Cracking the resistance of non-tuberculosis mycobacterium against existing antibiotics

A thesis submitted in partial fulfilment

of the requirements for the degree

of

Masters of Science

in Biological Sciences

at

The University of Waikato

by

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The University of Waikato

2015

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

Abstract

Mycobacterium avium (*M. avium*) is quickly becoming the most prevalent *Mycobacterium* infection in western countries. *M. avium* is an opportunistic pulmonary pathogen, infecting patients who often are vulnerable due to existing medical conditions. Antibiotic therapy of *M. avium* is long (generally 18 months) and often unsuccessful at eliminating the infection, promoting resistance to commonly used antibiotics, such as clarithromycin.

M. avium exists as a biofilm in lung infections, adding to the difficulty in eradicating the infection. My research investigated potential *M. avium* biofilm dispersing agents, as well as antibiotic synergy for improved *M. avium* eradication.

Our findings show that ten clinically isolated *M. avium* samples were all highly resistant (>16µg/mL) to antibiotics commonly used in the treatment of *M. avium* infections, both in planktonic and biofilm phenotypes. Some isolates were found to have MICs of >128µg/mL.

M. avium isolates differed in biofilm phenotype, and correspondingly, their susceptibility to antibiotics and potential biofilm dispersing agents.

No antibiotics tested during this research had significant bactericidal or bacteriostatic action on any *M. avium* isolates, in planktonic or biofilm phenotype.

The addition of 3.6mg/mL aspirin (acetylsalicylic acid) or 3.6mg/mL ibuprofen to planktonic and biofilm cells showed significant increase in *M. avium* cell death. Previously identified biofilm dispersing agents, such as mannitol, N-acetyl-L-cysteine, active manuka honey, methylglyoxal, Lipitor®, 2,4-dinitrophenol and EDTA, had no significant effect on dispersing *M. avium* biofilms or increasing their susceptibility to

antibiotics. A concentrations of 4mg/mL aspirin combined with 8mg/mL of EDTA had significant effects, sterilizing and completely eradicating *in vitro* three week old mature *M. avium* biofilms.

Future work should be conducted into the specific pathways aspirin and ibuprofen effect. Furthermore, development of effective agonists/antagonists of those pathways to disperse *M. avium* biofilms should be researched. Research into improved and novel *Mycobacterial* antibiotics are needed to develop drugs capable of successfully treating *M. avium* infections.

Acknowledgements

"The LORD is my strength and my song; he has become my salvation."

Psalm 118:14

Firstly, I would like to humbly thank my supervisor Dr. Ray Cursons, who has helped me beyond measure with my thesis. Thank you for dedicating your time, effort and brilliant mind to helping me achieve something worth writing about. I will always be grateful to have had you as my supervisor and mentor. Thank you.

I would like to thank the wonderful people of the Molecular Genetics and Proteins Department at the University of Waikato: Dr. Judith Burrows, Dr. Steve Bird, Dr. Linda Peters, Dr. Greg Jacobson, Olivia Patty, and Sari Karppinen. Thank you for everything you have done to make me laugh and support my education.

I would like to thank the organisations that have generously provided funding for my Masters Research, namely the Waikato Hospital Research Grant and the Dick Wilkins Scholarship, without which, I would not have been able to complete my Masters Degree.

Many thanks to Dr. Noel Karalus, and the Waikato Hospital for their collection and generous donation of *Mycobacterium avium* cultures and antibiotics for the use in this research. Without you, nothing would have been achieved!

Without the help of Helen Turner, I would have never been able to operate the SEM. Thank you for all of your training, tireless help, sample preparation and for showing me who I want to be like when I grow up.

With all of my heart I wish to thank my fellow Masters and Doctorate students; Kirsty Mayall, Nick Elliott, Sarah Hardie, Laura Bell, Grant Broomfield, Heng Zhang, Steve Evans, Ron Blaza, Cass Parker, Alice Wang, Nikki Webb, Brooke Wilson and Alice Chibnall. Thank you so much, I will remember these years for the rest of my life. Thank you for keeping my sanity in check, and together making it out the other side. I will miss your love of life and desire to achieve more out of the world.

To my family, I cannot express words for my gratitude or your generosity and love. Supporting me through five years of university, all I can say is how much I love you, and have missed you these years away from home.

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Abbreviations

Abbreviation	Term
°C	Degrees Celsius
3’	Three prime DNA end
5’	Five prime DNA end
ACES	N-(2-Acetamido)-2-aminoethanesulfonic acid.
AI	Autoinducer
aspirin	Acetylsalicylic acid
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pair(s)
DMF	*N*,*N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide
DSF	Diffusible Signalling Factors
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMA	Ethidium monoazide
et al.	And others
EtBr	Ethidium bromide
EtOH	Ethanol
HCl	Hydrochloric acid
ITS	Internal Transcribed Spacer
kb	Kilobase(s)
K. pneumoniae	*Klebsiella pneumoniae*
L	Litre(s)

M	Molar
M. avium	*Mycobacterium avium*
M. intracellulare	*Mycobacterium intracellulare*
M. tuberculosis	*Mycobacterium tuberculosis*
mA	Milliampere(s)
MAC	*Mycobacterium avium* Complex
mg	Milligram(s)
MIC	Minimum Inhibitory Concentration
mL	Millilitre(s)
mM	Millimolar
MPI	Ministry of Primary Industries
mQH2O	milliQ water
mRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Millivolt(s)
Na	Sodium
NaOH	Sodium hydroxide
NCBI	National centre for biotechnology information
ng	Nanogram(s)
nm	Nanometre(s)
NO	Nitric oxide
OD	Optical density
P. aeruginosa	*Psuedomonas aeruginosa*
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethalene glycol
pMol	Picomole(s)
QS	Quorum sensing

RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RT	Room temperature
RT-PCR	Real-time PCR
S. aureus	*Staphylococcus aureus*
S. epidermis	*Staphylococcus epidermis*
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
STC	2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride
subsp.	Sub-species
TPF	Triphenylformazan
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
Tween80	Polysorbate 80
U	Enzyme units
UV	Ultra violet
V	Volt(s)
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide
µL	Microlitre(s)
µm	Micrometre(s)

Chapter One

1. Literature Review and Introduction

1.1 Introduction

There are over 120 species of *Mycobacterium* known*,* including some of the most infectious pathogens (Zakham *et al.,* 2012). *Mycobacterium tuberculosis (M. tuberculosis)* is the cause of the devastating disease tuberculosis, however, other clinical species of *Mycobacteria such as Mycobacterium avium* (*M. avium*) and *Mycobacterium intracellulare* (*M. intracellulare*) also exist, and are gaining more attention for their emerging pathogenicity.

These two non-tuberculosis *Mycobacterium* often co-exist, and are extremely difficult to distinguish between. They are often studied in unison, and are referred to in literature as the *M. avium* complex (MAC). MAC are opportunistic pulmonary pathogens (Flores *et al.,* 2005) which commonly infect immunologically compromised individuals or patients with prior lung damage (Thoen *et al.,* 1981). *M. avium* is globally distributed, and isolates have been obtained from fresh and salt water as well as soils, house dust, bedding and rain water tanks (Inderlied *et al.,* 1993; Starkova *et al.,* 2013). MAC can infect both humans and animals, however, it has been suggested that it cannot easily be transmitted between humans due to its ubiquitous distribution and low clinical infection rate (Edwards *et al.,* 1969; Thoen *et al.,* 1981).

1.2 *Mycobacterium avium*

The *Mycobacterium* genus have an unusual cell wall composition, made up of long-chain mycolic acids and high G+C content DNA (Stinear *et al.,* 2004), as well as unique lipids such as lipoarabinomannan and trehalose dimycolate (Banerjee *et al.,* 2011; Ingen *et al.,* 2012). This hydrophobic cell wall makes permeability of antibiotics difficult and thus is a crucial factor in *Mycobacteria* drug resistance (Bosne-David *et al.,* 2000). *M. avium* is a gram-positive, slow growing organism (Starkova *et al.,* 2013), reaching a stationary phase (concentration of 10^8- 10^9cfu/mL) in Middlebrook 7H9 media after seven days (Do *et al.,* 2014). Colony growth in *M. avium* can be observed as smooth or rough colonies, with the rough phenotype correlating to increased resistance to antibiotics (Ingen *et al.,* 2012). The main source of MAC infection is thought to be from infected domestic birds, hence the species name "avium" (Kaevska *et al.,* 2011).

1.3 Diagnosis of MAC and *Mycobacterium avium*

For fully confirmable results colonies can take up to two weeks for substantial growth, which provides opportunity for contamination (Wu *et al.,* 2009). Identification of MAC infections are diagnosed via microbiological data, histopathic studies and observation of their pigment and growth characteristics (Wu *et al.,* 2009). The inefficient turnaround time for positive identification of Mycobacteria, however, resulted in efforts to find a faster diagnostic technique (Bensi *et al.,* 2013). In research today, the identification of MAC is most commonly performed via polymerase chain reaction (PCR) methods, where *M. avium* and *M. intracellulare* can be separately identified. In mycobacterial genomes, an insertion element called the IS region is particularly important for effecting the virulence of infectious species. IS regions are mobile DNA elements, roughly 1.5kb long, repeated throughout the genome, which encode for a transposase enzyme (Stinear *et*

al., 2004). They are often seen throughout bacterial species suggesting that they are easily exchanged between bacteria, however, in some mycobacterial species, they have remained conserved and can therefore be successfully used as identifying markers for species and sub-species (subsp.). MAC can be identified through IS regions IS*901,* IS*1245* and IS*1311* (Stinear *et al.,* 2004), and further classified into sub species of *M. avium* by the identification of IS regions. *M. avium* subsp. *hominissuis* contain several copies of IS*1245*, but lack IS*901*, whereas *M. avium* subsp. *avium* contain only one copy of IS*1245*, but multiple copies of IS*901* (Guerrero *et al.,* 1995; Johansen *et al.,* 2007; Domingos *et al.,* 2009; Kaevska *et al.,* 2011). Several other studies have also shown that other genetic markers can be used in the identification of MAC such as housekeeping genes, gyrA, gryB (Fukushima *et al.,* 2003*)* and hsp65 (Bensi *et al.,* 2013) as well as 16S-23S internal transcribed spacer (ITS) region, and 16S ribosomal RNA (rRNA) gene (Wu *et al.,* 2009; Radomski *et al.,* 2010 Hashemi-Shahraki *et al.,* 2013). Molecular targets DT1 and DT6 have also been used to identify *M. avium* (Iamsawat *et al.,* 2010).

1.4 *Mycobacterium avium* Infection, Aetiology and Pathophysiology

In New Zealand, MAC infections are the most common non-tuberculosis infections (Freeman *et al.*, 2007). Common symptoms of a MAC infection include cough, fever and weight loss, which are similar to other *Mycobacterium* infections (Tran *et al.,* 2014). MAC organisms can be isolated from sputum samples and have been described frequently as a low-grade infection, colonizing the lungs rather than becoming a true disease (Interlied *et al.,* 1993). However it is suggested that in developed countries where tuberculosis occurrence is low, MAC infections can be more common and lead to death if left untreated (Tran *et al.,* 2014). *M. avium* has also been isolated from broncho-alveolar lavage, bone marrow, faeces and blood samples (Bensi *et al.,* 2013).

The primary route of MAC infection in AIDS patients is the intestinal tract (Klatt *et al.,* 1987; Wallace & Hannah, 1988) followed by the respiratory tract (Agins *et al.,* 1989). MAC can also cause soft tissue infection and lymphadenitis, especially in patients under the age of five, which can result in surgical removal of the cervical lymph nodes (Freeman *et al.,* 2007; Kaevska *et al.,* 2011). This drastic procedure is often the last resort when the MAC infection does not respond to antibiotic treatment methods (Wu *et al.,* 2009). Left untreated, MAC infections eventually disseminate throughout the host. Unfortunately, due to its low person-to-person transmission rate, research into MAC infections and their effective treatment has often been side-lined (Wu *et al.,* 2009).

1.5 *Mycobacterium avium* Incidence

MAC infection occurs more commonly in adults (Inderlied *et al.*, 1993) especially in elderly patients, and more frequently in women than men (Freeman *et al.,* 2007). MAC infection is also more prominent in Caucasian ethnicities than any other (Inderlied *et al.,* 1993). The species composition of MAC populations in patients appears to differ based on environment (Inderlied *et al.,* 1993). The most common source of MAC infection seems to be from inhalation or ingestion of water (Radomski *et al.,* 2010). MAC has been isolated from water filtering systems, hospital water supplies, drinking water supplies, and natural aquatic ecosystems (Radomski *et al.,* 2010).

Before the emergence of AIDS, chronic lung infection was the most common symptom of MAC infection and only a handful of MAC cases were reported throughout the 1940's-1960's. (Feldman *et al.,* 1943; Crow *et al.,* 1957; Lewis *et al.,* 1960). Although the human to human transmission factor is low, MAC infections are more prevalent among AIDS patients and patients with previous lung damage (Freemen *et al.,* 2007). It is also suggested that HIV-infected patients who do not succumb to other illnesses will

eventually die due to disseminated MAC infection (Chaisson *et al*., 1992; Nightingale et al., 1992). Patients with hairy cell leukaemia, cystic fibrosis (Kilby *et al.,* 1992) and previous pulmonary disease are also more frequently infected with MAC (Bennett *et al.,* 1986; Bouza *et al.,* 1978; Etzkorn *et al.,* 1986). Patients with suppressed immune systems, such as organ transplant patients, are also at risk of MAC infection (Wu *et al.,* 2009).

1.6 Treatment of *Mycobacterium avium*

Several antibiotic agents are currently used to combat MAC infections, however, in depth studies into the minimum inhibitory concentration (MIC) of antibiotics and synergy between antibiotics that are clinically used to treat *M. avium* infections are lacking.

Drug therapy is the most common treatment of *M. avium* infections, yet it is an extremely long treatment course, that consists of several months of therapy, often with reoccurrence of the infection towards the end of the course. Ingen *et al.* (2012) reports that a recommended regime is a macrolide, ethambutol, and rifampicin administered over 18-24 months, with potential use of amikacin for the first 3-6 months. This is undesirable due to the toxicity and potential side effects from pro-longed antibiotic use.

1.7.1 Antibiotic MICs Against *Mycobacterium avium*

Clarithromycin and azithromycin are both effective macrolides against Mycobacteria (Inderlied, & Salfinger, 1995) with clarithromycin being the most commonly used primary antibiotic to treat *M. avium* (Park *et al.,* 2009). However, patients receiving long term macrolide treatment report resistance to macrolides occurring during therapy

(Meier *et al*., 1994; Nash & Inderlied, 1995; Wallace Jr *et al*., 1996). Park *et al.,* (2009) showed that out of 56 *M. avium* isolates tested, three isolates had MIC's of over 64µg/mL - although the majority had MICs of below 2µg/mL (53 out of 56). Similarly, Inagaki *et al.* (2011) showed that out of 245 isolates, 219 strains were clarithromycin susceptible with broth MICs of under 8µg/mL, while 26 strains had MICs of over 32µg/mL. Lee *et al.* (2011) determined that the clinically significant resistance to clarithromycin is an MIC above 32µg/mL. Clarithromycin has shown to be effective against *M. smegmatis, M. avium* and *M. tuberculosis,* potentially suggesting that they share a similar active site (Bosne-David *et al.,* 2000). Although clarithromycin is shown to currently be the most effective antibiotic against *M. avium*, monotherapy will lead to the development of resistance bacteria and more efficient multidrug- therapy regimes need to be investigated (Inagaki *et al.,* 2011).

In a study conducted by Homach *et al.* (2013) the MIC_{90} of antibiotics against 58 *M. avium* isolates grown in MGIT were determined. MIC_{90} was defined as the antibiotic concentration needed to inhibit 90% of the isolates. Clarithromycin was again found be the most effective antibiotic against *M. avium,* while Ofloxacin was found to be potentially ineffective at treating MAC infections (Homach *et al.,* 2013).

Table 1. MIC_{90} results from Homach *et al.* (2013).

Antibiotic	MIC_{90} (µg/mL)
Amikacin	≤20
Clarithromycin	≤4
Ethambutol	>25
Moxifloxacin	≤2
Ofloxacin	>10
Rifabutin	≤2
Rifampicin	>10

Homach *et al.* (2013) admit that *in vitro* data into *M. avium* drug susceptibility is severely lacking, with non-uniform methods of MIC analysis, and little research into the synergistic properties of combining antimycobacterial drugs. These reasons could account for the lack of uniform MIC's recorded for *M. avium.* Due to the wide variety of MIC values determined for *M. avium* isolates, Heifets (1988) suggested that extremely resistant isolates had broth MIC values of greater than 16µg/mL.

Ethambutol has also been used in the effective treatment of the MAC complex (Hoffner *et al.*, 1987) with evidence to suggest that it inhibits arabinan biosynthesis and disrupts the arabinogalactan structure within the cell wall (Takayama & Kilburn, 1989: Mikusovà *et al.,* 1995). Other studies suggest that ethambutol also has non-lethal effects which can alter cell wall permeability. (Bosne-David *et al.,* 2000). However, liver and vision problems are associated with long term ethambutol treatment. Jadaun *et al.* (2007) determined that only 35% of *M. avium* isolates tested had MICs of 4µg/mL. However, no direct data has been provided comparing *in vitro* MIC results to actual clinical outcomes (Homach *et al.,* 2013).

