
21	Grower's Selection - Yellow Pepper
22	Celery
23	Cucumber
24	Smoked Haddock
25	Salad Tomato
26	Casserole Vegetables - Carrot
27	Casserole Vegetables - Potato
28	Casserole Vegetables - Swede
29	Casserole Vegetables - Leek
30	Casserole Vegetables - White Onion
31	Swede
32	Mix Veg - Carrot
33	Mix Veg - Broccoli
34	Mix Veg - Cauliflower
35	Twin Pack - Baby Corn
36	Twin Pack - Fine Beans
37	Beansprouts
38	Plum
39	Potato
40	Radish
41	Red Apple
42	Orange Pulp
43	Orange Zest
44	Lemon Pulp
45	Lemon Zest
46	Banana
47	Green Apple
48	Red Pepper
49	Large Potato
50	New Potato
51	Parsnip
52	Sweet Potato
53	Pear
54	Onion
55	Whole Milk
56	Cottage Cheese
57	Single Cream
58	Organic Yogurt
59	Soil – Onion from allotment
60	Flat Cap Mushrooms
61	Shallot
62	Jersey Royal Potato
63	Beansprouts
64	Almonds
65	Dried Papaya
66	Dried Cranberry

Table 4-3 continued: All foods tested for non-tuberculous mycobacteria

No	Food samples tested
67	Pick and mix sweets (loose)
68	Bread (uncut from bakery)
69	Ham (loose)
70	Flat Leaf Parsley
71	Cherry
72	Ginger
73	Butter (Lurpak)
74	Cheese (Hard)
75	Stir Fry Chicken (from butcher)
76	Stir Fry Onion (from butcher)
77	Stir Fry Carrot (from butcher)
78	Stir Fry Marinade (from butcher)
79	Mixed Grill Beef Burger (from butcher)
80	Mixed Grill Herb Burger (from butcher)
81	Mixed Grill Herb Sausage (from butcher)
82	Mixed Grill Beef Sausage (from butcher)
83	Mixed Grill Pork Sausage (from butcher)

Uninoculated control plates, broth and equipment from all sample sets were incubated at both 30ºC and 37ºC for ten days. Unused swabs were also placed onto RGM plates, spread over the whole plate, and incubated at both 30ºC and 37ºC for ten days as shown in Table 4-4.

Table 4-4: Negative controls used in all sampling

No.	Controls
1	Control Broth 30ºC
2	Control Broth 37ºC
3	Control Plate 30ºC
4	Control Plate 30ºC
5	Water Filter
6	Scalpel
7	Methylated Spirit
8	Control Sponge (not used) 30ºC
9	Control Sponge (not used) 37ºC
10	Control Broth 30ºC
11	Control Broth 37ºC
12	Control Plate 30ºC
13	Control Plate 30ºC
14	Water Filter

Table 4-4 continued: Negative controls used in all sampling

No.	Controls
15	Scalpel
16	Methylated Spirit
17	Control Sponge (not used) 30ºC
18	Control Sponge (not used) 37ºC
19	Control Broth 37ºC
20	Control Broth 30ºC
21	Control Plate 37ºC
22	Control Plate 30ºC
23	Water Filter
24	Scalpel
25	Methylated Spirit
26	Sponge (unused)
27	Sponge (unused)

4.8.5 Methods of identification of all isolates recovered

4.8.5.1 Matrix-assisted laser/desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

All bacterial growth from all types of sample were sub-cultured onto Columbia blood agar to obtain pure cultures and identified using MALDI-TOF MS. For those isolates that were not able to be identified full extractions were performed and identification was attempted a second time using MALDI-TOF MS.

4.8.5.1.1 Preparation of sample for MALDI-TOF MS

A single colony was isolated and applied as a thin but visible film onto the MALDI target plate using a sterile wooden stick. This was overlaid with 2 µl of matrix (alpha-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid) and allowed to air dry. This is the suggested method as recommended by Bruker (Bruker, 2017).

For difficult to identify isolates or suspected mycobacteria, an extraction process had to be adhered to in order to disrupt cell wall structures. After numerous protocols

were evaluated, the method adopted was one recommended by Saleeb *et al*. Using a 1 µl loop, a visible amount of cells were suspended in 70% ethanol and briefly centrifuged. The supernatant was discarded and the pellet re-suspended in 50 µl formic acid, vortexed for five minutes and 50 µl 100% acetonitrile added and vortexed for a further five minutes. The sample was then centrifuged at maximum speed for two minutes and 2 µl placed onto the MALDI target plate, air-dried and overlaid with matrix as previously described (Saleeb *et al.*, 2011).

4.8.5.2 Gram-staining and staining for acid-fast bacilli

Gram-staining was performed on all isolates that could not be identified by MALDI-TOF MS. An auramine and/or Zeihl Neelsen stain was also performed in order to assess the likelihood of mycobacteria and eliminate any non-mycobacteria.

4.8.5.2.1 Staining of heat fixed films by auramine phenol for the detection by fluorescence of acid and alcohol-fast organisms

A 1 µl sterile plastic loopful of each isolate was spread over an individual slide, keeping away from the slide edges and avoiding making the smear too thick. The slides were then heat fixed by placing on a hot plate for two minutes. Auramine phenol stain was poured onto the heat fixed slide and left for 15 minutes then washed off with water. Acid-alcohol (1% v/v) was poured on and left for one minute, washed off and then the process was repeated for a further two minutes. This staged approach to decolourising the slides takes account of the fact that the first minute will elute the auramine phenol from the slide, consequently diluting the effect of the acid-alcohol. The second phase (two minutes) will therefore be at full strength. Acid-alcohol was washed off and the slide was counter stained with (0.02% w/v) aqueous solution of thiazine red for 30 seconds before being rinsed with water and allowed

to dry. Slides were examined using U.V microscopy using a x40 non-cover glass lens.

4.8.5.2.2 Staining of heat fixed films by Ziehl-Neelson for the detection of acid and alcohol-fast organisms

Slides were prepared and heat fixed as for auramine staining then flooded with strong carbol fuchsin. Using a lighted metal rod consisting of cotton wool soaked in industrial methylated spirit the slides were gently heated until just steaming and then allowed to stand for five minutes. Slides were heated again until steaming and left for a further five minutes before being rinsed well with water. Slides were then decolourised several times for 2-5 minutes with a 3% v/v acid-alcohol solution, rinsing with tap water between each application. To counter-stain, 1% methylene blue was added for 30 seconds before slides were rinsed with water and allowed to dry. Immersion oil was added to each slide and examined using a light microscope and x40 and x100 lenses.

4.8.5.2.3 Gram-staining of unidentified isolates to eliminate any non-mycobacteria

Slides were prepared and heat fixed as for auramine and ZN staining. Once the slides were cooled down crystal violet was added, left for 60 seconds, and then washed briefly with water. Gram's iodine was added and left for 60 seconds before being washed off with water. Slides were then flooded with 95% acetone for three seconds and washed off. Safranin was then added to slides as a counter-stain and left for 30 seconds before being washed off. Slides were then examined under the oil immersion lens (100x objective).

4.8.5.3 HAIN Genotyping protocol for the identification of clinically relevant mycobacterial species using BEEBlot G45 Automated Platform

All AFB positive isolates were sub-cultured onto Columbia blood agar and prepared for identification using HAIN Genotyping (HAIN Lifescience, 2015). Five commonly encountered control species previously typed by VNTR profiing at Public Health Colindale, UK and listed in Appendix 1, were also tested to validate the HAIN Genotyping results. These included *M. abscessus* subsp*. abscessus* (*n* = 1), *M. chelonae* (*n* = 1), *M. immunogenum* (*n* = 1), *M. abscessus* subsp*. massiliense* (*n* = 1) and *M. mucogenicum* (*n* = 1).

4.8.5.3.1 Sample preparation for extraction of DNA

A 10 µl loopful of pure bacteria was suspended in 1 ml of PCR grade water and centrifuged at 10,000g for 15 minutes. The supernatant was poured off and the pellet re-suspended in 300µl of PCR grade water. Tubes were then incubated for 20 minutes in a 90^{o}C heating block. At the end of the heating process, tubes were removed and placed directly into a sonicating water bath. After 15 minutes the tubes were centrifuged at 21,000g for five minutes and 200µl of the supernatant was transferred to a new, labelled 1.5 ml screw capped pointed tube for PCR.

4.8.5.3.2 Amplification of extracted mycobacterial DNA

Mastermix was made up using the following amounts per sample; 3.5 µl sterile water, 5.0 µl 10 x buffer, 1.0 µl $MgCl_2$, 35.0 µl primer nucleotide mix and 0.5 µl Taq DNA polymerase and gently vortexed. Capped 0.2 ml PCR tubes were labelled for each sample and 45 µl of mastermix was added to each one along with 5 µl of extracted DNA and briefly vortexed. Specific primer information is proprietary and not in the public domain (K. Geber 2017, personal communication, 31 July).

4.8.5.3.3 Running the Amplification Cycler.

Tubes were loaded into the block along with a contamination control sample containing 5 µl water in place of DNA, and the amplification profile was set as shown in Table 4-5. Once completed, the amplified samples were ready for the Hain genotyping.

Table 4-5: Amplification profile for DNA

Number of cycles	Temperature	Time
1	95°C	15 minutes
10	95°C	30 seconds
	58°C	2 minutes
20	95°C	25 seconds
	53°C	40 seconds
	70°C	40 seconds
1	70°C	8 minutes

4.8.5.3.4 Hybridisation Test Procedure

Common Mycobacteria (CM) or Additional Species (AS) test strips were removed from storage at 4°C and allowed to come to room temperature whilst the BEEBlot G45 automated platform was pre-warmed to 45°C. A volume of 20 µl of denaturation solution was added to each of the wells followed by 20 µl of amplified sample, carefully mixed by pipetting up and down and incubated at room temperature for five minutes. Stringent wash solution, hybridisation buffer, conjugate, substrate and rinse solutions were placed in the appropriate sections in the BBBlot and when prompted one strip was placed into each well, ensuring they were completely covered by the solution and the coated side facing upwards. Once the cycle was completed strips were removed and pasted onto a kit evaluation sheet by aligning

the bands CC and UC with respective lines on the sheet. This procedure was repeated for any samples requiring further identification with the Additional Species (AS) kit.

4.9 Results

From all of the testing performed, a total of 298 separate samples (Table 4-1 to 4.3) were taken from various locations comprising 83 food items, 64 water samples and 151 environmental areas. From these, 272 individual isolates were recovered (see Table 4-6 and Table 4-7) and 196 showed no growth. This included 92 from environmental samples, 161 from water sampling and 19 from foods. All control plates (*n* = 27) were negative after ten days' incubation.

Table 4-6: Description of all areas tested for NTM

Area	Growth	Identification
Environmental areas		
Washing up sponge	+	*M. peregrinum* x 2
	+	Gram-negative
Floor Dust	+	Gram-negative
Kitchen bench	+	Gram-negative
House phone	NG	
Toilet seat	NG	
Sink draining board	+	*Streptococcus oralis* x 2
	+	Gram-negative
Sofa (leather)	NG	
Hallway (door handles, surfaces, floor)	+	*Chryseobacterium* sp.
Bathroom (basin, showerhead)	+	Gram-negative
	+	*D. acidovorans* x 5
	+	*M. fortuitum*
	+	*Mycobacterium* species
Kitchen (basin, shelves, taps)	+	Gram-negative
Living room (table, sofa, mantelpiece, handle, light switch)	NG	
Chicken egg	NG	
Can of diet coca cola lid	NG	
Horse manure	+	Gram-negative
	+	*Chryseobacterium* sp.x 2
	+	Gram-negative
	+	*Myroides odoratus*
	+	*A. fumigatus* x 2
Countryside soil	+	*Chyrseobacterium oranimense*
	+	Gram-negative
	+	*Arthrobacer* sp.
	+	*M. peregrinum*
	+	*A. fumigatus*

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
	+	*Achromobacter* sp.
	+	*Chryseobacterium* sp.
Bus seats	+	Gram-negative
Cash machine	NG	
Computer desk	+	*M. peregrinum*
Window sill dust	+	*M. peregrinum* x 2
	+	*Fusarium* sp.
Laptop keyboard	NG	
House plant	+	*Achromobacter* sp.
	+	*M. peregrinum*
	+	*Mycobacterium* species
	+	*Fusarium* sp. X 2
	+	*Fusarium oxysporum*
Grass	NG	
Cigarette ash	NG	
Lab Coat Microbiology Research Dept.	+	Gram-negative
	+	*M. chelonae*
Medical Student Shirt	NG	
Medical Student ID Badge	NG	
Cigarette (Lambert and Butler) - Interior	+	*Bacillus* sp. X 2
Cigarette (Lambert and Butler) - Filter	NG	
Paediatric Outpatients Green Area Reception Desk (Not cleaned)	NG	
Paediatric Outpatients Red Area Children's Desk (Not cleaned)	NG	
C1 Consultants Desk	+	Gram-negative
C1 Sink	+	Gram-negative x 2
C1 Children's Desk & Chair	NG	
C1 BP Cuff	NG	
C1 Pillow	NG	
C1 Couch	NG	
C1 Couch Blue Roll	NG	
C2 Consultants Desk	NG	
C2 Sink	+	*D. acidovorans* x 2
C2 Children's Desk & Chair	NG	
C2 BP Cuff	NG	
C2 Couch	NG	
C2 Pillow	NG	
C2 Stethoscope	NG	
C3 Consultants Desk	NG	
C3 Sink	+	Gram-negative x 2
	+	***M. chelonae***
C3 Children's Desk & Chair	NG	
C3 BP Cuff	NG	
C3 Couch	NG	

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
C3 Pillow	NG	
C3 Children's Toys	NG	
C4 Consultants Desk	NG	
C4 Sink	+	*D. acidovorans*
C4 Children's Desk & Chair	NG	
C4 BP Cuff	NG	
C4 Couch	NG	
C4 Pillow	NG	
C4 Couch Blue Roll	NG	
C4 Stethoscope	NG	
C5 Consultants Desk	NG	
C5 Sink	+	*D. acidovorans*
	+	***M. chelonae***
C5 Children's desk & chair	NG	
C5 BP Cuff	NG	
C5 Couch	NG	
C5 Pillow	NG	
C5 Children's Toys	NG	
Treatment Room Desk	NG	
Treatment Room Couch	NG	
Treatment Room Sink	+	***M. chelonae***
	+	Gram-negative
	+	*M. chelonae*
	+	*D. acidovorans* x 2
Treatment Room Pillow	NG	
Treatment Room Cuff	NG	
Treatment Room Height Machine	NG	
Treatment Room Weight Machine	NG	
Treatment Room Nurse's Trolley	NG	
Treatment Room Children's Toys	+	Gram-negative
Treatment Room Child's Wheelchair	NG	
Adult C1 Patient chair	NG	
Adult C1 Bed	NG	
Adult C1 Desk	NG	
Adult C1 Sink	+	*Mycobacterium* species x 2
Adult C2 Patient chair	NG	
Adult C2 Bed	NG	
Adult C2 Desk	NG	
Adult C2 Sink	+	*Mycobacterium* species
Adult C3 Patient chair	NG	
Adult C3 Bed	NG	
Adult C3 Desk	NG	
Adult C3 Sink	+	*Mycobacterium* species
Adult C4 Patient chair	NG	
Adult C4 Bed	NG	
Adult C4 Desk	NG	

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
Adult C4 Sink	+	*D. acidovorans*
Adult C5 Patient chair	NG	
Adult C5 Bed	NG	
Adult C5 Desk	NG	
Adult C5 Sink	+	***M. chelonae***
Adult C6 Patient chair	NG	
Adult C6 Bed	NG	
Adult C6 Desk	NG	
Adult C6 Sink	+	*Mycobacterium* species x 2
Adult C7 Patient chair	NG	
Adult C7 Bed	NG	
Adult C7 Desk	NG	
Adult C7 Sink	NG	
Adult C8 Patient chair	NG	
Adult C8 Bed	NG	
Adult C8 Desk	NG	
Adult C8 Sink	NG	
Adult C8 Observation machine	NG	
Adult C9 Patient chair	NG	
Adult C9 Bed	NG	
Adult C9 Desk	NG	
Adult C9 Sink	+	*Mycobacterium* species x 2
Lung function room 1 sink	NG	
Lung function room 1 spirometer tubes	NG	
Lung function room 1 desk	NG	
Lung function room 1 chair	NG	
Lung function room 2 sink	+	*Mycobacterium* species
Lung function room 2 spirometer 1	NG	
Lung function room 2 spirometer 2	NG	
Lung function room 2 spirometer 3	NG	
Lung function room 2 HD pft chair/glass	NG	
Ward 52 C6 room sink	+	*Mycobacterium* species x 2
Ward 52 C6 showerhead	+	*D. acidovorans*
Ward 52 C7 room sink	NG	
Ward 52 C7 WC sink	+	*Mycobacterium* species
Ward 52 C7 armchair	NG	
Ward 52 C7 showerhead	+	*Mycobacterium* species x 2
Ward 52 C8 WC sink	+	*M. mucogenicum*
Ward 52 C8 room sink	+	*Mycobacterium* species
Ward 52 C8 room armchair	NG	
Ward 52 C8 room bathroom door handles	NG	
Ward 52 C8 room showerhead	+	*M. mucogenicum*
	+	*Mycobacterium* species
Ward 52 C10 room sink	+	*Mycobacterium* species

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
	+	*Mycobacterium* species
Ward 52 C10 WC sink	NG	
Ward 52 C10 showerhead	NG	
Ward 52 C10 O_2 wall mounts	NG	
Ward 52 C10 armchair	NG	
Ward 52 C10 window sill	NG	
Ward 52 C10 patient table	NG	
Ward 52 C10 bed	+	*M. peregrinum*
Ward 52 C11 WC sink	+	*D. acidovorans*
Ward 52 C11 room sink	+	*M. mucogenicum* x 2
Ward 52 C11 showerhead	+	*M. mucogenicum*
Ward 52 C11 armchair	NG	
Ward 52 Corridor observation machine	NG	
Ward 52 Corridor patient chair	NG	
Flower Petal	NG	
Flower Stem	NG	
Flower Soil	NG	
Flower Root	NG	
Waters		
House 1 bathroom hot	+	*Curtobacterium flaccumfaciens*
	+	***M. chelonae***
House 1 bathroom cold	+	*M. mucogenicum* x 2
House 1 Kitchen hot	NG	
House 1 kitchen cold	+	*M. mucogenicum*
	+	***M. chelonae* x 2**
House 1 toilet	+	*Microbacterium* sp.
	+	Gram-negative
	+	*M. chelonae* x 4
House 1 outdoor tap	+	*Curtobacterium flaccumfaciens*
	+	Gram-negative
	+	*Arthrobacter* sp.
House 2 bathroom hot	+	*Mycobacterium* species x 3
	+	Gram-negative
House 3 bathroom cold	+	*Mycobacterium* species
House 2 kitchen cold	+	*Mycobacterium* species
	+	*Mycobacterium* species
House 2 outdoor tap	NG	
House 2 shower hot	+	Gram-negative x 3
	+	*Chryseobacterium indologenes*
	+	*Mycobacterium* species
	+	Gram-negative
Pathology Dept. Freeman Hospital Male Bathroom Hot	+	Gram-negative
Pathology Dept. Freeman Hospital Male Bathroom Cold	+	***M. chelonae***

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
	+	Gram-negative
Pathology Dept. Freeman Hospital Female Bathroom Hot	+	Gram-negative
	+	*Chryseobacterium* sp.
Pathology Dept. Freeman Hospital Female Bathroom Cold	+	Gram-negative
	+	*M. mucogenicum*
	+	*Mycobacterium* species
Freeman Hospital Outpatient Drinking Water	+	*Mycobacterium* species
Pathology Dept. Freeman Hospital Kitchen Hot	NG	
Pathology Dept. Freeman Hospital Kitchen Cold	+	Gram-negative
Ward 52 CF Unit C6 Shower	+	*Mycobacterium* species x 2
Ward 52 CF Unit C6 WC Sink	+	*Mycobacterium* species x 5
Ward 52 CF Unit C6 Room Sink	+	*Mycobacterium* species x 2
Ward 52 CF Unit C8 Shower	+	*Mycobacterium* species x 3
Ward 52 CF Unit C8 WC Sink	+	*Mycobacterium* species
	+	Gram-negative
	+	*D. acidovorans* x 4
Ward 52 CF Unit C8 Room Sink	+	*D. acidovorans* x 4
Ward 52 CF Unit C10 Shower	+	*Mycobacterium* species x 2
	+	*D. acidovorans*
Ward 52 CF Unit C10 WC Sink	+	Gram-negative
	+	*Mycobacterium* species
Ward 52 CF Unit C10 Room Sink	+	***M. chelonae* x 3**
	+	*Mycobacterium* species
Ward 52 CF Unit C7 Shower	+	*M. chelonae* x 2
Ward 52 CF Unit C11 Shower	+	***M. chelonae* x 4**
	+	*Mycobacterium* species
Adult CF Clinic Waiting Rm Drinking Fountain	+	***M. chelonae* x 4**
	+	*Mycobacterium* species
Adult CF Clinic C1 sink	+	***M. chelonae***
	+	*Mycobacterium* species
	+	*Lactobacillus* sp.
Adult CF Clinic C5 sink	+	*Mycobacterium* species x 5
Adult CF Clinic C6 sink	+	*D. acidovorans* x 4
	+	*Mycobacterium* species
	+	*Flavobactrium lindanitolerans*
	+	*Fusarium* sp.
Adult CF Clinic C7 sink	+	*Mycobacterium* species x 2
Adult CF Clinic C8 sink	+	*Mycobacterium* species
	+	*D. acidovorans*
	+	*D. acidovorans*
Adult CF Clinic C9 sink	+	*Chryseobacterium indologenes*
	+	*D. acidovorans*

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
	+	*Chryseobacterium indologenes*
	+	Gram-negative
Paediatric C1 sink	+	*D. acidovorans* x 2
	+	***M. chelonae***
	+	*Mycobacterium* species
Paediatric C2 sink	+	*D. acidovorans* x 3
	+	*M. chelonae* x 2
Paediatric C3 sink	+	*D. acidovorans* x 2
	+	*M. chelonae* x 2
Paediatric C4 sink	+	***M. chelonae* x 2**
	+	*Mycobacterium* species
Paediatric C5 sink	+	*Mycobacterium* species
	+	*D. acidovorans*
	+	*M. chelonae* x 2
Paediatric treatment room sink	+	*D. acidovorans* x 2
Paediatric drinking water outpatients main waiting area	+	*D. acidovorans* x 2
	+	*Mycobacterium* species
	+	*M. chelonae*
	+	*Mycobacterium* species
Paediatric drinking water green (area 1)	+	***M. chelonae* x 3**
	+	*Mycobacterium* species
Paediatric drinking water red (area 2)	+	***M. chelonae* x 4**
	+	*Mycobacterium* species
Evian bottled water	NG	
Eden Falls bottled water	+	Gram-negative
	+	UNKNOWN
Asda Smart Price bottled water	+	Gram-negative
	+	Gram-negative
	+	Gram-negative
Highland Spring bottled water	NG	
Buxton bottled water	NG	
Ouseburn River	+	*Morganella morganii* x 2
	+	*Delftia acidovorans* x 2
	+	*Serratia liquefaciens*
	+	*Klebsiella oxytoca*
Paddy Freeman Pond, High Heaton	+	*Delftia acidovorans* x 2
	+	Gram-negative x 2
	+	*A. flavus* x 2
	+	*Serratia fonticola* x 2
Coca-Cola	NG	
Fanta Zero	NG	
Tango	NG	
Sun Magic Orange Juice	NG	
Sun Magic Pineapple Juice	NG	

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
Sun Magic Apple Juice	NG	
Volvic Touch of Fruit Cherry	NG	
Volvic Touch of Fruit Lemon/Lime	NG	
Ribena	NG	
Robinsons Fruit Shoot Orange	NG	
Robinsons Fruit Shoot Apple/Blackcurrant	NG	
Pepsi cola	NG	
Foods		
Brussel Sprouts	NG	
Salad Cress	+	*D. acidovorans*
Salad Cress Soil	NG	
Cheese	+	Gram-negative
Spring Onion - White	+	*D. acidovorans*
Spring Onion - Green	+	*D. acidovorans*
Strawberry	NG	
Raspberry	NG	
Sweet and Crunchy prepacked salad	+	*Serratia* sp.
Coleslaw prepacked mix	NG	
Iceberg Lettuce	NG	
Blackberry	NG	
Sliced Red Onions	NG	
Grape - Red	NG	
Grape - Green	NG	
Mushrooms	NG	
Savoy Cabbage	NG	
Grower's Selection - Red Onion	NG	
Grower's Selection - Courgette	NG	
Grower's Selection - Red Pepper	NG	
Grower's Selection - Yellow Pepper	NG	
Celery	+	*D. acidovorans*
Cucumber	NG	
Smoked Haddock	NG	
Salad Tomato	NG	
Casserole Vegetables - Carrot	NG	
Casserole Vegetables - Potato	NG	
Casserole Vegetables - Swede	+	*Burkholderia gladioli*
Casserole Vegetables - Leek	NG	
Casserole Vegetables - White Onion	+	*D. acidovorans*
Swede	NG	
Mix Veg - Carrot	NG	
Mix Veg - Broccoli	+	*Lactobacillus* sp.
Mix Veg - Cauliflower	NG	
Twin Pack - Baby Corn	NG	
Twin Pack - Fine Beans	NG	

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
Beansprouts	NG	
Plum	+	Gram-negative
Potato	NG	
Radish	+	*Pandoraea* sp.
Red Apple	NG	
Orange Pulp	NG	
Orange Zest	NG	
Lemon Pulp	NG	
Lemon Zest	NG	
Banana	NG	
Green Apple	NG	
Red Pepper	NG	
Large Potato	NG	
New Potato	+	Gram-negative
Parsnip	+	Gram-negative
Sweet Potato	+	Gram-negative
Pear	NG	
Onion	+	*Mycobacterium* species
Whole Milk	NG	
Cottage Cheese	NG	
Single Cream	NG	
Organic Yogurt	NG	
Soil – Onion from allotment	+	*M. mucogenicum*
Flat Cap Mushrooms	NG	
Shallot	NG	
Jersey Royal Potato	+	Gram-negative
Beansprouts	NG	
Almonds	+	UNKNOWN
Dried Papaya	NG	
Dried Cranberry	NG	
Hard Candy (pick and mix)	NG	
Brazil Nut Toffee (pick and mix)	NG	
Milk Bottle (pick and mix)	NG	
Fizzy Cola (pick and mix)	NG	
Gummy Bear (pick and mix)	NG	
Cherry Lips (pick and mix)	NG	
Bread (uncut from bakery)	NG	
Ham (loose)	NG	
Flat Leaf Parsley	NG	
Cherry	NG	
Ginger	+	UNKNOWN
Butter (Lurpak)	NG	
Cheese (Hard)	NG	
Stir Fry Chicken (from butcher)	NG	
Stir Fry Onion (from butcher)	NG	

Table 4-6 continued: Description of all areas tested for NTM

Area	Growth	Identification
Stir Fry Carrot (from butcher)	NG	
Stir Fry Marinade (from butcher)	NG	
Mixed Grill Beef Burger (from butcher)	NG	
Mixed Grill Herb Burger (from butcher)	NG	
Mixed Grill Herb Sausage (from butcher)	NG	
Mixed Grill Beef Sausage (from butcher)	NG	
Mixed Grill Pork Sausage (from butcher)	NG	
Controls		
Control Broth 30ºC	NG	
Control Broth 37ºC	NG	
Control Plate 30ºC	NG	
Control Plate 30ºC	NG	
Water Filter	NG	
Scalpel	NG	
Methylated Spirit	NG	
Control Sponge (not used) 30ºC	NG	
Control Sponge (not used) 37ºC	NG	
Control Broth 30ºC	NG	
Control Broth 37ºC	NG	
Control Plate 30ºC	NG	
Control Plate 30ºC	NG	
Water Filter	NG	
Scalpel	NG	
Methylated Spirit	NG	
Control Sponge (not used) 30ºC	NG	
Control Sponge (not used) 37ºC	NG	
Control Broth 37ºC	NG	
Control Broth 30ºC	NG	
Control Plate 37ºC	NG	
Control Plate 30ºC	NG	
Water Filter	NG	
Scalpel	NG	
Methylated Spirit	NG	
Sponge (unused)	NG	
Sponge (unused)	NG	

Those areas/isolates highlighted in bold were sent to Public Health, Colindale, UK to verify identities.

Out of 272 individual isolates recovered, 130 were identified as mycobacteria (65 unknown mycobacterial species), 118 were Gram-negative, 11 Gram-positive and 10 fungi. Three isolates were unable to be identified by any methods used, however although these were all AFB positive, they were not identified as mycobacteria by HAIN (see Table 4-7).

Using the GenoType Mycobacterium CM, a species-specific probe shows if members of the genus Mycobacterium are present, and in this case, further differentiation with GenoType Mycobacterium AS is recommended. Due to this, presumptuous identifications of *M. mucogenicum* could be given for some if not all of the 65 mycobacterial isolates that were not identified to species level. This is due to all but one of these being recovered from water samples/sinks where the majority of Mycobacterial isolates were either *M. chelonae* or *M. mucogenicum*. It could also be possible that had the HAINS CM test been repeated, many of these could be identified as *M. chelonae*. However, time and cost was a big factor in this, and the main aim was to try to isolate MABSC.

The most frequently isolated Gram negative species in this study (43.2% of all Gram-negatives identified), was *D. acidovorans*, commonly found in water or soil, rarely a cause of infection, and usually only in immunocompromised individuals (Chun *et al.*, 2009). All isolates in this study were found in either water, sinks or food (onions, celery, and cress). This species is not often reported in CF patients (Marchandin *et al.*, 2012).

Table 4-7: Species identification of recovered isolates in all areas tested

Species	*n*	Species	*n*
Gram-negative isolates	**118**	**Gram-positive isolates**	**11**
Achromobacter sp.	2	*Arthrobacter* spp.	2
B. gladioli	1	*Bacillus* spp.	2
D. acidovorans	51	*Curtobacterium flaccumfaciens*	2
Chryseobacterium sp.	9	*Lactobacillus* spp.	2
Flavobacterium lindanitolerans	1	*Microbacterium* sp.	1
Other Gram-negative (by Gram-stain)	45	*Streptococcus oralis*	2
Klebsiella oxytoca	1		
Morganella morganii	2	**Non-tuberculous mycobacteria**	**130**
Myroides odoratus	1	*M. chelonae*	46
Pandoraea sp.	1	*M. fortuitum*	1
Serratia spp.	4	*M. mucogenicum*	10
		M. peregrinum	8
Fungal isolates	**10**	*Mycobacterium spp.*	65
A. flavus	2		
A. fumigatus	3		
Fusarium spp.	5		
Unidentified (non-mycobacterial) isolates	**3**	**TOTAL ISOLATES**	**272**

4.9.1 Identification of all recovered isolates

All 272 isolates underwent analysis by MALDI-TOF MS, and 94 were given a score value of >2.0 or higher indicating secure Genus identification and probable species identification (Saffert *et al.*, 2011). A further 45 isolates were identified as non-mycobacteria by Gram stain, auramine and/or ZN stain.

4.9.1.1 HAIN Genotyping for the Identification of clinically relevant mycobacterial species using BEEBlot G45 Automated Platform

A total of 133 isolates (plus an additional five as controls) underwent HAIN Genotyping for common mycobacteria. Out of these 130 were positively identified as mycobacteria. Three isolates remained unidentified, although they were not identified as mycobacteria by HAIN.

4.9.1.2 Evaluation and interpretation of HAIN results

Positive bands were noted and species determined using the HAIN CM and AS evaluation charts as shown in Figure 1-9 and Figure 1-10.

4.10 Discussion

For many years, it has become apparent that due to the lack of evidence of person-to-person transmission of NTM, it was generally accepted that the source of NTM infection in humans is the environment. It is well documented that NTM are said to be ubiquitous inhabitants within the environment, sharing a variety of ecological habitats with humans, including water, household plumbing, hot tubs, spas, peat and soil (Embil *et al.*, 1997; De Groote *et al.*, 2006; Falkinham, 2009; Feazel *et al.*, 2009; Thomson *et al.*, 2013b; Thomson *et al.*, 2013a).

Discounting MABSC, other species of mycobacteria, e.g. *M. chelonae,* were found in abundance in many samples in this study. It cannot therefore be disregarded that the possibility of MABSC in the environment may possibly be overgrown in culture by other more plentiful mycobacterial species and thereby remain undetected. It could therefore be a challenge to detect MABSC if present in very small amounts (Ripoll *et al.*, 2009). Out of all the mycobacteria isolated in this study, 35.4% were identified as *M. chelonae*; all were isolated either from sinks within the CF units or from waterborne sources. However, despite the wide array of areas tested in this study, there were no MABSC isolated from any of the samples. This would imply that *M. chelonae* can easily be isolated in copious amounts within the environment, particularly in water sources.

Regardless of progress in understanding the ecology and epidemiology of NTM, there are still many unanswered questions. Although MABSC is understood to be acquired from the environment, reports of this are particularly lacking, and for those that do exist, their true credentials are questionable due to the identification of *M. chelonae* and MABSC still very much being confused. At the time of their writing, reports prior to 1992 identifying *M. abscessus* from the environment cannot be certain to be accurate, as *M. abscessus* and *M. chelonae* were not then designated

as separate species. Even some current reports cannot be taken at face value due to the identification methods used, for example, issues with HAIN Genotyping as described in Section 1.9.6. Although HAIN was used to identify a number of mycobacterial isolates in this study, control organisms, which had previously been identified by VNTR provided accurate results. A selection of *M. chelonae* from isolated species in this study (as shown by bold text in Table 4-6) were also sent to Public Health England, Colindale, UK to be verified by VNTR in order to confirm that the HAIN results were accurate.

Recent developments in HAIN Genotyping mean that there is now an updated technique on the market; however, at the time of this study this was not available. This method, known as HAIN Genotype NTM-DR VER 10, and shown in Figure 4-1, is said to provide reliable results and is able to differentiate between the three members of the MABSC complex (HAIN Lifescience, 2017). The banding patterns significantly differ to that of previous HAIN Genotype Mycobacterium CM for *M. abscessus* and *M. chelonae* as shown in Section 1.9.6 Figure 1-9.

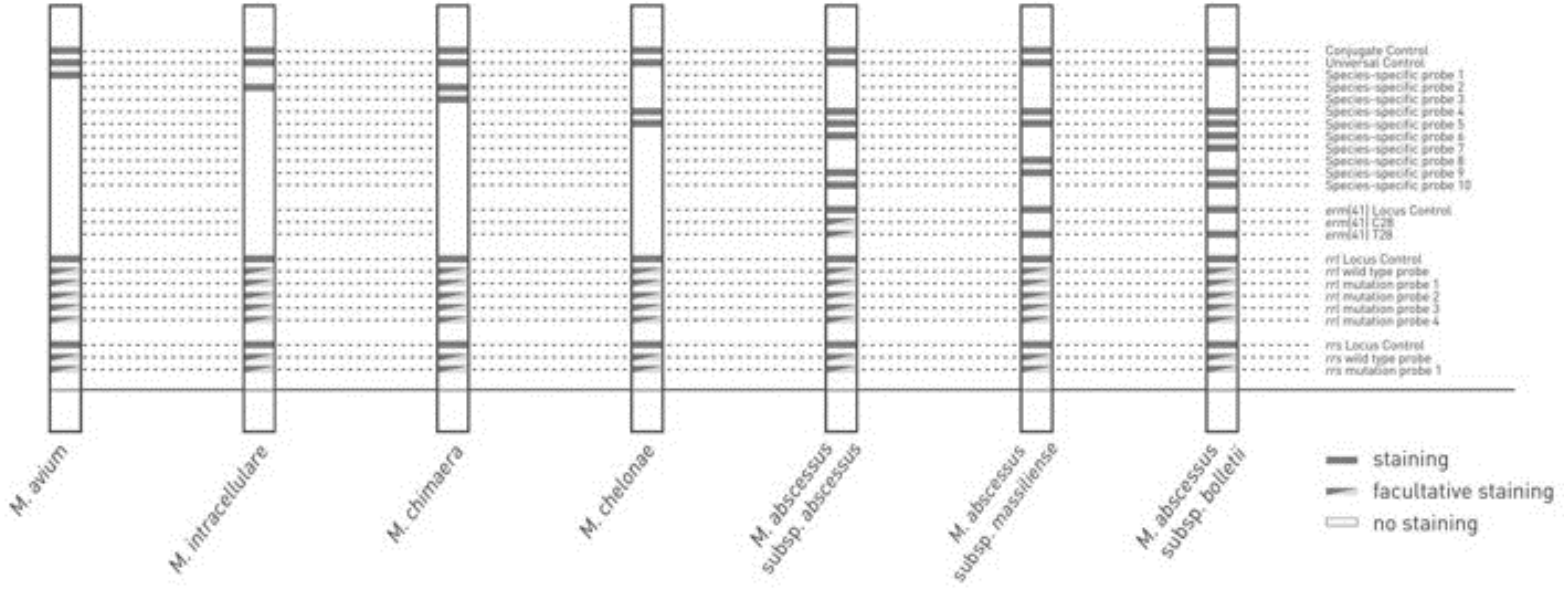


Figure 4-1: HAIN Genotype NTM-DR showing a difference in banding patterns between *M. abscessus complex* and *M. chelonae*

Recent evidence also now exists to confirm that there is widespread transmission of MABSC within the global CF community in a study performed by Bryant *et al*., 2016. Previously, the majority of reports published indicated that patients with CF were infected with strains thought to be acquired environmentally that were genetically dissimilar, however upon carrying out whole genome sequencing in a single CF centre in the UK, two clusters of patients were infected with identical or virtually identical MABSC isolates. Social network analysis suggested that these were likely to have been acquired from within the hospital setting via fomite transmission (Bryant *et al.*, 2013).

Whole genome sequencing from several CF centres globally was carried out on 1080 isolates from 517 individual patients in order to see if cross contamination could be the most plausible source of infection rather than independent acquisition from the environment. Although there were large genetic dissimilarities found between many isolates, multiple clades of almost undistinguishable isolates, mainly *M. abscessus* subsp*. abscessus* and *M. abscessus* subsp*. massiliense*, from areas that were very diverse geographically were identified. This suggests that the spread of circulating clones of MABSC is prevalent within the global CF patient community. The majority of patients were infected with clustered rather than unclustered isolates, and predominantly with *M. abscessus* subsp*. abscessus* clusters 1 and 2 and *M. abscessus* subsp*. massiliense* cluster 1. Individual transmission events were also able to be determined, as well as likely mechanisms of transmission between patients, and it was concluded that fomite spread and airborne transmission were feasible explanations (Bryant *et al*., 2016).

Despite this new evidence, no isolates of MABSC were isolated in this study from any hospital surfaces or equipment, and neither from rooms that were at the time

occupied by patients with CF, one known to be infected with *M. abscessus* subsp. *massilense.*

The presence of a vast number of genes and operons within the genome of MABSC that are involved in resistance to arsenic and encoding cysteine desulferases is undoubtedly a trademark of a soil or aquatic dwelling environmental organism (Ripoll *et al.*, 2009). However MABSC also contains many genes known to be involved in intracellular survival and is furnished with lipase encoding genes in order to obtain energy from eukaryotic host-derived lipids. It has far fewer ABC transporters or two-component sensor histidine kinases than *M. smegmatis*, signifying that MABSC may specialise in intracellular parasitism. An acceptable assumption is that MABSC have advanced to evade predators such as free living amoebas that share the same environment (Adekambi *et al.*, 2004). Soil dwelling amoebas are plentiful at soil-plant boundaries supporting the growth of plant parasites, including bacteria, which amoebas feed upon. As the MABSC genome encodes a large number of salicylate hydroxylases it enables MABSC to resist the salicylic acid mediated protection mechanisms of plants suggesting that MABSC resides in close contact with plants and so consequently also amoebas (Ripoll *et al.*, 2009).

Many reports predict a continuous rise in the incidence of interactions between NTM and humans, likely resulting in more clinical cases of environmentally-derived NTM (Prevots and Marras, 2015). This can be attributed to several factors, including the use of chlorine for the disinfection of drinking water, medical devices, and in industrial settings used to sterilise habitats, thereby selecting for NTM by reducing or eliminating competitors (Marras and Daley, 2002; Johnson and Odell, 2014). Another factor is the growing proportion of the population predisposed to environmental NTM infection, such as those individuals with immunosuppressive

disorders, for example increasing incidences of transplantations, AIDS, and simply age. Novel environmental mycobacteria will also continue to be identified as more rapid and sophisticated identification methods are developed (Primm *et al.*, 2004).

CHAPTER FIVE

Antibiotic susceptibility testing of rapidly-growing Mycobacteria with a focus on *Mycobacterium abscessus* complex

Introduction

5.1 Current treatment approaches to non-tuberculous mycobacterial infection

Treatment of NTM infections is either by drug therapy, surgery, or a combination of both of these, however treatments can be lengthy, complex, very costly and are recurrently accompanied by drug related toxicities and side effects. These are frequently somewhat severe and consequently signify a substantial healthcare concern (van Ingen *et al.*, 2010). Standard *M. tuberculosis* treatments are typically ineffective against NTM (Raju *et al.*, 2016), therefore for rapidly-growing mycobacteria, treatment regimens are predominantly based upon *in vitro* drug susceptibility testing. Results of the susceptibility testing can differ considerably between species due to huge variabilities in growth rates between species, as well as innate resistance to antibacterial drugs. Although there remains a lack of correlation between *in vitro* drug susceptibility results and *in vivo* treatment outcomes, susceptibility testing can still be beneficial to those patients who have failed to respond to first line treatments, or have suffered from reoccurrence of a prior NTM infection (Griffith *et al.*, 2007) and treatments should still be guided by drug susceptibility results (Floto *et al.*, 2016).

5.2 Recommended antibiotic treatments for pulmonary infection due to *Mycobacteria abscessus* complex in patients with cystic fibrosis

There can be a huge variation of treatments between patients with infection caused by MABSC. This is due to a lack of clinical trial data to support any particular treatment approach, but will largely consist of a preliminary intensive phase with an oral macrolide, usually azithromycin plus intravenous amikacin with one or more additional intravenous antibiotics. This is usually cefoxitin, tigecycline or imipenem

for between three to twelve weeks dependent on the patients' response to treatment and severity of infection, as well as their tolerance to particular drugs. This is followed by a continuation phase of an oral macrolide, clarithromycin or preferably azithromycin, and inhaled amikacin with the addition of two to three other antibiotics such as minocycline, linezolid, clofazimine or moxifloxacin (See Figure 5-1 and Table 5-1 below).

Clarithromycin has a slightly enhanced *in vitro* activity in comparison to azithromycin, however it would appear that clarithromycin also induces greater *erm*[41] gene expression therefore may be less effective than azithromycin against *M. abscessus* subsp*. abscessus*. Conversely, this is not the case for *M. abscessus* subsp*. massiliense* where both macrolides appear to be equally effective (Choi *et al.*, 2012). There is however conflicting published data regarding the impact of the *erm*[41] gene expression with each of these drugs, with Maurer *et al* reporting no significant differences in clarithromycin and azithromycin resistance in *M. abscessus* subsp*. abscessus* therefore suggesting that no preference should be given to either of these drugs in order to limit macrolide resistance (Maurer *et al.*, 2014b).

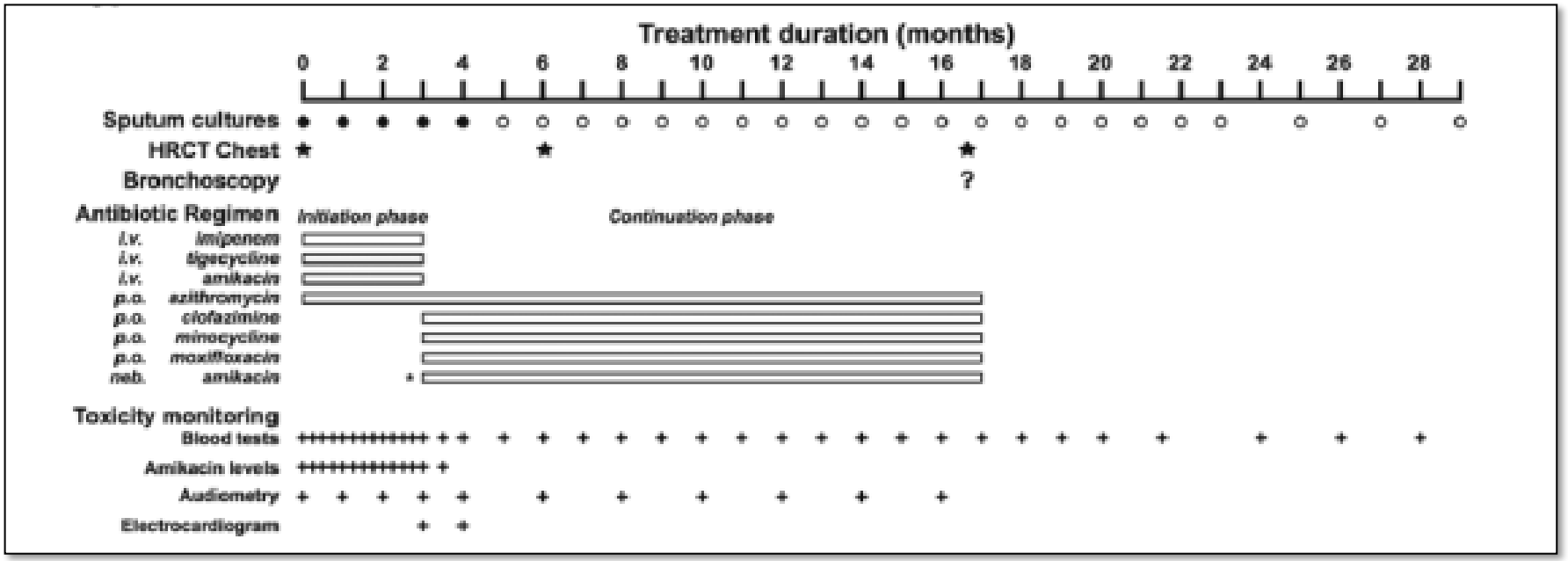


Figure 5-1: Typical MABSC treatment schedule as recommended by the US Cystic Fibrosis Foundation and European Cystic Fibrosis Society (Floto et al., 2016)

Table 5-1: Current guidelines for recommended antibiotic dosing regimens in the treatment of MABSC pulmonary disease in patients with cystic fibrosis (Floto *et al.*, 2016)

Antibiotic	Route	Dose suitable for children and adolescents	Dose suitable for adults
Amikacin	Intravenous*	Children: 15-30 mg/kg/dose once daily Adolescents: 10-15 mg/kg/dose once daily Maximum dose 1500 mg daily	10-30 mg/kg once daily OR 15 mg/kg/day in 2 doses Daily to 3 x weekly dosing
	Nebulised*ɬǂ	250-500 mg/dose once or twice daily	250-500 mg once or twice daily
Azithromycin	Oral	Children: 10-12 mg/kg/dose once daily Adolescents : As adult dose Maximum dose 500 mg	250-500 mg once daily
Cefoxitin	Intravenous	50 mg/kg/dose three times daily Maximum dose 12 g a day	200 mg/kg/dose in three divided doses daily Maximum dose 12 g a day
Clarithromycin	Oral	7.5 mg/kg/dose three times daily Maximum dose 500 mg	500 mg twice daily
	Intravenous	Not recommended	
Clofazimine	Oral ɬ¥	1-2 mg/kg/dose once daily Maximum dose 100 mg	50-100 mg once a day
Imipenem	Intravenous	15-20 mg/kg/dose twice daily Maximum dose 1000 mg	1 g twice daily
Linezolid	Oral **	< 12 years old: 10 mg/kg/dose three times daily ≥12 years old: 10 mg/kg/dose once or twice daily Maximum dose 600 mg	600 mg once or twice daily
	Intravenous **	< 12 years old: 10 mg/kg/dose three times daily ≥12 years old: 10 mg/kg/dose once or twice daily Maximum dose 600 mg	600 mg once or twice daily
Moxifloxacin	Oral	7.5-10 mg/kg/dose once daily Maximum dose 400 mg daily	400 mg once daily
Minocycline	Oral	2 mg/kg/dose once daily Maximum dose 200 mg daily	100 mg twice daily
Tigecycline	Intravenous ɬπ	8-11 years old: 1.2 mg/kg/dose three times daily Maximum dose 500 mg ≥12 years old: 100 mg loading dose then 50 mg once or twice daily	100 mg loading dose then 50 mg once or twice daily

*Adjust dose according to levels. The usual starting dose is 15 mg/kg aiming for peak level of 20-30 µg/ml and trough levels of <5-10 µg/ml

** Usually given with high dose (100 mg daily) pyridoxine (vitamin B_6) to reduce the risk of cytopaenias

ɬ As tolerated

ǂ Mixed with saline

¥ Only available in the USA via an Investigational New Drug Application (IND) application to the Food and Drug Administration (FDA)

π Many practitioners recommend pre dosing with one or more anti-emetics before dosing and/or gradual dose escalation from 25 mg daily to minimise nausea and vomiting

Clinical improvement in patients suffering from infection with MABSC would appear to be frequently unsuccessful (Maurer *et al.*, 2014a), and culture conversion is not attainable for a great many patients, although data on treatment outcomes is still very limited. MABSC exist in the lungs in many forms, including within macrophages and in biofilms posing vast difficulties to access, therefore creating a great challenge to systemically administered antibiotics (Cipolla *et al.*, 2015).

5.3 Liposomal amikacin for inhalation in patients with non-tuberculous mycobacterial pulmonary disease

Increasing attention is now being paid to the possible use of Arikace, liposomal amikacin for inhalation (LAI), for the treatment of NTM infection in patients with cystic fibrosis (CF) *via* delivery of the antibiotic in higher concentrations (Olivier *et al.*, 2016). Liposomes are microscopic membranous vesicles with an aqueous centre that can be used as a transporter for pharmaceutical drugs (Rose *et al.*, 2014). These vesicles can incorporate lipid-soluble drugs into the membrane and water-soluble drugs into their aqueous spaces and release their contents by interacting with cells by adsorption, endocytosis, lipid exchange, or fusion. The drugs are then dispersed into the body at a close proximity to the site of the lung infection.

5.3.1 Arikace clinical trials completed and in progress for use against non-tuberculous mycobacterial infection

In a randomised placebo controlled phase 2 clinical trial (clinical trial identifier NCT01315236), patients received a daily dose of 590 mg of LAI (or placebo) in addition to their ongoing treatments. Results indicated that LAI was effective in attaining negative sputum culture for NTM caused by MAC, however this was not observed for those patients with MABSC (Biller *et al.*, 2015; Olivier *et al.*, 2016).

Other clinical trials involving arikace ongoing at present are “Open-label Safety Extension Study Assessing Safety and Tolerability of LAI in Patients Who Participated in Study INS-212” (NCT02628600) and Phase 3 “Study to Evaluate Efficacy of LAI When Added to Multi-drug Regimen Compared to Multi-drug Regimen Alone” (NCT02344004) which is due for completion in October 2016 (ClinicalTrails.gov., 2016).

5.4 Antibiotic resistance mechanisms in *Mycobacteria abscessus* complex

MABSC has multiple innate antibiotic resistance mechanisms (Figure 5-2), but the most important development has been the discovery of an inducible erythromycin methylase resistance gene (*erm*^{41}) (Nash *et al.*, 2009). The *erm*^{41} gene marginally differs between *M*. *abscessus* complex subspecies with *M*. *abscessus* subsp. *abscessus* having a complete *erm*^{41}gene with ten sequevars (Brown-Elliott *et al.*, 2015). Sequevars with nucleotide T28 are associated with a complete fully functional gene and inducible clarithromycin resistance, and sequevars with nucleotide C28 are associated with a non-functional gene and linked to clarithromycin susceptibility. In *M*. *abscessus* subsp. *bolletii*, the *erm*^{41} gene is similar to T28 in *M*. *abscessus* subsp. *abscessus*; however, the *erm*^{41} gene of *M*. *abscessus* subsp. *massiliense* is known to have two deletions, rendering it non-functional. Sequence analysis of the *erm*^{41} gene has been used to classify *M*. *abscessus* complex subspecies (Rubio *et al.*, 2015).

M. abscessus subsp. *abscessus* may initially appear susceptible to macrolides, however upon exposure to these drugs resistance can develop. Until inducible resistance due to *erm*^{41} was defined, clarithromycin was effectively the drug of choice for *M. abscessus* subsp. *abscessus*. It was then revealed that although a

strain may seem to be susceptible after three days *in vitro* incubation, due to induction of the synthesis of a methyltransferase, clarithromycin resistance occurred if incubation was extended to fourteen days, (Nash *et al.*, 2009). Similarly, this could also occur if the isolate was pre-incubated with clarithromycin (Maurer *et al.*, 2014b).

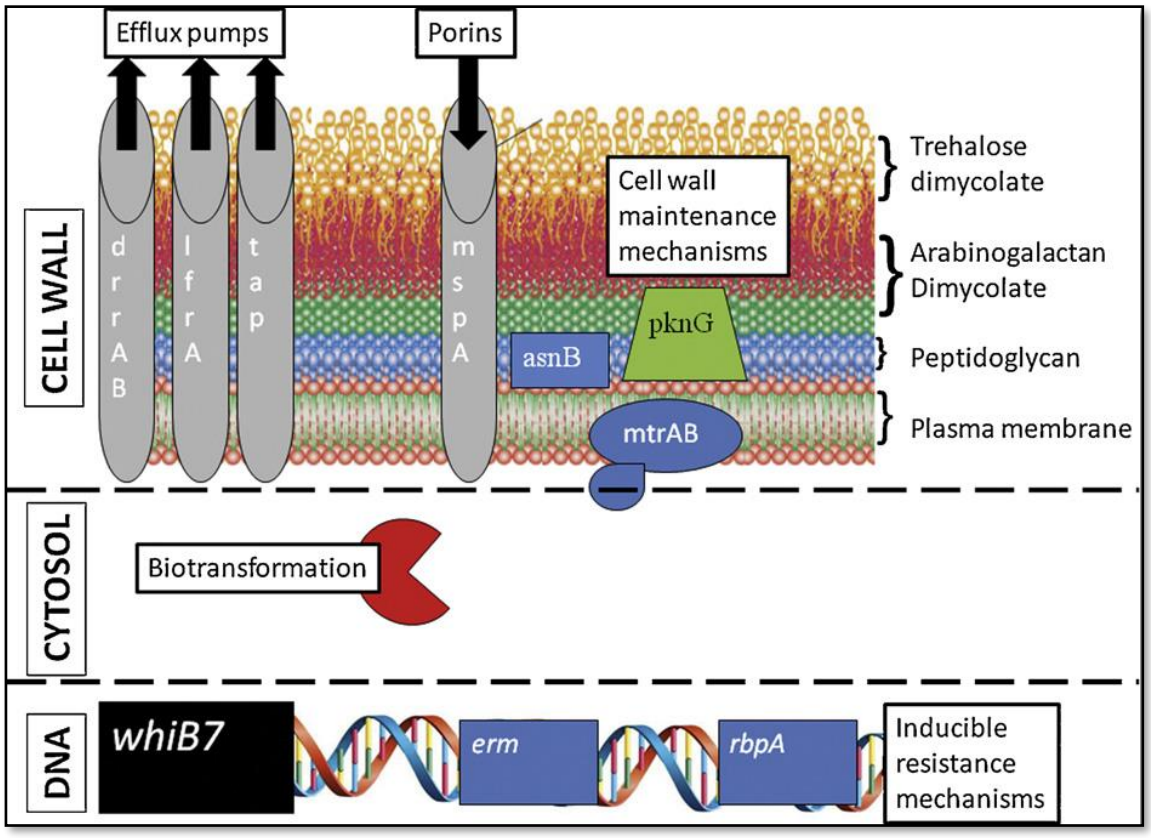


Figure 5-2: Mycobacterial cell wall and mechanisms of drug resistance (van Ingen *et al.*, 2012)

As shown in Figure 5-2, the mechanisms of drug resistance can derive from the high content of lipids, as well as many mechanisms that control cell wall content including low porin count, efflux pumps, active biotransformation by cytosolic enzymes and inducible resistance mechanisms (van Ingen *et al.*, 2012).

5.4.1 Macrolide resistance

Resistance to macrolides occurs by post transcriptional methylation of the 23S rRNA, a component of the large 50S subunit which prevents the drug from attaching (Nessar *et al.*, 2012). Macrolides such as azithromycin, clarithromycin, erythromycin and telithromycin are a class of bacteriostatic antibiotics that target the rRNA, preventing ribosomal translocation by binding reversibly to the P site of the 50S subunit of the ribosome and preventing peptidyltransferase from adding the peptidyl group attached to the tRNA to the next amino acid consequently inhibiting protein synthesis. Changes or modifications to the 50S ribosomal subunit, the target binding site for macrolides, will confer resistance to macrolides (Menninger, 1995).

M. abscessus subsp. *abscessus* and *M. abscessus* subsp*. bolletii* are both known to carry the inducible full length *erm*^{41} gene, while *M. abscessus* subsp*. massiliense* has a truncated *erm*^{41} gene that is dysfunctional due to two distinctive deletions at bases 64-64 and 159-432 resulting in macrolide susceptibility (Kim *et al.*, 2010a). Conflicting reports however have claimed that strains of *M. abscessus* subsp*. massiliense* do exist harbouring a full length functional *erm*^{41} gene said to have possibly emerged by means of horizontal gene transfer from either *M. abscessus* subsp*. abscessus* or *M. abscessus* subsp*. bolletii (Choi et al., 2012; Shallom et al., 2013)*

While having a full length *erm*^{41} gene typically results in macrolide resistance, some strains of *M. abscessus* subsp*. abscessus* with a full length *erm*^{41} are non-functional due to a T to C modification at position 28 (T28C) that likely results in an altered conformation of *erm*^{41}. This results in a failure to bind adequately to domain V, the region where macrolides bind to the ribosome (Bastian *et al.*, 2011).