Isoniazid is commonly used in the treatment of tuberculosis, although its activity against *M. avium* is not as effective, as well as isoniazid having hepatotoxic effects and being poorly tolerated by some patients reduces its potential use in MAC infections (Primm & Franzblau, 2007).

Fluoroquinolones, such as moxifloxacin and ciprofloxacin, are also commonly used in the treatment of *Mycobacterium.* Their activity is against the bacterial enzyme DNA gyrase, inhibiting the separation of DNA strains and thus cell replication.

Amikacin is a last resort aminoglycoside, used when all other antibiotics have failed in MAC treatment. It causes permanent ototoxicity and thus is not commonly used, to avoid this side-effect. Amikacin binds to the 30s bacterial ribosomal subunit causing

messenger RNA (mRNA) to be misread, corrupting protein synthesis. Brown-Elliot *et al.* (2013) studied the *in vitro* MIC of amikacin against MAC isolates. Of the isolates 96.2% had MICs of ≤32µg/mL, however, this data was across MAC isolates and not specifically for *M. avium* isolates. Interestingly, increased exposure to amikacin resulted in a repeatable point mutation (A to G) at position 1408 within the 16S rRNA of all MAC isolates (Brown-Elliot *et al.,* 2013). Ingen *et al.* (2012) showed that with the addition of clofazimine (often used as a replacement for rifampicin in MAC infections), showed synergy with amikacin, reducing the MIC of MAC isolates from above 16µg/mL, to 4µg/mL.

1.7.2 Antibiotic Resistance in *Mycobacterium avium*

MAC is resistant to most traditional antimycobacterial agents such as those used to treat tuberculosis (Mizuguchi *et al.,* 1983; Good, 1985; Primm & Franzblau, 2007; Tran *et al.,* 2014). The complex cell wall structure aids in antibiotic inhibition (Rastogi *et al.*, 1989; Rastogi *et al.*, 1990) as well as the ability of MACs to produce three types of lactamases (Mizuguchi *et al.,* 1985), making them resistant to classical β-lactam class antibiotics (Flores *et al.,* 2005). MAC isolates from infected AIDS patients were found to be more resistant to antibiotics than other isolates from non-AIDS patients (Inderlied *et al.,* 1993).

The maintenance of the cell wall is attributed to *Mav2520* and *pks12*, and disruption of these genes leads to increased permeability of the cell wall and increases the susceptibility of *M. avium* bacteria to lipophilic antibiotics (Ingen *et al.,* 2012). The discovery of polyketide synthase (Pks12) in *M. avium* strains has been shown to increase drug resistance. Matsunaga *et al.* (2012) showed that Pks12 is positively correlated with drug tolerance and also suggest that Pks12 mediates drug resistance through

intracellular drug accumulation. It is well known that mutations in the 23S rRNA can affect membrane permeability (Lee *et al.,* 2011) and transmembrane proteins, such as drug efflux pumps which are associated with bacteria resistance (Fierro *et al.,* 1988; Coleman *et al.,* 1994). Ingen *et al.* (2012) reported the finding of drug efflux pumps, suggesting that are found across all *Mycobacteria* species, and confer resistance to rifampicin, tetracyclines, and macrolides. In *M. avium* isolates with high levels of resistance to clarithromycin, point mutations at 2058 or 2059 within the V domain of the 23S rRNA gene have been identified (Inagaki *et al.,* 2011; Christianson *et al.,* 2013). This is similar to macrolide resistance associated with point mutations at 2275 or 2275 within the 23S rRNA gene in *M. intracelullare* (Iamsawat *et al.,* 2010). The genome of *M. avium* is circular and comprised of single stranded DNA, approximately 5.4 x 10^6 base pairs (bp) in length (Inderlied *et al.,* 1993). However, extra chromosomal DNA in self-replicating plasmids are common in MACs. (Crawford *et al.* 1984; Crawford *et al.* 1986). An insertion sequence in these plasmids was reported in antibiotic resistant pathogenic *M. avium* strains (Kunze *et al.,* 1991) while this insertion sequence was absent in non-resistant strains.

The use of non-lethal, cell wall-disrupting factors have provided a way around drug resistance, acting by disrupting the layering of cell wall components. This was demonstrated by Bosne-David *et al.* (2000) who reversed clarithromycin resistance in *M. tuberculosis* through the addition of sub-inhibitory cell wall inhibitors. Reduced concentrations of antibiotics, although sub-lethal, may influence the components of the cell wall, and therefore reduce cell competence leading to eventual cell death (Bosne-David *et al.,* 2000).

Despite the increasing resistance and presence of highly macrolide resistant *M. avium* isolates, no new drugs targeting *Mycobacteria* have been developed since the 1960s (Primm & Franzblau, 2007).

1.8 Biofilms

It is surmised that in nature, bacteria do not freely exist in planktonic populations. Rather, they survive via aggregating together and forming a community surrounded by a matrix of polysaccharides, protein and extracellular DNA (Estrela & Abraham, 2010; Islam *et al.,* 2012) which allows the community of bacteria to adhere to a surface (Taraskiewicz *et al.,* 2013). This biofilm formation is a survival mechanism for bacteria in natural environments, which offers protection against environmental conditions as well as enhanced gathering of nutrients in sub-optimal conditions (Hall- Stoodley *et al.,* 2004). Biofilms are dynamic, constantly moving to allow aeration and nutrient gathering to all members of the biofilm population which can be comprised of a single species, or a community of micro-organisms (Islam *et al.,* 2012; Taraskiewicz *et al.,* 2013). The extracellular matrix of the biofilm in pathogenic, biofilm forming bacteria allows for defence against a host's immunological response, and chronic infection with biofilms causes a similar response as inflammation and tissue damage (Islam *et al.,* 2012). Biofilms are often formed in response to environmental stresses (Geier *et al.,* 2008).

1.8.1 Biofilm Formation

The formation of biofilms can be grouped into three stages. The first stage begins when planktonic cells attach to a surface. Following this, the cells begin to form an extracellular matrix which cause clumping of single cells into aggregates (figure 1). Studies have shown that at this early biofilm stage antibiotics are still effective in clearing the aggregating cells and prevent development of mature biofilms (Taraskiewicz

et al., 2013). After attachment and aggregation, biofilm maturation occurs, with the cells shifting from a planktonic to sedentary lifestyle (Islam *et al.,* 2012).

However, after the three stages of biofilm formation, detachment occurs. This is when bacteria cells within the biofilm are released and return to a planktonic lifestyle, often forming biofilms where they come into contact with another un-colonised surface resulting in disseminated infection in patients.

Biofilm formation occurs via cell signalling, or quorum-sensing (QS). QS occurs when a threshold concentration of an autoinducer (AI), a molecule or small peptide, is reached and planktonic cells begin to adhere to a surface and change gene expression to promote biofilm conditions (Schuster *et al.,* 2003; Taraskiewicz *et al.,* 2013). The most studied AIs include autoinducer-1 and -2 (AI-1, AI-2), *N*-acyl-L-homoserine lactones (AHL1-6) or small AI peptides (AIP) (Estrela & Abraham, 2010). Due to the nature of biofilms generally being a mixed species population, QS molecules and AIs can be detected across a wide range of bacterial species (Keller & Surette, 2006). QS in bacterial populations is extremely complex, with multiple regulatory pathways being involved (Wagner *et al.,* 2004). QS has been implicated as the same mechanism that reverses the formation process, promoting biofilm dispersal (Taraskiewicz *et al.,* 2013). This complexity may explain why previous studies into blocking QS mechanisms does not completely eradicate biofilm formation.

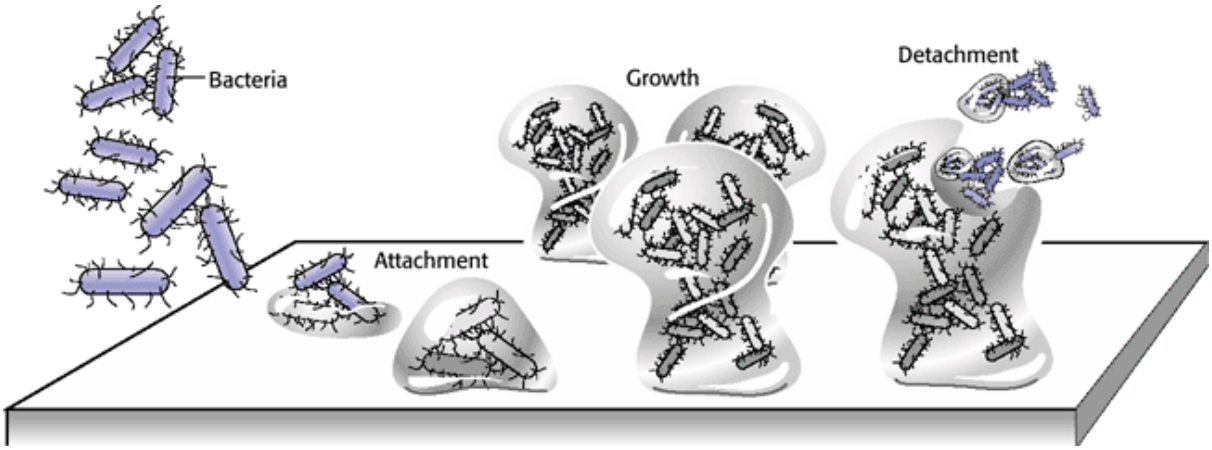


Figure 1. Moholkar, K. & Ziran, B. Illustration of the biofilm bacterial colonization process. From Vermeer and the invention of seeing (p. 572) by Bryan Jay Wolf, 2006, Rockwood & Green's Fractures in Adults 6th Ed. Philadelphia: Lippincott Williams & Wilkins.

1.8.2 Biofilms in Clinical Settings

The clinical significance of biofilms is becoming more widely recognised as a hindrance to the effective treatment of bacterial infections. Both antibiotic resistance and the ability to continuously re-infect patients can be attributed to the formation of biofilms (Keays *et al.,* 2008: Estrela & Abraham, 2010). Such developments in cystic fibrosis, (Keays *et al.,* 2008) have shown that antibiotic resistance of respiratory infections plays a vital role in the chronic reinfection and inability to eradicate infection. The formation of biofilms on diverse surfaces such as implants, contact lenses, medical devices, wounds, lung epithelium and other tissues (Ramage *et al.,* 2007; Islam *et al.,* 2012), means that biofilms have become increasingly prevalent within clinical settings and can prove to be challenging and costly to treat. Catheters are a potentially dangerous source of infection with biofilms colonising tubes that feed directly into the host (Falkinham, 2007) and resisting any antibiotic effort to clear them. The release of planktonic cells through dispersal phase of a biofilm can be effectively treated, however, actual eradication of a biofilm within a patient is rarely achieved (Taraskiewicz *et al.,* 2013). It has also been shown that planktonic cultures have a much lower resistance to

antibiotics with biofilms showing between 10-100 fold increases in resistance (Keays *et al.,* 2008). This suggests that original MIC's of broth cultivated bacteria is inaccurate in representing actual dosage required to treat infections *in vitro*. Although it is still largely unknown why biofilms exhibit much higher MICs (Estrela & Abraham, 2010), it has been shown that cystic fibrosis patients who received antibiotics which fought biofilm formation had improved clinical outcomes (Keays *et al.,* 2008).

1.8.3 Antibiotic Resistance Mechanisms of Biofilms

Physically, biofilms provide natural antibiotic resistance purely via 'safety in numbers' as seen below (figure 2). Extracellular matrix results in a slower penetration of antibiotics, allowing bacterial cells to elicit stress responses such as up-regulation of antibiotic pump expression (McNabe *et al.,* 2011) and effectively avoid antibiotic induced cell death. Furthermore, the microenvironment within the biofilm is altered, thus altering conditions at which the antibiotics interact with bacterial cells. Subtle changes in pH, temperature and oxygen availability can affect the kill rate of antibiotics (Taraskiewicz *et al.,* 2013). These three mechanisms allow for the development of biofilm persister cells that lead to redevelopment of biofilms after antibiotic treatment. It is suggested that these persister cells exist in particularly nutrient depleted parts of the biofilm,and in their starved state, adopt a dormancy-like stationary metabolism that is highly resistant to antibiotics (as most antibiotics act on actively dividing cells) (McNabe *et al.,* 2011; Taraskiewicz *et al.,* 2013).

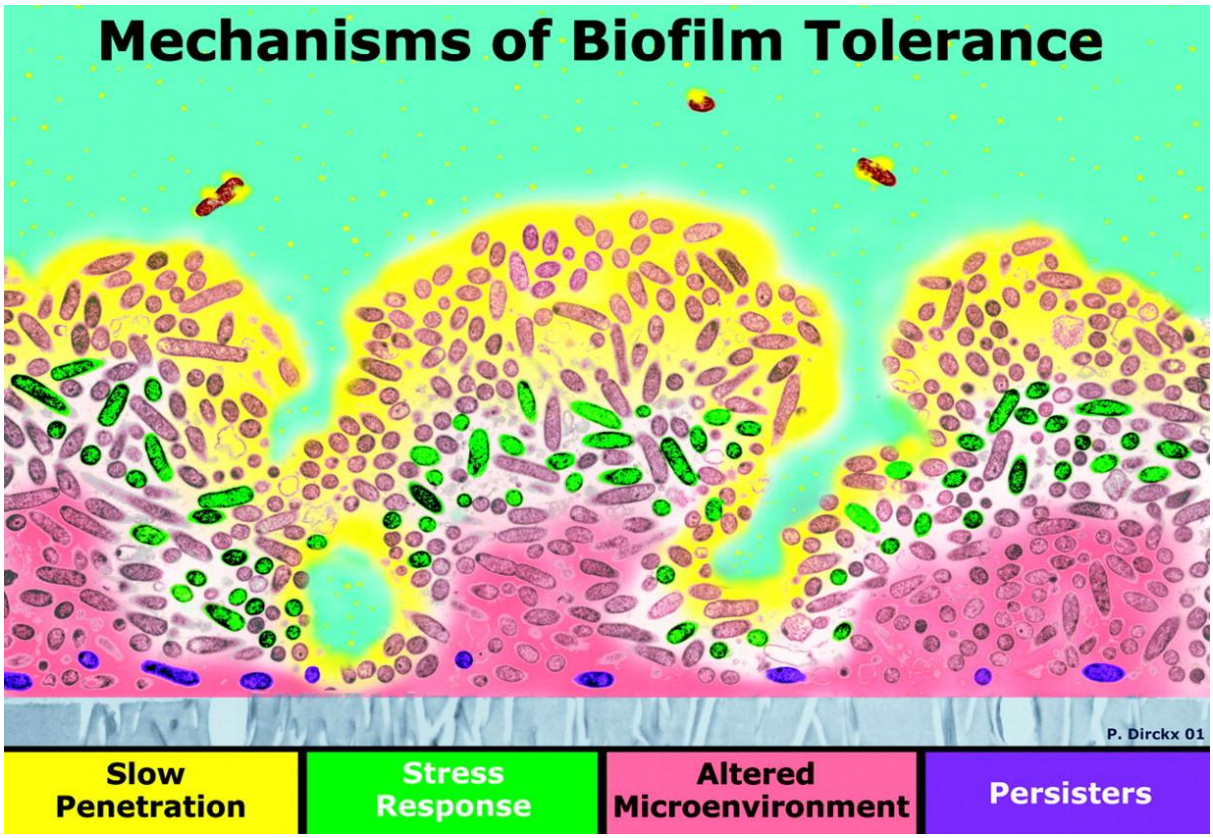


Figure 2. Chambless *et al*., (2006), Four possible mechanisms of biofilm antibiotic resistance, Retrieved January 6, 2015, from Journal of Applied Environmental Microbiology website: http://aem.asm.org/content/72/3/2005/F1.expansion.html

This structure of a biofilm plays a vital role in their antibiotic resistance and especially to high dosages of drugs (Estrela & Abraham, 2010). The control of biofilms can be separated into three categories; Metabolites interfering with the stabilization and formation of biofilms by interfering with AIs; the addition of cleaving-enzymes that compromise the polysaccharide and/or DNA elements of the biofilm matrix; and the disruption of biofilm formation signalling molecules that cause dispersal of the biofilm, reverting cells back into a planktonic lifestyle (Estrela & Abraham, 2010).

1.8.4 Biofilm Dispersal Agents

Recent focus has also shifted onto compounds that cause biofilm dispersal, especially in clinical settings where biofilms have emerged as being extremely difficult to treat. A non-toxic biofilm dispersing agent which would be able to be pumped through catheters to clear and prevent biofilms would demonstrate extreme efficiency in hospitals. The development of biofilms on implants is also a great hindrance to patients, and an orally taken biofilm dispersing agent could greatly decrease the post-operation complications with implants.