5.4.2 Aminoglycoside resistance

Aminoglycosides, such as kanamycin, amikacin or tobramycin are key bactericidal drugs in the treatment of MABSC, targeting the 16S rRNA in the ribosome, and inhibiting protein synthesis by interfering with the proof reading process and causing errors in synthesis with premature termination (Shakil *et al.*, 2008). A spontaneous single A to G mutation at position 1408 (A1048G) affecting the 16S rRNA of *M. abscessus* subsp*. abscessus* isolates was reported to be associated with resistance to aminoglycosides (Prammananan *et al.*, 1998). As *M. abscessus* subsp*. abscessus* only has one copy of the rRNA operon, this makes the occurrence of a single mutation more probable (Sassi and Drancourt, 2014). Many other single mutations affecting the 16S rRNA instigating resistance to aminoglycosides (T1406A, C1409T and G1491T) have been reported (Nessar *et al.*, 2012).

5.4.3 The role of the mycobacterial cell wall in antibiotic resistance

The role of the complex hydrophobic mycobacterial cell wall shown in Chapter 1, Figure 1-1 has been widely observed, and many of its properties play a large part in the poor diffusion of antibiotics as discussed in Chapter 1 (Jarlier and Nikaido, 1990). Genomic analysis has also revealed the presence of other prospective drug resistance elements such as putative β-lactamases, aminoglycoside phosphotransferases and aminoglycoside acetyltransferases (Ripoll *et al.*, 2009).

5.4.4 Efflux pumps conferring antibiotic resistance in mycobacteria

The use of efflux pumps as a means of eradicating antimicrobial compounds was first defined in 1978 (Levy and McMurry, 1978) followed by active membrane-bound antimicrobial transport proteins conferring antibiotic resistance to tetracycline's being described in *Escherichia coli* by means of expulsion from the cell (Ball *et al.*,

1980). Prior to this they were known only for upholding a role of protecting against toxicities by maintaining cell homeostasis by pumping toxic agents out of the cell (Alvarez-Ortega *et al.*, 2013). They are now known to be involved in antibiotic resistance in mycobacteria with numerous being discovered (Szumowski *et al.*, 2013) contributing to resistance of drugs such as isoniazid, rifampicin, clofazimine, ciprofloxacin, linezolid, tetracycline and streptomycin (Nessar *et al.*, 2012; Rodrigues *et al.*, 2013; Pal *et al.*, 2014; Fonseca *et al.*, 2015). The genome of MABSC encodes for several proteins involved in antimicrobial efflux systems including members of the major facilitator superfamily (MFS), ATP binding cassette (ABC) transporters, mycobacterial membrane protein large (MmpL) family of transporters and a multidrug resistance Stp protein comparable to that defined in the resistance to spectinomycin and tetracycline in *M. tuberculosis* (Ramon-Garcia *et al.*, 2007).

5.4.5 Other mechanisms known to contribute to mycobacterial drug resistance

MABSC contains a 23319 base pair mercury resistance plasmid identical to the pMM23 mercury resistance plasmid of *M. marinum* (Stinear *et al.*, 2008) which carries a mercury resistance operon flanked by two genes MAB_p04c and MAB_p10, encoding site-specific recombinases (Ripoll *et al.*, 2009). However, although mercury resistance has been studied comprehensively in other species, additional research is needed to elucidate mercury resistance in mycobacteria.

Mycobacterial resistance to ethambutol is thought to occur due to mutations in the embCAB operon that encodes arabinosyl transferases, recognised targets of ethambutol (Palomino and Martin, 2014). Mutations conferring nucleotide

substitutions at amino acid residue 306 in embB is reported to cause ethambutol resistance (Sreevatsan *et al.*, 1997).

In sequences of conserved regions known as quinolone resistance-determining regions (QRDR) in the DNA gyrase subunits GyrA and GyrB it was revealed that the presence of alanine at position 83 within GyrA QRDR and arginine and asparagine at positions 447 and 464 respectively, within GyrB QRDR confer resistance to fluoroquinolones in MABSC*, M. avium*, *M. intracellulare*, *M. marinum* and *M. chelonae* (Guillemin *et al.*, 1998)

MABSC is known to produce numerous enzymes that can potentially damage or modify antibiotics, causing their inactivation. These include an Ambler class A ß-lactamase, a rifampin ADP-ribosyl transferase, an aminoglycoside 2′-N-acetyltransferase and at least twelve homologs of aminoglycoside phosphotransferases, four homologs of monooxygenases potentially involved in resistance to rifampin and tetracyclines, two FolP homologs conferring resistance to cotrimoxazole, one homolog of UDP-N-acetylglucosamine 1-carboxyvinyltransferase MurA conferring resistance to fosfomycin, and two homologs of 23S rRNA methylases conferring resistance to macrolides (Vester and Douthwaite, 2001). MABSC also produce enzymes that could modify aminoglycoside drugs by transferring acetyl or phosphate residues on crucial positions within the antibiotic, causing their inactivation (Ripoll *et al.*, 2009).

5.5 Challenges of diagnosis and treatment of *Mycobacteria abscessus*

As it is difficult to distinguish between colonisation and true infection, the decision of whether to treat MABSC infection or not embraces many challenges. Antibiotic susceptibilities can greatly differ between MABSC subspecies, particularly members of the MABSC, and monotherapy will often fail to produce a cure.

Current multidrug therapy recommendations remain contentious, with success for very few patients and failure for the majority (Griffith *et al.*, 2007) as well as high rates of resistance being reported for many of these antibiotics (Wallace *et al.*, 2001; Yang *et al.*, 2003; Chihara *et al.*, 2010; Bastian *et al.*, 2011).

5.6 Aims and objectives

Standard therapies for both MABSC and *M. tuberculosis* as well as additional antimicrobials that are not generally used for treatment of mycobacterial infection were evaluated in order to establish the minimum inhibitory concentrations and establish whether any may be used for the successful treatment of MABSC.

5.7 Materials

5.7.1 Bacterial strains used in antimicrobial testing of non-tuberculous mycobacteria

A collection of 100 NTM isolates from 94 adult and paediatric patients were tested, with the exception of one isolate of *M. abscessus* subsp*. bolletii* that was derived from an environmental sample. Clinical isolates were derived from CF sputum samples by standard methods and previously stored at -20^{o}C in glycerol/skimmed milk. These included *M. abscessus* subsp*. abscessus* (*n* = 56)*, M. chelonae* (*n* = 23), *M. abscessus* subsp*. massiliense (n* = 7), *M. abscessus* subsp*. bolletii* (*n* = 3), *M. fortuitum* (*n* = 3), *M. llatzerense* (*n* = 2), *M. salmoniphilum* (*n* = 2), M. immunogenum (*n* = 1), *M. intracellualare* (*n* = 1), *M. mucogenicum* (*n* = 1), and *M. septicum* (*n* = 1). Further details can be found in Appendix 1.

The species and subspecies identity of all strains had been previously confirmed by sequencing of at least two of three housekeeping genes (*rpoB, hsp*65 and *sodA*) using previously described methods (Blauwendraat *et al.*, 2012). NCTC control strains were also used; *S. aureus* NCTC 6571, *C. perfringens* NCTC 8797 and *E. coli* NCTC 10418. All frozen isolates were subcultured on Columbia agar with 5% horse blood prior to testing.

5.7.2 Growth media

Mueller-Hinton agar (CM0337) and Mueller-Hinton broth (CM0405) were supplied from Oxoid Ltd, Basingstoke, UK and prepared according to manufacturer's instructions. Blood agar was prepared from Columbia agar powder (CM0331) (Oxoid) and 5% defibrinated horse blood supplied by TSC Biosciences, Buckingham, UK.

5.7.3 Antimicrobials

Rifampicin was supplied by Duchefa Biochemie BV, Haarlem, The Netherlands, moxifloxacin was obtained from Bayer HealthCare Pharmaceuticals, Berlin, Germany, azithromycin from Aspire Pharma Ltd, Petersfield, UK, meropenem from Fresenius Kabi Ltd, Cheshire, UK, tobramycin from Medimpex UK Ltd, London, UK, ciprofloxacin was obtained from Fannin, Northamptonshire, UK, doripenem from Janssen-Cilag, Buckinghamshire, UK and tigecycline was obtained from Pfizer, Hampshire, UK. All other antimicrobials were all purchased from Sigma-Aldrich, Poole, UK

5.7.4 Equipment

All equipment as described in previous sections 2.3.4 and 3.10.6.

5.8 Methods

5.8.1 Preparation of the medium for agar dilutions for a range of single antimicrobials against rapidly-growing mycobacteria

Mueller Hinton agar was made up according to manufacturer's instructions where 90 ml of sterile deionised water was added to 3.8 g agar. These were autoclaved at 116^{o}C for 10 minutes. Each antimicrobial was dissolved in 20 ml of sterile distilled water, with the exception of clarithromycin, clofazimine and rifampicin, which were first dissolved in 200 µl of N-Methyl-2-pyrrolidone then added to 19.8 ml sterile distilled water. The dissolved antimicrobials were filter sterilised and diluted down to 10 x the strength of each target concentration with sterile distilled water and 10 ml was added to 90 ml Mueller-Hinton agar giving a total volume of 100 ml. For each target concentration, five plates were prepared in sterile Petri dishes and allowed to set.

5.8.2 Investigation of various antimicrobials using agar-based dilutions against rapidly-growing mycobacteria isolates from patients with cystic fibrosis

The following ranges of antimicrobials were used in the initial MIC agar dilution testing, and were selected based on known breakpoints published by the European Committee on Antimicrobial Susceptibility Testing Guidelines (EUCAST, 2014) and a review of the current literature; amikacin 4 – 128 mg/L, azithromycin 0.125 – 4 mg/L, cefoxitin 2 – 64 mg/L, chloramphenicol 2 – 64 mg/L, ciprofloxacin 0.25 – 8 mg/L, clarithromycin 0.5 – 16 mg/L, clindamycin 0.125 – 4 mg/L, clofazimine 0.125 – 4 mg/L, doripenem 0.5 – 16 mg/L, doxycycline 0.25 – 8 mg/L, erthyromycin 0.25 – 8 mg/L, fusidic acid 0.125 – 4 mg/L, gentamicin 2 – 64 mg/L, imipenem 1 – 32 mg/L, isoniazid 0.125 – 4 mg/L, kanamycin 0.125 – 4 mg/L, linezolid 0.5 – 16 mg/L,

meropenem 0.5 – 16 mg/L, metronidazole 0.5 – 16 mg/L, minocycline 8 – 256 mg/L, moxifloxacin 1 – 32 mg/L, rifampicin 4 – 128 mg/L, streptomycin 0.5 – 16 mg/L, teicoplanin 0.25 – 8 mg/L, tigecycline 0.125 – 4 mg/L, tobramycin 0.125 – 4 mg/L, trimethoprim-sulfamethoxazole 0.125 – 4 mg/L, spectinomycin 0.125 – 4 mg/L and resorcinol 0.0625 – 2 mg/L. All tests were performed in duplicate on separate occasions.

5.8.3 Bacterial strains and culture onto medium containing single antimicrobial agents

A suspension containing 2 ml sterile saline (0.85%) and turbidity equivalent to a 0.5 McFarland standard (approx. 1.5 x 10^8 CFU/ml) was prepared for each isolate. For the rough colony types, where clumping occurred, vortexing with three sterile 3 mm glass beads for 10 minutes effectively dispersed all clumps. Each medium type was inoculated using a multipoint inoculator with a 1 µl aliquot of each isolate (i.e. approx. 1.5 x 10^5 CFU). Results were recorded after five days incubation at 30^{o}C with the exception of metronidazole, which was incubated for five days anaerobically at 37^{o}C. Mueller-Hinton control plates were set up and each one inoculated with 20 of the 100 mycobacterial isolates. *S. aureus* and *E. coli* were included to serve as control organisms and *C. perfringens* as an anaerobic control.

5.9 Results

The data for all of the mycobacterial isolates tested and antimicrobial agents are shown in Table 5-2 below with the MIC_{50} and MIC_{90} of *M. abscessus* subsp*. abscessus, M. chelonae and M. abscessus* subsp*. massiliense* shown in Table 5-3. The agar dilution method used follows the principle of establishing the lowest concentration of the serially diluted antibiotic concentration that completely inhibits bacterial growth.

Results show that the majority of antimicrobials were ineffective against *M. abscessus* subsp*. abscessus*, at the ranges tested.

From the recommended treatment regimes, the results for *M. abscessus* subsp*. abscessus* show that cefoxitin had a range of MIC 8->64 mg/L. All but two isolates had a MIC of 8-64 mg/L with a MIC_{50} 32 mg/L and MIC_{90} 64 mg/L. Clarithromycin had a range of MIC <0.5->16 mg/L, with 24 (42.9%) isolates having a MIC <0.5 mg/L, MIC_{50} 2 mg/L and MIC_{90} 16 mg/L. Tigecycline had a range of MIC <0.125->4 mg/L with 24 (42.9%) isolates having a MIC ≤2 mg/L and both the MIC_{50} and MIC_{90} 4 mg/L. Although minocycline had a range of <8 - >256, both MIC_{50} and MIC_{90} were >256 mg/L with only 15 (26.8%) isolates having a MIC of 256 mg/L or less, and only one with a MIC of <8 mg/L. Results for moxifloxacin showed that 53 isolates (94.6%) had a MIC >4 mg/L, and for linezolid 46 (82.1%) isolates had a MIC ≥16 mg/L. All isolates had a MIC >128 mg for amikacin, >4 mg/L for clofazimine and 49 (87.5%) isolates had a MIC >16 mg/L for imipenem. Azithromycin had a range of 0.5->4 mg/L, with 43 isolates (76.8%) >4 mg/L and MIC_{50} and MIC_{90} both >4 mg/L.

Results for *M. abscessus* subsp*. massiliense* show for all recommended guideline treatments clarithromycin was the only one antibiotic that seemed to show any activity with a MIC range of <0.5->16 mg/L with 6/7 (85.7%) isolates having a MIC

<0.5 mg/L and only one isolate had a MIC >16 mg/L. Tigecycline had a range of 0.5-4 mg/L with 5/7 isolates MIC 4 mg/L.

M. abscessus subsp*. massiliense* isolates showed greater susceptibility than *M. abscessus* subsp*. abscessus* for azithromycin with both MIC_{50} and MIC_{90} of 4 mg/L (MIC range 1->4 mg/L) with only one isolate having a higher MIC than 4 mg/L whereas only 23.2% (13/56) of *M. abscessus* subsp*. abscessus* had a MIC 4 mg/L or lower for azithromycin.

The three isolates of *M. abscessus* subspecies *bolletii* tested showed resistance to all antimicrobials tested except clarithromycin with two isolates having a MIC <0.5 mg/L and one isolate MIC 4mg/L. All other antimicrobials appear to be ineffective demonstrating resistance.

Clarithromycin also demonstrated superior results to all other antimicrobials tested for *M. chelonae* with a range of <0.5-8 mg/L, 19/23 (82.6%) isolates having a MIC <0.5 mg/L, and MIC_{50} <0.5 mg/L and MIC_{90} 2 mg/L.

Mycobacteria fortuitum showed susceptibility to tigecycline with two isolates having a MIC <0.125 mg/L and one MIC 0.5 mg/L. However, ciprofloxacin demonstrated three differing MIC's, <0.125 mg/L, 0.5 mg/L and >4 mg/L and moxifloxacin ranged from <0.125 mg/L to >4 mg/L.

For species where there were only one or two isolates tested, for example *M. salmoniphilum* and *M. llatzerense*, it is not possible to determine accurate susceptibility profiles, for example for the two isolates of *M. salmoniphilum* conflicting MIC's were shown for streptomycin, 4 mg/L and <0.5 mg/L. As with *M. llatzerense*, MIC's of 1 mg/L and <0.5 mg/L were shown for meropenem.

The three control strains shown in Table 5-4 demonstrated MIC's within the acceptable range for each antimicrobial tested according the EUCAST guidelines (EUCAST, 2014).

Table 5-2: Range of MIC's of NTM species using a selection of twenty-nine single antimicrobials (mg/L)

	Antimicrobial	Mycobacterial species										
		M. abscessus	*M. massiliense*	*M. bolletii*	*M. chelonae*	*M. fortuitum*	*M llatzerense*	*M.salmoniphilum*	*M. mucogenicum*	*M.immunogenum*	*M. septicum*	*M intracellulare*
		n = 56	*n* = 7	*n* = 3	*n* = 23	*n* = 3	*n* = 2	*n* = 1	*n* = 1	*n* = 2	*n* = 1	*n* = 1
Recommended treatments	Amikacin	>128	>128	64 - >128	>128	64 - >128	64	<4	>128	>128	128	>128
	Azithromycin	0.5->4	1->4	2 - >4	1->4	>4	1 - 2	0.25	>4	1	>4	1
	Cefoxitin	8 - >64	16 - 64	64 - >64	>64	16 - 64	4	<2 - 4	64	16	>64	>64
	Clarithromycin	<0.5 - >16	<0.5 - >16	<0.5 - 4	<0.5 - 8	<0.5 - >16	<0.5	>16	<0.5	<0.5	>16	>16
	Clofazimine	>4	>4	>4	>4	>4	>4	<0.125	>4	>4	2	>4
	Imipenem	1->16	2->16	>16	>16	16 - >16	1	>16	2	16	16	>16
	Linezolid	<0.5 - >16	8 - >16	4 - >16	16 - >16	>16	<0.5	<0.5	>16	>16	>16	16
	Minocycline	<8 - >256	<8 - >256	64 - >256	<8 - >256	<8 - 32	<8	<8	>256	<8	128	32
	Moxifloxacin	1 - >4	>4	1 - >4	<0.125 - >4	<0.125 - >4	>4	0.5	>4	2	0.25	>4
	Tigecycline	<0.125 - >4	0.5 - 4	0.125 - 4	<0.125 - 1	0.5 - <0.125	<0.125	<0.125	2	1	<0.125	0.25
	Chloramphenicol	32 - >64	>64	>64	64 - >64	>64	8	>64	>64	>64	>64	>64
	Ciprofloxacin	4 - >4	>4	<0.125 - >4	<0.125 - >4	<0.125 - >4	0.5	4	>4	1	1	>4
	Clindomycin	>4	>4	>4	>4	>4	>4	0.25 - 0.5	>4	>4	>4	>4
	Doripenem	8 - >16	>16	16 - >16	>16	16 - >16	16	2	>16	>16	16	>16
	Doxycycline	2 - >8	>8	>8	<0.25 - >8	8 - >8	1 - 2.	<0.5	>8	1	>8	>8
	Erythromycin	1 - >8	>8	8 - >8	1 - >8	>8	8	1	>8	8	>8	4
	Fusidic Acid	1 - >4	>4	<0.125 - >4	4 - >4	>4	1 - 2.	1	2	>4	>4	>4
	Gentamicin	16 - >64	64 - >64	4 - >64	32 - 64	16 - 32	4 - 8.	32	>64	32	8	16
	Isoniazid	>4	>4	>4	2 - >4	>4	>4	<0.125	>4	>4	>4	>4
	Kanamycin	2 - >4	>4	<0.125 - >4	4 - >4	>4	0.5	4	2	>4	>4	>4
	Meropenem	2 - >16	>16	<0.5 - >16	8 - >16	8 - >16	<0.5 - 1	<0.5	2	8	8	>16
	Metronidazole	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
	Resorcinol	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2	>2
	Rifampicin	<2 - >128	64 - >128	>128	>128	32 - >128	64 - 128	<2 - 4	>128	>128	>128	>128
	Teicoplanin	4 - >8	>8	<0.25 - >8	>8	>8	8 - >8	4	>8	>8	>8	>8
	Tobramycin	16 - >16	>16	<0.5 - >16	8 - >16	>16	16	<0.5	>16	16	16	8
	Trim-Sulf*	<0.125->4	2->4	2 - >4	4->4	>4	<0.125	4	0.25	1	0.5	>4
	Spectinomycin	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8	>8
	Streptomycin	16 - >16	>16	>16	>16	>16	8	<0.5 - 4	>16	>16	>16	>16

* Trimethoprim/Sulfamethoxazole

Table 5-3: MIC's, MIC_{50} and MIC_{90} of *M. abscessus* subsp*. abscessus, M. abscessus* subsp*. massiliense and M. chelonae* using a selection of twenty-nine single antimicrobials (mg/L)

	Antimicrobial	*M. abscessus* subspecies *abscessus* *n* = 56			*M. chelonae* *n* = 23			*M. abscessus* subspecies *massiliense* *n* = 7		
		RANGE	MIC_{50}	MIC_{90}	RANGE	MIC_{50}	MIC_{90}	RANGE	MIC_{50}	MIC_{90}
Recommended treatments	Amikacin	>128	>128	>128	>128	>128	>128	>128	>128	>128
	Azithromycin	0.5->4	>4	>4	1->4	2	>4	1->4	4	4
	Cefoxitin	8 - >64	32	64	>64	>64	>64	16 - 64	64	64
	Clarithromycin	<0.5 - >16	2	16	<0.5 - 8	<0.5	2	<0.5 - >16	<0.5	<0.5
	Clofazimine	>4	>4	>4	>4	>4	>4	>4	>4	>4
	Imipenem	1->16	>16	>16	>16	>16	>16	2->16	>16	>16
	Linezolid	<0.5 - >16	>16	>16	16 - >16	16	>16	8 - >16	>16	>16
	Minocycline	<8 - >256	>256	>256	<8 - >256	128	>256	<8 - >256	>256	>256
	Moxifloxacin	1 - >4	>4	>4	<0.125 - >4	>4	>4	>4	>4	>4
	Tigecycline	<0.125 - >4	4	4	<0.125 - 1	1	1	0.5 - 4	4	4
	Chloramphenicol	32 - >64	>64	>64	64 - >64	>64	>64	>64	>64	>64
	Ciprofloxacin	4 - >4	>4	>4	<0.125 - >4	>4	>4	>4	>4	>4
	Clindamycin	>4	>4	>4	>4	>4	>4	>4	>4	>4
	Doripenem	8 - >16	>16	>16	>16	>16	>16	>16	>16	>16
	Doxycycline	2 - >8	>8	>8	<0.25 - >8	>8	>8	>8	>8	>8
	Erythromycin	1 - >8	>8	>8	1 - >8	>8	>8	>8	>8	>8
	Fusidic Acid	1 - >4	>4	>4	4 - >4	>4	>4	>4	>4	>4
	Gentamicin	16 - >64	>64	>64	32 - 64	32	64	64 - >64	>64	>64
	Isoniazid	>4	>4	>4	2 - >4	4	>4	>4	>4	>4
	Kanamycin	2 - >4	>4	>4	4 - >4	>4	>4	>4	>4	>4
	Meropenem	2 - >16	>16	>16	8 - >16	>16	>16	>16	>16	>16
	Metronidazole	>16	>16	>16	>16	>16	>16	>16	>16	>16
	Resorcinol	>2	>2	>2	>2	>2	>2	>2	>2	>2
	Rifampicin	<2 - >128	>128	>128	>128	>128	>128	64 - >128	>128	>128
	Teicoplanin	4 - >8	>8	>8	>8	>8	>8	>8	>8	>8
	Tobramycin	16 - >16	>16	>16	8 - >16	8	16	>16	>16	>16
	Trim-Sulf*	<0.125->4	>4	>4	4->4	>4	>4	2->4	>4	>4
	Spectinomycin	>8	>8	>8	>8	>8	>8	>8	>8	>8
	Streptomycin	16 - >16	>16	>16	>16	>16	>16	>16	>16	>16

* Trimethoprim/Sulfamethoxazole

Table 5-4: Control Organisms, range of antimicrobial tested and EUCAST MIC ranges (mg/L)

Recommended Treatment	Range Tested	MIC of Control	*S. aureus* NCTC 6571	*C. perfringens* NCTC 8797	*E. coli* NCTC 10418
Amikacin	4-128	16			8-16
Azithromycin	0.125-4	1	0.5-2		
Cefoxitin	2-64	4	1-4		
Clarithromycin	0.5-16	0.5	0.125-0.5		
Clofazimine*	0.125-4	>4			
Imipenem	1-32	2			2-8
Linezolid	0.5-16	4	1-4		
Minocycline	8-256	<8	0.06-0.5		
Moxifloxacin	0.125-4	0.125	0.016-0.125		
Tigecycline	0.125-4	1			1-2
Other Antimicrobials					
Chloramphenicol	2-64	8	2-16		
Ciprofloxacin	0.125-4	1			0.5-1
Clindamycin	0.125-4	0.25	0.06-0.25		
Doripenem	0.5-16	2			1-2
Doxycycline	0.25-8	0.5	0.125-0.5		
Erythromycin	0.25-8	0.5	0.25-1		
Fusidic Acid	0.125-4	0.25	0.06-0.25		
Gentamicin	2-64	4			2-4
Isoniazid*	0.125-4	>4			
Kanamycin	0.125-4	2			0.25-8
Meropenem	0.5-16	2			2-8
Metronidazole**	0.5-16	4		0.125-0.5	
Resorcinol**	0.0625-2	>2			
Rifampicin*	4-128	4			
Teicoplanin	0.25-8	0.5	0.25-1		
Tobramycin	0.125-4	1	0.125-1		
Trim-Sulf*	0.125-4	0.125	≤0.5		
Spectinomycin	0.125-4	>8			8-64
Streptomycin*	0.5-16	16			2-16

* *M. tuberculosis* treatments

** Reported as effective against *M. ascessus* in paper by Chopra et al, 2007

Where range tested was higher/lower than control MIC, this was due to the range for that antimicrobial being specifically chosen due to journal reports of a MIC using MABSC.

5.10 Discussion

It is well documented that a reliable and successful antimicrobial regimen for MABSC infection, in particular *M. abscessus* subsp*. abscessus*, which is the most pathogenic and chemotherapy resistant of all rapidly growing NTM, still remains to be identified (Jeon *et al.*, 2009; Nessar *et al.*, 2012; Pang *et al.*, 2013). Effective treatments are particularly challenging as there are no definitive guidelines as to which antimicrobial(s) should be given and it is widely held that they are resistant to all first line anti-tuberculosis agents such as streptomycin, isoniazid, ethambutol, rifampicin and pyrazinamide, resulting in very limited therapeutic choices and a high treatment failure rate (Oh *et al.*, 2014; Talati *et al.*, 2008).

It has been suggested that any treatment regimen containing clarithromycin is more effective in patients with *M. abscessus* subsp*. massiliense* pulmonary disease than in those with *M. abscessus* subsp*. abscessus*. Clarithromycin inducible resistance shown in *M. abscessus* subsp*. abscessus* clinical strains is partly accountable for the lack of success of antibiotic therapies containing clarithromycin (Koh *et al.*, 2011). Based on this, the CLSI guidelines recommended that the incubation period of MABSC strains, in cases where results at day 3 may indicate susceptibility, should ideally be extended up to 14 days (Clinical and Laboratory Standards Institute, 2011). It is evident from the results in Table 5-2 and Table 5-3 that the MIC of *M. abscessus* subsp*. massiliense* (both MIC_{50} and MIC_{90} <0.5 mg/L) is notably lower than those shown for *M. abscessus* subsp*. abscessus* (MIC_{50} 2 mg/L and MIC_{90} 16 mg/L) inferring that inducible resistance may be occurring.

There is no current reliable evidence that antibiotic treatment is always beneficial, however the UK CF Trust Antibiotic Working Group specify that ATS criteria for diagnosis should be followed (Floto *et al.*, 2016). This specifies that minimum evaluation is to include a high-resolution computed tomography (HRCT) scan, three

or more sputum samples positive for acid-fast bacilli, and exclusion of other disorders. In those with CF who have suspected NTM infection, it is essential to first treat their typical pathogens and then evaluate whether anti-mycobacterial therapy is justified (Griffith *et al.*, 2007). There is no consensus on standardised NTM treatments, and these can vary depending on many factors such as the extent of the infection, the type of NMT isolated, underlying conditions, differing *in-vitro* and *in-vivo* effectiveness, and the toxicity of many NTM therapies, particularly with long-term use.

From the MICs obtained, the majority of NTMs tested in this study could be successfully treated, however, *M. abscessus* subspecies *abscessus* remains elusive to almost all antibiotics tested. Clarithromycin (MIC_{50} 2 mg/L and MIC_{90} 16 mg/L) and tigeclycline (MIC_{50} and MIC_{90} both 4 mg/L) were the most promising, however for infections involving *M. abscessus* subsp*. abscessus*, antibiotic regimens based on *in vitro* susceptibilities are not shown to be active *in vivo* (Oh *et al.*, 2014). Encouraging synergistic *in vitro* activity for tigecycline with clarithromycin has been shown in a study by Huang *et al* (2013), supporting the results obtained in this study (Huang *et al.*, 2013). None of the other antibiotics tested were effective against *M. abscessus* subsp*. abscessus.*

Antibiotic sensitivities would not appear to be routinely performed upon first isolation of MABSC, and from the results obtained in this study none of the recommended treatments are effective for every strain, and can range from very low to extremely high MIC's, for example minocycline with a range of <8 - >256 mg/L which would not be suitable for patient treatments. For species where only a small number of isolates were tested, for example *M. fortuitum*, the MIC was different for each of the three isolates, this would indicate that it is huge challenge to obtain a typical and

reliable strategy when considering treatments, and each isolate should be looked at individually as MIC's can vary dramatically within the same species or subspecies.

This study would appear to verify that it is only the most commonly routinely used antimicrobials which have any effect on MABSC, for example clarithromycin, in particular against *M. abscessus* subsp*. abscessus*. None of the frontline *M. tuberculosis* antimicrobials tested have shown to have effects on MABSC likely to be clinically relevant, however many of these did seem to exhibit activity against *M. salmoniphilum*, for example clofazimine and isoniazid, again reinforcing that species must be correctly identified as successful treatments can be so varied between species.

Amikacin, a semi-synthetic aminoglycoside derivative of kanamycin, is acknowledged as one of the fundamental antimicrobials in the treatment of MABSC and it is typically reported that MABSC exhibit *in vitro* susceptibility to this antimicrobial (Novosad *et al.*, 2016).However the results in this study contradict this and all isolates tested were shown to be resistant. The *Mab_3168c* gene involved in cell wall synthesis could feasibly be making the MABSC cells less penetrable to amikacin as well as acetylating the antimicrobial, rendering it inactive, and this could conceivably explain the results in this study. In a report by Tsai *et al* (2013), it is claimed that to further confirm that the *Mab_3168c* gene conferred resistance to amikacin, it was introduced into another mycobacteria species; *M. smegmatis*. This established that the amikacin MIC value was two-fold higher. It was also suggested that the inactivation of *Mab_3168c* may change the structure of the MABSC cell wall, resulting in a change in colony morphotype, reduced cellular aggregation and the ability to survive inside macrophages as well as increased susceptibility to amikacin (Tsai *et al.*, 2013).

Chopra *et al*, (2011), showed both metronidazole, a nitroimidazole class antibiotic mainly used for the treatment of anaerobic bacteria, and the antiseptic resorcinol were effective against *M. abscessus* subsp*. abscessus*, reporting MIC's of <0.015 mg/L and 0.09 mg/L respectively. However these results were based on only one type strain of *M. abscessus* subsp*. abscessus* (ATCC 19977) and no clinical isolates were tested (Chopra *et al.*, 2011). Results from this study do not support these findings, and using a range taken either side of the MIC reported by Chopra *et al* (2011), resorcinol has an MIC of >2 mg/L for all isolates tested, using a concentration range between 0.0625-2 mg/L, and metronidazole has a MIC of >16 mg/L for all isolates tested, using a range between 0.5-16 mg/L. A similar study was conducted by Mukherjee *et al* (2012), but even at the highest concentration tested (2mM), metronidazole was ineffective against *M. abscessus* subsp*. abscessus* as reported by Chopra *et al* (2011) (Mukherjee *et al.*, 2012).

Incomplete information can be very misleading to clinicians who are looking at trying novel treatments for any patient. An example of how testing of only one isolate cannot be reliably used for patient treatment plans is in this study where only three isolates of *M. fortuitum* were tested. Results for ciprofloxacin showed three varying MIC's of <0.125 mg/L, 0.5 mg/L and >4 mg/L. For the same antibiotic, the three isolates of *M. abscessus* subsp*. bolletii* had MIC's of between <0.125->4 mg/L.

MABSC, in particular *M. abscessus* subsp*. abscessus*, exhibit resistance to almost all known Food and Drug administration (FDA) approved antimicrobials, and this combined with the lack of novel antibiotics being discovered is hampering efforts to improve current treatment plans.

A suggestion could be to perform antimicrobial synergy testing, for example using checkerboard assays, time kill curves or multiple-combination bactericidal testing of currently recommended antimicrobial treatments alongside others that are not

ordinarily used for MABSC treatments, but could possibly display synergy when combined. This however may still not provide a standard treatment regime as many isolates of MABSC have such varying antimicrobial susceptibility profiles.

New anti-tuberculosis drugs such as the diarylquinoline Bedaquiline, formerly TMC207 and R207910, and the first drug in a novel class appropriate for the treatment of multi-drug resistant tuberculosis since Rifampin in 1971, was FDA approved in December 2012 with very promising results (Philley *et al.*, 2015). This drug has a novel mechanism of action and structurally differs from all other available *M. tuberculosis* front line agents by interfering with the bacterial energy metabolism. It works by inhibiting mycobacterial adenosine 5'-triphosphate (ATP) synthase, by binding to subunit c of the enzyme that is vital for the generation of energy in *M. tuberculosis* (Mahajan, 2013).

In a report by Philey *et al* (2015), Bedaquiline appears to be effective for the treatment of not only multi-drug resistant tuberculosis, but also against MABSC and MAC, however larger studies are required in order to substantiate results as this report only consisted of ten patients, six with MAC and four with MABSC (Philley *et al.*, 2015). Pang *et al* (2017) also more recently reported that Bedaquiline had moderate *in vitro* activity against NTM with MIC_{50} and MIC_{90} of 0.03 m/L and 16 mg/L for MAC and MIC_{50} and MIC_{90} of 0.13 mg/L and >16 mg/L for MABSC (Pang *et al.*, 2017).

Further studies on resistant strains are urgently required and it should also be noted that the treatment of MABSC cannot reliably be guided by *in vitro* susceptibility testing. The ATS guidelines state that "At present, there is no reliable or dependable antibiotic regimen, even based on *in vitro* susceptibilities, including parenteral agents, to produce a cure for *M. abscessus* lung disease" (Floto *et al.*, 2016), and this was also noted in a report by Jeon et al, where it was indicated "The optimal

therapeutic regimen and duration of treatment for *M. abscessus* lung disease has not been established" (Jeon *et al.*, 2009). It is vital that clinicians distinguish the MABSC subspecies of any patient isolate, and if *M. abscessus* subsp*. abscessus* is identified, is the *erm*^{41} gene active.

The accessibility of MABSC genomes and the development of genetic methods in order to study MABSC represent major advancements in this field, particularly for *M. abscessus* subsp*. abscessus*, the most virulent and antibiotic resistant of the MABSC subspecies however it is evident from this study and all published literature that more effective antimicrobials are urgently needed.

CHAPTER SIX

Final discussion and future research

Final discussion and future research

6.1 Review and final discussion

This thesis is centred on MABSC, the development of a novel culture medium for the isolation of MABSC in patients with CF, methods of identification, the acquisition of MABSC and *in vitro* susceptibility studies. Current culture methods are fraught with difficulties, particularly for the polymicrobial samples often presented from patients with CF where the overgrowth of more rapidly-growing bacterial species can prevent MABSC from being detected in any given sample. Improvements in the isolation of MABSC are urgently required in order to offer the possibility of a more rapid and accurate diagnosis.

MABSC is an important pathogen responsible for an extensive array of infections and is now thought to be the most prominent Mycobacterium alongside MAC in patients with CF. The major threat of MABSC is its intrinsic resistance to almost all known antimicrobials, particularly *M. abscessus* subsp*. abscessus* which is said to be one of the most resistant microorganisms known (Nessar *et al.*, 2012).

Many changes in the nomenclature and taxonomic classification of MABSC over the years have made diagnosis and identification problematic. Many current journal articles still refer to *M. chelonae-abscessus* complex (Simmon *et al.*, 2011), and many dispute the existence of *M. abscessus* subsp*. Massiliense* (Leao *et al.*, 2011).

In Chapter Two a novel culture medium (RGM) was developed for the isolation of MABSC in patients with CF. Various enrichment and selective agents were evaluated against isolates of MABSC and frequently encountered Gram-negative species seen in patients with CF and a combination of the most effective agents resulted in the formulation of RGM medium. This was evaluated and compared against currently used culture methods both liquid and solid, using samples from

patients with CF in many centres across the UK, Europe and the United States with very promising results. From the analysis, it can be concluded that RGM medium offers a superior option compared with any of the other selective agars for isolation of rapidly-growing mycobacteria from the sputum of patients with CF. It is also anticipated that RGM medium will be made commercially available in due course.

Chapter three looks at the possibility of incorporating chromogenic or fluorogenic substrates into RGM medium in order to improve it further, and a variety of these were investigated. Unfortunately, none of these substrates provided any improved specificity or sensitivity for the detection of MABSC or were able to differentiate between MABSC and Gram-negative bacteria found commonly in the sputum of patients with CF, therefore were not suitable for inclusion into the medium. Also investigated was the possibility of differentiating between MABSC and less pathogenic *M. chelonae*; however, none of the substrates investigated were able to provide such a distinction.

Chapter four discussed the acquisition of MABSC in patients with CF, and investigated whether this is undeniably an environmentally acquired pathogen, or is it transferred via person-to-person transmission. Although numerous areas and sources were investigated, no isolates of MABSC were isolated from the environment. *M. chelonae* was frequently isolated, particularly in water samples, however this is not now commonly viewed as a pathogenic species and it may well be that due to the many taxonomic changes, any *M. chelonae* that were considered pathogenic previously were in fact MABSC isolates. There are still very few reports substantiating person-to-person transmission of MABSC and the majority of articles will still maintain that MABSC is acquired only from the environment.

Chapter five is centered on the current treatment approaches to MABSC. Progress in this area of research has been inadequate and slow, and it is believed that once

MABSC is acquired it can still not be referred to as a curable disease. It is very much unknown why some patients can be seen to improve with therapy and some cannot, however the correct identification is imperative, *as M. abscessus* subsp. *massiliense* would appear to be more straightforwardly treatable than *M. abscessus* subsp*. abscessus* or more the rarely isolated *M. abscessus* subsp*. bolletii*.

More effective treatments are urgently required as presently there is no optimum therapeutic regimen or ideal time period of treatment in order to produce a cure for MABSC infection.

6.2 Future Research

The field of research associated with MABSC is large, offering many opportunities that could be explored for future research:

- One line of interest would be in the continued investigation of RGM medium in order to establish whether it could be of benefit in the isolation of slow-growing mycobacteria affecting patients with CF such as MAC. This was briefly investigated by Plongla *et al*. (University of North Carolina, School of Medicine in Chapel Hill, USA). They demonstrated that extended incubation of RGM for 28 days afforded the isolation of slow-growing species including MAC, *M. gordonae*, *M. arupense/nonchromogenicum* and *M. nebraskense* (Plongla *et al.*, 2017).
- In addition, a liquid version of RGM could be developed for use in the MGIT system to replace what is currently used, as regular contamination of more rapidly-growing species still remains a problem in this area with many cultures of patient samples having to be abandoned.
- There is a large amount of further antibiotic work that could be undertaken, including Multiple Combination Bactericidal Testing of both currently administered and previously untested antimicrobials as well as novel compounds and antimicrobials in combinations of two, three and even four with the anticipation that previously untested combinations may demonstrate synergy against multi-resistant MABSC isolates, in particular *M. abscessus* subsp*. abscessus*. As the techniques currently employed to differentiate between the subspecies of MABSC are complex, not all laboratories are able to do this therefore a more rapid, cost effective method is required.

- More research is needed into how MABSC is acquired by patients with CF. Whole genome sequencing has provided limited evidence of person-to-person transmission where it was identified to be indirect rather than direct, however there are still currently very few reports of this and the majority of publications maintain MABSC is acquired environmentally.

In conclusion, the amount of exploration that is still required with regards to MABSC is significant, and there is scope for a large amount of research that could still be undertaken.

CHAPTER SEVEN

Appendices

Appendices

Appendix 1: All isolates used in the development and evaluation of RGM medium, chromogenic and fluorogenic substrate testing and antimicrobial susceptibility testing throughout this thesis

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
1000	*M. abscessus* complex (chimeric)	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1001	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			5.7.1
1002	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
1003	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
1004	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1005	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
1007	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1008	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1		5.7.1
1009	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1		5.7.1
1010	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1 3.10.2		
1013	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15	3.10.1 3.10.2		
1014	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1015	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1016	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1019	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1020	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1021	*M. abscessus* subsp. *abscessus*	CF patient, UK				5.7.1
1023	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1		
1024	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			5.7.1
1025	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
1026	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1 3.10.2		5.7.1
1027	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1 3.10.2		

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
1029	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15	3.10.1 3.10.2	4.8.5.3	5.7.1
1030	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15	3.10.1		
1032	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1 3.10.2		5.7.1
1033	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
1034	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1035	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1036	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1037	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.10 2.4.12 2.4.15			5.7.1
1038	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1039	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1040	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1041	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1042	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1043	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.10 2.4.12 2.4.15			5.7.1
1044	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1045	*M. abscessus* subsp. *abscessus*	Paediatric CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.2		5.7.1
1046	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1047	*M. abscessus* subsp. *abscessus*	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1048	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9 2.4.15			5.7.1
1049	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			5.7.1
1050	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1051	*M. abscessus* subsp. *abscessus*	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
1052	*M. abscessus* subsp. *abscessus*	Paediatric CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.2		5.7.1
1053	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.2		5.7.1
1054	*M. abscessus* subsp. *abscessus*	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
1055	*M. abscessus* subsp. *abscessus*	Adult CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15 2.5.6	3.10.2		5.7.1
1056	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.9			5.7.1
1058	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1061	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1062	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1063	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1064	*M. absessus* complex (chimeric)	CF patient, Ireland	2.4.15			5.7.1
1065	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.71
1066	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1067	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1068	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1069	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1070	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1071	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1072	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1073	*M. abscessus* subsp. *abscessus*	CF patient, UK		3.10.1		5.7.1
1074	*M. abscessus* subsp. *abscessus*	CF patient, Ireland	2.4.15			5.7.1
1075	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			5.7.1
1076	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			5.7.1
1078	*M. abscessus* subsp. *abscessus*	CF patient, UK	2.4.15			
2000	*M. chelonae*	CF patient, UK	2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
2001	*M. chelonae*	CF patient, UK	2.4.15			5.7.1
2002	*M. chelonae*	CF patient, UK	2.4.15	3.10.1 3.10.2		5.7.1
2003	*M. chelonae*	CF patient, UK	2.4.15			5.7.1
2004	*M. chelonae*	CF patient, UK	2.4.15	3.10.1	4.8.5.3	
2005	*M. chelonae*	CF patient, UK	2.4.15	3.10.1		5.7.1
2007	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			5.7.1
2008	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			5.7.1
2009	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			
2010	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			5.7.1
2011	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			5.7.1
2012	*M. chelonae*	CF patient, UK	2.4.9 2.4.15	3.10.1		5.7.1
2013	*M. chelonae*	CF patient, UK	2.4.9 2.4.15	3.10.1		
2014	*M. chelonae*	CF patient, UK	2.4.9 2.4.15	3.10.1 3.10.2		
2015	*M. chelonae*	CF patient, UK	2.4.9 2.4.15			
2016	*M. chelonae*	CF patient, Ireland				5.7.1
2017	*M. chelonae*	CF patient, Ireland				5.7.1
2018	*M. chelonae*	CF patient, Ireland				5.7.1
2019	*M. chelonae*	CF patient, Ireland				5.7.1
2020	*M. chelonae*	CF patient, Ireland				5.7.1
2021	*M. chelonae*	CF patient, Ireland				5.7.1
2022	*M. chelonae*	CF patient, Ireland				5.7.1
2023	*M. chelonae*	CF patient, Ireland				5.7.1
2024	*M. chelonae*	CF patient, Ireland				5.7.1
2025	*M. chelonae*	CF patient, Ireland				5.7.1
2026	*M. chelonae*	CF patient, Ireland				5.7.1
2027	*M. chelonae*	CF patient, Ireland				5.7.1
2028	*M. chelonae*	CF patient, Ireland				5.7.1
2029	*M. chelonae*	CF patient, Ireland				5.7.1
2047	*M. chelonae*	CF patient, UK	2.4.15			
2048	*M. chelonae*	CF patient, UK	2.4.15			
2049	*M. chelonae*	CF patient, Ireland	2.4.15			
3000	*M. abscessus* subsp. *massiliense*	CF patient, UK	2.4.15			
3001	*M. abscessus* subsp. *massiliense*	CF patient, UK	2.4.15	3.10.1		
3002	*M. abscessus* subsp. *massiliense*	CF patient, UK	2.4.15	3.10.2		

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
3003	*M. abscessus* subsp. *massiliense*	CF patient, UK		3.10.1		
3004	*M. abscessus* subsp. *massiliense*	CF patient, UK	2.4.15			5.7.1
3005	*M. abscessus* subsp. *massiliense*	CF patient, UK		3.10.1		
3006	*M. abscessus* subsp. *massiliense*	CF patient, UK		3.10.1		
3007	*M. abscessus* subsp. *massiliense*	CF patient, UK		3.10.1 3.10.2		
3009	*M. abscessus* subsp. *massiliense*	CF patient, UK	2.4.9 2.4.15	3.10.1	4.8.5.3	5.7.1
3010	*M. abscessus* subsp. *massiliense*	Adult CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
3011	*M. abscessus* subsp. *massiliense*	Adult CF patient, UK	2.4.2 2.4.15 2.5.6	3.10.2		5.7.1
3012	*M. abscessus* subsp. *massiliense*	Adult CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.1 3.10.2		5.7.1
3013	*M. abscessus* subsp. *massiliense*	Adult CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.1 3.10.2		5.7.1
3014	*M. abscessus* subsp. *massiliense*	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.2		5.7.1
3015	*M. abscessus* subsp. *massiliense*	Paediatric CF patient, UK	2.4.2 2.4.15	3.10.1 3.10.2		
3016	*M. abscessus* subsp. *bolletii*	Paediatric CF patient, UK	2.4.2 2.4.10 2.4.12 2.4.15	3.10.1 3.10.2		5.7.1
3017	*M. abscessus* subsp. *bolletii*	Environmental isolate from sink. CF unit, RVI, Newcastle upon Tyne, UK	2.4.2 2.4.10 2.4.12 2.4.15 2.5.6	3.10.1 3.10.2		5.7.1
3019	*M. abscessus* subsp. *bolletii*	CF patient, Ireland	2.4.15			5.7.1
3020	*M. abscessus* subsp. *massiliense*	CF patient, Ireland	2.4.15			
4008	*M. llatzerense*	CF patient, UK	2.4.15			5.7.1
4009	*M. llatzerense*	CF patient, UK	2.4.15			5.7.1
4010	*M. mucogenicum*	CF patient, UK	2.4.15		4.8.5.3	5.7.1
4011	*M. salmoniphilum*	CF patient, UK	2.4.15			
4013	*M. salmoniphilum*	CF patient, UK	2.4.9 2.4.15			5.7.1
4014	*M. salmoniphilum*	CF patient, UK	2.4.9 2.4.15			5.7.1

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
4015	*M. immunogenum*	CF patient, UK	2.4.9 2.4.15		4.8.5.3	5.7.1
4016	*M. intracellulare*	CF patient, UK	2.4.9			5.7.1
4017	*M. fortuitum*	CF patient, UK. PHE, UK	2.4.15			5.7.1
4018	*M. fortuitum*	CF patient, UK. PHE, UK	2.4.15			5.7.1
4019	*M. fortuitum*	CF patient, UK. PHE, UK	2.4.15			5.7.1
4048	*M. septicum*	CF patient, UK				5.7.1
462213	*Morganella morganii*	CF patient, UK	2.4.15			
7000	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15			
7001	*Burkholderia cenocepacia*	CF patient, UK	2.4.15	3.10.1		
7002	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15	3.10.1		
7003	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15			
7004	*Pseudomonas aeruginosa*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7005	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15			
7006	*Burkholderia multivorans*	CF patient, UK	2.4.15	3.10.1		
7007	*Inquilinus limosus*	CF patient, UK	2.4.14 2.4.15	3.10.1 3.10.2		
7008	*Delftia acidovorans*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7009	*Burkholderia cenocepacia*	CF patient, UK		3.10.2		
7010	*Achromobacter xylosoxidans*	CF patient, UK		3.10.2		
7011	*Inquilinus limosus*	CF patient, UK	2.4.15	3.10.2		
7012	*Burkholderia cenocepacia*	CF patient, UK	2.4.15	3.10.2		
7013	*Burkholderia multivorans*	CF patient, UK	2.4.15			
7014	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15			
7015	*Achromobacter xylosoxidans*	CF patient, UK		3.10.2		
7016	*Burkholderia multivorans*	CF patient, UK	2.4.15			
7017	*Burkholderia multivorans*	CF patient, UK	2.4.15	3.10.2		
7018	*Pandoraea norimbergenis*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7019	*Serratia marcescens*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7020	*Serratia marcescens*	CF patient, UK	2.4.14 2.4.15	3.10.2		
7021	*Achromobacter xylosoxidans*	CF patient, UK	2.4.15			
7022	*Burkholderia multivorans*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7023	*Achromobacter xylosoxidans*	CF patient, UK		3.10.1		
7024	*Burkholderia multivorans*	CF patient, UK		3.10.2		
7026	*Burkholderia stabilis*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7027	*Achromobacter xylosoxidans*	CF patient, UK		3.10.2		
7029	*Burkholderia multivorans*	CF patient, UK	2.4.15			
7031	*Ochrobactrum* sp.	CF patient, UK	2.4.15	3.10.2		