Nitric oxide (NO) has been found to disperse biofilms in gram-positive bacteria-particularly in *Psuedomonas aeruginosa* (*P. aeruginosa*) (Nabloa *et al.,* 2005; Schlag *et al.,* 2007; Hetrick *et al.,* 2009). The addition of chelating agents, such as ethylenediaminetetraacetic acid (EDTA), have been shown to have some biofilm dispersing activity (Ramage *et al.,* 2007; Liaqat *et al.,* 2010; Robertson *et al.,* 2012; Cavaliere *et al.,* 2014). Acetylsalicylic acid (aspirin) combined with EDTA showed dramatic effects on established *P. aeruginosa* biofilms, completely eradicating existing biofilms (Al-Bakri *et al.,* 2009). N-acetyl-L-cysteine and ibuprofen were shown to decrease biofilm formation in *Escherichia coli* (*E. coli)* strains (Naves *et al.,* 2010). Certain fatty acids have also been found to possess biofilm dispersal capabilities across species, and interestingly, induce biofilm dispersal in species that do not intrinsically produce these fatty acids (Davies & Marques, 2009). Several fatty acids from the opportunistic pathogen *Stenotrophomonas maltophila* have been found to be diffusible signalling factors (DSF) that caused a reduction in biofilm formation, and inhibited swarming in mixed-culture populations (Huang & Wong*,* 2007). In addition to this, enzymes have also been explored for their antibiofilm properties. The addition of DNase I to *P. aeruginosa* biofilms in cystic fibrosis patients showed a decrease in thickness of the sputum, and is thought to act on the extracellular DNA exposed in the biofilm matrix, weakening the

biofilm (Whitchurch *et al.,* 2002). Lysostaphin, an endopeptidase, was also found to effectively penetrate biofilms of *Staphylococcus aureus* (*S. aureus*) and *Staphylococcus epidermis* (*S. epidermis*) (Walencka *et al.,* 2005; Kokai-Kun *et al.,* 2009; Belyanksky *et al.,* 2011). Alpha-amylases were also found to have anti-biofilm activity on *S. aureus* (Craigen *et al.,* 2011). Alginate lyase, and lactonase were reported to cause liquefaction of *P. aeruginosa* biofilm matrixes (Alkawash *et al.,* 2006; Kiran *et al.,* 2011). Mannitol at 10-40mM concentrations was found to revert persister cell phenotype in *P. aeruginosa*, increasing their sensitivity to tobramycin by up to 1,000 fold (Barraud *et al.,* 2013).

Furthermore, attempts have been made to combine quorum-quenching compounds with antibiotic treatment for biofilms. The purpose of these attempts were to prevent single cells from disseminating and re-establishing a biofilm. Although the host immune system should recognise and effectively destroy single pathogenic cells, underlying conditions that lead to the biofilm formation, such as autoimmune diseases and previous lung damage often means that the body is not capable of clearing biofilms (Estrela & Abraham, 2010). Quorum- quenching compounds prevent the accumulation and eventual biofilm formation of planktonic cells by disrupting QS. Several compounds have been identified through previous studies such as patulin and penicillic acid which greatly increase sensitivity of *P. aeruginosa* to conventional antibiotics (Wagner *et al.,* 2004; Abraham, 2005; Liaqat *et al.,* 2010). El-Mowafy *et al.* (2014) found aspirin to be effective at inhibiting QS pathways in *P. aeruginosa* which lead to a decrease in the pathogens virulence and biofilm formation. Furthermore, the surfactant protein, PLUNC, has been found to be able to inhibit the formation of *P. aeruginosa* biofilms *in vitro* (Gakhar *et al.,* 2010).

Although the identification of biofilm dispersing agents has progressed, many of these compounds are specific for certain species of bacteria, and cannot be generalised to

other species. Compounds that cause detrimental effects in one species of bacteria may enhance the antibiotic resistance in another, therefore careful study of each potential biofilm dispersing agent must be conducted. Furthermore, many compounds which prevent biofilm formation have been identified, but have no clinical significance as they are ineffective at treating existing mature biofilms (Estrela & Abraham, 2010). The complexity of biofilm metabolism and regulation has been studied in few bacterial species, and even so, is not completely understood.

1.8.5 Biofilms in *Mycobacterium avium*

Previous research has uncovered the major role of biofilms in bacterial resistance. The aggregation of cells within a matrix provides many advantages. One being antibiotic resistance, with the share size of a biofilm acting as a barrier to the innermost cells preventing antibiotics from reaching the innermost cells. It has been shown that MAC occupies biofilms in nature and infection (Carter *et al.,* 2003; Ingen *et al.,* 2012). In a study investigating *M. avium* resistance and biofilm growth in catheters conducted by Falkinham (2007) it was observed that there was an increase in antibiotic resistance of *M. avium* grown in biofilms compared to planktonic *M. avium* grown in suspension. This supports previous research into increased drug resistance in biofilm phenotype. McNabe *et al.* (2011) found that both sub-inhibitory concentrations of streptomycin and tetracycline increased biofilm formation of *M. avium* grown *in vitro,* and could possibly exacerbate MAC infection in patients receiving antibiotic treatment with these drugs. In contrast, their study found that ampicillin, moxifloxacin and rifampicin has no effect on biofilm growth. McNabe *et al.* (2011), found that biofilm formation in *M. avium* conferred resistance to clarithromycin and ethambutol, both antibiotics that are used as primary drugs in MAC infection. Falkinham (2007) surmised that the physical defence of

a biofilm is not the cause of antibiotic resistance, but prolongs the time it takes antibiotics to reach the cells. This allows time for physiological changes within the *M. avium* cells to occur, conferring antibiotic resistance to the cells. This agrees with other research which suggests that the naturally slow growing *M. avium* is further stunted in a biofilm phenotype, increasing the individual cells resistance to antibiotics by reducing their metabolism (Ingen *et al.,* 2012). Interestingly *M. smegmatis* biofilms, which are thought to share many characteristics with *M. avium* biofilms, have a strict dependence on extra-cellular iron to form biofilms (Islam *et al.,* 2012).

1.8.6 Biofilm Genetics

It is estimated that roughly twenty percent of bacterial genes are expressed differently in biofilms (Schuster *et al.,* 2003; Estrela & Abraham, 2010) which could explain the difficulties in exploring the communication within, and regulation of biofilms. Due to development of high throughput sequencing, the ability to mine genome and transcriptomes for genes and RNA involved in biofilm processes has become more easily accessible. Little research into the gene pathways that effect biofilm formation in *M. avium* currently exists.

1.8.7 Biofilm Genetics Within *Mycobacterium avium*

The regulator LysR has been associated with biofilm formation in *M. avium* (McNabe *et al.,* 2011). LysR has been found to regulate the synthesis of mycolyldiacylglycerol (Islam *et al.,* 2012). It has also been found that mutant *M. avium* lacking specific attachment genes are unable to form biofilms on epithelial cells of rats or PVC surfaces (Yamazaki *et al.,* 2006). Biofilm formation related genes *guaB2*, and *gtf* have been identified in previous studies, and are associated with glycopeptolipid (GPL) and lipoarabinomannan

(LAM) production – both are compounds used in *M. avium* cell wall synthesis (Freeman *et al.,* 2006; Yamazaki *et al.,* 2006; McNabe *et al.,* 2011). *M. avium* isolates with mutant GPL had reduced biofilm formation but no adverse effect on planktonic populations (Yamakazi *et al.,* 2006). Yamazaki *et al.* (2006) identified three hypothetical membrane transport proteins (tmptA, tmptB and tmptc) in *M. smegmatis,* which are also highly conserved in *M. avium* and have a proposed function in transporting precursor GPL proteins from the inner membrane. Deletion of tmpt gene cluster resulted in impaired biofilm function in *M. smegmatis* (Yamakazi *et al.,* 2006). Geier *et al.* (2008) showed that AI-2, when added to planktonic *M. avium* cultures, induced a biofilm phenotype. However, they suggested that conversely to other research, AI-2 did not affect QS in *M. avium,* rather causing biofilm formation due to inducing an oxidative stress response. Currently there is no research into potential detachment mechanisms, mechanisms for antibiotic resistance, and little research into signalling or metabolic pathways associated with *M. avium* biofilms.

1.9 Methodology

Colorimetric methods are often used to visually identify active metabolising cells.

For example 2,3-diphenyl-5-thienyl-(2)-tetrazolium chloride (STC), is an oxidation-reduction dye that has been used in previous experiments for accurate identification of positive culture growth, and for drug susceptibility testing (Park *et al.,* 2009). The molecule does not affect *M. avium* growth or antibiotic activity, but produces a dark pink precipitate via cell growth therefore effectively showing active cell metabolism. Similarly, the reduction of yellow 2,3,5 triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) produces a colour change from colourless to red in viable cells (Mohammadzadeh *et al.,* 2006; Rodríguez *et al.,* 2011). This allows rapid and accurate

detection of viable cells under observation through a microscope, and has already been used in previous *M. avium* and bacterial MIC studies (Rodríguez *et al.,* 2011).

Ethidium monoazide (EMA) has been commonly used with *Mycobacteria* to visually differentiate between live/dead cells. In bacterial cells EMA is only able to bind to chromosomal DNA of dead bacteria (Riedy *et al.,* 1991; Takashi *et al.,* 2009). Ethidium bromide (EtBr) also performs this function and is able to be used to visualise live/dead bacteria. However, unlike EMA that irreversibly binds to the DNA (Nogva *et al.,* 2003; Rudi *et al.,* 2005), EtBr reversibly intercalates with DNA in dead cells and therefore cells have to be visualised as soon as possible to retain accurate results (Tomchick & Mandel, 1964; Waring, 1965).

MTT (3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) are both colometric reagents used in colometric assays - particularly to asses cell viability (Mohammadzadeh *et al.,* 2006; Singh *et al.,* 2011; Trafny *et al.,* 2013). Both compounds have already been used to assess cell viability in *Mycobacteria* and *M. avium* (Primm & Franzblau, 2007; Singh *et al.,* 2011).The water soluble yellow MTT is reduced to formazan which is an insoluble purple crystal by actively metabolising cells (Mshana *et al.,* 1998). A solubilizing solution can be added to dissolve the formazan, allowing the absorbance of the coloured solution to be analysed on a Broad Smart Spec ™ 3000 spectrophotometer (Mosmann, 1983; Mohammadzadeh *et al.,* 2006; Trafny *et al.,* 2013). XTT is often used over MTT due to the reduced XTT being water soluble therefore eliminating the need for a solubilising solution (Primm & Franzblau, 2007).

1.10 Research Objectives

The objective of this research is to investigate possible options in the treatment of *M. avium*. This was to be done by conducting experiments into both the antibiotic sensitivity of *M. avium* isolates from the Waikato Hospital, and their response to potential biofilm dispersing agents.

1.10.1 Aims

1) To identify the MIC's and potential antibiotic resistant strains of *M. avium* from hospital isolates.

2) To identify synergy between antibiotic combinations, and antibiotics with potential biofilm dispersing agents, which result in effective sterilization of *M. avium* isolates.

3) To determine the pathways involved with consequential bacterial biofilm formation and the effect of previously identified biofilm dispersing agents on *M. avium*.

1.10.2 Hypotheses

1) Several hospital strains will have increased MICs and increased antibiotic resistance in planktonic form.

2) *M. avium* biofilms will have greatly increased MICs in comparison to their planktonically grown counterparts.

3) The addition of biofilm dispersing agents to biofilms will reduce their MIC to antibiotics.

4) The biofilm dispersing agents will effect gene expression.

Chapter Two

2. Methods and Materials

All following methods were carried out at the Molecular Genetics Laboratory (C.2.03) within the University of Waikato under PC1 and PC2 conditions. All techniques were carried out using aseptic technique, and all work stations were disinfected with virkon, or 70% ethanol and laboratory consumables were disinfected with virkon, trigene, and disposed of in accordance to Ministry of Primary Industries (MPI) standard protocol.

2.1.1 *Mycobacterium avium* Isolate Collection and Preparation

This research was conducted using 12 *M. avium* isolates collected from the Waikato Hospitals isolate library. All 12 isolates were collected from a wide range of patients treated by the Waikato Hospital. The isolates were grown in the Waikato Hospital Microbiology laboratory before being transported at room temperature to the Molecular Genetics Laboratory (C.2.03) at the University of Waikato. The isolates were stored at -4 degrees Celsius (°C) temperatures until the research began.

Isolate Origin

Isolate 1: Submandibular lymph node

Isolate 2: Submandibular lymph node

Isolate 3: Sputum

Isolate 4: Sputum

Isolate 5: Cervical lymph node tissue

Isolate 6: Lesion on neck

Isolate 7: Cervical lymph node tissue

Isolate 8. Sputum

Isolate 9: Unknown

Isolate 10: Unknown

Isolate 11: Sputum

Isolate A: Sputum

2.1.2 Culture Storage Protocol

All *M. avium* cultures were stored at -80°C to provide an original stock that could be resuscitated and grown up if working cultures became contaminated. In a sterile 15mL falcon tube, 0.75mL of sterile glycerol was added to 7.5mL of inoculum provided by the Waikato Hospital. This solution was then divided and pipetted into 1.5mL cryotubes and stored within a -80°C freezer.

2.1.3 Culture Methods

All *M. avium* cultures were grown in 50mL vented tissue culture flasks, using 10mL of BHI + 199 + 1/100 ACES buffer media (final pH 6.5). Cultures were subbed into fresh, sterile media every 14 days to prevent contamination and to maintain a population of actively metabolising cells. All cultures were incubated at 37°C to promote and maintain growth, unless otherwise stated.

2.1.4 Media Preparation

All media was prepared using autoclaved 15-18 megohm-cm double distilled deionised water (mQH2O) (Barnstead double distilled/deionisation system). All recipes for media can be found in appendix one (page 146).

2.2 Antibiotic Preparation

All antibiotic solutions were reconstituted using autoclaved 15-18 megohm-cm double distilled deionised water (mQH2O) (Barnstead double distilled/deionisation system), unless stated otherwise. All antibiotic solution recipes can be found in appendix two (page 150). All solutions were prepared in 15mL falcon tubes, under sterile conditions and filter sterilized using 0.22µm millipore filters and 10mL syringes.

The antibiotics used in this research were kindly purchased by Noel Karalus from the Waikato Hospital. The antibiotics provided were as follows; Klacid ® IV 500mg (Clarithromycin); DBL ™ 500mg in 2mL (Amikacin); Augmentin (Co-Amoxiclav); Rifadin ® 600mg (Rifampicin); DOXY 100 ™ 100mg (Doxycycline); Aspen Ciprofloxacin 200mg in 100mL (Ciprofloxacin); Cefoxitin 100mg; Avelox® 400mg (Moxifloxacin); Ethambutol; Rifinah ™ (Rifampicin with Isoniazid).

2.3 Biofilm Dispersal Agents Preparation

All biofilm dispersal agents were prepared using autoclaved 15-18 megohm-cm double distilled deionised water (mQH2O) (Barnstead double distilled/deionisation system), unless stated otherwise. All recipes for biofilm dispersal agents can be found in appendix

three (page 152). All solutions were prepared in 15mL falcon tubes, under sterile conditions and filter sterilized using 0.22µm millipore filters and 10mL syringes.

2.4.1 MTT Protocol

Firstly, 10mLs of 5mg/mL MTT solution was prepared in a 15mL falcon tube and 50mL of 20% SDS, 50% DMF MTT Solubilising solution was prepared in a sterile 50mL falcon tube, as per their recipes found in appendix one (page 147). Fresh MTT was added to the culture at a 1:3 volume (e.g. 167µL MTT added per one mL of culture). The MTT was pipetted up and down four times to ensure the solutions were thoroughly mixed. The culture, plus MTT, was incubated in the absence of light at 37°C for four hours. After the four hour incubation, MTT solubilising solution was added to the culture at a 2:3 volume (e.g. 666µL MTT Solubilising solution added per one mL of culture). The MTT Solubilising solution was pipetted up and down four times to ensure that the solutions were thoroughly mixed. Samples were then incubated in the absence of light overnight at 37°C to ensure complete solubilising of the purple formazan crystals. Sample optical density (OD) was then measured on a spectrophotometer to determine the amount of formazan present in each sample.

2.4.2 Measuring ODs of MTT Treated Samples

Sample OD was measured on a spectrophotometer at a wavelength of 570 nanometres (nm). A control of one mL sterile media, 167µL MTT and 666µL MTT solubilising solution was used to blank the machine. The samples were thoroughly mixed before being loaded into curvettes and read at a wavelength of 570nm on a spectrophotometer. The spectrophotometer was blanked every five samples to ensure minimal machine error.

2.5 *Mycobacterium avium* Biofilm Cultivation

In contrast to previous studies using Middlebrook 7H9 media, Brain heart infusion (BHI) +199 media was determined to have the greatest rate of *M. avium* growth, forming mature biofilms within three days of sub-culturing, and therefore was used for all planktonic and biofilm experiments. A 1/100 dilution of 1M ACES buffer (pH 6.5) was added to the BHI +199 media to ensure that any bactericidal or bacteriostatic activity was not due to an extreme pH change, caused by an antibiotic, biofilm dispersing agent, or solutions made up in dimethyl sulfoxide (DMSO).

For antibiotic and biofilm dispersal agent experiments, 24 well polystyrene culture plates were used to efficiently control large numbers of samples (figure 3). Biofilms were cultivated by placing a 12mm sterilized glass coverslip into the bottom of each well to aid in the adherence of the biofilms onto the glass surface and promote their growth over planktonic growth. One mL of inoculated media was then added to every well. To ensure consistency between biofilm and planktonic experiments, planktonic cells were also cultured in 24 well plates, without 12mm glass coverslips. Antibiotic or biofilm dispersal agents were then added to wells, or to the inoculated media, in order to maintain accurate concentrations by reducing pipetting error.