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
7032	*Burkholderia vietnamiensis*	CF patient, UK	2.4.14 2.4.15	3.10.1 3.10.2		
7034	*Burkholderia cenocepacia*	CF patient, UK	2.4.15	3.10.1		
7036	*Burkholderia cenocepacia*	CF patient, UK	2.4.15	3.10.1 3.10.2		
7037	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15 2.5.6	3.10.1 3.10.2		
7038	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			
7039	*Pseudomonas aeruginosa*	Non-CF parent of CF patient, UK	2.4.7 2.4.15 2.5.6			
7040	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			
7041	*Pseudomonas aeruginosa*	CF patient, Denmark	2.4.7 2.4.15			
7042	*Pseudomonas aeruginosa*	Paediatric CF patient, Melbourne, Australia	2.4.7 2.4.15			
7043	*Pseudomonas aeruginosa*	Adult CF patient, Brisbane, Australia	2.4.7 2.4.15			
7044	*Pseudomonas aeruginosa*	Adult CF patient, Hobart, Australia	2.4.7 2.4.14 2.4.15			
7045	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7046	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7047	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7048	*Pseudomonas aeruginosa*	Paediatric CF patient, Seattle, USA	2.4.7 2.4.15			
7049	*Pseudomonas aeruginosa*	Paediatric CF patient, Seattle, USA	2.4.7 2.4.15			
7050	*Pseudomonas aeruginosa*	Paediatric CF patient, Seattle, USA	2.4.7 2.4.15			
7051	*Pseudomonas aeruginosa*	Paediatric CF patient, Seattle, USA	2.4.7 2.4.15			
7052	*Pseudomonas aeruginosa*	Paediatric CF patient, Seattle, USA	2.4.7 2.4.15			
7053	*Pseudomonas aeruginosa*	ATCC 15692 Infected wound	2.4.7 2.4.15			
7054	*Pseudomonas aeruginosa*	Human burn isolate, UK	2.4.7 2.4.15			
7055	*Pseudomonas aeruginosa*	Clinical non-CF patient, UK	2.4.7 2.4.15			
7056	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
7057	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7058	*Pseudomonas aeruginosa*	CF patient, Lisbon, Portugal	2.4.7 2.4.15			
7059	*Pseudomonas aeruginosa*	CF patient, Lisbon, Portugal	2.4.7 2.4.15			
7060	*Pseudomonas aeruginosa*	Non-CF bronchiectasis patient, UK	2.4.7 2.4.15			
7061	*Pseudomonas aeruginosa*	Non-CF urine sample, Poland	2.4.7 2.4.15			
7062	*Pseudomonas aeruginosa*	Keratitis eye isolate, UK	2.4.7 2.4.15			
7063	*Pseudomonas aeruginosa*	Chronic CF patient, Boston, USA	2.4.7 2.4.15			
7064	*Pseudomonas aeruginosa*	CF patient, Denmark	2.4.7 2.4.15			
7065	*Pseudomonas aeruginosa*	Non-CF clinical patient, Leuven, Belgium	2.4.7 2.4.15			
7066	*Pseudomonas aeruginosa*	Non-CF burn, Michigan, USA	2.4.7 2.4.15			
7067	*Pseudomonas aeruginosa*	Lake water, Lake Tamaco, Japan	2.4.7 2.4.15			
7068	*Pseudomonas aeruginosa*	LMG 14084: Water, Bucharest, Romania	2.4.7 2.4.15			
7069	*Pseudomonas aeruginosa*	Hospital environment, Prague, Czech Republic	2.4.7 2.4.15			
7070	*Pseudomonas aeruginosa*	CF patient, Hobart, Australia	2.4.7 2.4.15			
7071	*Pseudomonas aeruginosa*	Tobacco plant, Philippines	2.4.7 2.4.15			
7072	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7073	*Pseudomonas aeruginosa*	ICU acute infection, Spain	2.4.7 2.4.15			
7074	*Pseudomonas aeruginosa*	COPD patient, USA	2.4.7 2.4.15			
7075	*Pseudomonas aeruginosa*	ICU acute infection, France	2.4.7 2.4.15			
7076	*Pseudomonas aeruginosa*	Keratitis, Manchester, UK	2.4.7 2.4.15			
7077	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7078	*Pseudomonas aeruginosa*	Community acquired pneumonia, UK	2.4.7 2.4.15			
7079	*Pseudomonas aeruginosa*	CF patient, Germany	2.4.7 2.4.15			
7080	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
7081	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
7082	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
7083	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
7084	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
7085	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
7086	*Ralstonia mannitolilytica*	CF patient, UK	2.4.15			
76024	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76150	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76525	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76773	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76831	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76832	*M. avium*	CF patient, UK. PHE, UK	2.4.18			
76147	*M. intracellulare*	Non-CF patient, UK. PHE, UK	2.4.18			
76223	*M. intracellulare*	Non-CF patient, UK. PHE, UK	2.4.18			
76017	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
76089	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
76463	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
77041	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
77042	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
77043	*M. tuberculosis* complex	Non-CF patient, UK. PHE, UK	2.4.18			
8001	*Achromobacter xylosoxidans*	CF patient, UK	2.4.7 2.4.14 2.4.15			
8002	*Achromobacter* sp.	CF patient, UK	2.4.9 2.4.15			
8003	*Acinetobacter* sp.	CF patient, UK	2.4.7 2.4.15	3.10.2		
8004	*Burkholderia multivorans*	CF patient, UK	2.4.7 2.4.14 2.4.15 2.5.6			
8005	*Burkholderia stabilis*	CF patient, UK	2.4.9 2.4.15			
8006	*Burkholderia cenocepacia* IIIA	CF patient, UK	2.4.7 2.4.15			
8007	*Burkholderia cepacia* (G1)	CF patient, UK	2.4.7 2.4.14 2.4.15	3.10.2		
8008	*Burkholderia contaminans*	CF patient, UK	2.4.7 2.4.14 2.4.15	3.10.2		
8009	*Pseudomonas aeruginosa*	NCTC 10662	2.4.7 2.4.14 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
8010	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.14 2.4.15			
8011	*Pseudomonas aeruginosa*	CF patient, UK	2.4.9 2.4.15	3.10.2		
8012	*Pseudomonas aeruginosa*	CF patient, UK	2.4.9 2.4.15	3.10.2		
8013	*Pseudomonas aeruginosa*	CF patient, UK	2.4.9 2.4.15	3.10.2		
8014	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			
8015	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			
8016	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15 2.5.6			
8017	*Pseudomonas aeruginosa*	CF patient, UK	2.4.9 2.4.15			
8018	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15			
8019	*Pseudomonas aeruginosa*	CF patient, UK	2.4.7 2.4.15 2.5.6			
8020	*Pandoraea apista*	CF patient, UK	2.4.7 2.4.14 2.4.15	3.10.2		
8021	*Pandoraea pnomenusa*	CF patient, UK	2.4.7 2.4.14 2.4.15	3.10.2		
8022	*Stenotrophomonas maltophilia*	CF patient, UK	2.4.7 2.4.15	3.10.2		
8023	*Stenotrophomonas maltophilia*	CF patient, UK	2.4.7 2.4.15	3.10.2		
8024	*Stenotrophomonas maltophilia*	CF patient, UK	2.4.15			
8025	*Elizabethkingia miricola*	CF patient, UK	2.4.9 2.4.15	3.10.2		
8029	*Stenotrophomonas maltophilia*	NCTC 10257	2.4.9 2.4.14 2.4.15			
8030	*Acinetobacter baumannii*	ATCC 19606	2.4.9 2.4.14 2.4.15			
8031	*Providencia rettgeri*	NCTC 7475	2.4.7 2.4.15			
8032	*Citrobacter freundii*	NCTC 9750	2.4.7 2.4.15			
8033	*Streptococcus pyogenes*	NCTC 8309	2.4.7 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
8034	*Streptococcus salivarius*	NCTC 8618	2.4.7 2.4.15			
8035	*Streptococcus gordonii*	NCTC 7865	2.4.7 2.4.15			
8036	*Escherichia coli*	NCTC 10418	2.4.7 2.4.14 2.4.15	3.10.1		
8037	*Raoultella planticola*	NCTC 9528	2.4.7 2.4.14 2.4.15			
8038	*Enterobacter cloacae*	NCTC 11936	2.4.7 2.4.14 2.4.15 2.5.6			
8039	*Serratia marcescens*	NCTC 10211	2.4.7 2.4.15			
8040	*Streptococcus pneumoniae*	NCTC 12977	2.4.7 2.4.15			
8064	*Burkholderia ambifaria*	LMG 19467	2.4.15			
8065	*Burkholderia ambifaria*	LMG 19182	2.4.15			
8066	*Burkholderia anthina*	LMG 20980	2.4.15			
8067	*Burkholderia anthina*	LMG 20983	2.4.15			
8068	*Burkholderia cenocepacia*	LMG 18832	2.4.15			
8069	*Burkholderia cenocepacia*	LMG 18830	2.4.15			
8070	*Burkholderia cenocepacia*	LMG 18828	2.4.15			
8071	*Burkholderia cenocepacia*	LMG 18829	2.4.15			
8072	*Burkholderia cenocepacia*	LMG 18863	2.4.15			
8073	*Burkholderia cenocepacia*	LMG 16656	2.4.15			
8074	*Burkholderia cepacia*	LMG 18821	2.4.15			
8075	*Burkholderia cepacia*	LMG 1222	2.4.15			
8076	*Burkholderia dolosa*	LMG 21820	2.4.15			
8077	*Burkholderia dolosa*	LMG 18943	2.4.15			
8078	*Burkholderia multivorans*	LMG 18824	2.4.15			
8079	*Burkholderia multivorans*	LMG 18822	2.4.15			
8080	*Burkholderia multivorans*	LMG 16660	2.4.15			
8081	*Burkholderia multivorans*	LMG 17588	2.4.15			
8082	*Burkholderia multivorans*	LMG 13010	2.4.15			
8083	*Burkholderia pyrrocinia*	LMG 21824	2.4.15			
8084	*Burkholderia pyrrocinia*	LMG 14191	2.4.15			
8085	*Burkholderia stabilis*	LMG 14294	2.4.15			
8086	*Burkholderia stabilis*	LMG 18870	2.4.15			
8087	*Burkholderia vietnamiensis*	LMG 18835	2.4.15			
8088	*Burkholderia vietnamiensis*	LMG 10929	2.4.15			
8089	*Burkholderia vietnamiensis*	LMG 16232	2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
9001	*Staphylococcus aureus*	ATCC 6571	2.4.7 2.4.14 2.4.15	3.10.1		
9002	*Staphylococcus aureus* MRSA	NCTC 11939	2.4.7 2.4.15			
9003	*Enterococcus faecalis*	NCTC 775	2.4.7 2.4.14 2.4.15			
9004	*Staphylococcus epidermidis*	NCTC 11047	2.4.7 2.4.15			
9005	*Enterococcus faecium*	NCTC 7171	2.4.7 2.4.15			
9006	*Bacillus subtilis*	NCTC 9372	2.4.7 2.4.15			
9007	*Enterobacter aerogenes*	CF patient, UK	2.4.7 2.4.15			
9008	*Neisseria flavescens*	CF patient, UK	2.4.7 2.4.15			
9009	*Moraxella catarrhalis*	CF patient, UK	2.4.7 2.4.15			
9010	*Haemophilus influenzae*	NCTC 11931	2.4.7 2.4.15			
9011	*Candida albicans*	ATCC 90028	2.4.7 2.4.14 2.4.15 2.5.6			
9012	*Candida glabrata*	NCPF 3943	2.4.7 2.4.15 2.5.6			
9013	*Aspergillus fumigatus*	CF patient, UK	2.4.7 2.4.15 2.5.6			
9014	*Aspergillus fumigatus*	CF patient, UK	2.4.7 2.4.15			
9015	*Aspergillus terreus*	CF patient, UK	2.4.7 2.4.15 2.5.6			
9016	*Scedosporium apiospermum*	CF patient, UK	2.4.7 2.4.15 2.5.6			
9017	*Scedosporium prolificans*	CF patient, UK	2.4.7 2.4.15 2.5.6			
9018	*Geosmithia argillacea*	CF patient, UK	2.4.7 2.4.15 2.5.6			
9059	*Candida albicans*	CF patient, UK	2.4.15			
9110	*Staphylococcus aureus* MRSA	14 EMRSA15 1758/98 MRSA European Collection	2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
9111	*Staphylococcus aureus* MRSA	15 EMRSA15 14956 MRSA European Collection	2.4.15			
9112	*Staphylococcus aureus* MRSA	3 FIN 7481 (E14) MRSA European Collection	2.4.15			
9113	*Staphylococcus aureus* MRSA	4 FIN 54511 (E6) MRSA European Collection	2.4.15			
9114	*Staphylococcus aureus* MRSA	5FIN 54518 (E7) MRSA European Collection	2.4.15			
9115	*Staphylococcus aureus* MRSA	6FRA 462 MRSA European Collection	2.4.15			
9116	*Staphylococcus aureus* MRSA	7 FRA 920 MRSA European Collection	2.4.15			
9117	*Staphylococcus aureus* MRSA	8 FRA 95034 (4b) MRSA European Collection	2.4.15			
9118	*Staphylococcus aureus* MRSA	9 GER 131/98 MRSA European Collection	2.4.15			
9119	*Staphylococcus aureus* MRSA	10 GER 1966/97 MRSA European Collection	2.4.15			
9120	*Staphylococcus aureus* MRSA	11 GER 2594 1/97 MRSA European Collection	2.4.15			
9121	*Staphylococcus aureus* MRSA	12 GER 2594 2/97 MRSA European Collection	2.4.15			
9122	*Staphylococcus aureus* MRSA	13 EMRSA15 1729/98 MRSA European Collection	2.4.15			
9123	*Staphylococcus aureus* MRSA	14 EMRSA15 1758/98 MRSA European Collection	2.4.15			
9124	*Staphylococcus aureus* MRSA	15 EMRSA15 14956 MRSA European Collection	2.4.15			
9125	*Staphylococcus aureus* MRSA	16 EMRSA15 12484/98 MRSA European Collection	2.4.15			
9126	*Staphylococcus aureus* MRSA	17 EMRSA15 14185/98 MRSA European Collection	2.4.15			
9127	*Staphylococcus aureus* MRSA	18 EMRSA15 16822/98 MRSA European Collection	2.4.15			
9128	*Staphylococcus aureus* MRSA	19 EMRSA 19972/98 MRSA European Collection	2.4.15			
9129	*Staphylococcus aureus* MRSA	20 EMRSA 20460/98 MRSA European Collection	2.4.15			
9130	*Staphylococcus aureus* MRSA	21 EMRSA15 21268/98 MRSA European Collection	2.4.15			
9131	*Staphylococcus aureus* MRSA	22 EMRSA15 21698/98 MRSA European Collection	2.4.15			
9132	*Staphylococcus aureus* MRSA	23 EMRSA15 2501/98 MRSA European Collection	2.4.15			
9133	*Staphylococcus aureus* MRSA	24 EMRSA15 6323/98 MRSA European Collection	2.4.15			
9134	*Staphylococcus aureus* MRSA	25 EMRSA16 00036/98 MRSA European Collection	2.4.15			
9750	*Citrobacter freundii*	NCTC 9750	2.4.15			
PHE1	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.5.6 2.4.15			
PHE10	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
PHE11	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE12	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE13	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE14	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE15	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE16	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE17	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE18	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE19	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE2	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE20	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE21	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE25	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE27	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE29	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE3	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE30	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE31	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE33	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE34	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE35	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE36	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE37	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE38	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
PHE39	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE4	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE40	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE41	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE42	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE43	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE44	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE45	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE46	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE47	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE48	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE5	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE50	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE53	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE55	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE59	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE61	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE62	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE63	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE66	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE74	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE78	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE8	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.15			
PHE80	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			

Isolate Reference	Species	Isolate Origin	Chapter 2	Chapter 3	Chapter 4	Chapter 5
PHE81	*M. chelonae*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE9	*M. chelonae*	CF patient, UK. PHE, UK	2.4.15			
PHE90	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			
PHE99	*M. abscessus* subsp. *abscessus*	CF patient, UK. PHE, UK	2.4.9 2.4.15			

Pseudomonas aeruginosa **International Reference Panel**: Gram-negative isolate reference numbers **7039 – 7079** *Pseudomonas aeruginosa* International Reference Panel (De Soyza *et al.*, 2013)

NCTC: National Collection of Type Cultures, Colindale, UK.

ATCC: American Type Culture Collection, Manassas, United Sates.

CF Patient, UK: Isolates from Microbiology Department, Freeman Hospital, Newcastle upon Tyne, UK from patients with CF

CF Patient, Ireland: Isolates from St Vincent's University Hospital, Dublin, Ireland from patients with CF

NCPF: National Collection of Pathogenic Fungi, Colindale, UK.

PHE Isolates: Public Health England Laboratory, Freeman Hospital, Newcastle upon Tyne UK from patients with CF

LMG: LM-UGent hosts the BCCM/LMG Bacteria Collection, which maintains over 20000 strains, representing some 700 genera and 4.500 species. Available at http://bccm.belspo.be/about-us/bccm-lmg

MRSA European Collection: A collection of 25 strains representing the most frequently encountered MRSA types isolated in Europe were provided by Public Health England, Colindale, London, United Kingdom, as freeze-dried cultures. The collection included strains isolated in Belgium, Finland, France, Germany, and the United Kingdom. Available at http://jcm.asm.org/content/42/10/4519.long

Appendix 2: Ingredients and how to prepare 500 ml RGM medium

QUANTITY	PRODUCT	SUPPLIER	PRODUCT CODE
5 g	Bacteriological Agar nº. 1 (LP0011)	Oxoid	LP0011B
2 g	Yeast Extract	bioMérieux	03904110
50 ml	10 x Strength Middlebrook Broth Base	(see below)	
375 ml	Deionised H_2O		

Autoclave (while in autoclave, prepare the OADC supplement and Antimicrobials)

Make up 50 ml OADC Supplement using 50 ml previously sterilised and cooled deionised H_2O (prepared as required)

Add in:

QUANTITY	PRODUCT	SUPPLIER	PRODUCT CODE
2.5 g	Bovine Serum Albumin	Sigma-Aldrich	A2153
1 g	Dextrose	Sigma-Aldrich	G7528
0.002 g	Catalase	Sigma-Aldrich	C9322
31.5 µl	Oleic Acid	Sigma-Aldrich	O1008

Filter sterilise

Dissolve the following each in 5 ml deionised H_2O except the Amp B which is first dissolved in 200 µl NM2P and then added to 4.8 ml deionised H_2O

QUANTITY	PRODUCT	SUPPLIER	PRODUCT CODE
0.036 g	Colistin methanesulfonate	bioMérieux	N/A 45% pure
0.2 g	Fosfomycin	Sigma-Aldrich	P5396
0.0125 g	Glucose-6-Phosphate	Sigma-Aldrich	G7879
0.0025 g	Amphotericin B	Duchefa Biochemie BV	A0103.0005
0.016 g	C390	Biosynth	16100/3

Once agar is cooled to 50ºC, add 50 ml of OADC supplement and the 5 x 5 ml of the above antimicrobials and G-6-P (Total volume 500 ml). Pour plates.

1 Litre Middlebook Broth Base (10 x strength)

Dissolve the following in 960 ml deionised H_2O

QUANTITY	PRODUCT	SUPPLIER	PRODUCT CODE
5 g	Ammonium Sulphate	BDH	100333B
5 g	L-Glutamic Acid	Sigma-Aldrich	G2128
25 g	Disodium Phosphate	Sigma-Aldrich	S7907
10 g	Monopotassium Phosphate	Sigma-Aldrich	P5655
1 g	Sodium Citrate	BDH	102425M
0.5 g	Magnesium Sulphate	Sigma-Aldrich	M1880
0.005 g	Calcium Chloride	BDH	22311.297
0.005 g	Biotin*	Sigma-Aldrich	B4501
0.01 g	Copper Sulphate**	Sigma-Aldrich	C1297
0.01 g	Zinc Sulphate	Sigma-Aldrich	Z4750
0.01 g	Pyridoxine	Sigma-Aldrich	P9755
0.4 g	Ferric Ammonium Citrate	Sigma-Aldrich	F5879
40 ml	Glycerol	Sigma-Aldrich	G7757

Check pH is 6.6 +/-0.2 and adjust accordingly
Split into 100 ml volumes and autoclave. Store in fridge until required.

* 0.01g (10mg) Biotin dissolved in 1ml water and 500µl used (0.005g)
** 0.01g (10mg) Copper Sulphate dissolved in 1ml water and 500µl used (0.005g)

Appendix 3: Antimicrobial supplement for use in MB/BacT bottles

Bottle A: 10 mL Middlebrook 7H9 broth (bioMérieux ref: 259797) supplemented with selective supplement (bioMérieux reference: 259760 containing: Reconstitution Fluid: oleic acid, glycerol, bovine serum albumin, and amaranth in water. MB/BacT® Antibiotic Supplement Lyophilized supplement with: amphotericin B, azlocillin, nalidixic acid, polymyxin B, trimethoprim, and vancomycin).

Bottle B: 10 mL Middlebrook 7H9 broth (bioMérieux ref: 259797) supplemented with Reconstitution Fluid: oleic acid, glycerol, bovine serum albumin, and amaranth in water containing in-house selective formulation comprising fosfomycin, C390, colomycin, glucose-6-phosphate and amphotericin.

Appendix 4: Ethical approval for clinical samples used in the evaluation of RGM

This PhD form is part of an ongoing research project into the development of improved diagnostic methods for the detection of pathogenic bacteria. The project has formal approval from the Research and Development Department of the Newcastle Upon Tyne Hospitals NHS Trust (Project registration number: 02999). The project does not require specific approval from the research ethics committee and this has previously been endorsed by the head of the Research and Development Office (Newcastle Upon Tyne Hospitals NHS Trust) and the Chair of the local research ethics committee, subject to a number of conditions that must be strictly adhered to. These conditions are:

- Clinical samples used in this study will only be those that are sent to the laboratory for routine investigation i.e. no additional samples will be requested specifically for the study.

- All samples will be anonymised by Biomedical Scientists who have legitimate access to patient identifiable information and who are processing the samples as part of their routine workload before they are passed to the student.

- Any additional test(s) performed on the sample would always be within the scope of the test that has been requested by the clinician. Test that require specific informed consent e.g. HIV testing would not be performed.

- The fact that extra tests may be performed on patient samples would in no way impact on the routine processing of the samples using the standard operating procedures of the laboratory or the reporting of results.

- Any data obtained from additional tests would not be reported and would not be allowed to impact on patient management in any way. Data would be used solely for the evaluation of methods within the project.

- On occasion, evaluations may be sponsored by commercial companies interested in the performance of new diagnostic products. On such occasions, no patient identifiable data will be released to such companies.

These agreed criteria allow the routine assessment of new laboratory methods (such as a new culture medium or a new piece of instrumentation) without the requirement of applying for formal ethical approval. Such evaluations are frequent and are part of normal laboratory development. The laboratory is positively discouraged from sending trivial requests to the ethics committee for projects for which we already have clear guidance as they have a high workload of genuine requests to consider.

In the later stages of the project, it is possible that:

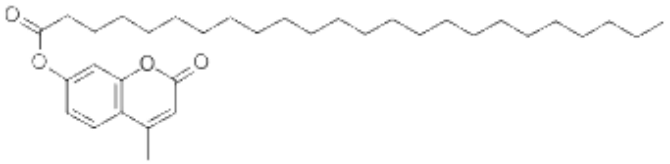
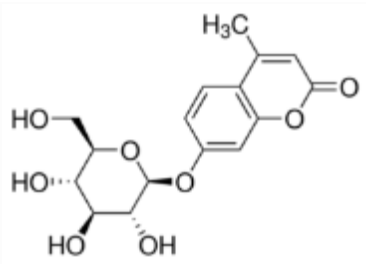
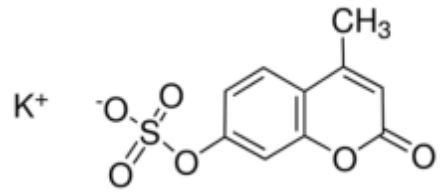
- Additional samples may not be requested specifically for the purposes of this project.

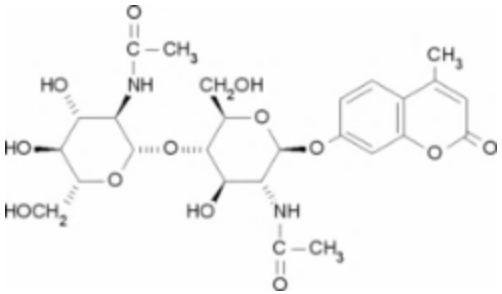
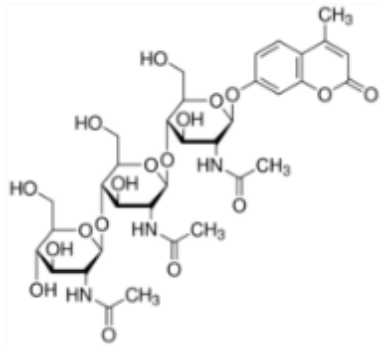
- Clinical information may be required from patient's notes to assess the clinical significance of pathogens that are isolated.

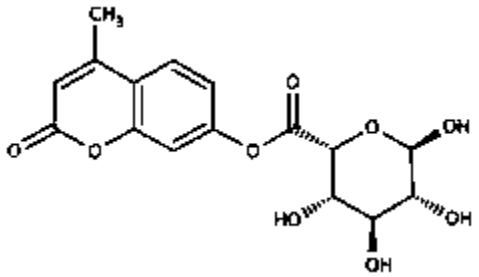
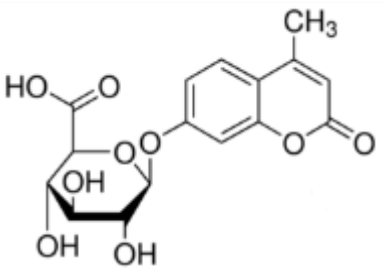
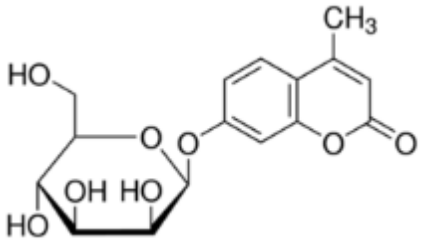
No such work will be carried out unless formal approval is provided by the local research ethics committee following the standard application process. It is highly likely that informed written patient consent will be required in such instances.

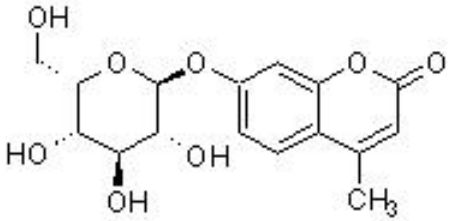
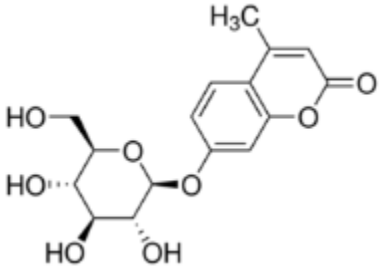
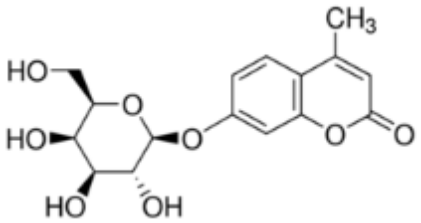
Prof. John Perry
Clinical Scientist
Head of Clinical Microbiology Research
Newcastle Upon Tyne Hospitals NHS Trust

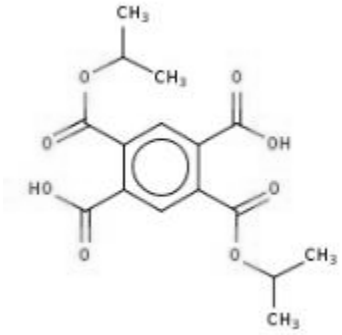
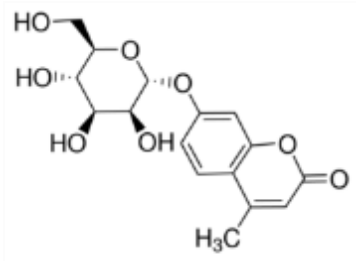
Appendix 5: Structures of substrates used for fluorogenic testing in Chapter 3

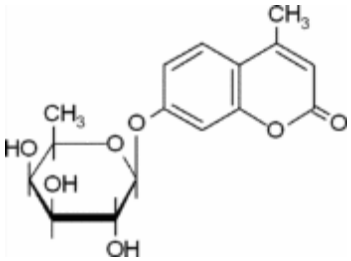
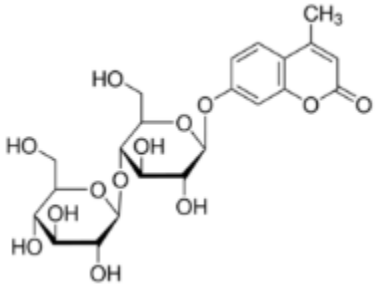
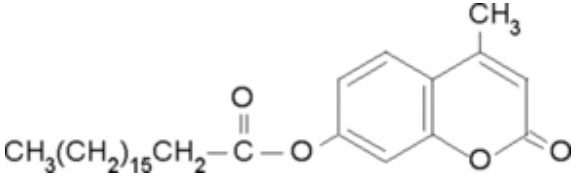
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl lignocerate		526.79	$C_{34}H_{54}O_4$
4-Methylumbelliferyl β-D-glucopyranoside		338.31	$C_{16}H_{18}O_8$
4-Methylumbelliferyl sulfate		294.32	$C_{10}H_7KO_6S$

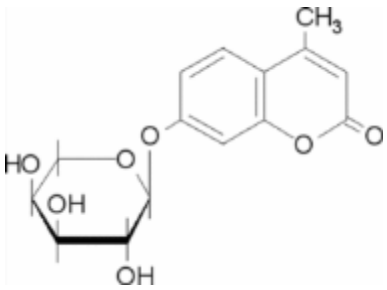
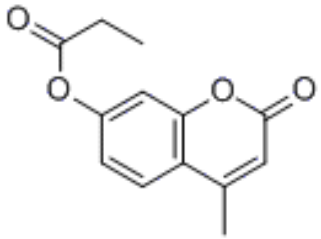
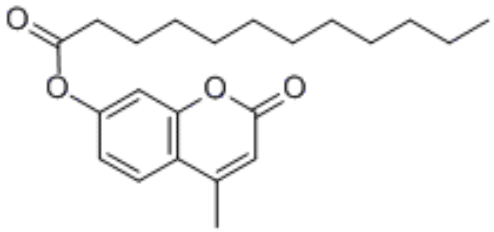
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl β-D-N,N'-diacetylchitobioside hydrate		582.55	$C_{26}H_{34}N_2O_{13}\cdot xH_2O$
4-Methylumbelliferyl β-D-N,N'-triacetylchitotriose		785.75	$C_{34}H_{47}N_3O_{15}$

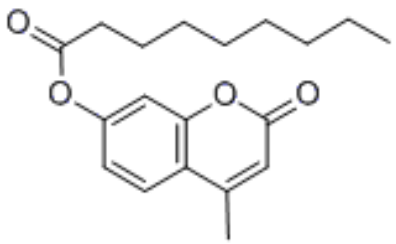
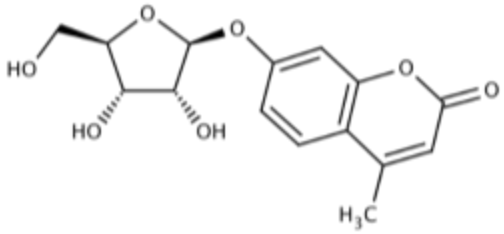
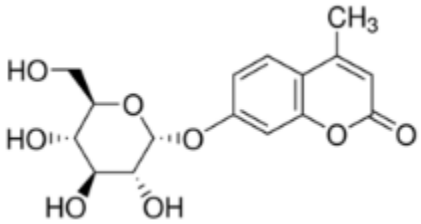
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl α-L-iduronide		352.30	$C_{16}H_{16}O_9$
4-Methylumbelliferyl α-D-glucuronide		352.30	$C_{16}H_{16}O_9$
: 4-Methylumbelliferyl β-D-mannopyranoside		338.31	$C_{16}H_{18}O_8$

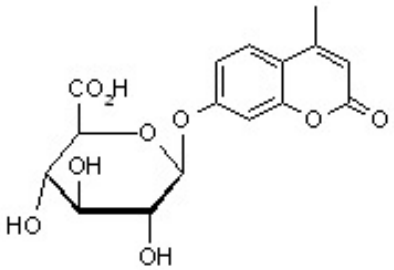
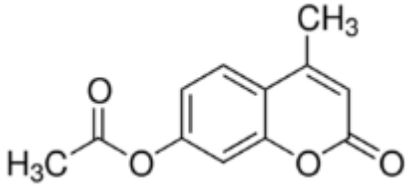
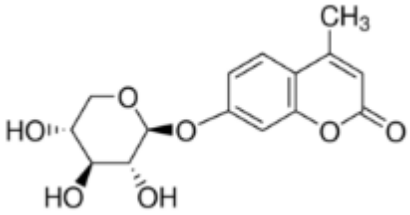
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl α-L-idopyranoside		338.31	$C_{16}H_{18}O_8$
4-Methylumbelliferyl β-D-glucoside		338.31	$C_{16}H_{18}O_8$
4-Methylumbelliferyl β-D-galactoside		338.31	$C_{16}H_{18}O_8$

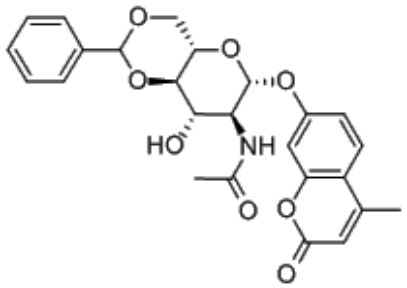
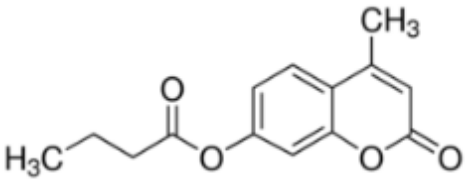
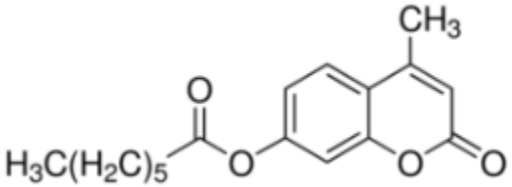
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl α-D-galactoside		338.31	$C_{16}H_{18}O_8$
4-Methylumbelliferyl α-D-mannopyranoside		338.30	$C_{16}H_{18}O_8$

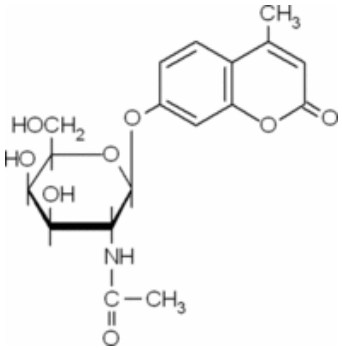
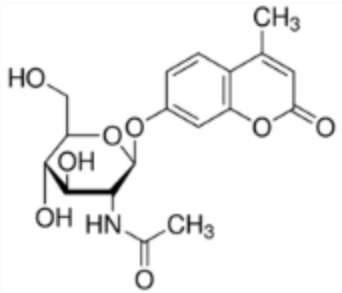
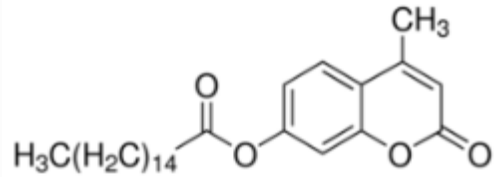
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl β-D-fucoside		322.31	$C_{16}H_{18}O_7$
4-Methylumbelliferyl β-D-cellobioside		500.45	$C_{22}H_{28}O_{13}$
4-Methylumbelliferyl stearate		442.63	$C_{28}H_{42}O_4$