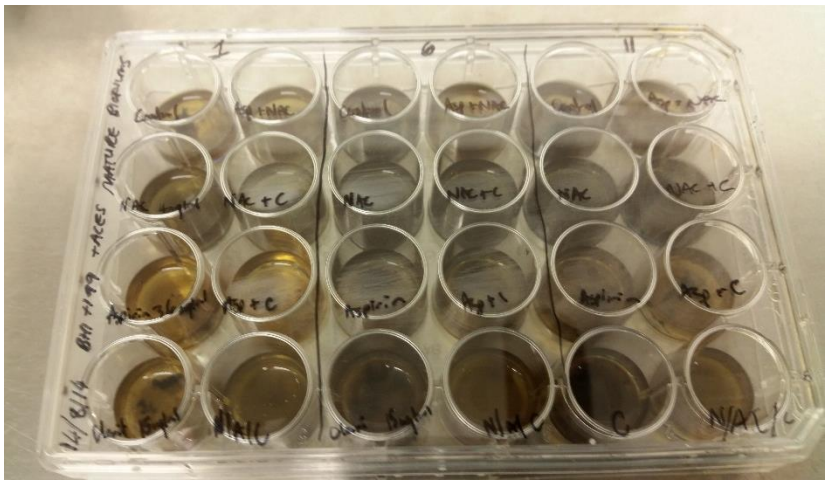


Figure 3. Polystyrene 24 well plate used to grow *M. avium* biofilms. The purple colouration in the wells is due to the formation of insoluble formazan crystals during MTT analysis.

2.5.1 Glass Versus Polystyrene

For effective biofilm formation in following experiments, the best biofilm growth promoting surface had to be determined. This was done by measuring the difference in growth between the two surfaces, using both MTT and crystal violet staining methods.

For MTT growth determination a glass or polystyrene bead was placed into a 1.7mL sterile eppendorf tube. To the tube 0.1mL of inoculated BHI+199 media was added and left to grow over 24 hours at 37°C. After incubation, 16µL of 5mg/mL MTT solution was added and mixed thoroughly, then left to continue incubating at 37°C for a further four hours. After four hours, 90µL of MTT solubilising solution was added to the solution. After mixing well, the tubes were incubated at 37°C overnight and the OD of each solution read at a 570nm wavelength via spectrophotometer. A control solution repeating all conditions described, however, replacing inoculated media with sterile media was also conducted.

For growth analysis via crystal violet staining, one glass or polystyrene bead was placed into a 1.7mL sterile eppendorf tube and 0.1mL of inoculated BHI+199 media was added

to each tube, and left to grow over 24 hours at 37°C. After incubation, 100µL of crystal violet stain was added and left to develop at room temperature (RT) for 15 minutes. Then each bead was washed three times with sterile water, ensuring all of the crystal violet stain had been removed. After washing, 1mL of 90% EtOH was added to the tube, to dissolve the stain from the attached biofilm cells. The OD of each solution read at a 570nm wavelength via spectrophotometer, as per recommended in previous research (Mshana *et al.,* 1998; Trafny *et al.,* 2013). A solution, replacing inoculated media with sterile media was treated in the exact same conditions as described above, and was used as a control.

In both experiments, the control was used as a blank for the spectrophotometer. Both experiments were repeated.

2.5.2 Aspirin Longevity Experiment Design

In each sample, one mL of media was inoculated with 100µL of 1/10 diluted inoculum. Then to one sample, 36µL of 100mg/mL aspirin stock solution was added and mixed thoroughly. The other sample was left untreated to act as a control. Both samples were then incubated over ten days at 37°C. Every day, 100µL was removed from both the aspirin treated, and non-treated samples and spread using aseptic technique onto a BHI + 199 + 5% horse serum agar plate. The plates were then incubated at 37°C and checked for growth on a daily basis.

2.5.3 Antibiotic Titration

A dilution series was made from 128µL/mg to 1µL/mg concentration of antibiotics according to the following figure;

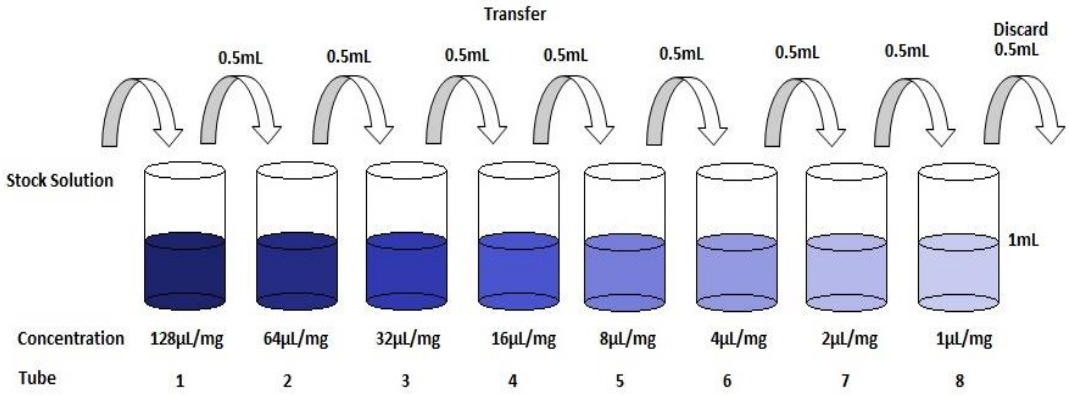


Figure 4. Standard dilution series used to prepare concentration gradients of antibiotics.

2.6 PCR Protocols

All PCRs were performed using BIORAD T100™ Thermo Cycler PCR machines, and reaction mixes made in single 200µl PCR tubes (Axygen). The PCR reaction mixes were prepared in a DNA free UV hood, to minimise potential contaminants, DNases and RNases. A negative control was prepared in every experiment, replacing DNA with DNase and RNase free water. PCR masters mixes were prepared in bulk, aliquoted out and frozen at -20°C, and used when needed

Table 2. Primers used in PCR reactions. Tmpt1 and tmpt2 are two separate primer sets for isolating a gene encoding a hypothetical SAM-dependent methyltransferase.

Target	Primer	Sequence (5'-3')	Product length (bp)	Recommended Annealing Temperature	Reference
Inosine-5-monophosphate dehydrogenase	GuaB2 F	TCA CCT GCC GCC CCG ACA ACA CGC TGC CCC	1000	74.8	McNabe *et al.,* 2011
Inosine-5-monophosphate dehydrogenase	GuaB2 R	GGC ACC CGG CCC TCG ATG CCC TCG GGC ACC	1000	76.5	McNabe *et al.,* 2011
Glycosyl transferase	Gtf F	ATG GAG GGCGCC GAC GTG CCC	374	69.6	McNabe *et al.,* 2011
Glycosyl transferase	Gtf R	AGG ATC GCG GTG ATG CTG CCC	374	64.7	McNabe *et al.,* 2011
Internal Transcribed Spacer Region *IS*311	*IS*311 F	GCG TGA GGC TCT GTG GTG AA	608	60.0	Tran *et al.,* 2014
Internal Transcribed Spacer Region *IS*311	*IS*311 R	ATG ACG ACC GCT TGG GAG AC	608	59..6	Tran *et al.,* 2014

DT1 Region	DT1 F	CGT TGG CTG GCC ATT CAC GAA GGA GT	269	64.9	Tran *et al.,* 2014
DT1 Region	DT1 R	CGT TGG CTG GCC ATT CAC GAA GGA GT	269	67.0	Tran *et al.,* 2014
SAM- dependent methyltransferase	Tmpt2 F	GGC ATC GAA TGG AAC AGG GT	374	57.7	Denkin *et al.,* 2005
SAM- dependent methyltransferase	Tmpt2 R	TCT CAA CCA ACG CAC GAG AT	374	56.6	Denkin *et al.,* 2005
SAM- dependent methyltransferase	Tmpt1 F	CAC CCT GTT CCA TTC GAT GC	187	56.4	Denkin *et al.,* 2005
SAM- dependent methyltransferase	Tmpt1 R	GAT CAC CCA GTA CTT GCC GA	187	56.8	Denkin *et al.,* 2005
16S-23S rRNA internal transcribed spacer (ITS)	MAI F	TTG GGC CCT GAG ACA ACA CT	146	59.97	Tran *et al.,* 2014
16S-23S rRNA internal transcribed spacer (ITS)	MAI R	GGC GTT CAT CGA AAT GTG TAA TT	146	60.04	Tran *et al.,* 2014

2.6.1 Identification PCR

All isolates were identified by PCR and gel electrophoresis as to their correct classification, using MAI primers (table 2) taken from Tran *et al.* (2014). One µL of genomic DNA was added to 19µL of master mix per PCR reaction tube.

The cycle used was touchdown PCR, as per below.

Step		Temperature	Time
TAQ activation		95°C	15 minutes
Cycle x 10 Dropping 1°C Per cycle	Denaturation	95°C	20 seconds
	Annealing	65°C-55°C	15 seconds
	Extension	72°C	30 seconds
Cycle x35	Denaturation	95°C	20 seconds
	Annealing	55°C	20 seconds
	Extension	72°C	30 seconds
Final Extension		72°C	10 minutes

2.6.2 SAUTYPE PCR

SAUTYPE PCR was then performed to distinguish if there were any genetic differences between the isolates and potentially give an explanation for the differences in observed phenotype.

TAQ polymerase was added to the master mix at a ratio of 0.25µL:50µL TAQ polymerase to total PCR reaction mix volume. Two µL MB01 Genomic DNA was added to every 18µL of master mix. For this PCR, primer concentration was doubled (2µL:50µL primer to total volume).

SAUtype PCR cycle was used as below.

Step	Temperature	Time
TAQ activation	95°C	15 minutes
	25°C	5 seconds
	25°C-60°C	Increasing 0.1°C per second until 60°C is reached
	60°C	30 seconds
Cycle x2	95°C	60 seconds
	50°C	15 seconds
	50°C-25°C	Decreasing 0.1°C per second until 25°C is reached
	25°C-50°C	Increasing 0.1°C per second until 50°C is reached
	50°C	30 seconds
Cycle x 39	95°C	15 seconds
	48°C	60 seconds
	65°C	2 minutes
Final Extension	65°C	10 minutes

2.6.3 Subtype Identification PCR

Subtype identification was done using DT1 and IS*1311* Primers (table 2), and a PCR cycle, adapted from Tran *et al.* (2014).

Step		Temperature	Time
TAQ activation		95°C	10 minutes
Cycle x35	Denaturation	95°C	20 seconds
	Annealing	60°C	40 seconds
	Extension	72°C	35 seconds
Final Extension		72°C	10 minutes

2.6.4 Real Time PCR

Real time PCR was carried out using the Quanta Biosciences qScript™ One-Step SYBR ® Green qRT-PCR Kit, and this PCR cycle as follows.

Step		Temperature	Time
TAQ activation		95°C	10 minutes
Cycle x40	Denaturation	95°C	15 seconds
	Annealing	55°C	15 seconds
	Extension	72°C	20 seconds
Final Extension		80°C	10 minutes

2.6.4.1 Quanta Biosciences qScript™ One-Step SYBR ® Green qRT-PCR Kit Method

Quanta Biosciences qScript™ One-Step SYBR ® Green qRT-PCR Kit was used for real time PCR from the three RNA samples (acquired as per 2.12.2). For a 50µL reaction mix, the following quantities apply.

Reaction Ingredients	Per Reaction	Concentration
One-Step SYBR® Green Master Mix	25µL	1X
Forward Primer	~	200-500nM
Reverse Primer	~	200-500nM
Nuclease Free H2O	~	Make up to 50µL
RNA Template	5-10µL	~
qScript One-Step RT	1µL	1X

The reaction mix was prepared in a PCR cabinet with the RNA added to the reaction mix in a sterile, RNase free environment. The qRT-PCR was the carried out on a cycle as described above. After the qRT-PCR had completed the products were then run out on a 1% agarose gel as described in 2.7.1 and 2.7.2.

2.6.5 Primer Reconstitution

The primers were reconstituted with TE buffer to give a final concentration of 200pMol, and aliquoted into 0.6mL eppendorf tubes, and stored at -20°C. By aliquoting out the primers it prevents contamination of the entire primer stock solution and provided sterile reserves in case issues arose during PCR. From the 200pMol primer stocks a 20pMol concentration was made by dilution and was added to the PCR mix.

2.7 Gel Electrophoresis Protocol

Electrophoresis was used to visualise PCR reactions and determine base pair (bp) number of PCR products via the use of a 100bp ladder.

2.7.1 Agarose Gel Preparation

Into a conical flask 35mL 1X Super Buffer was added before 0.35g of agarose (hyagrose) was mixed in to create a 1% agarose gel. The solution was microwaved at medium heat for 2 minutes, with occasional stirring to prevent boiling over, in order to melt the agarose. Molten agarose gel was cooled via running the conical flask under cold water and 2µL of 10mg/mL EtBr was added and mixed thoroughly via stirring. EtBr was added for following visualisation and imagine of the gel. The molten agarose gel was then poured into a gel rig, well combs added and left to set. Once set, well combs were removed.

2.7.2 Gel Electrophoresis

Previously made, solidified, 1X Super Buffer gel was loaded into a Thermo Fischer Scientific ™ Owl Separation Systems ™ small gel electrophoresis tank. Sufficient 1X Super

Buffer was added to the tank until the gel was covered with 2-5mL of 1X Super Buffer. Five µL of 100bp ladder was added to a well in the gel in order to be able to accurately read the correct bp size of resulting PCR products. Approximately 2µL of 6X binding dye was mixed thoroughly with 10µL of PCR product before being loaded into a well on the agarose gel. Gel electrophoresis tank was sealed and electrodes adjusted so that the wells were closest to the negative electrode. Gels were run for 10 minutes at 200V.

2.7.3 Gel Visualisation and Imaging

After completion of gel electrophoresis, the gel was safely removed from the gel electrophoresis tank, and placed onto a UV light box. Under protective conditions, the UV light was turned on, and allowed the visualisation of the DNA bands within the gel. These were then able to be photographed via a computer program Scion X Imaging, and saved for future purposes. All gels were disposed of by soaking in bleach to destroy DNA products and autoclaving, as in accordance with MPI protocol.

2.8 Microscopy

Microscopy was used to physically view *M. avium* bacteria and assess growth, death and viability using stains. A Zeiss Fluorescence Axiostar microscope was used to observe samples.

2.8.1 Auramine-O Double Stain Protocol

Staining protocol was adapted from Ichijo *et al.* (2010). The supernatant was removed from biofilm via sterile pipette, ensuring that the biofilm remains undisturbed and intact. A 1mL aliquot of ~2.0% formaldehyde to PBS solution was added to the biofilm

and left for 10 minutes. The formaldehyde: PBS solution was removed and 0.5mL of the auramine-O stain was added to the biofilm, then left to develop for 15 minutes in the absence of light. The auramine-O stain was removed and biofilm was washed with 1mL sterile water. Following the removal of the sterile water, 1mL of acid alcohol methylene blue counter stain was added to the biofilm and left to develop in the absence of light for 10 minutes. The acid alcohol methylene blue counter stain was then removed and biofilm washed with sterile water, so to remove all unused counter stain. The biofilms on glass coverslips were then mounted on microscope slides and viewed under blue light.

2.8.2 SEM Cell Fixative Preparation

The supernatant was removed from each well, carefully to not disturb the biofilm growing on the glass coverslip. Each sample was washed with 1mL of PBS, again carefully to not disturb the biofilm. Then 1mL of 4% gluteraldehyde was added to every sample and then incubated at RT for four hours. In order to fix and kill the cells within the biofilm, making them safe to work with. Following the incubation, the 4% gluteraldehyde was removed and biofilm was washed with 1mL PBS. The biofilm was then saturated with 50% EtOH, and left at RT for an hour. The 50% EtOH was then removed and replaced with 75% EtOH and left at RT for another hour. This process of removing the previous EtOH concentration and saturating the biofilm with another EtOH concentration, then leaving for an hour was repeated through 50%, 75%, 90%, 95% and finally 100% concentrations of EtOH. Once the biofilm samples had been saturated with 100% EtOH for an hour, they were ready to proceed to critical point drying technique used to prepare samples for SEM imaging. The series of steps increasing the EtOH

concentration were carried out in an attempt to replace the H20 within the cells with EtOH, essentially fixing them and preserving their structure.

2.8.3 SEM Sample Preparation, Critical Point Drying and Mounting

Sample preparation, critical point drying, and sample mounting for SEM imaging protocol was performed by the University of Waikato's SEM technician, Helen Turner.

2.8.4 2,3,5 Triphenyl Tetrazolium Chloride Indicator

Oxidation- reduction indicator TTC is used to identify bacteria metabolism (Rodríguez *et al.,* 2011) with the bacteria showing red under normal lighting conditions at a magnification of 40x. The TTC was prepared according to appendix one, and used in a ratio of 10µL of TTC to 1mL sample.

2.8.5 Fluorescein Diacetate (FDA) and Ethidium Bromide (EtBr) Double Stain Protocol for Live/Dead Cells

For every sample, the concentration of FDA must be 0.025%, and EtBr 0.005%, according to Netuschil *et al.* (2014). With this double stain, actively metabolising cells will fluoresce green and dead cells will fluoresce red with the EtBr being able to enter dead cells.