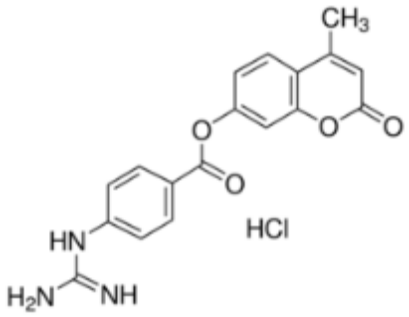
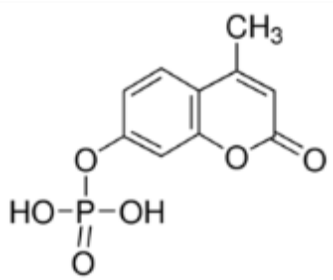
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl α-L-arabinopyranoside		308.28	$C_{15}H_{16}O_7$
4-Methylumbelliferyl propionate		232.23	$C_{13}H_{12}O_4$
4-Methylumbelliferyl laurate		358.50	$C_{22}H_{30}O_4$

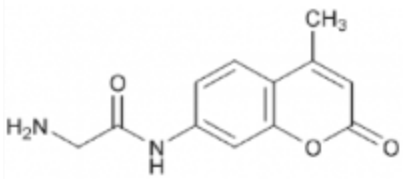
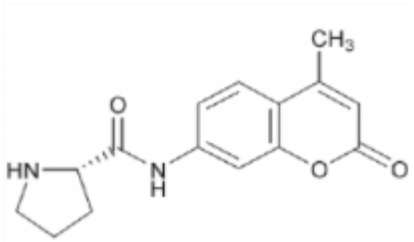
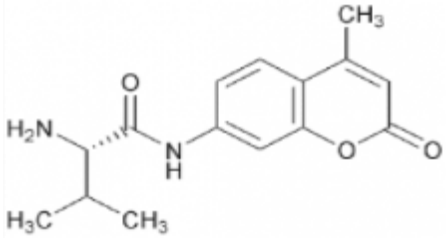
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl nonanoate		316.40	$C_{19}H_{24}O_4$
4-Methylumbelliferyl riboside/4- Methylumbelliferyl β-D-ribofuranoside		308.30	$C_{15}H_{16}O_7$
4-Methylumbelliferyl α-D-glucopyranoside		338.30	$C_{16}H_{18}O_8$

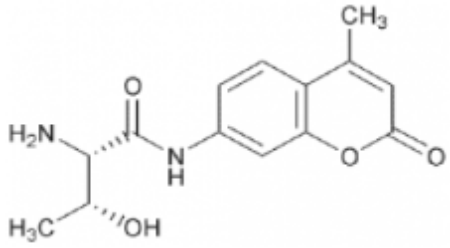
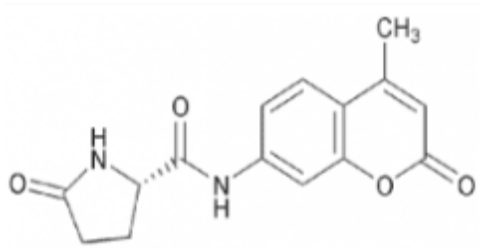
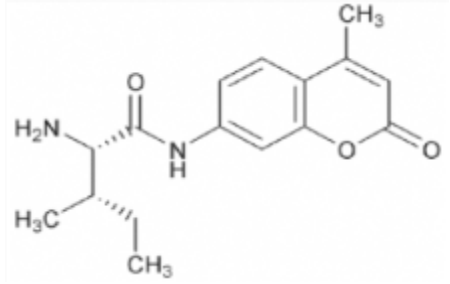
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl β-D-glucuronide		352.30	$C_{16}H_{16}O_9$
4-Methylumbelliferyl acetate		218.21	$C_{12}H_{10}O_4$
4-Methylumbelliferyl 7-β-D-xyloside		308.28	$C_{15}H_{16}O_7$

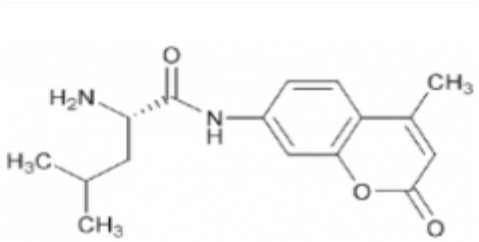
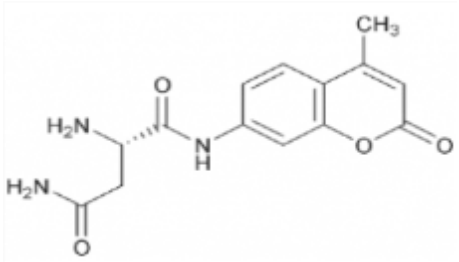
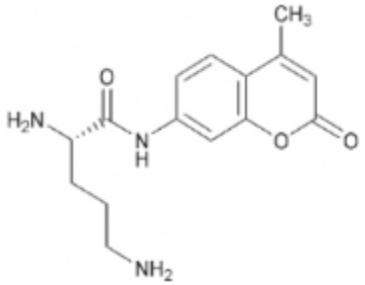
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl acetamido-4,6-0-benzylidene-2-deoxy-β-D-glucopyranoside		467.47	$C_{25}H_{25}NO_8$
4-Methylumbelliferyl butyrate		246.26	$C_{14}H_{14}O_4$
4-Methylumbelliferyl heptanoate		288.34	$C_{17}H_{20}O_4$

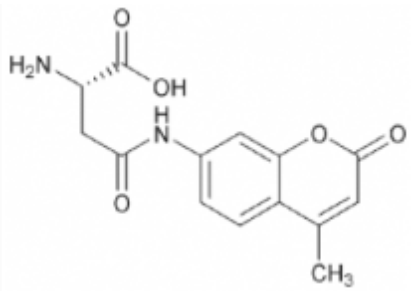
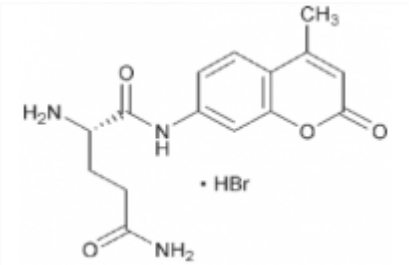
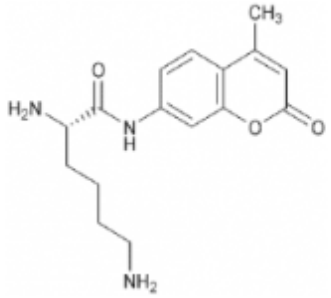
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl N-acetyl-β-D-galactosaminide		379.36	$C_{18}H_{21}NO_8$
4-Methylumbelliferyl N-acetyl-β-D-glucosaminide		379.36	$C_{18}H_{21}NO_8$
4-Methylumbelliferyl palmitate		414.58	$C_{26}H_{38}N_3O_4$

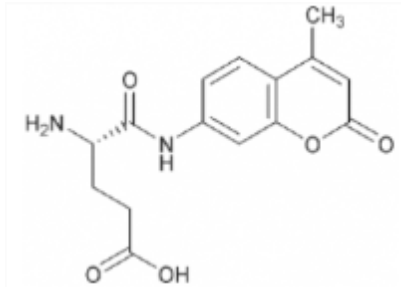
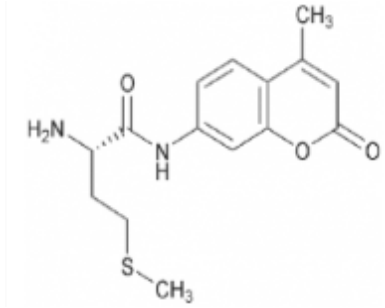
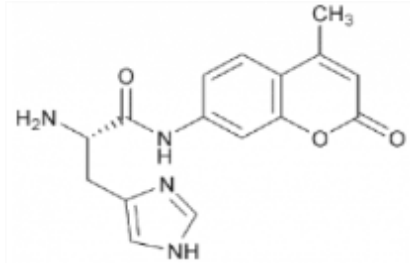
Name	Chemical Structure	Molecular Weight	Molecular Formula
4-Methylumbelliferyl p-guanidinobenzoate hydrochlorate hydrate		373.79	$C_{18}H_{15}N_3O_4 \cdot HCl \cdot xH_2O$
4-Methylumbelliferyl phosphate		256.15	$C_{10}H_9O_6P$

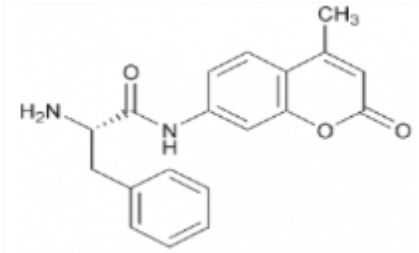
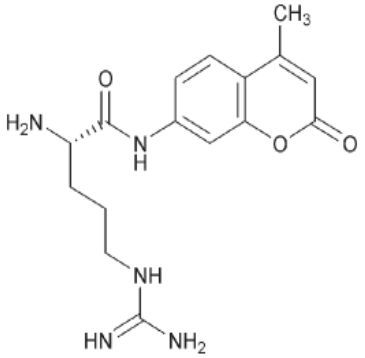
Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Gly-AMC.Hbr		232.24	$C_{12}H_{12}N_2O_3$
H-Pro-AMC hydrobromide salt (Prolyl)		272.30	$C_{15}H_{16}N_2O_3$
H-Val-AMC		274.32	$C_{15}H_{18}N_2O_3$

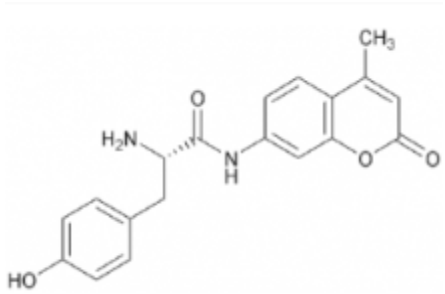
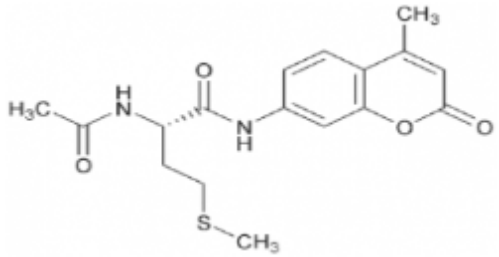
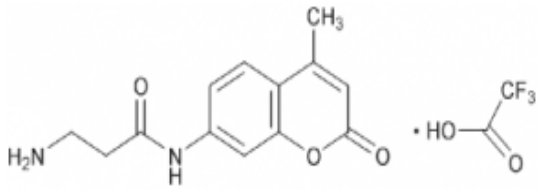
Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Thr-AMC		276.29	$C_{14}H_{16}N_2O_4$
Pyr-AMC		286.29	$C_{15}H_{14}N_2O_4$
H-Ile-AMC.TFA		288.35	$C_{16}H_{20}N_2O_3$

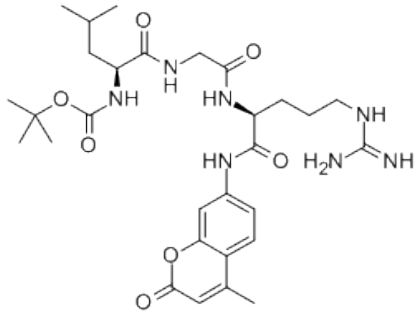
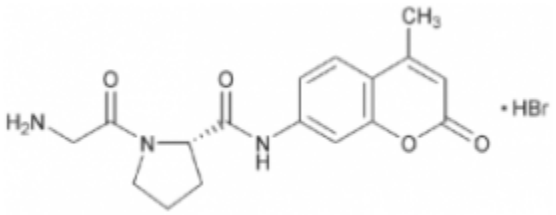
Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Leu-AMC.HCl		288.35	$C_{16}H_{20}N_2O_3$
H-Asn-AMC.TFA		286.29	$C_{14}H_{15}N_3O_4$
H-Orn-AMC.2HCl		289.33	$C_{15}H_{19}N_3O_3$

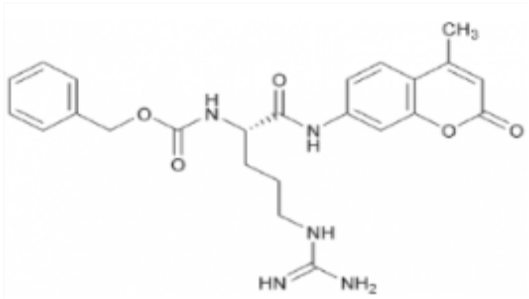
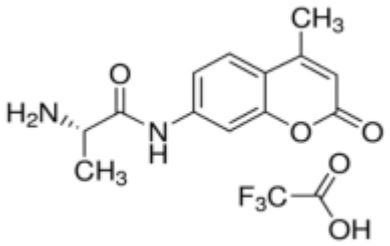
Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Asp(AMC)-OH		290.28	$C_{14}H_{14}N_2O_5$
H-Gln-AMC		303.32	$C_{15}H_{17}N_3O_4$
H-Lys-AMC.acetate		303.36	$C_{16}H_{21}N_3O_3$

Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Glu-AMC		304.30	$C_{15}H_{16}N_2O_5$
H-Met-AMC acetate salt		306.39	$C_{15}H_{18}N_2O_3S$
H-His-AMC		312.33	$C_{16}H_{16}N_4O_3$

Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Phe-AMC.TFA		322.36	$C_{19}H_{18}N_2O_3$
H-Arg-AMC.2HCl		331.38	$C_{16}H_{21}N_5O_3$

Name	Chemical Structure	Molecular Weight	Molecular Formula
H-Try-AMC		338.36	$C_{19}H_{18}N_2O_4$
Ac-Met-AMC		348.42	$C_{17}H_{20}N_2O_4S$
H-β-Ala-AMC.TFA		360.29	$C_{13}H_{14}N_2O_3.C_2HF_3O_2$

Name	Chemical Structure	Molecular Weight	Molecular Formula
Boc-Leu-Gly-Arg-AMC acetate salt		601.70	$C_{29}H_{43}N_7O_7$
H-Gly-pro-AMC.HBr		410.27	$C_{17}H_{19}N_3O_4$.HBr

Name	Chemical Structure	Molecular Weight	Molecular Formula
Z-Arg-AMC.HCl		465.51	$C_{24}H_{27}N_5O_5$
L-alanine-AMC trifluoroacetate salt		360.29	$C_{13}H_{14}N_2O_3 \cdot C_2HF_3O_2$

Appendix 6: Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation

Species	4MA	4MPr	4MB	4MH	4MN	4MLa	4MPgh	4MPa	4MSt	4MLi	4Mara	4Mxyl	4Mribs
A. xylosoxidans	-263	76	97	-10	325	1316	-4	4	-20	-72	-61	-30	-14
A. xylosoxidans	-112	-40	-23	123	80	319	-112	1	-17	-64	-246	-18	-14
A. xylosoxidans	-113	-64	48	95	-16	977	-1	-4	-21	-81	-222	-12	251
B. cenocepacia	-473	-738	-1102	434	-1089	1156	-247	291	41	-85	-177	-59	400
B. cenocepacia	-844	-872	-895	-1193	-94	248	-488	-108	-120	-127	-317	6	231
B. multivorans	-689	-600	-481	-547	-48	672	-417	-57	-69	-88	-1286	-57	-15
B. multivorans	-959	-763	-532	327	-689	2817	-474	1060	484	-14	-1220	-62	-9
B. multivorans	-500	-568	-698	-283	-262	2002	-207	994	143	-40	-348	-6	-6
B. stabilis	144	298	394	1028	435	2017	-261	3828	2428	48	20	113	143
B. vietnamiensis	-212	-165	-320	77	-17	-52	-120	8	-23	-75	-75	26	773
D. acidovorans	-44	-19	-94	118	83	-120	94	133	7	15	-141	90	-19
I. limosus	-344	-126	7	-18	-288	26	-79	21	-8	-43	159	53	272
P. norimbergenis	48	-39	100	323	56	179	31	32	5	-63	79	16	57
P. aeruginosa	703	534	331	-52	594	2222	-169	3897	2592	-51	-20	36	167
S. marcesens	-951	-696	2184	1769	115	2894	-314	4515	2998	134	2278	-28	402
M. abscessus	-480	-289	-165	62	380	1566	-21	970	277	-67	90	158	537
M. abscessus	-342	-293	-224	-34	-354	1315	11	313	94	-117	99	32	217
M. abscessus	-331	-245	-174	-6	521	1455	-21	500	191	6	370	-1	179
M. abscessus	-467	-220	-202	-20	-379	1449	-3	672	151	6	78	16	30
M. abscessus	-402	-172	-264	-3	-200	1453	15	1076	330	100	346	11	260
M. abscessus	-211	-236	-249	75	111	1147	22	940	207	-87	448	41	148
M. abscessus	-264	-216	-246	-49	149	967	-5	740	168	1	245	69	8
M. abscessus	-310	-112	-462	58	-74	1188	-23	474	288	-91	-62	-1	-8
M. abscessus	-70	-2	57	367	516	1651	-104	1196	377	30	37	30	83
M. abscessus	-211	-247	-14	276	1030	1731	1	859	193	-72	372	43	17
M. abscessus	-425	-340	-147	55	490	1453	-89	382	183	-93	309	63	32
M. abscessus	-337	-359	-78	24	471	1523	-83	671	156	-104	137	53	87

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	4MA	4MPr	4MB	4MH	4MN	4MLa	4MPgh	4MPa	4MSt	4MLi	4Mara	4Mxyl	4Mribs
M. bolletii	-366	-275	-179	21	399	1468	0	780	184	-101	63	5	16
M. bolletii (ENV)	-334	-256	-99	79	-2004	1374	-23	738	226	-87	152	25	17
M. chelonae	-257	-254	-268	-27	386	1465	-42	986	292	-93	351	113	81
M. chelonae	-467	-144	-159	38	-286	1306	-9	313	124	-100	-59	-17	-3
M. chelonae	-793	-416	-536	-53	1	1376	-95	681	194	-91	393	-12	42
M. chelonae	-412	-203	-360	-89	-10	1197	5	204	119	-44	161	4	14
M. chelonae	-579	-244	-248	-126	80	1573	-78	995	243	0	283	84	195
M. chelonae	-819	-318	-351	-209	102	895	-120	1344	347	14	326	54	150
M. massiliense	-119	21	-90	352	449	1457	68	585	219	27	261	51	31
M. massiliense	-238	-153	18	188	289	1817	69	609	216	-82	617	100	151
M. massiliense	-53	-206	205	313	251	1613	-2	745	250	-105	475	52	250
M. massiliense	0	30	42	262	-1661	1647	54	1454	332	-94	594	43	68
M. massiliense	72	12	120	333	-1611	1709	16	526	157	-113	370	47	80
M. massiliense	82	57	158	329	-1332	1761	58	695	376	-114	232	16	20
M. massiliense	-20	-10	104	225	1003	1580	104	605	146	-75	368	12	143
M. massiliense	-321	-337	100	261	84	1630	86	1632	261	-53	337	49	220
S. aureus + Ctrl	122	63	-298	-120	-246	-65	86	12	-14	-118	5069	-33	169
E. coli + Ctrl	42	-151	8	-661	-154	18	44	77	24	-84	18	-11	168
Neg Ctrl	-112	85	-52	-143	24	44	-78	14	7	-103	-33	-27	-2
Neg Ctrl	-141	92	-87	-36	-75	10	3	-17	-3	-1	14	-4	-12
Neg Ctrl	251	-177	139	179	51	-54	75	2	-4	105	18	30	12
Neg Ctrl Totals	-2	0	1	-1	1	0	-1	-2	0	1	-2	-1	-2

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	4Mribf	4Mfuc	4Mglupα	4Mglupβ	4Mmanα	4Mgalα	4Mgalβ	4Mgluβ	4MIdo	4Manβ	4Mcurβ	4MIdu	4MNgal
A. xylosoxidans	-54	-3	168	32	-21	6	851	9	-4	-28	6	4	-839
A. xylosoxidans	-53	-7	-49	4	-18	3	871	-33	-7	-23	-7	1	-725
A. xylosoxidans	2715	-10	56	75	-20	2	955	-8	-7	-20	-7	1	-761
B. cenocepacia	356	-8	-543	-4	-8	-8	-55	-54	-24	-56	-42	-8	-105
B. cenocepacia	510	-10	-1353	156	-8	-2	896	142	-7	0	-26	-5	-112
B. multivorans	-67	-11	-1483	-120	2	-4	-173	-57	-37	-62	-34	-10	-203
B. multivorans	-69	-15	-1369	-115	-2	-7	-197	-74	-26	-78	-31	-13	-210
B. multivorans	-63	-13	-630	-38	-11	-6	567	-73	-29	-82	-47	-23	-173
B. stabilis	1207	-1	318	33	-15	4	1206	-11	1	-26	-5	3	-195
B. vietnamiensis	-31	-5	-308	176	-15	-2	608	-5	-15	-23	-2	0	-191
D. acidovorans	-42	-3	-505	50	-23	-3	1342	69	-6	-8	144	-13	-313
I. limosus	1566	10	163	670	57	3	1062	1099	-1	-37	2	2	192
P. norimbergenis	185	2	478	43	-6	7	1232	18	6	-6	13	10	-112
P. aeruginosa	6393	3	-242	33	-13	-1	1173	-2	-3	-16	-9	-2	-220
S. marcesens	6296	1	-1400	3735	313	218	1395	7186	-4	41	258	279	2201
M. abscessus	39	408	239	1657	511	20	1701	2267	30	187	137	23	38
M. abscessus	108	56	388	1256	803	80	2083	1612	44	185	120	25	125
M. abscessus	216	355	488	1690	709	53	1485	1287	79	243	145	27	130
M. abscessus	48	3	126	1047	853	11	1669	293	15	35	23	7	-93
M. abscessus	545	8	573	2885	1250	94	2482	3181	42	300	179	142	115
M. abscessus	221	94	678	3930	1630	120	2336	3774	46	319	108	26	196
M. abscessus	581	160	579	1499	381	13	2191	3030	38	171	2	30	123
M. abscessus	6	27	-139	450	637	11	1880	1156	16	40	57	9	83
M. abscessus	586	37	787	307	275	35	1683	248	24	35	55	15	185
M. abscessus	31	81	815	977	999	29	2160	4600	60	409	192	40	247
M. abscessus	143	2	406	294	245	8	1682	845	30	89	110	32	94
M. abscessus	101	156	419	860	11	10	2391	1234	29	138	71	19	241
M. bolletii	35	-1	431	673	378	38	2644	2049	41	242	161	27	182
M. bolletii (ENV)	28	-1	180	132	247	7	1665	207	27	35	55	18	164

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	4Mribf	4Mfuc	4Mglupα	4Mglupβ	4Mmanα	4Mgalα	4Mgalβ	4Mgluβ	4MIdo	4Manβ	4Mcurβ	4MIdu	4MNgal
M. chelonae	289	14	93	794	600	8	1692	838	23	78	89	25	103
M. chelonae	-49	-6	-122	256	705	3	1445	1243	24	107	66	14	22
M. chelonae	240	-6	506	703	447	7	1806	357	12	33	65	15	54
M. chelonae	-23	-5	90	94	524	7	1558	277	12	19	47	11	17
M. chelonae	177	59	422	1902	665	7	2072	897	16	53	-56	10	-25
M. chelonae	278	26	-228	1284	1877	9	1870	1453	20	54	57	20	25
M. massiliense	63	148	603	286	186	4	1923	341	13	30	46	13	191
M. massiliense	445	90	940	546	431	54	1766	1371	53	234	157	42	193
M. massiliense	126	88	920	905	376	25	3239	3539	56	373	205	43	279
M. massiliense	358	36	1236	1289	932	36	2673	2525	68	353	193	42	409
M. massiliense	104	86	718	592	821	13	2253	717	36	75	88	24	272
M. massiliense	-29	84	413	542	339	7	1814	1174	36	-74	85	19	305
M. massiliense	158	51	1194	1677	732	66	2900	1793	46	256	174	37	404
M. massiliense	229	169	899	3043	1009	59	3265	4899	58	346	208	39	349
S. aureus + Ctrl	4659	273	56	85	-3	4	4418	37	9	-16	295	22	18
E. coli + Ctrl	3292	6	214	51	-8	3	1211	-2	-1	-26	-3	-6	-50
Neg Ctrl	-53	3	74	30	-4	2	38	-4	-14	-11	-2	-14	45
Neg Ctrl	-54	-3	-541	16	3	3	-1	-8	9	7	1	5	-45
Neg Ctrl	108	1	468	-46	1	-5	-37	12	6	4	1	8	-1
Neg Ctrl Totals	1	1	1	-1	0	1	1	0	1	0	1	-1	0

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	4MNglu	4Mcel	Pro	Val	Thr	Ile	Leu	Orn	Gln	MetH	His	Phe	ArgH
A. xylosoxidans	-2552	-1	501	34	33	17	140	85	180	118	-10	137	69
A. xylosoxidans	-2490	-7	678	37	17	14	315	-26	441	81	-2	211	235
A. xylosoxidans	-2876	-8	827	33	53	2	309	-46	2432	117	898	247	181
B. cenocepacia	-1312	-20	-166	10	3	-26	2584	-432	2615	429	56	2620	1873
B. cenocepacia	-1030	13	677	18	3	5	275	-199	2786	79	-18	661	-39
B. multivorans	-1478	-16	1107	-5	-44	-19	269	-474	45	50	-55	213	-35
B. multivorans	-1677	-18	199	3	-33	-11	140	-462	-137	83	-55	112	-28
B. multivorans	-875	-14	676	8	-19	-7	199	-297	-54	32	-53	155	89
B. stabilis	-113	3	5361	89	27	46	1449	13	521	323	73	1199	657
B. vietnamiensis	-526	-2	8241	350	372	3933	9014	1800	918	3257	122	527	7279
D. acidovorans	-2906	22	19	-54	24	20	415	-226	362	3029	-40	282	166
I. limosus	-344	5	6920	1571	453	2130	10040	101	1772	735	214	9669	1189
P. norimbergenis	-79	3	1482	70	40	38	1151	108	1313	260	7	392	560
P. aeruginosa	-690	-9	14	40	7	2	357	-68	902	4	-45	180	2566
S. marcesens	-1764	568	8106	2409	7816	3954	3774	1336	7833	8388	2634	6456	1562
M. abscessus	-25	11	1912	1053	1693	742	7800	2081	1742	3513	2391	8235	6669
M. abscessus	4	19	3960	1905	1182	1659	7824	2694	1191	3799	4715	6870	6055
M. abscessus	443	16	1055	851	2954	504	7221	1522	1963	4452	1378	7995	7088
M. abscessus	-293	6	903	425	729	380	5480	1458	572	931	975	6159	4791
M. abscessus	-1544	8	2025	1086	1920	516	7371	2312	1558	4413	2238	7459	6680
M. abscessus	-1028	30	3812	1975	2519	1321	8144	3532	2688	4110	3848	8522	7451
M. abscessus	-2442	14	3229	1010	1865	1315	7554	2156	2101	4633	4916	8670	7414
M. abscessus	-184	11	1261	2414	781	1189	7694	2087	666	1629	853	7031	4900
M. abscessus	296	9	1448	747	784	855	7442	2514	2113	4010	2739	8652	7874
M. abscessus	430	18	1351	1124	1216	594	7932	1752	1431	4489	3477	7887	6230
M. abscessus	62	-3	43	56	37	22	2157	421	161	2623	4123	2106	7672
M. abscessus	773	23	2771	1630	1956	1110	8390	2858	2718	5403	2655	8422	7237
M. bolletii	434	7	1048	726	1352	395	6843	2675	1803	2497	2950	7573	6873
M. bolletii (ENV)	395	4	46	93	20	149	1907	142	-13	1459	1598	1703	670
M. chelonae	216	2	305	215	211	158	5149	1612	770	2622	3550	5838	7399
M. chelonae	160	1	1618	743	561	736	5781	215	1203	683	1346	5153	5034

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	4MNglu	4Mcel	Pro	Val	Thr	Ile	Leu	Orn	Gln	MetH	His	Phe	ArgH
M. chelonae	149	0	151	742	105	231	2335	717	951	1838	932	4829	6504
M. chelonae	-41	5	454	550	65	302	4535	840	136	310	658	1328	5771
M. chelonae	-1850	3	451	288	363	179	4705	1696	1190	4828	4585	6968	7430
M. chelonae	242	12	1555	830	1199	821	7769	2950	2433	2169	3118	8523	8559
M. massiliense	497	6	695	481	466	414	4904	805	1120	4485	2678	7033	4991
M. massiliense	541	34	3224	3238	2242	1696	8548	4182	2260	2144	3283	8972	8106
M. massiliense	820	25	2945	1494	3310	904	8144	3096	2978	5381	4652	8947	8793
M. massiliense	1173	20	1774	800	1061	413	5820	1792	1952	3468	1903	7875	7413
M. massiliense	522	3	584	370	791	210	5014	691	702	4917	4331	7636	7006
M. massiliense	851	15	3047	2310	2632	1308	9453	2916	2053	2665	2498	8185	6412
M. massiliense	764	36	3361	1719	2476	1343	8952	4050	3248	3495	3660	9337	8488
M. massiliense	996	36	3912	3344	3880	2140	9366	4222	3516	5689	4634	9463	8472
S. aureus + Ctrl	-371	-3	28	10	28	22	346	46	31	78	60	398	615
E. coli + Ctrl	-228	-3	3	34	14	12	48	-23	-39	9	18	27	36
Neg Ctrl	-95	-1	-1	26	-20	-3	18	-3	-21	-4	-2	3	-5
Neg Ctrl	-32	-3	-20	-66	-2	-12	-3	-99	17	3	0	-14	-8
Neg Ctrl	127	4	19	39	21	14	-15	102	3	1	1	11	14
Neg Ctrl Totals	0	1	-2	-1	-2	0	0	1	-1	0	-1	0	1

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	Try	MetA	Ala	AAMC	GlyP	Boc	4MPh	4MSu	123	124	521	266	MR291	7N3CA
A. xylosoxidans	87	20	-13	1470	-484	19	170	-730	-1	-0.5	4	-1.5	-0.5	1951
A. xylosoxidans	151	18	-4	2083	-302	18	267	-991	-0.5	-0.5	-4.5	0.5	0.5	1422
A. xylosoxidans	274	16	30	1476	-272	19	2346	152	-1.5	1	-2.5	0.5	1	3119
B. cenocepacia	2239	5	639	1884	-474	354	4276	-1464	-1	0.5	-1.5	1.5	0	-31
B. cenocepacia	519	11	826	3302	-310	10	4283	-1568	0	0	-0.5	1.5	0.5	456
B. multivorans	145	0	1776	1334	-1425	3	2900	945	0	0.5	-4.5	1	0.5	3015
B. multivorans	79	-4	1646	2417	-1590	16	4856	580	-1.5	0.5	-6	1	0.5	506
B. multivorans	105	-4	1250	2206	-1136	18	5545	-227	-1	0	-2.5	2.5	0	1551
B. stabilis	489	6	938	4254	-108	18	6010	-643	-0.5	0	-5	-1.5	0.5	240
B. vietnamiensis	425	3	44	4082	1282	13	3024	606	0.5	0	-1.5	-1	-0.5	-594
D. acidovorans	410	-9	44	2847	-143	15	109	5787	0	1.5	-5	-2.5	1	2266
I. limosus	10049	1	280	4763	-82	310	61	-1137	-2.5	-0.5	1.5	9	1	-1082
P. norimbergenis	299	31	23	3378	-59	3	298	-930	0	0.5	1.5	2	0	-2077
P. aeruginosa	121	9	1530	483	-330	82	-7	278	1	0	-9.5	5.5	0.5	-3469
S. marcesens	8601	11	7935	8628	2683	4273	4412	-1302	1	0	-3	8	2	2229
M. abscessus	6793	12	1321	4949	-17	412	106	2415	0	1.5	1	7	2.5	-2113
M. abscessus	6495	51	1413	7074	297	610	117	1327	0.5	2.5	-1.5	9	17.5	-418
M. abscessus	6874	36	2334	4362	197	553	102	826	0.5	1	0	10	13.5	-1977
M. abscessus	3972	14	1186	7027	-106	111	120	740	-0.5	1	-0.5	8	9.5	-120
M. abscessus	6776	8	2510	7037	-44	388	174	3452	0	0.5	-3	29	14.5	-1012
M. abscessus	7510	20	3662	7862	337	843	84	1889	-1	2	-1	12	9	-42
M. abscessus	6534	21	3303	5955	437	1173	113	2562	0.5	0.5	0	21	5.5	-214
M. abscessus	6416	4	332	2410	-147	102	97	-302	0	1	-7.5	-4	3	3052
M. abscessus	6354	17	2195	3457	462	431	62	-278	0.5	0.5	-0.5	80.5	2.5	3778
M. abscessus	5928	60	2968	4968	651	927	170	2544	-0.5	1	4	15	8	-2798
M. abscessus	2570	19	1796	4609	-175	147	70	-124	0	0.5	-1	7.5	6.5	-3376
M. abscessus	7553	51	3096	3670	360	1329	233	1718	0.5	1.5	-0.5	10.5	5.5	-3561
M. bolletii	5848	8	1427	2931	-137	391	171	1923	-0.5	0.5	-1	51	3.5	-3288
M. bolletii (ENV)	3053	12	1795	4540	186	227	108	802	0	1	-4	14	8.5	-521
M. chelonae	4645	1	2477	4779	-2	362	102	2021	0.5	1	-6.5	16.5	6.5	-3266
M. chelonae	791	6	1109	3080	-249	58	67	-768	0.5	0	-3.5	7	3	-1873
M. chelonae	4318	1	1849	4014	-430	56	224	3067	1.5	1.5	-2	7	3.5	-578

Average spectrophotometer readings for fluorogenic substrates after 72 hours incubation continued

Species	Try	MetA	Ala	AAMC	GlyP	Boc	4MPh	4MSu	123	124	521	266	MR291	7N3CA
M. chelonae	695	3	1155	5710	-103	102	111	-741	0.5	2	0.5	13.5	5	-371
M. chelonae	7174	5	4458	5629	365	528	207	2929	0.5	0	-2	15.5	6.5	-789
M. chelonae	7961	17	2993	5928	104	395	134	3800	0	2	-4.5	27	4.5	-885
M. massiliense	5088	13	1424	1789	108	467	91	1928	-0.5	1.5	-4.5	1.5	7.5	-970
M. massiliense	7288	59	1941	4033	483	646	95	1315	-0.5	0.5	6.5	20.5	2.5	-3831
M. massiliense	8544	37	2526	6277	596	970	266	1918	0	1	5	18	2	-3647
M. massiliense	3297	24	2017	5596	616	589	191	4392	0	1.5	5.5	20.5	2	-3574
M. massiliense	8300	42	2052	4718	527	605	175	2337	0.5	1.5	1	3.5	2	-1842
M. massiliense	6832	28	954	1566	369	381	76	3445	1	0.5	3	5	1.5	-3671
M. massiliense	8555	33	1804	7052	125	460	213	1491	0.5	1.5	2	28.5	5	-2222
M. massiliense	8514	42	3318	6362	800	1111	228	3180	0.5	2.5	4.5	13.5	5.5	-573
S. aureus + Ctrl	293	15	15	8	70	173	6158	-1504	0	0	-2	3.5	0.5	6359
E. coli + Ctrl	17	11	2	1964	85	-11	6368	-1566	-1	-0.5	2	2.5	1	6545
Neg Ctrl	15	2	18	33	114	-3	7	-281	-0.5	0.5	0.5	0	0.5	191
Neg Ctrl	-14	-4	-22	-24	-171	1	-4	58	0	0.5	-2.5	-2	-1	203
Neg Ctrl	-2	1	3	-9	56	2	-4	222	0	-0.5	1	3	-0.5	-394
Neg Ctrl Totals	0	-1	-2	1	-1	0	-1	-1	-1	1	-1	1	-1	1

M. abscessus: M. abscessus subsp*. abscessus*
M. bolletii : M. abscessus subsp*. bolletii*
M. massiliense : M. abscessus subsp*. massiliense*

Key : 4MA : 4-Methylumbelliferyl acetate, 4MPr : 4-Methylumbelliferyl propionate, 4MB: 4-Methylumbelliferyl butyrate, 4MH: 4-Methylumbelliferyl heptanoate, 4MN : 4-Methylumbelliferyl nonanoate, 4MLa : 4-Methylumbelliferyl laurate, 4MPgh : 4-Methylumbelliferyl p-guanidinobenzoate hydochlorate hydrate, 4MPa : 4-Methylumbelliferyl palmitate, 4MSt : 4-Methylumbelliferyl stearate, 4MLi : 4-Methylumbelliferyl lignocerate, 4Mara : 4-Methylumbelliferyl α-L-arabinopyranoside, 4Mxyl : 4-Methylumbelliferyl 7-β-D-xyloside, 4Mribs : 4-Methylumbelliferyl riboside, 4Mribf : 4-Methylumbelliferyl β-D-ribofuranoside, 4Mfuc : 4-Methylumbelliferyl β-D-fucoside, 4Mglupα : 4-Methylumbelliferyl α-D-glucopyranoside, 4Mglupβ : 4-Methylumbelliferyl β-D-glucopyranoside, 4Manα : 4-Methylumbelliferyl α-D-mannopyranoside, 4Mgalα : 4-Methylumbelliferyl α-D-galactoside, 4Mgalβ : 4-Methylumbelliferyl β-D-galactoside, 4Mgluβ : 4-Methylumbelliferyl β-D-glucoside, 4MIdo : 4-Methylumbelliferyl α-L-idopyranoside, 4Manβ : 4-Methylumbelliferyl β-D-mannopyranoside, 4Mcurβ : 4-Methylumbelliferyl β-D-glucuronide, 4Midu : 4-Methylumbelliferyl α-L-iduronide, 4MNgal : 4-Methylumbelliferyl N-acetyl-β-D-galactosaminide, 4MNglu : 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide, 4Mcel : 4-Methylumbelliferyl β-D-cellobioside, Pro : H-Pro-AMC hydrobromide salt (Prolyl), Val : H-Val-AMC, Thr : H-Thr-AMC, Ile : H-Ile-AMC.TFA, Leu : H-Leu-AMC.HCl, Orn : H-Orn-AMC.2HCl, Gln : H-Gln-AMC, MetH : H-Met-AMC acetate salt, His : H-His-AMC, Phe : H-Phe-AMC.TFA, ArgH : H-Arg-AMC.2HCl, Try : H-Try-AMC, MetA : Ac-Met-AMC, Ala : H-β-Ala-Amc.TFA, AAMC : L-alanine AMC trifluoroacetate salt, GlyP : H-Gly-pro-AMC.HBr, Boc : Boc-Leu-Gly-Arg-AMC acetate salt, 4MPh : 4-Methylumbelliferyl phosphate, 4MSu : 4-Methylumbelliferyl sulfate, SR123 : , SR124 : , S521 : , SR266 : , MR291 : Methyl Red 291, 7N3CA : 7-nitrocoumarin-3-carboxylic acid.

CHAPTER EIGHT

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CHAPTER NINE

Publications and Conference Proceedings

Patents, conference proceedings and publications relating to this thesis

9.1 Patents

S. Orenga, A. Perry, J.D Pery, C.L Preece. (2016). Enrichment and selective culture of mycobacteria. France. WO2016124863 A1

9.2 Conference Proceedings

A. Perry, J.D. Perry, C.L Preece, D. Tierney, S. Peart, F.K. Gould: Antimicrobial activity of cysteamine against antibiotic resistant pathogens isolated from cystic fibrosis and non - CF patients including *Mycobacterium abscessus* complex from lung transplant recipients. 36th ISHLT Annual Meeting, WASHINGTON DC; 04/2016

A. Perry, C.L Preece, M. Permain, C. Williams, D. Tierney, A. Robb, S. Bourke, C. O'Brien, J.D Perry: RGM medium for isolation of mycobacteria from respiratory samples of patients with cystic fibrosis (CF). Inaugural Manchester Cystic Fibrosis Conference, Manchester, UK; 04/2016

C.L Preece, A. Perry, A.L Jones, S.P Cummings, M.F Thomas, M. Brodlie, C.J O'Brien, S.J Bourke, J.D Perry: Evaluation of various culture media for improved detection of rapidly growing mycobacteria from the sputum of patients with cystic fibrosis. 29th North American Cystic Fibrosis Conference, Phoenix, Arizona; 10/2015

C.L Preece, A. Perry, J.D. Perry, S.P. Cummings, A.L. Jones, S.J. Bourke: Comparison of two chromogenic media for isolation of *Staphylococcus aureus* from respiratory samples of patients with cystic fibrosis. European Cystic Fibrosis Conference, Brussels, Belgium; 06/2015

C.L Preece: A novel culture medium for recovery of rapidly-growing mycobacteria from the sputum of patients with cystic fibrosis. Northumbria University Post Graduate Research Conference, Northumbria University; 05/2015

R. Plonga, C.L Preece, J.D Perry, P.H Gilligan: Evaluation of RGM medium for detection and identification of non-tuberculous mycobacteria from patients with cystic fibrosis. ASM Microbe, Boston; 06/2016

A. Perry, C.L Preece, S. Bourke, S. Doe, C. O'Brien, M. Brodlie, M. Thomas, A. Robb, J.D Perry: RGM: A new medium for isolation of rapidly-growing mycobacteria from respiratory samples of patients with cystic fibrosis. 15 months experience of routine use for CF respiratory samples. CF Consortium Meeting, Liverpool, UK; 11/2016

36th ISHLT
Annual Meeting
WASHINGTON DC
April 2016

Antimicrobial activity of cysteamine against antibiotic resistant pathogens isolated from cystic fibrosis (CF) and non-CF patients including *Mycobacterium abscessus* complex from lung transplant recipients

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Introduction

Background: **Lung transplantation for cystic fibrosis (CF) patients colonised with pan resistant bacteria, in particular *Mycobacterium abscessus* complex (MABSC) may preclude them from lung transplantation due to poor outcomes. Treatment of MABSC generally consists of a three drug regime including nephrotoxic/ototoxic amikacin. Oral cysteamine is licenced for the treatment of cystinosis and has recently been reported as having good antimicrobial and anti-biofilm activity against CF pathogens including MABSC. In addition it has mucolytic activity and reduces viscoelasticity of sputum.**

Purpose of the study: We evaluated the antimicrobial activity of this compound against a collection of 31 strains of CF and non- CF pathogens including isolates from lung transplant recipients. We also evaluated the interaction of cysteamine with 12 anti-pseudomonal agents against 4 strains of *P. aeruginosa* (Pa).

Materials and Methods

Microdilution MIC's against 12 MABSC were performed and bactericidal activity was assessed by subculture. MIC's were performed against a further collection of 19 strains including: Pa (*n*=3), *Achromobacter* spp. (*n*=2) and 1 each of the following: *Burkholderia multivorans*, *Burkholderia cenocepacia*, *Ralstonia mannitolylitica*, *Stenotrophomonas maltophilia*, *Pandoraea pulmonicola* and *Inquilinus limosus*. NCTC strains of *E coli*, *S aureus*, *C albicans*, *E faecalis* and 4 carbapenemase-producing Enterobacteriacae (CPE) were also evaluated.

The Multiple Combination Bactericidal Test (MCBT) was utilized to assess synergy by incorporating cysteamine at 500 mg/L with 12 agents at the systemic concentration. The four strains of Pa selected were only susceptible to colomycin.

References

Cysteamine (cystaran®), a novel mucoactive antimicrobial & anti-biofilm agent for the treatment of cystic fibrosis, Chiron et al. Orphanet Journal of Rare Diseases (2014) 9:139 http://www.ojrd.com/content/9/1/139

Cysteamine as a Future Intervention in Cystic Fibrosis Against Current and Emerging Pathogens: A Potent Ex-vivo Study Confirming its Antimicrobial and Mucoactive Potential in Sputum. Devereux et al. EBioMedicine (2015) Vol 2, Issue 10, 1507-1512

Pharmacokinetics of enteric-coated cysteamine bitartrate in healthy adults: a pilot study. Gungabissoon et al. British Journal of Clinical Pharmacology (2010) 170–262

Table 1: Minimum inhibitory concentrations (MIC) mg/L and minimum bactericidal concentrations (MBC) mg/L of all 31 isolates tested.

SPECIES	Ref	MIC (mg/L)	MBC (mg/L)
Gram negatives			
Achromobacter sp.	3355	512	512
Achromobacter sp.	3744	256	512
Acinetobacter sp.	3741	512	512
B. cenocepacia	3453	128	128
B. multivorans	3764	512	512
C. freundii complex	3797	512	512
E. coli	NCTC 10418	512	512
I. limosus	3652	256	256
K. pneumoniae	3796	512	512
K. pneumoniae	3809	512	512
P. aeruginosa	NCTC 10662	256	512
P. aeruginosa	3808	64	512
P. aeruginosa	3411	512	512
P. pulmonicola	3651	512	1024
R. mannitolytica	3304	256	256
S. maltophilia	3501	256	512
Gram positives			
C. albicans	NCTC 90028	1024	1024
E. faecalis	NCTC 775	512	1024
S. aureus	NCTC 6571	512	1024
Mycobacteria			
M. abscessus	1007	256	1024
M. abscessus	1021	256	512
M. abscessus	1042	256	512
M. abscessus	1043	1024	512
M. abscessus	1052	512	1024
M. abscessus	1058	512	1024
M. abscessus	1064	256	1024
M. balletii	3016	512	1024
M. balletii	3019	256	>1024
M. massiliense	3000	256	1024
M. massiliense	3009	512	1024
M. massiliense	3020	256	1024

Results

The MIC's of 12 MABSC ranged from 256-1024 mg/L with bactericidal activity against 8 strains at 1024 mg/L and 512 mg/L for 3 strains. One strain was inhibited at 256 mg/L but no bactericidal activity observed at 1024 mg/L. The MIC's of the 19 other isolates ranged from 64-1024 mg/L with bactericidal activity ranging from 128-1024 mg/L for all except *C. albicans* (see Table 1). Cysteamine at 500mg/L was bactericidal against all 4 strains of Pa and no antagonism was observed with antipseudomonal agents in the MCBT (see Table 2).

Table 2: Antibiotics tested against four strains of *P. aeruginosa* at EUCAST breakpoint as single agents and in combination with cysteamine at 500 mg/L (no antagonism was observed).

Antibiotics	Antibiotics alone at EUCAST breakpoint				Antibiotics in combination with 500 mg/L cysteamine			
(cysteamine 500 mg/L)	Pa 1	Pa 2	Pa 3	Pa 4	Pa 1	Pa	Pa 3	Pa 4
Timentin	R	R	R	R	S	S	S	S
Ceftazidime	R	R	R	R	S	S	S	S
Ciprofloxacin	R	R	R	R	S	S	S	S
Tazocin	R	R	R	R	S	S	S	S
Colomycin	S	S	S	S	S	S	S	S
Meropenem	R	R	R	R	S	S	S	S
Aztreonam	R	R	R	R	S	S	S	S
Tobramycin	R	R	R	R	S	S	S	S
Doripenem	R	R	R	R	S	S	S	S
Temocillin	R	R	R	R	S	S	S	S
Fosfomycin	R	R	R	R	S	S	S	S
Co-trimoxazole	R	R	R	R	S	S	S	S
Cysteamine	S	S	S	S	S	S	S	S

Conclusions

A study by Devereux *et al, 2015* demonstrated cysteamine has *in vitro* properties potentially therapeutically beneficial in cystic fibrosis (CF). In this study they investigated the antimicrobial and mucolytic activity of cysteamine against the complex biologic matrix of CF sputa. The results of our study are comparable with those of other studies that demonstrate that cysteamine has potential as an antimicrobial agent for the treatment of severe infection caused by pan resistant bacteria and may therefore have potential for treatment of post lung transplant patients with or without CF and warrants further evaluation. The tolerability and antimicrobial activity of cysteamine is due to be assessed in CF patients.

POSTER NUMBER 1010

RGM: A new medium for isolation of Rapidly Growing Mycobacteria from respiratory samples of patients with cystic fibrosis

Inaugural Manchester Cystic Fibrosis Conference 21st April, 2016

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Background

Rapidly-growing mycobacteria may be significant pathogens in patients with cystic fibrosis (CF) leading to a decline in lung function. In the European CF population, the predominant species of mycobacteria are rapidly-growing species, especially *Mycobacterium abscessus* complex (MABSC) which consists of *M. abscessus* subsp. *abscessus*, subsp. *massiliense* and subsp. *bolletii*. The CF Trust recommend submission of sputum for AFB culture annually. RGM medium (see Figure 1) is a novel, highly-selective agar medium for the isolation of rapidly-growing mycobacteria from patients with CF. All CF respiratory samples are inoculated direct onto the agar with no decontamination required. Although not yet commercially available, this medium can be provided by the Freeman Hospital Microbiology department. We report here, our experience of using RGM medium for culture of respiratory samples from patients with CF over a 15-month period. We compared these results to traditional AFB cultures performed at Newcastle PHE laboratory, Freeman Hospital during the same time period.

Materials & Methods

1. Between December 2014 and February 2016, 4408 clinical samples from 625 patients with CF were cultured onto RGM medium. The main specimen types were sputa (n = 2443) and cough swabs (n = 1557). Culture plates were incubated for 10 days at 30°C and read after 4, 7 and 10 days of incubation.

Colonies were identified using MALDI-TOF mass spectrometry (Bruker) and mycobacteria were confirmed to species level using *rpoB*, *hsp65* and *sodA* sequence cluster analysis, Colindale, UK .

2: We extracted data from the laboratory database for frequency of submission of sputum samples sent to PHE Newcastle during the same time period for AFB culture .

Results (see table 1)

1. **Fifty six of 625 patients** submitted at least one sample from which mycobacteria were recovered **(prevalence: 8.9%)**. Of the patients who were colonized or infected, the implicated species included *M. abscessus* complex (60.7%), *M. chelonae* (16.1%), *M. fortuitum* (1.8%), *M. mucogenicum* (1.8%) and *Mycobacterium* species (19.6%). Sputum samples were more likely to yield mycobacteria than cough swabs (8.5% versus 1.9% positivity, respectively). However, for 15 patients, mycobacteria were first isolated by culture of cough swabs.

2. Of 625 patients screened using RGM between 21/11/14 - 13/02/16, for 412 patients **(66%) no sample was sent for AFB culture.** For 213 patients, 469 samples were cultured for AFB. Seventy three **(15.5%)** cultures were contaminated and no report could be issued with respect to isolation of AFB. **22 patients were positive for AFB (prevalence 10.8%).**

Table 1. Mycobacteria recovered from 4408 respiratory samples submitted by patients with CF using RGM medium and comparison with surveillance using formal AFB culture.

	Number of samples and patients (n) tested using RGM medium.						No. of patients tested (n) using formal AFB culture
	Total	Cough Swabs	Sputa	BAL	Miscellaneous	Patients	
	4408	2443	1557	131	277	625	213[a]
Total mycobacteria	195	46	133	4	12	56	22[b]
M. abscessus complex	168	40	114	2	12	34	13
M. chelonae	10	2	8	0	0	9	3
M. fortuitum	2	0	2	0	0	1	0
M. avium complex*	0	0	0	0	0	0	6
M. mucogenicum	1	0	1	0	0	1	0
*M. tuberculosis**	0	0	0	0	0	0	1
Other *Mycobacterium* species	14	4	8	2	0	11	0

* RGM is not designed for isolation of slow growing mycobacteria

a: 469 samples cultured from 213 patients.
b: Two different *Mycobacterium* spp isolated from one patient sample

Figure 1: RGM medium showing pure growth of *M. abscessus* smooth colonies (left) and rough colonies (right)

Conclusions

- **Culture on RGM medium can be embedded into routine laboratory methods and allows the culture of all respiratory samples (including cough swabs) without decontamination of samples.**
- **This approach allowed detection of mycobacteria in samples from 56 patients whereas routine surveillance of the same patient population (n = 625) using formal AFB culture allowed detection of mycobacteria in only 22 patients. This was mainly due to the fact that sputum samples were not submitted for formal AFB culture from two thirds of this patient group during the 15-month period.**
- **Mycobacteria can be recovered from cough swabs using RGM medium in patients who are not producing sputum whereas such samples are not deemed appropriate for AFB culture.**
- **Our experience is that use of RGM medium offers a simple and straightforward method for enhanced surveillance of Nontuberculous Mycobacteria (NTM) disease in patients with CF. Annual submission of samples for formal AFB culture may still be necessary to recover slow-growing mycobacteria.**

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Evaluation of various culture media for improved detection of rapidly growing mycobacteria from the sputum of patients with cystic fibrosis

29th Annual North American Cystic Fibrosis Conference, Oct 2015, Phoenix, Arizona

Preece, C.L*.[1,2], Perry, A[1], Jones, A.L.[2], Cummings, S.P.[2], Bourke, S.J.[3], Robb, A.[3], Thomas, M.F.[4], Brodlie, M[4], O'Brien C.J[4]. & Perry, J.D.[1,2]
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Introduction

Background: Isolation of non tuberculous mycobacteria (NTM) from the sputum of patients with cystic fibrosis (CF) is particularly challenging due to the overgrowth of many more rapidly growing species that colonise the lungs of patients with CF[1,2]. Fungi and Gram negative bacteria can quickly overgrow and conceal any mycobacteria[2], with decontamination protocols also potentially reducing the yield of mycobacteria[1]. Recently, extended incubation of *Burkholderia cepacia* selective agar (BCSA) has been recommended as a suitable and effective culture method for rapidly growing NTM from the sputum of patients with CF[3], however variable cepacia media offer inconsistent results in terms of isolation rates of NTM. With this in mind, a number of cepacia media were evaluated against both Middlebrook selective medium and a novel RGM medium[4]

Materials & Methods

Five commercially available pre-poured media for the isolation of *Burkholderia cepacia* complex (BCC) were assessed and compared with two media designed specifically for the isolation of mycobacteria (RGM medium and Middlebrook 7H11 selective agar). All seven media were challenged with 147 isolates of rapidly-growing mycobacteria, 43 isolates of BCC and 142 isolates belonging to other species. Each isolate was suspended in 1 ml of saline (0.85%) to a turbidity equivalent to a McFarland 0.5 standard (approximately 1.5 x 10^8 CFU/ml) and a multipoint inoculator was used to deliver inocula of approximately 1 µl per spot (i.e. approximately 1.5 x 10^5 CFU). All media were incubated for 10 days at 30°C.

Results

Evaluation of seven selective agars for supporting the growth of mycobacteria.

Clear differences were revealed between the five different brands of BCSA in terms of their ability to support the growth of mycobacteria. For example, on Cepacia selective agar (bioMérieux) 95.9% of mycobacteria generated growth within 4 days of incubation compared with only 40.1% of isolates on Oxoid *B. cepacia* agar (Table 1). After ten days of incubation, ten isolates had still not grown on Oxoid *B. cepacia* agar including *M. abscessus* complex (MABSC) (*n* = 4), *M. chelonae* (*n* = 3), *M. llatzerense* (*n* = 2) and *M. mucogenicum* (*n* = 1). All isolates were recovered on Cepacia selective agar (bioMérieux) whereas other brands of BCSA failed to support the growth of between four and eight isolates. All isolates were recovered on Middlebrook 7H11 selective agar and RGM medium.

Evaluation of seven selective agars for inhibition of non-mycobacteria.

Table 2 and Figure 1 provides insights into the selectivity of the seven selective media with 185 non-mycobacteria. All of the five brands of BCSA showed effective inhibition of *P. aeruginosa*, which is an essential attribute of such media. Inhibition of other species was more variable however. For example, of 28 isolates of *S. aureus* (mainly methicillin-resistant strains), 21 (75%) were able to grow on BD OFPBL medium whereas only three isolates were able to grow on Oxoid B. cepacia agar and bioMérieux BCSA. All brands of media for isolation of BCC showed a poor ability to inhibit the growth of fungi – particularly *Aspergillus* spp. and yeasts. Overall, bioMérieux BCSA showed the greatest selectivity and BD OFPBL showed the weakest selectivity among the five brands tested.

Although Middlebrook selective medium is designed specifically for the isolation of mycobacteria from clinical samples, the growth of other non-mycobacterial species was relatively common with 73 out of 186 (39.25%) isolates able to grow. Overall, its selectivity was inferior to the two most selective media for BCC, although it was able to inhibit the growth of *Aspergillus fumigatus*. RGM medium was by far the most selective of all of the agars tested, with 90% of non-mycobacteria inhibited including all fungi and Gram-positive bacteria.

Table 1: Percentage of mycobacteria recovered on various selective agars at 30°C

	n	BCSA	Cepacia selective agar	B. cepacia agar	Cepacia medium	OFPBL	RGM medium	Middlebrook 7H11 agar
		bioMérieux	bioMérieux	Oxoid	BD	BD	N/A	E&O Laboratories
		33631	44347	PO0938	256180	254481	N/A	PP4084
MABSC	**94**							
Day 4		92.6	96.8	37.4	96.8	93.6	58.5	98.9
Day 7		98.9	98.9	91.5	98.9	98.9	93.9	98.9
Day 10		98.9	100	93.7	100	98.9	100	100
M. chelonae	**43**							
Day 4		97.7	100	9.3	95.3	100	100	100
Day 7		100	100	69.8	95.3	100	100	100
Day 10		100	100	93	97.7	100	100	100
Other species*	**10**							
Day 4		10	70	10	70	40	90	70
Day 7		10	80	60	70	40	90	70
Day 10		10	100	70	70	70	100	100
Total mycobacteria	**147**							
Day 4		86.4	95.9	40.1	94.6	91.8	68.6	97.3
Day 7		94.6	98	33	95.9	95.2	88.4	97.3
Day 10		94.6	100	93.2	97.3	97.3	100	100

* Other species of mycobacteria included *M. fortuitum*, *M. wolinskyi*, *M. llatzerense*, *M. immunogenum* and *M. mucogenicum*.