The one mL samples were transferred to 1.5mL eppendorf tubes and centrifuged for 20 seconds at 8,000rmp to force the bacterial cells to the bottom of the eppendorf tube. 700µL of supernatant was removed, and the pellet re-suspended in the remaining 300µL. For a 300µL sample, 6µL of FDA was added and left to incubate at RT for 30 minutes in complete absence of light. After FDA incubation, 1.5µL of EtBr was added to

the 300µL samples and incubated for a further 10 minutes at RT in complete absence of light. Then 10µL of the sample was mounted onto microscope slides and visualised under blue florescent light at 40x and 100x magnification. Percentage of red stained cells was determined visually.

Further 100µL of each sample was taken and spread onto BHI + 199 + HS plates with a glass spreader. The plates were then incubated for 7 days at 37°C to determine if the staining produced accurate results.

2.9 DNA Extraction Protocol

DNA extraction of all the isolates was carried out in order to perform further downstream analysis.

The complete contents of a culture flask containing 10mLs of *M. avium* culture was transferred to a sterile 15mL falcon tube. The falcon tube was then centrifuged at 3,500rmp for 10 minutes at 4°C. The supernatant was carefully removed via pipette and the pellet re-suspended with 5mL of acetone, then incubated at RT for 10 minutes. The contents were then centrifuged at 3,500rpm for a further 10 minutes at 4°C, before all the supernatant was carefully removed. The pellet was then re-suspended in 0.5mL SDS Tris buffer and transferred to a bead beating tube. The samples were then beat at 6.5 speed on (machine), following this procedure.

Action	Amount of Time
Bead Beater	25 seconds
Rest	1 minute
Bead Beater	35 seconds
Rest	1 minute
Bead Beater	35 seconds

This step is vital in the physical lysing of the tough mycobacterium cell wall. Rests between bead beating steps were needed to prevent the build-up of heat from friction.

The samples were then centrifuged at 13,000rpm for 2 minutes to remove the foam that had formed from bead beating. To each sample, 10µL of proteinase K was added and the samples were left to incubate in a thermomixer overnight at 55°C at 800rpm.

Following overnight incubation, 0.5mL of phenol chloroform was added to the samples and mixed thoroughly on a rotator wheel for 5 minutes. The samples were then centrifuged for a further 10 minutes at 13,000rpm, before the supernatant was transferred into a new sterile 1.7mL eppendorf tube. To the supernatant, 100µL lysing SDS buffer, 100µL 5M NaOH and 80µL of CTAB buffer was added. The samples were then incubated at 65°C for 10 minutes at 800rpm via thermomixer. Then an equal volume of chloroform was added to the sample and shaken vigorously for 30 seconds. The samples were then centrifuged at 13,000rpm for 10 minutes. The top layer of supernatant was transferred to a new 1.7mL eppendorf tube and an equal volume of isopropyl alcohol was added. The sample was left to precipitate overnight at -20°C.

Following the overnight incubation, the samples were centrifuged for 15 minutes at 13,000rpm. The supernatant was removed and discarded and the remaining pellet was washed with 1mL of 70% EtOH. Once all the EtOH had been removed the pellet was left

to dry. Following drying, the pellet was re-suspended in 150µL TE buffer and analysed for purity on the nanodrop.

The DNA was then stored at -4°C until required for downstream applications.

2.10 Bead Beater Tube Preparation

Capped tubes were filled with 0.1mm zirconia beads until they reached the line surrounding the widest part of the cone bottom. Eight 2.5mm glass beads were then added to the tube. Bead beater tubes were used in both DNA and RNA extraction protocols.

2.11 Restriction Enzyme Digest Protocol

Into a 1.7mL eppendorf tube, 1µL of sample DNA (with a concentration of 200-400ng/µL), 2µL of 10X Buffer 4, 1µL of MB01 restriction enzyme and 16µL of sterile mQH2O was pipetted. The sample was then incubated at 37°C at 700rpm for 12 hours. Following this the temperature was then increased to 65°C for 10 minutes to deactivate the MB01 enzyme. The digested DNA was then stored at 4°C until required for downstream applications.

2.12.1 RNA Preparation

For each RNA sample, four 1.5mL cultures of isolate 6 were cultured and treated before being used for RNA extraction. A total of 9mL of culture was needed to ensure enough RNA was isolated for downstream applications.

Into a sterile falcon tube, 20mL BHI+ 199 media was added. One mL of working culture was used to inoculate the 20mL media. The inoculated media was then distributed into 1.5mL aliquots into yellow screw top tubes. For the biofilm RNA samples, a glass coverslip was added to the bottom of the tube to promote biofilm formation over planktonic growth. These tubes were then incubated for three days at 37°C to promote growth. Into the tubes that would serve as the aspirin RNA isolates, 54µL of 100mg/mL aspirin was added. All the samples were then incubated over night at 37°C and the RNA was harvested the following morning via section 2.12.2.

2.12.2 RNA Extraction Protocol

The supernatant from the biofilm, and biofilm treated with aspirin samples was carefully removed and discarded. To the aspirin and biofilm samples, 0.5mL of 5M GITC was added to aid in the preservation of RNA throughout the extraction process. Five to ten 2.5mm glass beads were then added to aid in the breaking down of the biofilm, and the samples were vortexed for 20-30 seconds. To remove the build-up of foam the samples were then centrifuged at 2,500rpm for 5 minutes at 4°C.

The planktonic samples were then pipetted into 1.7mL eppendorf tubes and were centrifuged for 1 minute at 13,200rpm. Following this, the supernatant was removed and 0.5mL of GITC was added to the tube, then the pellet was re-suspended in the GITC.

Bead beater tubes were then prepared according to previously described protocol (section 2.10). All samples (aspirin treated biofilm, biofilm and planktonic cells) were then added to bead beater tubes and then beaten at 6.5 speed, following this procedure.

Action	Amount of Time
Bead Beater	30 seconds
Rest	1 minute
Bead Beater	35 seconds
Rest	1 minute
Bead Beater	40 seconds

The samples were then immediately put on ice and centrifuged at 13,200rpm for 1 minute at 4°C to remove the build-up of foam.

The correlating samples were all pooled into three 1.5mL eppendorf tubes, to give three 1mL sample for aspirin treated biofilm, a further three 1mL sample for biofilm, and repeated for planktonic cells.

To each tube containing 1mL sample, 100µL of 2M NaAc (at pH 4) was added. This was to lower the pH and maintain DNA at the interface and not in the supernatant. Furthermore, to each 1mL sample, 750µL of RNA grade water saturated phenol (at pH 4.3) was added. All the samples were then violently shaken for 20 seconds, before being placed on the rotator wheel for 10 minutes. Following their rotation, 200µL of bromo-chloropropopane was added to each sample. The samples were then violently mixed for a further 20 seconds, and placed back on the rotor wheel for 5 minutes. Afterwards, the samples were then centrifuged for 10 minutes at 12,200rpm at 4°C.

The top layer of supernatant was then transferred to 1.7mL eppendorf tubes using a fine sterile pipette tip. 0.1mL of bromo-chloropropopane was added to each sample and violently mixed for a further 20 seconds to ensure the proteins within the sample were being degraded. The samples were then centrifuged for 9 minutes at 12,200rpm at 4°C.

The supernatant was removed and an equal volume of isopropanol was added. The total RNA was left to precipitate overnight at -20°C.

After precipitation, the total RNA was washed with 70% EtOH, centrifuged to remove all remaining EtOH. Then the RNA pellet was re-suspended in TE Buffer and stored at -80°C.

2.12.3 Transcriptome Analysis

RNA samples were then sent to the Beijing Genomics Institute (http://www.genomics.cn/en/index) for further transcriptome processing, quality control and analysis.

Chapter Three

3. Results

3.1 PCR Results

3.1.1 DNA Quality

The following results were recorded on Thermo Fischer Scientific ™ Nanodrop 2000 ™ Spectrophotometer.

Table 3. DNA quality and concentration after DNA extraction was performed on isolates.

Isolate	260/280	230/260	Concentration ng/µL
1	1.93	1.82	57.2
2	1.99	2.02	649.2
3	2.02	0.76	33.8
4	1.81	0.49	6.6
5	2.01	2.10	450.4
6	2.04	2.11	518.2
7	2.02	2.19	651.4
8	1.85	2.03	300.5
9	1.89	2.01	457.3
10	1.99	2.06	522.7
11	2.05	1.99	316.4
A	1.83	1.92	583.3

3.1.2.1 PCR Identification

PCR with MAI forward and reverse primers, showed that all isolates, except 3 and 4 were *M. avium*. This was also confirmed by Waikato Hospital. It is likely that isolates 3 and 4 are other *Mycobacterium* species found in MAC infections.

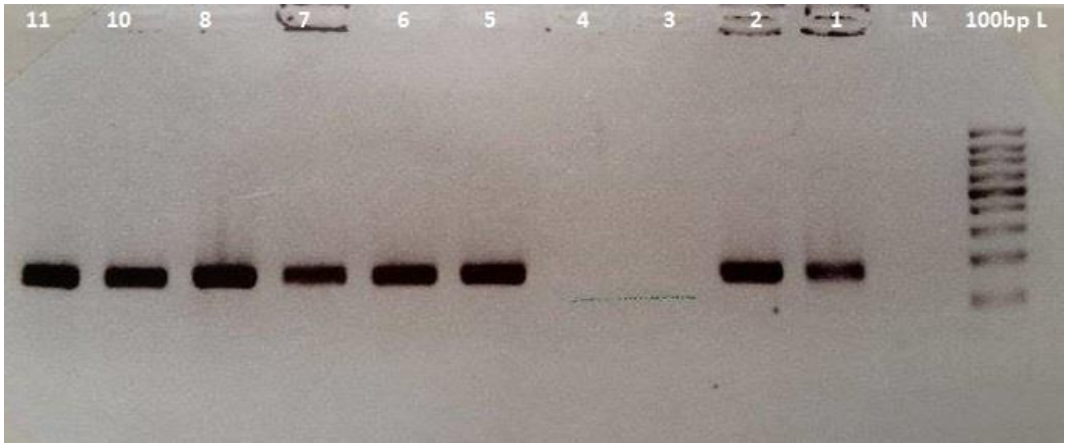


Figure 5. Gel electrophoresis photo showing the band size produced from performing a PCR on the extracted DNA with MAI primers. The expected band size produced, 146bp, can be seen in this picture. The isolate numbers are shown across the top, corresponding with their band size, as well as N (negative control), showing no contamination.

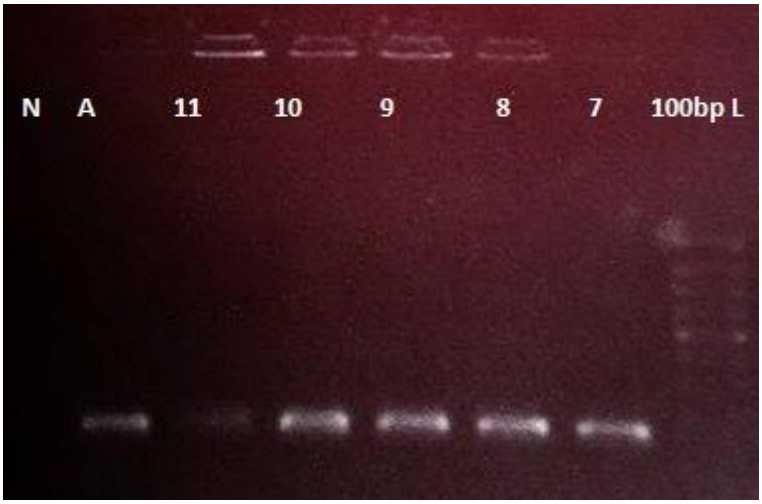


Figure 6. Gel electrophoresis photo showing the band size produced from performing a PCR on the extracted DNA with MAI primers. This PCR was repeated 6 months after the first identification PCR. The expected band size produced, 146bp, can be seen in this picture. The isolate numbers are shown across the top,

corresponding with their band, as well as N (negative control), showing no contamination in the PCR reaction mix.

3.1.2.2 PCR Subspecies Identification

Observed differences in isolate phenotype, in both broth media and biofilm formation on glass coverslips, was confirmed by SAUTYPE PCR and DT1 and IS*1311* primer sets.

3.1.2.2.1 SAUTYPE PCR

SAUTYPE PCR showed that all isolates differed in their genomic composition, with exception to isolates 6 and 11. No two isolates had the same restriction digest fragmentation pattern and SAUTYPE PCR results as seen in figure 7 below.

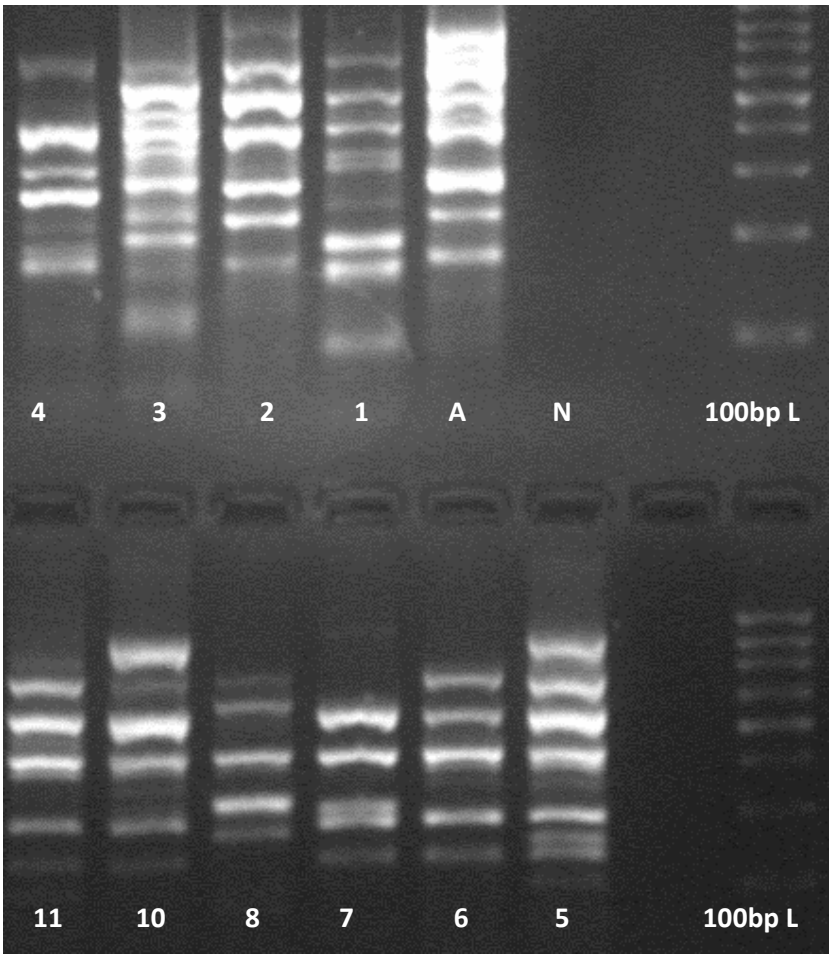


Figure 7. Gel image showing PCR with STG primers and MBO1 restricted DNA of all isolates.

3.1.2.2.2 DT1 and IS*1311* Identification

PCR samples positive for DT1 should show fragments at ~269bp and PCR samples positive for IS*1311* should show fragments ~608bp in size.

As seen in figure 8, isolate 1 and 8 shared similar PCR fragmentation, with a fragment size ~269bp and a very faint fragment ~608bp. Isolates 3 and 4 shared similar PCR fragments, with both band sizes at ~269bp, ~608bp and ~800bp. Isolates 5, 6 and 11 showed similar PCR fragment amplification. All other isolates showed a single band at ~608bp.

Therefore, isolates 2, 5, 6, 7, 10, 11 and A are either *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *hominissuis*. Isolates 1 and 8 are *M. avium* subsp. *avium*. Isolate 9 remains unknown. Isolates 3 and 4 are confirmed as not *M. avium*.

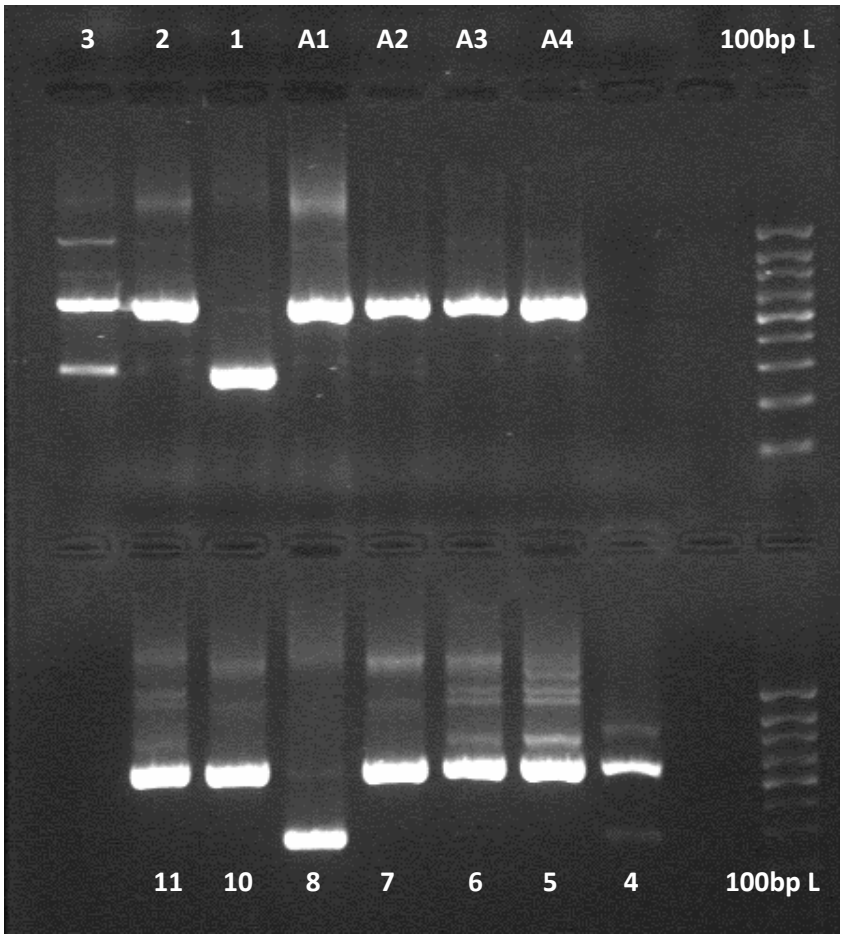


Figure 8. Gel image showing subtype identification PCR carried out with DT1 and IS*1311* primer sets.

3.2 Planktonic Cell Experiments

Experiments were conducted on planktonic *M. avium* cell populations to determine their MICs in broth, using the MTT analysis method.

Clarithromycin was most effective at inhibiting planktonic *M. avium* (absorbance dropping from 0.868 in the control to 0.529 at a 10µg/mL concentration). Ciprofloxacin also showed a decreasing trend (absorbance dropping from 0.687 in the control to 0.569 at a 20µg/mL concentration). Amikacin, cefoxitin and doxycycline, all showed an increase in cell metabolism at a 2.5µg/mL concentration, and no significant cell inhibition at higher drug concentrations (figure 9).

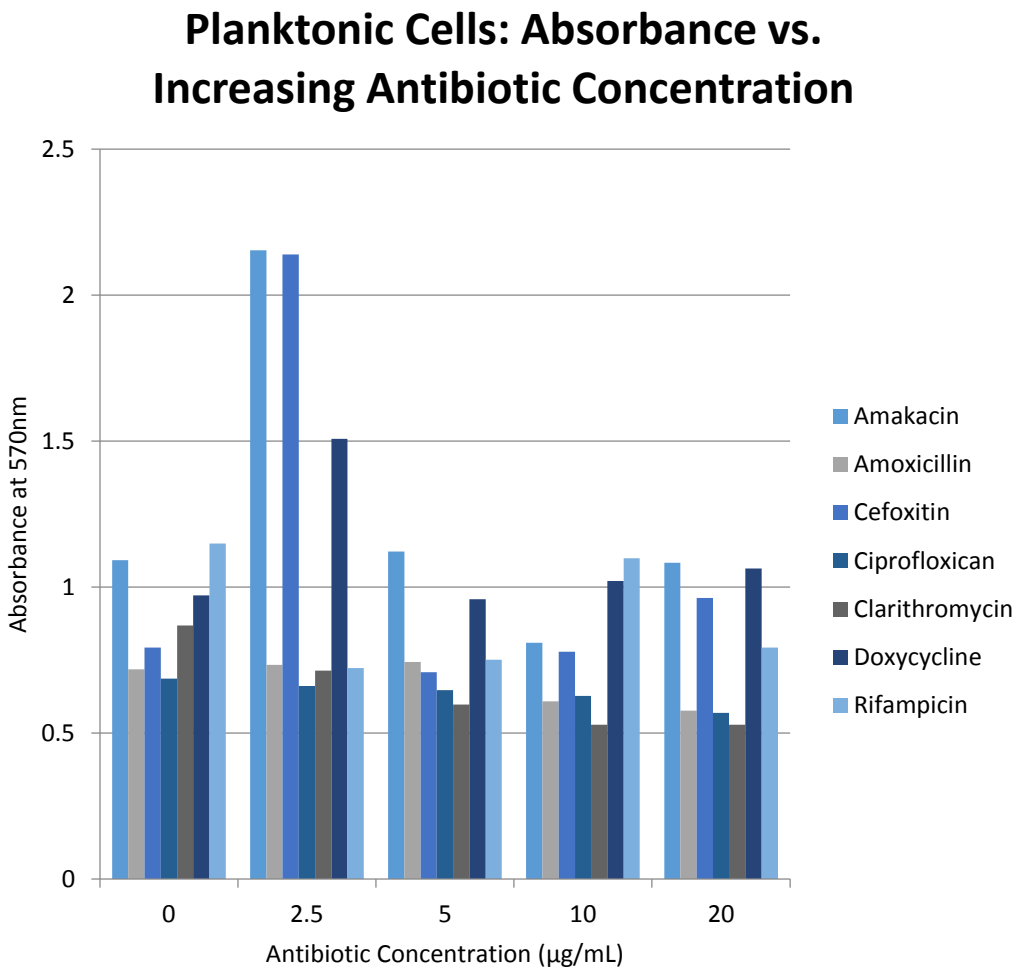


Figure 9. MTT ODs absorbance values for *M. avium* planktonic cells treated with varying concentrations of antibiotics (0µg/mL to 20µg/mL).

The experiment was repeated, however 25µg/mL of doxycycline was also added. Compared to the control (absorbance = 2.289), OD increased with the addition of 25µg/mL of doxycycline (absorbance = 2.386). Again, clarithromycin and cefoxitin alone were the most effective antibiotics against *M. avium* isolate 6. Amoxicillin at 2.5µg/mL combined with 25µg/mL of doxycycline showed synergy, ciprofloxacin at 10µg/mL with 25µg/mL doxycycline and clarithromycin at all concentrations combined with 25µg/mL doxycycline showed significant synergy, compared to their absorbance values without 25µg/mL doxycycline (figure 10). However, 5µg/mL of cefoxitin alone had the most

effect on inhibiting *M. avium* questioning the usefulness of doxycycline being used as a synergistic antibiotic in treatment of *M. avium* infection.

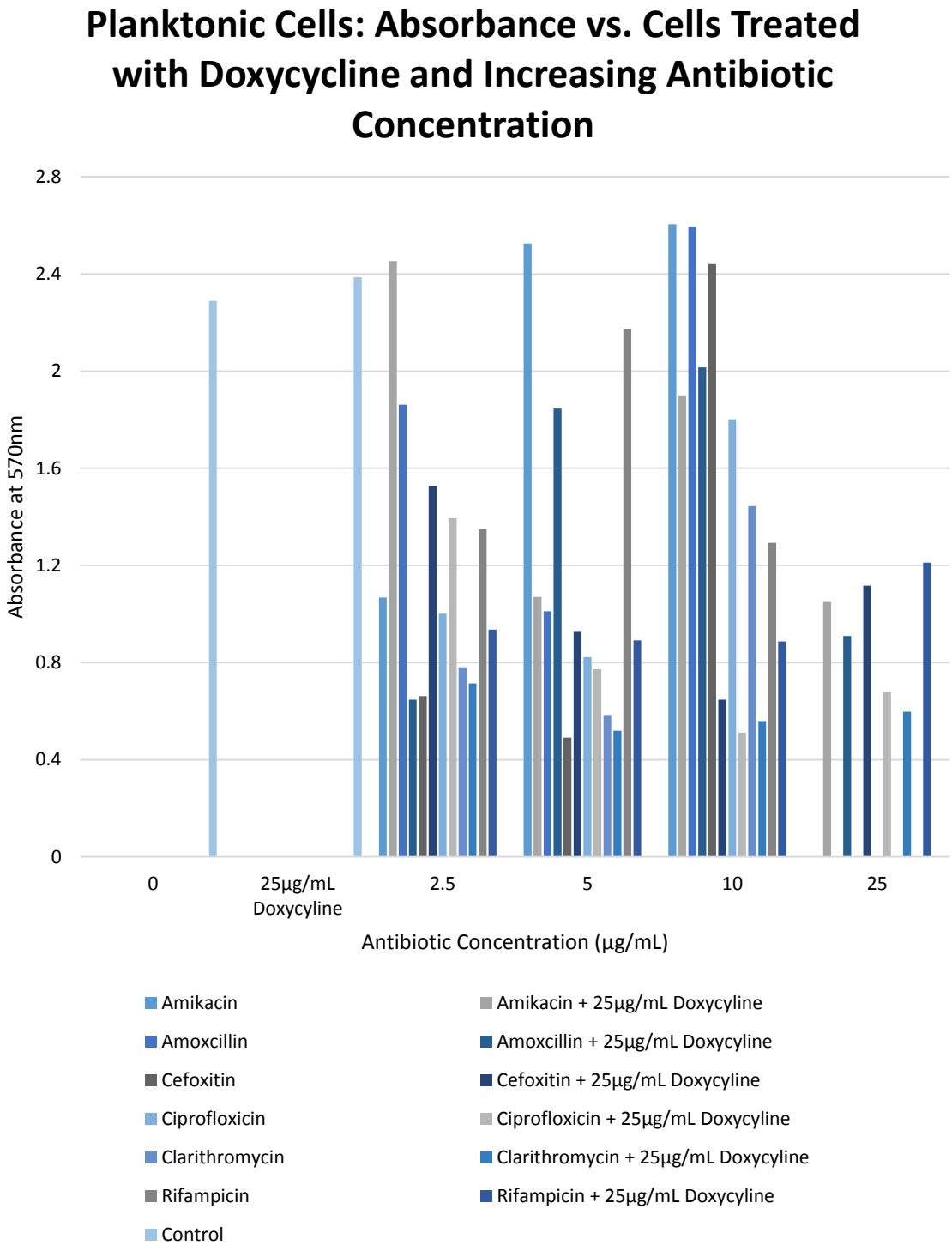


Figure 10. MTT ODs of *M. avium* isolate 6 planktonic cells, grown overnight, treated with varying concentrations of single antibiotics, and antibiotics combined with 25µg/mL of doxycycline.

All isolates were found to be resistant to concentrations of 15µg/mL, with no antibiotics achieving complete sterilization. Rifinah ™ (rifampicin + isoniazid), rifampicin and doxycycline consistently increased planktonic cell populations across all isolates, with absorbance values that exceeded the control (figure 11). Aspirin combined with ibuprofen (both at 3.6mg/mL concentrations) consistently reduced planktonic cell metabolism across all isolates, proving more effective than clarithromycin which was the most effective out of the antibiotics.

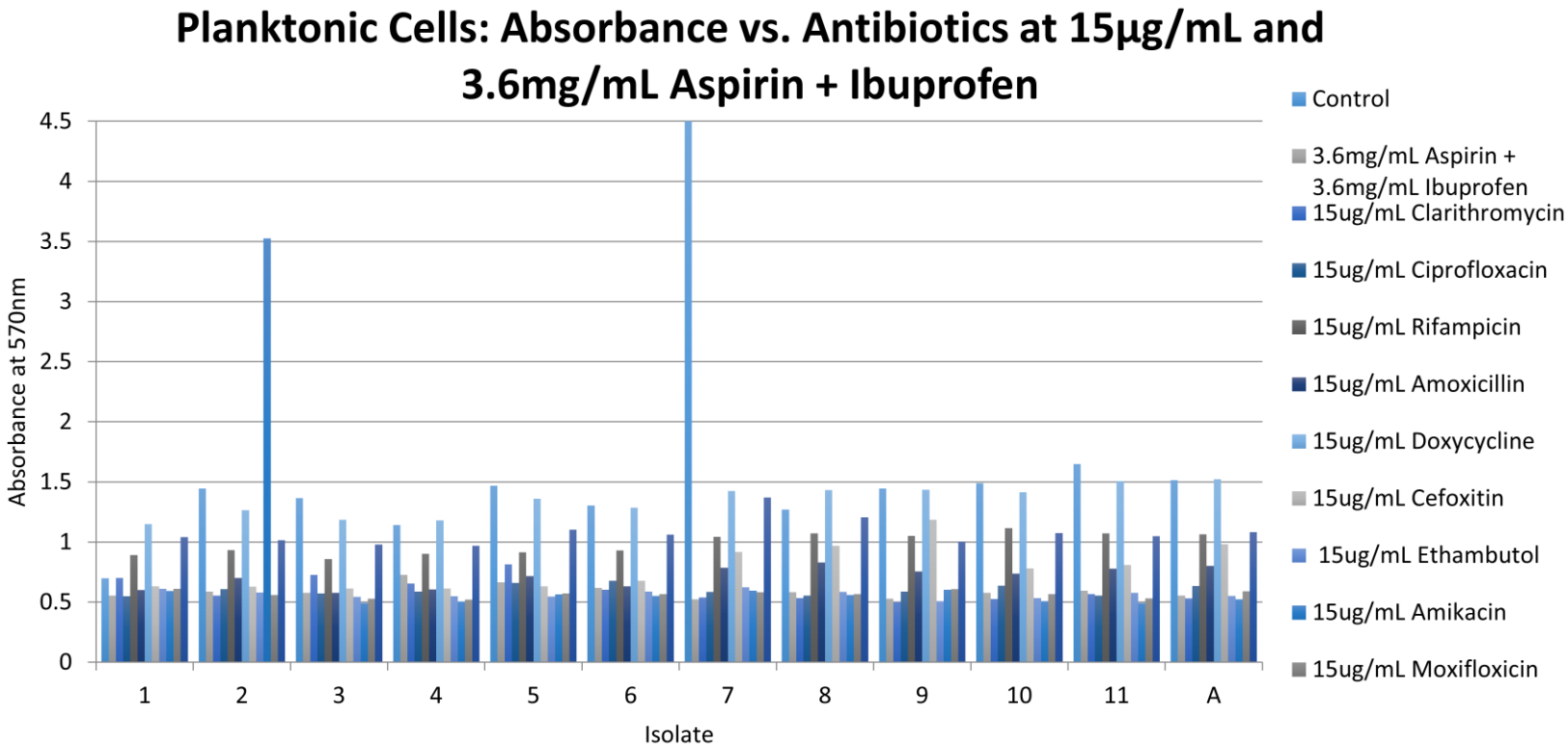


Figure 11. *M. avium* planktonic cells treated with varying antibiotics at 15µg/mL as well as 3.6mg/mL of aspirin and ibuprofen. The 15µg/mL amikacin result for isolate 2 could be the result of a contamination. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore, these absorbance values have been doubled to reflect this

Aspirin at concentrations of 3.6mg/mL had substantial inhibitory effects across all isolates (figure 12). This inhibitory effect improved when aspirin (3.6mg/mL) was combined with 15µg/mL clarithromycin. N-acetyl-L-cysteine had weaker effects, even promoting growth in isolate 1 compared to the control.

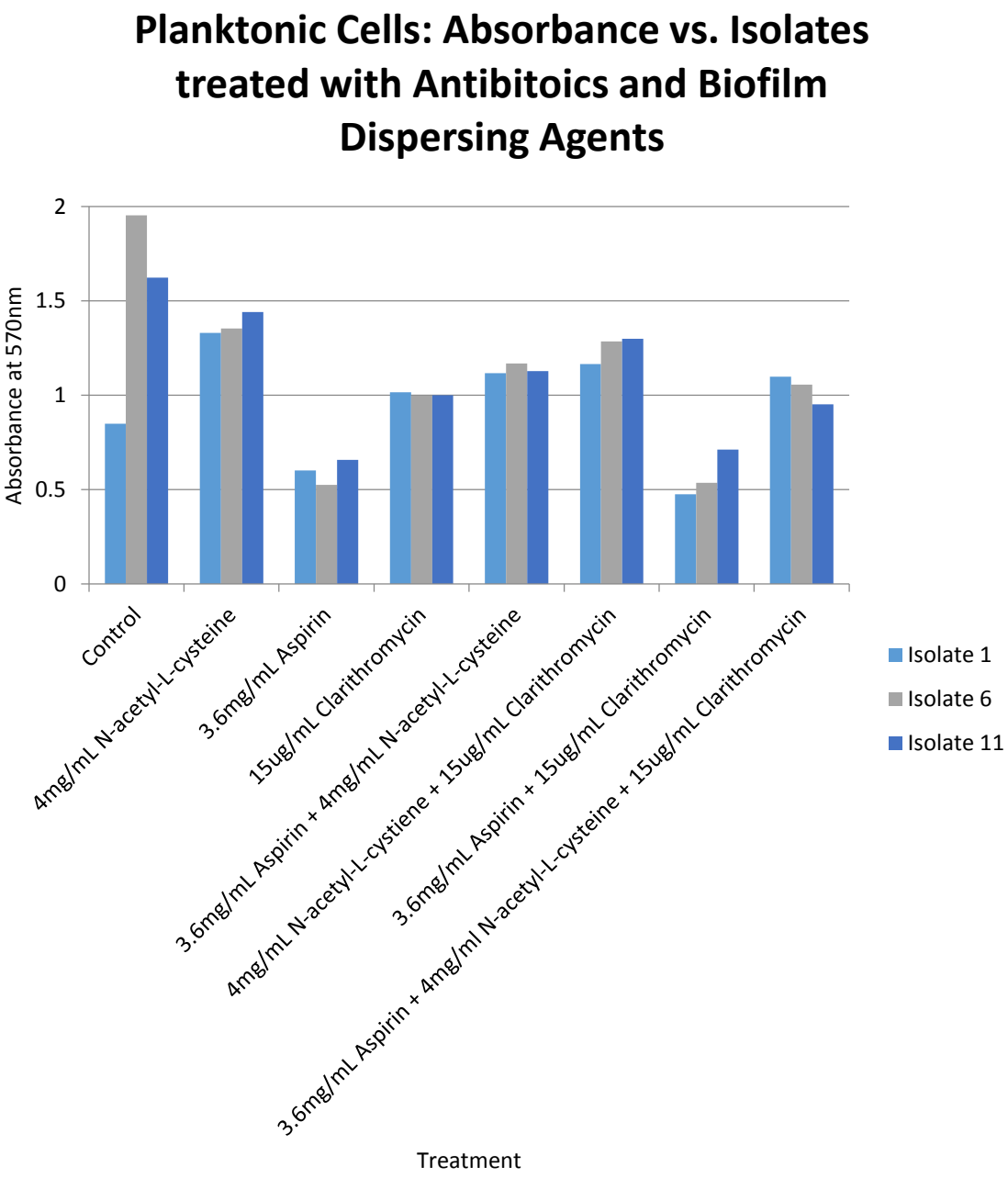


Figure 12. Corresponding MTT ODs of *M. avium* planktonic cells from three isolates treated with antibiotics and potential biofilm dispersing agents.