Table 2: Number of non mycobacteria isolates recovered on various selective agars after ten days of incubation at 30°C

	n	BCSA	Cepacia selective agar	B. cepacia agar	Cepacia medium	OFPBL	RGM medium	Middlebrook 7H11 agar
		bioMérieux	bioMérieux	Oxoid	BD	BD	N/A	E&O Laboratories
		33631	44347	PO0938	256180	254481	N/A	PP4084
Gram negatives	**141**	**54**	**60**	**55**	**59**	**72**	**18**	**63**
Enterobacteriaceae	11	2	0	2	2	6	0	1
A. xylosoxidans	8	3	3	3	3	8	2	3
Acinetobacter sp.	2	0	0	0	0	0	0	0
B. cepacia complex	43	37	40	36	37	41	11	39
D. acidovorans	1	1	0	0	0	1	0	0
E. miricola	1	1	1	1	1	1	0	1
H. influenzae	1	0	0	0	0	0	0	0
I. limosus	3	0	2	0	2	0	1	2
M. catarrhalis	1	0	0	0	0	0	0	0
N. flavescens	1	1	1	1	1	1	1	1
Ochrobactrum sp.	1	0	1	1	1	1	0	1
P. aeruginosa	33	0	2	1	2	2	0	2
Pandoraea spp.	3	3	3	3	3	3	2	3
R. mannitolilytica	7	6	6	6	6	6	0	7
S. maltophilia	4	0	1	1	0	5	0	5
Gram positives	**35**	**3**	**11**	**3**	**14**	**21**	**0**	**7**
B. subtilis	1	0	0	0	0	0	0	0
Enterococcus spp.	2	0	0	0	0	0	0	0
S. aureus	28	3	11	3	14	21	0	7
Streptococcus spp.	4	0	0	0	0	0	0	0
Yeast and Fungi	**9**	**5**	**8**	**9**	**8**	**8**	**0**	**3**
A. fumigatus	2	2	1	2	2	2	0	0
A. terreus	1	1	1	1	1	1	0	1
Candida spp.	3	2	3	3	3	3	0	1
E. rugifoleum	1	0	0	1	0	0	0	0
S. apiospermum	1	0	1	1	1	1	0	1
S. prolificans	1	0	1	1	1	1	0	0
Total	**185**	**62**	**79**	**67**	**81**	**101**	**18**	**73**

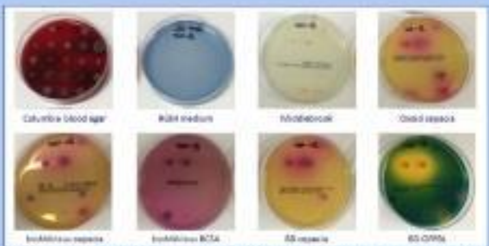

Figure 1: Growth of non mycobacteria at day 7 on various selective media. Species on Columbia blood agar control plate *Achromobacter* sp. (*n* = 2), *Acinetobacter* sp. (*n* = 1), BCC (*n* = 2), *P. aeruginosa* (*n* = 11), *C. freundii* (*n* = 1), *M. morganii* (*n* = 1), *S. maltophilia* (*n* = 2)

Conclusions

RGM medium supported the growth of all isolates of mycobacteria and was more selective against other bacteria and fungi than any other culture medium. Media for the isolation of BCC varied in their ability to support the growth of BCC and mycobacteria as well as their ability to inhibit other flora. A notable feature of RGM medium was its ability to prevent the growth of yeast, fungi and Gram positive bacteria with no isolates of these demonstrating growth within the ten days of incubation.

From our analysis we conclude that RGM medium offers a superior option than any of the other from the sputum of patients with CF.

It is anticipated that RGM medium will be made commercially available in due course but until then, the authors are committed to making the culture medium freely available to clinical laboratories who wish to independently verify the findings of this study.

Acknowledgements

The authors are grateful for the support provided by staff of the Freeman Hospital Microbiology Department, Newcastle upon Tyne, UK. We are also grateful to St. Vincent's University Hospital, Dublin, Republic of Ireland, and Public Health England, Newcastle upon Tyne, UK, for contributing isolates of mycobacteria.

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2. [illegible]
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4. [illegible]

POSTER NUMBER 376

Comparison of two chromogenic media for isolation of *Staphylococcus aureus* from respiratory samples of patients with cystic fibrosis

38th European Cystic Fibrosis Conference. June 2015, Brussels, Belgium

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Introduction

Background: *Staphylococcus aureus* is a significant pathogen in pulmonary infections associated with cystic fibrosis (CF). Isolation of *S. aureus* from sputum may be challenging due to the large number of other bacteria and fungi commonly present, including Gram-negative species that frequently possess resistance to multiple antimicrobials. It is therefore necessary to use a culture medium that has a high degree of selectivity for *S. aureus*. Isolation of *S. aureus* is further complicated in CF by the increasing occurrence of small-colony variants (SCV)[1]. Children and adolescents are often colonised with *S. aureus*, however SCV are predominantly found to be associated with advancing age, and also said to be linked to chronic and recurrent infections[2]. These SCV can be challenging to detect largely due to their reduced growth rate, atypical pin point colony morphology and uncharacteristic biochemical properties[3]. They may consequently remain undetected when using conventional methods and their frequency may be largely underestimated in patients with CF[4].

Purpose of the study: chrom ID *S. aureus* ELITE is a new chromogenic agar medium for the isolation of *S. aureus* that has improved selectivity. It is also optimised, by the inclusion of specific growth factors, for the isolation of auxotrophic strains of *S. aureus* (SCV). The aim of this study was to compare the performance of chrom ID *S. aureus* ELITE with an established chromogenic medium for the isolation of *S. aureus* from respiratory samples from patients with CF.

Materials & Methods

Two chromogenic agar based media, chromID *S. aureus* (chromID) and chromID *S. aureus* ELITE (chromID ELITE) (bioMérieux, Marcy-l'Étoile, France), were compared. These pre-prepared media were evaluated with 231 respiratory samples which included cough swabs (n = 108) and sputum samples (n = 123) from 171 distinct patients with CF. Sputum samples were homogenized (1:1) with sputasol, and 10 µL was cultured onto each medium. The clinical material from cough swabs was dispersed into 2 mL of saline (0.85%) and 10 µL was cultured onto each medium. Plates were incubated at 37°C and read at 20 h, 48 h, and 72 h and any colonies showing expected colouration were identified using MALDI-TOF MS (Bruker, UK).

Results

S. aureus was isolated from 52 samples (22%) (See Table 1). The sensitivity of chromID was 62% after 20 h of incubation, rising to 79% after 72 h. chromID ELITE showed a sensitivity of 79% after 20 h, rising to 92% after 72 h (P = 0.12). Fewer false positives were encountered on chromID ELITE (n = 68) compared with chromID (n = 146) Table 2 shows the numbers of each species generating coloured colonies on the two chromogenic media after 72 h incubation.

Figure: *S. aureus* isolated from sputum is shown as prominent pink colonies on chromID ELITE (a) and green colonies on chromID (b)

Table 1: Sensitivity of two chromogenic media for isolation of *S. aureus*

	chromID S. aureus			chromID S. aureus ELITE		
	20 h	48 h	72 h	20 h	48 h	72 h
Number of positive specimens	32	39	41	41	43	48
Sensitivity (%)	62	75	79	79	83	92
False Positives	23	102	146	9	38	68
Positive Predictive Values	58	28	22	82	53	41

Table 2: Coloured colonies recovered on two chromogenic media after 72 h incubation

Species	chromID	chromID ELITE	Species	chromID	chromID ELITE
A. radicidentis	1	0	*K. pneumoniae*	1	0
A. xylosoxidans	1	8	*K. rhinophila*	1	0
B. cereo	25	13	*L. lactis*	1	0
B. cenocepacia	0	2	*M. luteus*	14	1
B. cereus	1	1	*Microbacterium sp.*	1	1
Bacillus spp.	1	2	*M. osloensis*	0	1
B. multivorans	0	1	*N. perflava*	1	0
Brevibacterium sp.	1	0	*Ochrobactrum sp.*	4	0
C. albicans	13	0	*P. aeruginosa*	7	11
C. indologenes	5	4	*R. mucilaginosa*	18	2
C. parapsilosis	9	0	*S. epidermidis*	19	4
E. faecium	0	1	*S. haemolyticus*	1	1
G. adiacens	2	0	*S. hominis*	2	0
G. haemolysans	0	1	*S. maltophilia*	12	8
Gemella sp.	4	0	*S. pasteuri*	0	1
L. limosus	1	4	*S. warneri*	0	1
			False Positives	146	68

Conclusions

chromID ELITE is an effective medium for the isolation of *S. aureus* with challenging clinical samples that frequently contain small colony variant isolates of *S. aureus* as well as a diversity of other species regularly isolated from CF patients including many with antibiotic resistance. It was notable that after 18 h incubation, chromID ELITE displayed equivalent sensitivity to chromID that had been incubated for 72 h. An additional advantage of chromID ELITE was that fewer false positive colonies were recovered. In terms of sensitivity and specificity, chromID ELITE is a superior option to chromID for recovery of *S. aureus* from respiratory samples from patients with CF.

Acknowledgements

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ABSTRACT NUMBER 61

Northumbria University Post Graduate Conference. May 2015

The Newcastle upon Tyne Hospitals NHS Foundation Trust

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A novel culture medium for recovery of rapidly-growing mycobacteria from the sputum of patients with cystic fibrosis

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Introduction

1

Background: Non-tuberculous mycobacteria (NTM) are ubiquitous environmental organisms found in soil and water that are increasingly associated with pulmonary infection in patients with cystic fibrosis (CF). Infection with NTM is difficult to recognise and treat [1] with prevalence in sputum estimated at 7 to 24% for patients with CF [2]. The predominant species of NTM within the CF population in the UK, Europe is *Mycobacterium abscessus* complex (MABSC) [3-5] which **comprises** three subspecies: *Mycobacterium abscessus*, *Mycobacterium bolletii* and *Mycobacterium massiliense*. Infection with MABSC is associated with a decline in lung function [3,6] and may cause severe complications post lung transplantation [7].

Purpose of the study: Detection of NTM is routinely achieved by culture of sputum onto both solid media, e.g. Lowenstein–Jensen medium [8], and liquid broth, e.g. the mycobacterial growth indicator tube (MGIT). Culture in a liquid broth medium can provide more rapid results as well as an increased yield of mycobacteria [9]. However the rate of contamination with non-mycobacterial species is greater and many samples are discarded due to the overgrowth of faster growing microorganisms that frequently colonize the lungs of CF patients. *Burkholderia cepacia* selective agar (BCSA) has been recommended as a convenient and effective culture medium for the isolation of rapidly-growing NTM, however, not all NTM will grow on BCSA and overgrowth, particularly by fungi and Gram-negative bacteria, remains a problem [10]. A novel selective culture medium (RGM medium) was developed and evaluated for the isolation of rapidly-growing NTM from the sputum of children and adults with CF.

Materials and Methods

2

A total of 115 isolates of rapidly-growing mycobacteria and 98 other bacteria and fungi were inoculated onto RGM medium after initial testing with a selection of growth factors and antimicrobials. These were assessed for growth at 30°C for seven days. A total of 502 consecutive sputum samples were collected from 210 patients with CF. Each sample was homogenized and cultured onto both RGM medium and BCSA (bioMérieux, France). Cultures were incubated for 10 days at 30°C and read at four, seven and ten days. All colonies on both media were identified using MALDI-TOF MS (Bruker, UK) and any NTM confirmed by *rpoB*, *hsp65* and *sodA* gene sequencing (Public Health, Colindale, UK)

Table 1: Mycobacteria recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BSCA) and RGM medium.

SPECIES	TOTAL	BCSA		RGM	
	(either medium)	*n*	Sensitivity (%)	*n*	Sensitivity (%)
M. abscessus	31	13	42	31	100
M. massiliense	11	3	33	11	100
M. chelonae	6	1	17	5	83
M. avium	2	0	0	2	100
M. llatzerense	2	0	0	2	100
M. salmoniphilum	2	0	0	2	100
M. mucogenicum	1	0	0	1	100
Total mycobacteria	55	17	31	54	98

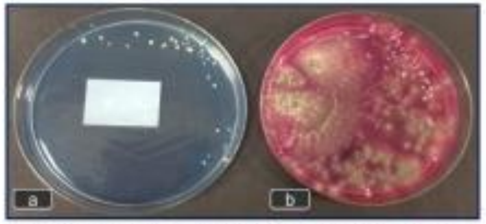

FIGURE: *M. abscessus* isolated from sputum shown as prominent white colonies on RGM medium (a) and unable to be detected on BCSA due to overgrowth of fungi and yeasts (b)

Table 2: Other species recovered from culture of 502 sputum samples on *Burkholderia cepacia* selective agar (BSCA) and RGM medium.

SPECIES	BCSA	RGM	SPECIES	BCSA	RGM
Number of isolates (n)			Number of isolates (n)		
Fungi and Yeasts	226	0	Gram negative	136	46
A. kalrae	2	0	*Pseudomonas* spp.	32	2
A. fumigatus	69	0	*B. cepacia* complex	30	18
A. terreus	6	0	*S. maltophilia*	24	0
Yeasts	121	0	*Achromobacter* spp.	21	18
E. dermatitidis	23	0	Enterobacteriaceae	14	2
S. apiospermum	5	0	*I. limosus*	4	2
Gram positive	57	0	*Ochrobactrum* spp.	4	0
Staphylococcus spp.	29	0	*Pandoraea* spp.	3	3
Enterococcus spp.	14	0	*A. hoeffii*	1	0
Streptococcus spp.	8	0	*M. radiotolerans*	1	0
G. adiacens	2	0	*R. radiobacter*	1	0
L. paracasei	2	0	*Sphingomonas* sp.	1	0
M. luteus	1	0	*D. acidovorans*	0	1
R. cyriacigeorgica	1	0	Total non-mycobacteria	419	46

Results

3

A total of 114 out of 115 mycobacteria grew on RGM medium within seven days of incubation, and only one isolate of *M. abscessus* failed to grow, but did grow on sub culture. Of 98 non-mycobacteria inoculated onto RGM medium, 92/98 (94%) were completely inhibited including all Gram-positive and fungal isolates. All 213 isolates grew well on control media.

Results with sputum samples showed that out of 502 samples tested from 210 distinct patients, 55 samples from 33 distinct patients yielded NTM giving an overall prevalence of 15.7%. Twenty one of the 210 patients were colonised with MABSC (prevalence: 10%). Table 1 shows the numbers of each species recovered by the two media. RGM medium enabled the detection of NTM from 54 of 55 positive samples whereas BCSA recovered NTM from 17 of 55 positive samples (sensitivity: 98% *vs.* 31%; $P \leq 0.0001$). For patients who had NTM found in their sputum ($n = 33$), 23 were detected using RGM only, one was detected using BCSA only (*M. chelonae*) and nine were detected using both media ($P \leq 0.0001$). A notable feature of RGM medium was its ability to prevent the growth of fungi and Gram-positive bacteria and no isolates of either of these groups were recovered from 502 sputum samples during ten days of incubation (Table 2).

Conclusions

4

RGM medium offers a simple and effective culture method for the isolation of rapidly-growing mycobacteria in sputum samples from patients with CF. This method eradicates the requirement of lengthy decontamination steps of samples prior to culture, which could potentially effect the viability of NTM. The availability of this medium allows for the systematic screening of all sputum samples routinely referred for culture from patients with CF.

Acknowledgements

5

The authors are grateful for the support provided by staff of the Freeman Hospital Microbiology Department, Newcastle upon Tyne, UK. We are also grateful to St. Vincent's University Hospital, Dublin, Republic of Ireland, and Public Health England, Newcastle upon Tyne, UK, for contributing isolates of mycobacteria.

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References

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SUNDAY-373

Evaluation of RGM Medium for Detection and Identification of Nontuberculous Mycobacteria from Patients with Cystic Fibrosis

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ABSTRACT (REVISED)

Objectives: We evaluated the identification of NTM on a novel selective medium (RGM) using MALDI-TOF MS and to determine the recovery of NTM on this medium from CF respiratory specimens. **Methods:** 41 archived isolates were grown on RGM and *Burkholderia cepacia* selective agar (BCSA). After 72-96h incubation at 30°C, archived isolates were identified by MALDI-TOF MS. 869 consecutive CF respiratory samples, including 177 specimens with concomitant AFB cultures (AFBC), were inoculated directly on RGM and BCSA and were observed for 4 weeks. MALDI-TOF MS or sequencing of 16s rRNA gene was used to identify the organisms. **Results:** In MALDI-TOF MS study, all NTM grew on both media could be correctly identified by MALDI-TOF MS, except 1 *M. immunogenum* on BCSA. RGM medium showed a significantly higher sensitivity (96.9%) for detection of mycobacteria in 869 samples than BCSA (34.7%) ($P = < 0.0001$). The sensitivity of RGM medium (93.2%) was also significantly higher than that of formal AFB culture (47.7%) when compared using a subset of 177 samples ($P = 0.0001$). **Conclusion:** RGM has a higher recovery rate of NTM and is more selective than BCSA. MALDI-TOF MS can be used to identify mycobacteria on RGM.

INTRODUCTION

- Nontuberculous mycobacteria (NTM) are pathogens in patients with cystic fibrosis (CF). Overgrowth of cultures with bacteria or fungi is a challenge for the recovery of NTM.
- RGM medium (RGM) is a novel selective agar containing OADC supplement and 4-antimicrobial mixture, and is designed to improve detection of NTM.
- We evaluated the identification of NTM on this medium using MALDI-TOF MS (bioMérieux) and recovery of NTM on RGM from CF respiratory specimens.

MATERIALS AND METHODS

- MALDI-TOF MS Study: 41 archived isolates (Table 1) were grown on RGM and *Burkholderia cepacia* selective agar (BCSA). After 72-96h incubation at 30°C, archived isolates were identified by MALDI-TOF MS.
- Clinical specimens: 869 Respiratory specimens (Table 2) from 493 CF patients were prospectively collected from December 2015 to April 2016. Specimens were directly plated on RGM and BCSA. On RGM and BCSA, growth was observed at 4, 7, 10, 14, 21 and 28 days of incubation at 30°C in air. AFB culture (177 samples from 159 patients) was inoculated into liquid (MGIT) and solid media (LJ) after double decontamination with NALC-NaOH and oxalic acid. AFBC was examined weekly for 8 weeks.
- Organism identification: MALDI-TOF MS (bioMérieux, Durham, NC) was used to identified RGM and bacteria. For mycobacteria, spectra were analyzed using bM Vitek MS research use-only system (RUO) with SARAMIS v4.12 database. Confidence value of ≥ 75% is acceptable. Sequencing of 16s rRNA gene was used to identified slow-growing mycobacteria.

RESULTS

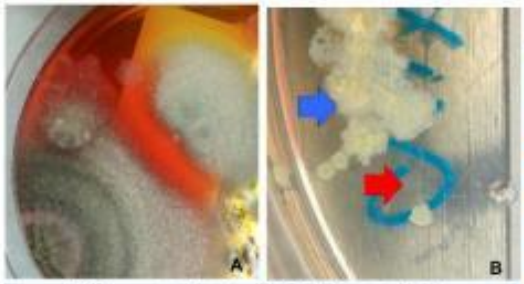

Figure 1: A) Overgrowth of mold on BCSA B) Colonies of *M. abscessus* complex (blue arrow) and *M. avium* complex (red arrow) on RGM

TABLE 1: Growth and identification using MALDI-TOF MS on 41 archived isolates grown on RGM and BCSA

Organism (n)	RGM		BCSA	
	Growth	ID	Growth	ID
M. abscessus subsp. *abscessus* (11)	11	11	10	10
M. abscessus subsp. *massiliense* (11)	11	11	11	11
M. chelonae (5)	5	5	5	5
M. fortuitum complex (6)	6	6	2	2
M. immunogenum (3)	3	3	3	2
M. mucogenicum (5)	5	5	1	1

TABLE 2: Type of specimens

Specimen Type	BCSA	AFBC
Total	869	177
Sputum/ tracheal aspirate	507 (58.4%)	133 (75.1%)
BAL fluid/ bronchial wash	48 (5.5%)	40(22.6%)
Deep pharyngeal swab	314 (36.1%)	4 (2.3%)

TABLE 3: Mycobacteria recovered on RGM, BCSA and AFBC from respiratory specimens of patients with CF

Organism	4-week RGM N(%)	Sensitivity (%)	4-week BCSA N(%)	Sensitivity (%)	Total N(%)	P-value	4-week RGM N(%)	Sensitivity (%)	4-week BCSA N(%)	Sensitivity (%)	8-Week AFBC N(%)	Sensitivity (%)	Total N(%)	P-value*
Mycobacteria	95 (10.9)	96.9	34 (3.9)	34.7	98 (11.3)	<0.0001	41 (23.2)	93.2	7 (3.9)	15.9	21 (11.9)	47.7	44 (24.9)	0.0001
M. abscessus complex	48 (5.5)	100	30 (3.4)	62.5	48 (5.5)	<0.0001	23 (13.0)	95.8	4(2.3)	16.7	16 (9.0)	66.7	24 (13.6)	0.046
M. chelonae	16(1.8)	100	1(0.1)	6.3	16(1.8)	0.0003	4(2.3)	100	2(1.1)	50.0	0	-	4(2.3)	0.134
M. immunogenum	8(0.9)	72.7	3(0.3)	27.3	11(1.3)	0.228	2(1.1)	66.7	1(0.6)	33.3	0	-	3(1.7)	0.479
M. avium complex	8(0.9)	100	0	-	8(0.9)	0.013	5(2.8)	-	0	-	5(2.8)		6(3.9)	0.489
M. mucogenicum	8(0.9)	100	0	-	8(0.9)	0.013	4(2.3)	-	0	-	0	-	4(2.3)	0.134
M. gordonae	4(0.5)	100	0	-	4(0.5)	0.134	3(1.7)		0	-	0	-	3(1.7)	0.248
M. arupense/ nonchromogenicum	1(0.1)	100	0	-	1(0.1)	-	0	-	0	-	0	-	0	-
M. nebraskense	1(0.1)	100	0	-	1(0.1)	-	0	-	0	-	0	-	0	-
M. lentiflavum	1(0.1)	100	0	-	1(0.1)	-	0	-	0	-	0	-	0	-

TABLE 4: Other species recovered on RGM, BCSA and AFBC from CF respiratory specimens

Organism	4-week RGM N(%)	4-week BCSA N(%)	Total N(%)	4-week RGM N(%)	4-week BCSA N(%)	8-Week AFBC N(%)	Total N(%)
Bacteria and fungi	95 (10.9)	243 (28.1)	254 (29.2)	22 (12.4)	87 (49.2)	73 (41.2)	89 (50.3)
Burkholderia spp.	42(4.8)	54(6.2)	54(6.2)	9(5.1)	9(5.1)	Overgrowth on AFBC •MGIT 6 (3.4) •LJ 45 (25.4) •Both 21 (11.9)	
Other nonfermenters	31(3.6)	57(6.6)	63(7.5)	9(5.1)	23(12.9)		
Enteric Gram-negative	1(0.1)	5(0.6)	5(0.6)	1(0.6)	2(1.1)		
Gram-positive	1(0.1)	6(0.7)	7(0.8)	0	2(1.1)		
Molds	0	55(6.3)	55(6.3)	0	25(14.1)		
Candida spp.	7(0.8)	55(6.3)	56(6.4)	0	21(11.9)		
Trichosporon spp.	13(1.5)	11(1.3)	14(1.6)	3(1.7)	5(2.8)		

*between 8-week AFBC and 4-week RGM

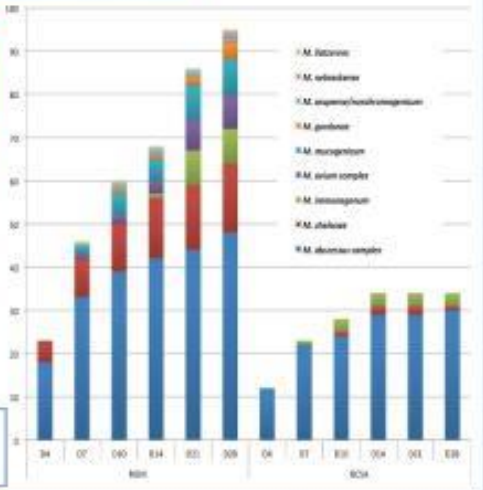

Figure 2: Recovery of mycobacteria on RGM and BCSA at different time points

CONCLUSIONS

- RGM has a higher NTM recovery rate and is more selective than BCSA. MALDI-TOF MS can be used to identify mycobacteria on RGM. There is a potential to obviate the AFBC. Significance of mycobacteria other than *M. abscessus* complex in CF needs to be studied. More samples are needed to justify direct recovery of *M. avium* complex on this medium.

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RGM: A new medium for isolation of Rapidly Growing Mycobacteria from respiratory samples of patients with cystic fibrosis
15 months experience of routine use for CF respiratory samples

CF Consortium meeting
29th November, 2016

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Background:
Non-tuberculous mycobacteria (NTM) are ubiquitous environmental organisms found in soil and water that are increasingly associated with pulmonary infection in patients with cystic fibrosis (CF). Infection with NTM is difficult to recognize and treat with prevalence in sputum estimated at 7 to 24% for patients with CF. The predominant species of NTM within the CF population in the UK, and several other European countries, is *Mycobacterium abscessus* complex (MABSC) which comprises three subspecies: *Mycobacterium abscessus* subsp. *abscessus*, *Mycobacterium abscessus* subsp. *bolletii* and *Mycobacterium abscessus* subsp. *massiliense*. Infection with MABSC is associated with a decline in lung function and it may cause severe complications post lung transplantation. There is also convincing evidence that the prevalence of infection by MABSC is increasing in the CF population.

New CF guidelines (2016)

Recommendation 1: The potential for cross infection with NTM (particularly *M. abscessus* complex) between individuals with CF should be minimized by following National Infection Control Guidelines.

Recommendation 2: Cultures for NTM should be performed annually in spontaneously expectorating individuals with a stable clinical course.

Recommendation 3: In the absence of clinical features suggestive of NTM pulmonary disease, individuals who are not capable of spontaneously producing sputum do not require screening cultures for NTM.

Recommendation 4: The CF Foundation and the ECFS recommend that culture and smears for acid fast bacilli from sputum should be used for NTM screening.

Recommendation 5: The CF Foundation and the ECFS recommend against the use of oro-pharyngeal swabs for NTM screening.

Purpose of the study:
We performed an audit of TB culture results from Newcastle PHE and up to 24% cultures were abandoned due to overgrowth of other CF pathogens. We therefore designed an in house media to overcome this problem. RGM is a new selective agar for isolation of NTM. This media is now routinely used in our laboratory for all CF respiratory samples including cough swabs. We have reviewed our data after 15 months of routine use.

Methods: Sputa are digested with sputasol, 10 µl is cultured onto ¼ RGM, cough swabs are cultured direct. Plates are incubated at 30°C for 10 days. Isolates are Initially identification by AFB stain and MALDI MS TOF (Bruker); first and second isolates are confirmed by *rpoB/sodA/hsp65* sequence cluster analysis and VNTR strain typing at Colindale.

Results: see table 1.

Table 1. Mycobacteria recovered from 4408 respiratory samples submitted by patients with CF using RGM medium and comparison with surveillance using formal AFB culture.

	Number of samples and patients (*n*) tested using RGM medium.						No. of patients tested (*n*) using formal AFB culture
	Total	Cough Swabs	Sputa	BAL	Miscellaneous	Patients	
	4408	2443	1557	131	277	625	213[a]
Total mycobacteria	195	46	133	4	12	56	22[b]
M. abscessus complex	168	40	114	2	12	34	13
M. chelonae	10	2	8	0	0	9	3
M. fortuitum	2	0	2	0	0	1	0
M. avium complex*	0	0	0	0	0	0	6
M. mucogenicum	1	0	1	0	0	1	0
*M. tuberculosis**	0	0	0	0	0	0	1
Other *Mycobacterium* species	14	4	8	2	0	11	0

Discussion The new CF guidelines do not recommend cough swabs for NTM culture as they are unsuitable for TB culture systems. Using RGM agar NTM was first detected from 15 patients using cough swabs. NTM were detected from more than twice as many patients (56 vs. 22) by routine use of RGM rather than relying on annual submission of specimens for formal AFB culture There is the potential for cross infection with MABSC and if an annual screening sample is all that is submitted then patients not known to be colonized may act as reservoirs for MABSC. The prevalence of MABSC is higher than *B cepacia* complex (BCC) and these species are isolated using BCC selective agars used routinely for all CF samples yet routine culture for NTM is not performed. RGM is a highly selective agar that can be incorporated into routine use for all CF respiratory samples for the isolation of NTM and without the need for expensive, laborious TB culture systems.

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