Furthermore aspirin (at a concentration of 3.6mg/mL) showed synergistic effects on planktonic *M. avium* cells when combined with 15µg/mL clarithromycin (figure 13). The average absorbance value across all confirmed *M. avium* isolates dropped from 1.34 in the untreated control to 0.6 in the aspirin and clarithromycin treated isolates. Both aspirin (3.6mg/mL) and ibuprofen (3.6mg/mL) had more effect on planktonic *M. avium* isolates than 15µg/mL clarithromycin. Unlike aspirin, the addition of 3.6mg/mL ibuprofen did not increase the effectiveness of aspirin combined with clarithromycin (figure 13).

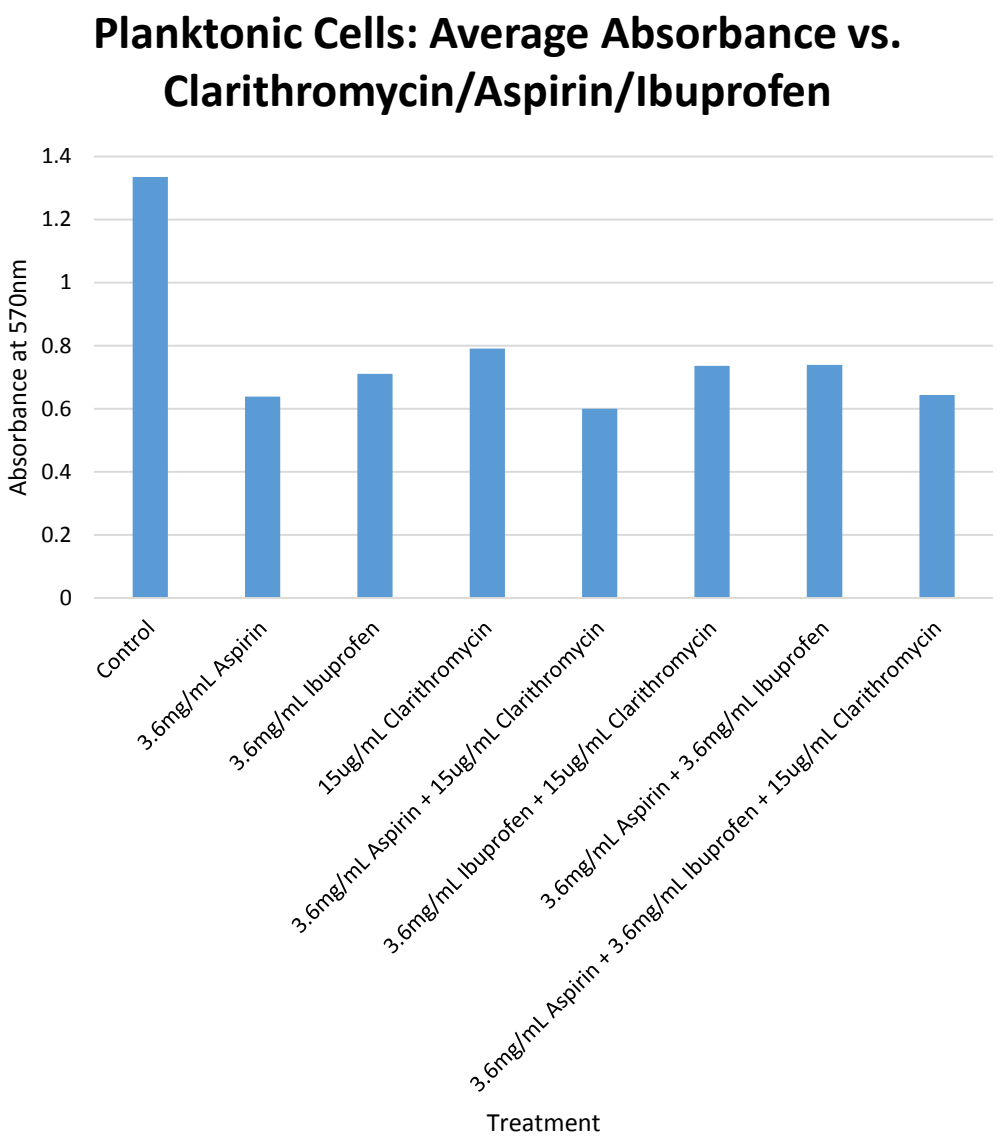


Figure 13. MTT average ODs of *M. avium* planktonic cells taken from four day old mature biofilms and treated with antibiotics and potential biofilm dispersing agents.

3.2.1 Aspirin Effectiveness Against Planktonic *Mycobacterium avium* Cells

A dilution series was made and MTT analysis performed on the planktonic cells. Aspirin effectivity against planktonic *M. avium* populations decreased with decreasing concentration (table 4).

Table 4. MTT ODs on *M. avium* isolate 6 planktonic cells, grown overnight, treated with varying concentrations of aspirin.

Dilution factor	Aspirin Concentration	OD absorbance at 570nm
Control	0mg/mL	0.706
1:2	1.8mg/mL	0.125
1:4	0.9mg/mL	0.281
1:8	0.45mg/mL	0.457
1:16	0.225mg/mL	0.560
1:32	0.1125mg/mL	0.634

This experiment was then repeated with a 1.8mg/mL aspirin concentration and different antibiotics. No significant synergy was found between aspirin and antibiotics (table 5). However, aspirin had a tenfold decrease in planktonic cell population compared to the control.

Table 5. MTT ODs on *M. avium* isolate 6 planktonic cells, grown overnight, treated with 1.8mg/mL aspirin and 5µg/mL of antibiotics that previously showed effect on *M. avium* planktonic cells.

Conditions	OD absorbance at 570nm
Control (0mg/mL)	1.727
Aspirin (1.8mg/mL)	0.174
Aspirin (1.8mg/mL) +5µg/mL Clarithromycin	0.354
Aspirin (1.8mg/mL) + 2.5µg/mL Amikacin	0.184
Aspirin (1.8mg/mL) + 5µg/mL Cefoxitin	0.228
Aspirin (1.8mg/mL) + 5µg/mL Ciprofloxacin	0.196

The experiment was repeated on four day old planktonic cell populations using decreasing aspirin concentrations and combined antibiotic therapies (figure 14). Aspirin again showed a dependency on concentration for effective inhibition of *M. avium* growth. A minimal dosage of aspirin (0.45mg/mL) combined with 5µg/mL of clarithromycin showed similar levels of activity against planktonic cells as 1.8mg/mL concentration of aspirin alone. Aspirin at 0.9mg/mL combined with 5µg/mL of clarithromycin and 5µg/mL of ciprofloxacin also showed the same level of inhibition to *M. avium* planktonic cell metabolism (figure 14).

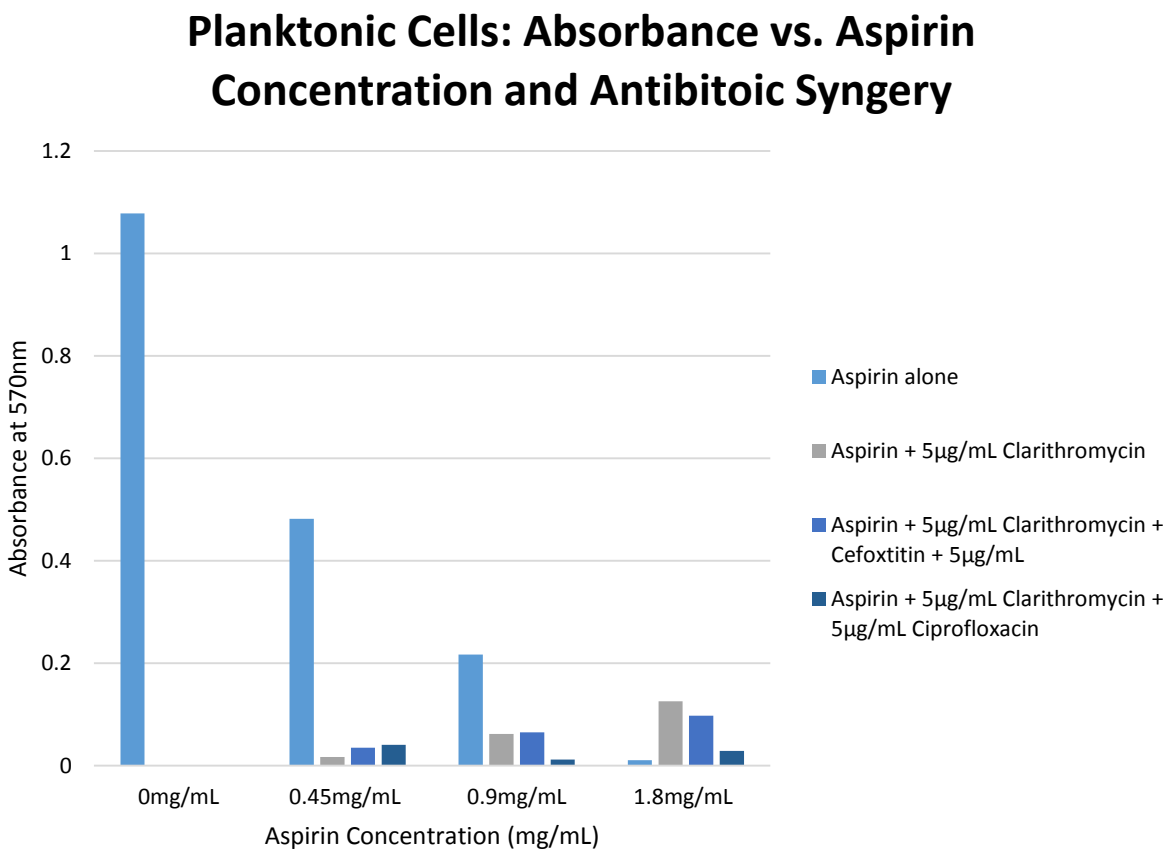


Figure 14. MTT ODs from *M. avium* planktonic cells treated with increasing concentrations of aspirin, and a combination of antibiotics.

3.2.2 Antibiotic and Biofilm Dispersal Agents MICs With TTC Indicator on *Mycobacterium avium* Planktonic Cells

TTC was then used to determine relative MICs of planktonic cells. MIC was determined by 0% red colouration in the cell populations, indicating complete sterilization.

Amikacin, amoxicillin, ciprofloxacin, clarithromycin and moxifloxacin all had complete sterilization at concentrations of 128µg/mL, while no MIC could be determined for cefoxitin, doxycycline, ethambutol, rifampicin and Rifinah ™. Aspirin had complete eradication at 4mg/mL, while ibuprofen sterilized at 1mg/mL (table 6).

Table 6. TTC Colorimetric deduced MICs of *M. avium.*

Treatment	Concentration of Treatment							
Antibiotic	1µg /mL	2µg /mL	4µg /mL	8µg /mL	16µg/ mL	32µg /mL	64µg /mL	128µg /mL
Amikacin	Red	Red	Red	Red	Red	Red	Red	Clear
Amoxicillin	Red	Red	Red	Red	Red	Red	Red	Clear
Cefoxitin	Red	Red	Red	Red	Red	Red	Red	Red
Ciprofloxacin	Red	Red	Red	Red	Red	Red	Red	Clear
Clarithromycin	Red	Red	Red	Red	Red	Red	Red	Clear
Doxycycline	Red	Red	Red	Red	Red	Red	Red	Red
Ethambutol	Red	Red	Red	Red	Red	Red	Red	Red
Moxifloxacin	Red	Red	Red	Red	Red	Red	Red	Clear
Rifampicin	Red	Red	Red	Red	Red	Red	Red	Red
Rifinah ™	Red	Red	Red	Red	Red	Red	Red	Red

Biofilm Disruptor	0.075 mg/mL	0.125 mg/ mL	0.25 mg/ mL	0.5mg /mL	1mg/ mL	2mg/ mL	4mg/ mL	8mg/ mL
Aspirin	Red	Red	Red	Red	Red	Red	Clear	Clear
Ibuprofen	Red	Red	Red	Red	Clear	Clear	Clear	Clear

3.2.3 FDA and EtBr Staining

Although no results produced a completely sterilized sample, across multiple isolates planktonic cells treated with 3.6mg/mL aspirin showed the greatest percentage of red fluorescing cells (88.9% average), thus cell death. For isolates 9 and 10, all samples showed growth on plates, except for those treated with 3.6mg/mL aspirin.

Again, isolate differed in susceptibility to both aspirin and ibuprofen combined with antibiotics. The average percentage of dead cells from the ten confirmed *M. avium* isolates showed that 3.6mg/mL of aspirin was most effective against planktonic cells (figure 15). However, aspirin combined with all other antibiotics produced 65% and higher dead *M. avium* planktonic cells within the sample.

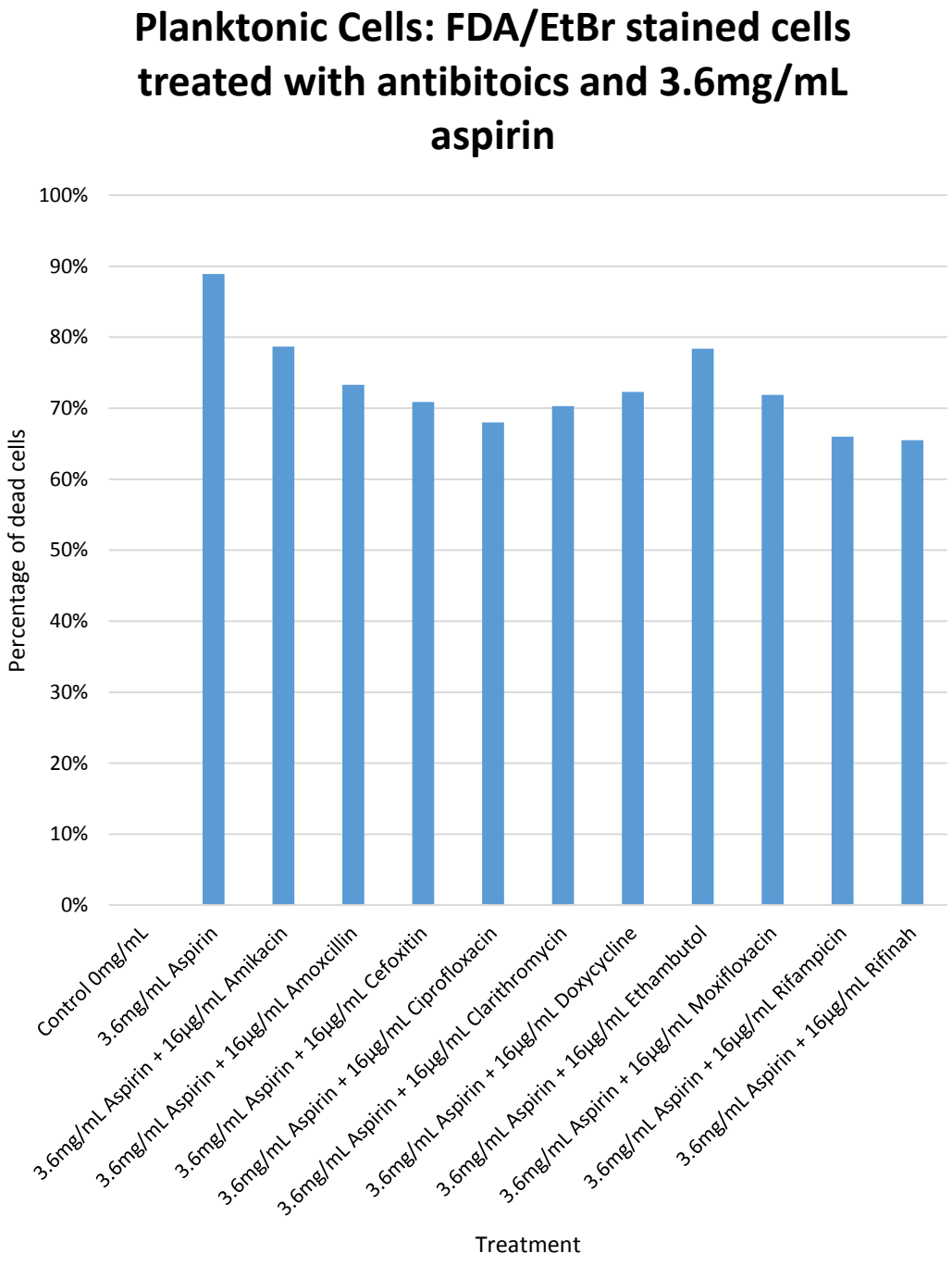


Figure 15. Average percentage of planktonic cell death on 10 *M. avium* isolates treated with 3.6mg/mL aspirin in combination with 16ug/mL concentration of antibiotics. The percentage of dead cells indicates the amount of cells fluorescing red under blue light, after FDA/EtBr staining.

Ibuprofen at 1mg/mL did not show as significant percentages of dead cells as 3.6mg/mL aspirin. Ibuprofen combined with ethambutol and ciprofloxacin showed the greatest

increase in red stained cells, while both rifampicin and Rifinah™ had the least effect (figure 16).

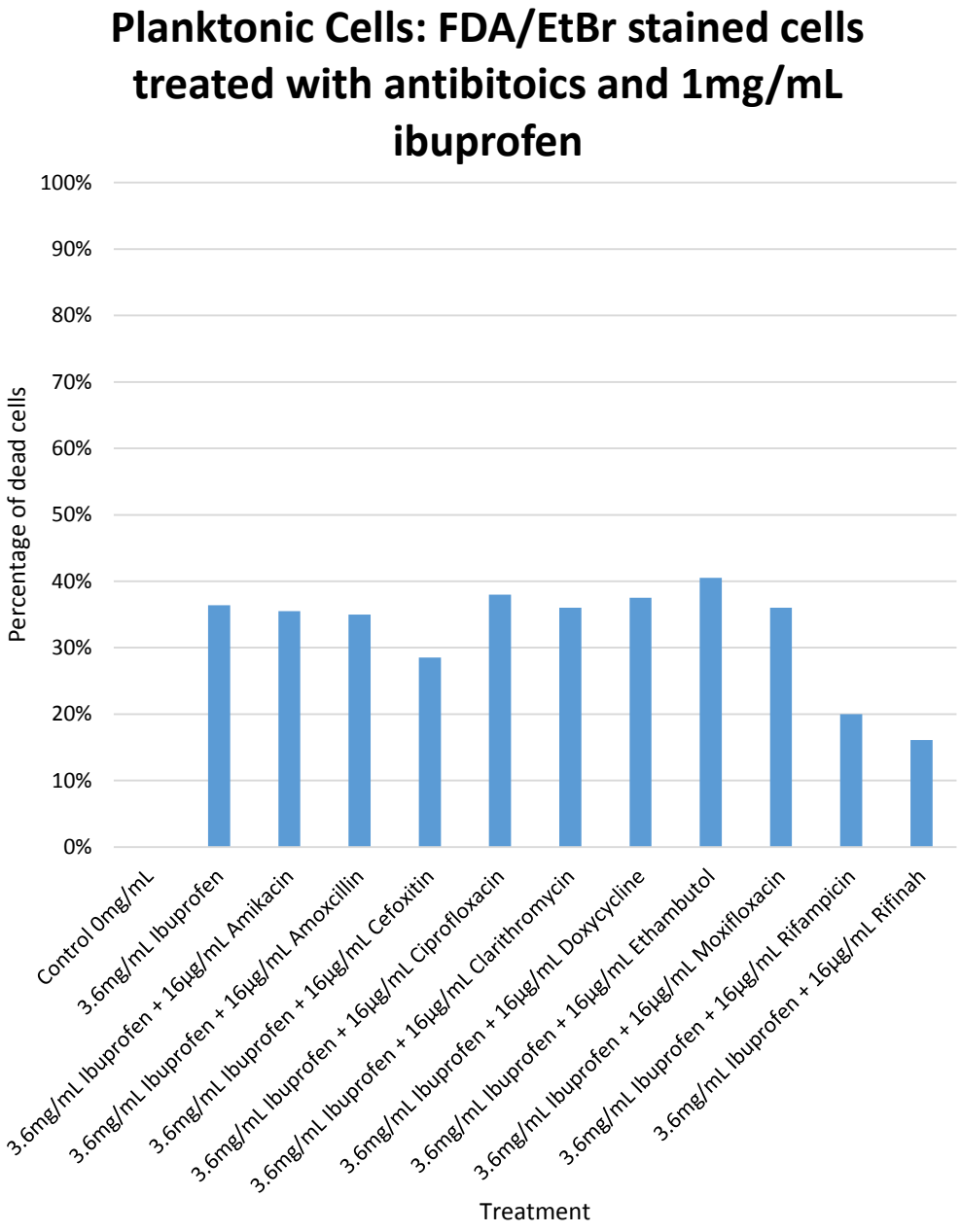


Figure 16. Average percentage of planktonic cell death on 10 *M. avium* isolates treated with 1mg/mL ibuprofen in combination with 16ug/mL concentration of antibiotics. The percentage of dead cells indicates the amount of cells fluorescing red under blue light, after FDA/EtBr staining.

3.3 Biofilm Formation

Experiments were conducted on mature biofilm populations of *M. avium* to ascertain the MICs of *M. avium* in biofilm phenotype.

3.3.1 Optimal Biofilm Growth Media

Middlebrook 7H9 media gave the best MTT OD absorbance readings at 570nm (1.036), suggesting that *M. avium* is best cultured in this media.

Table 7. Comparison of different growth media, and the optical density of resulting MTT analysis. Brain Heart Infusion combined with Middlebrook 7H9 media gave the best growth results.

Growth Media	OD at 570nm
Middlebrook 7H9 + 3% Glycerol	0.008
Middlebrook 7H9 + Tween80	0.001
199 Media + 3% Glycerol	0.492
BHI Media + Middlebrook 7H9	1.036
199 Media + 3% Glycerol + Nutrient Broth	0.309

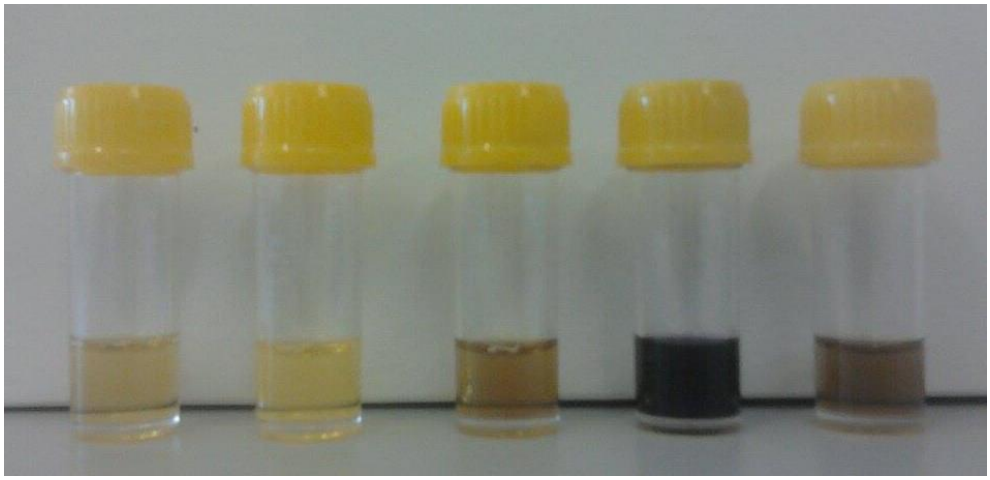


Figure 17. MTT analysis of different growth media. From left to right; Middlebrook 7H9 + 3% Glycerol; Middlebrook 7H9 + Tween80; 199 Media + 3% Glycerol; BHI Media + Middlebrook 7H9; 199 Media + 3% Glycerol + Nutrient Broth.

However, in contrast to the MTT results, under auramine-O acid alcohol methylene blue counter stain, the most efficient biofilm producing media was Middlebrook 7H9 with Tween80 (figure 18).

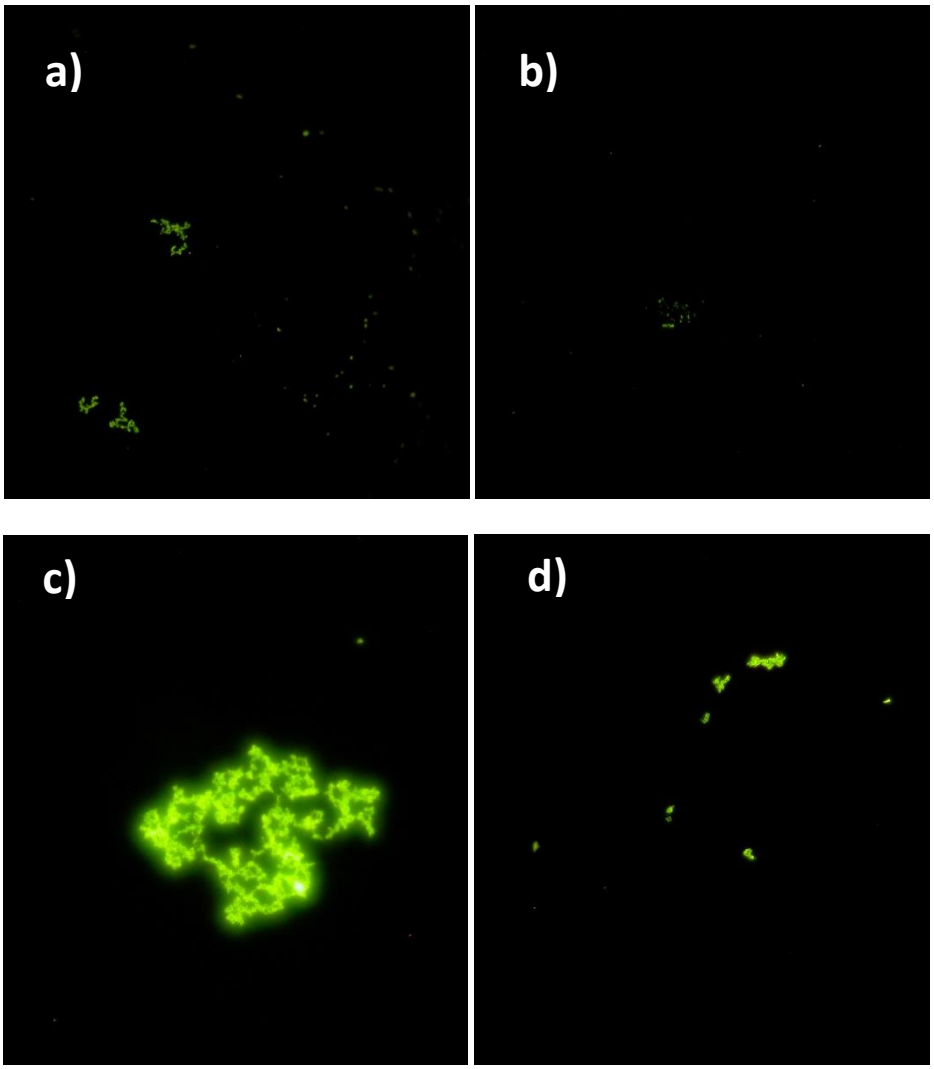


Figure 18. Microscope images of auramine-O, acid alcohol methylene blue counter stained biofilms, performed on isolate 6 under various media conditions after being grown overnight. A) Biofilm grown in 199 Media + 3% glycerol + Nutrient Broth media. B) Biofilm grown in Middlebrook 7H9 media + 3% glycerol. C) Biofilm grown in Middlebrook 7H9 media + Tween80. D) Biofilm grown in Brain Heart Infusion media + Middlebrook 7H9 media.

3.3.2 Glass Versus Polystyrene Biofilm Growth Promotion

In comparison between the optimal surfaces for biofilm development crystal violet staining showed that the glass beads had increased biofilm growth over polystyrene beads.

Table 8. Crystal Violet Staining OD absorbance readings at 570nm comparing glass and polystyrene beads.

Surface Type	OD at 570nm
Polystyrene Bead	0.035
Polystyrene Bead Repeat	0.115
Glass Bead	0.227
Glass Bead Repeat	0.180

3.3.3 Biofilm Phenotype Observations

Definite phenotypes of biofilm were observed between isolates. Isolates 6 and A1 (shown as A in the figure below) formed iris-like coverings across the whole glass cover slip, whereas isolates 8 and 11 clumped in the middle of the coverslip. Furthermore, isolates A1, A2 and A3 (both strains from the same patient) formed iris-like coverings across the glass coverslip as well as having clumps of biofilm accumulating across the coverslip.

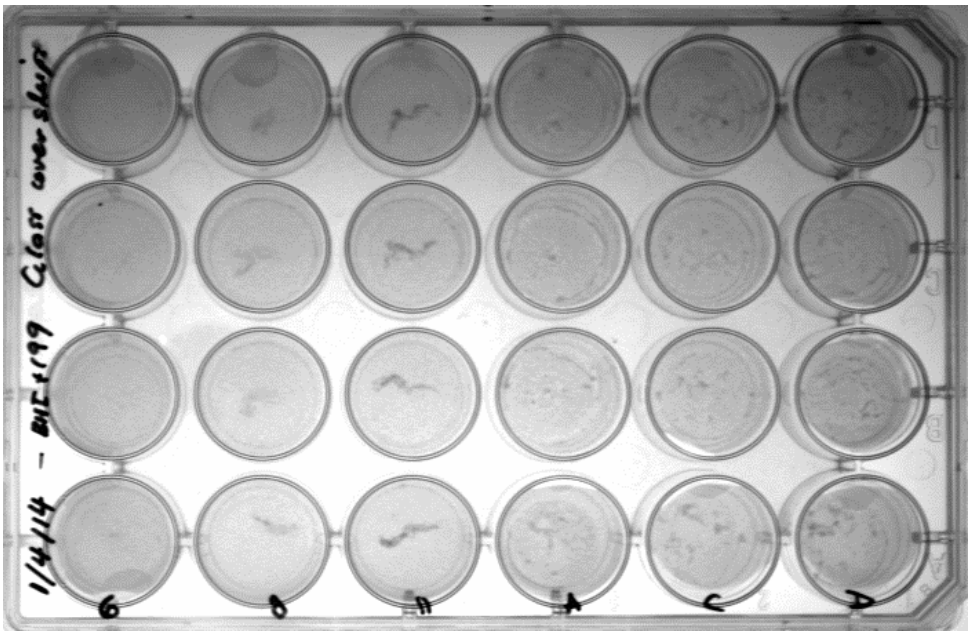
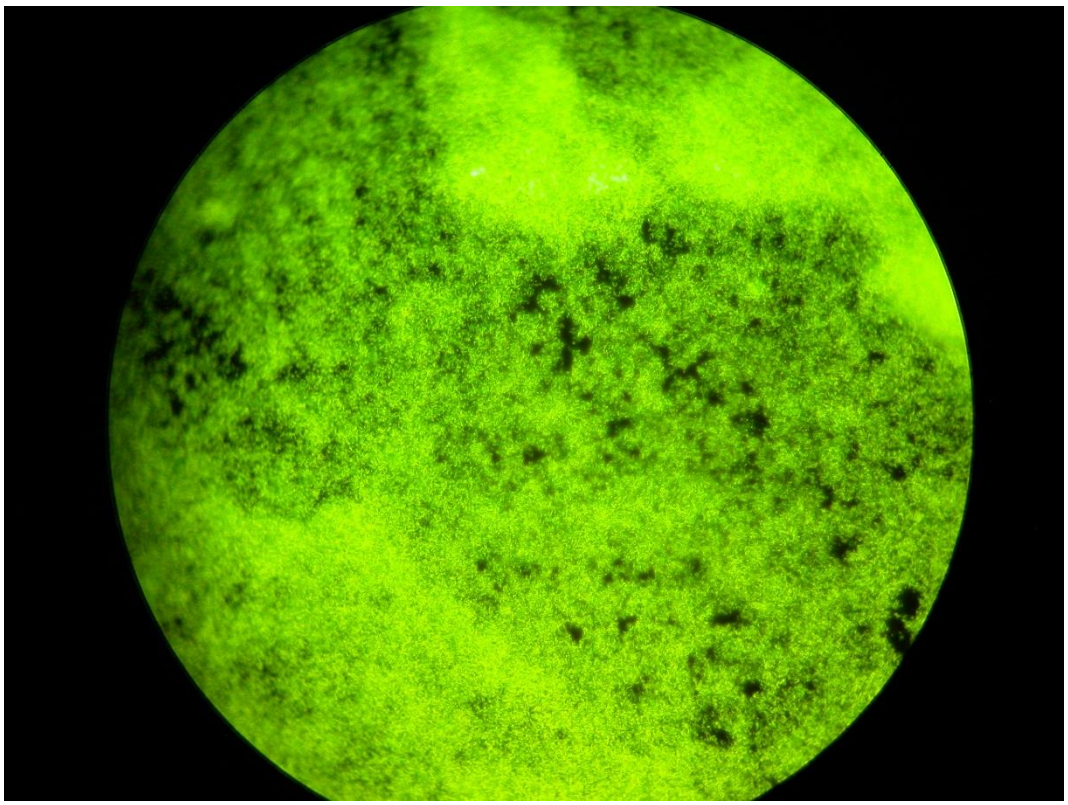
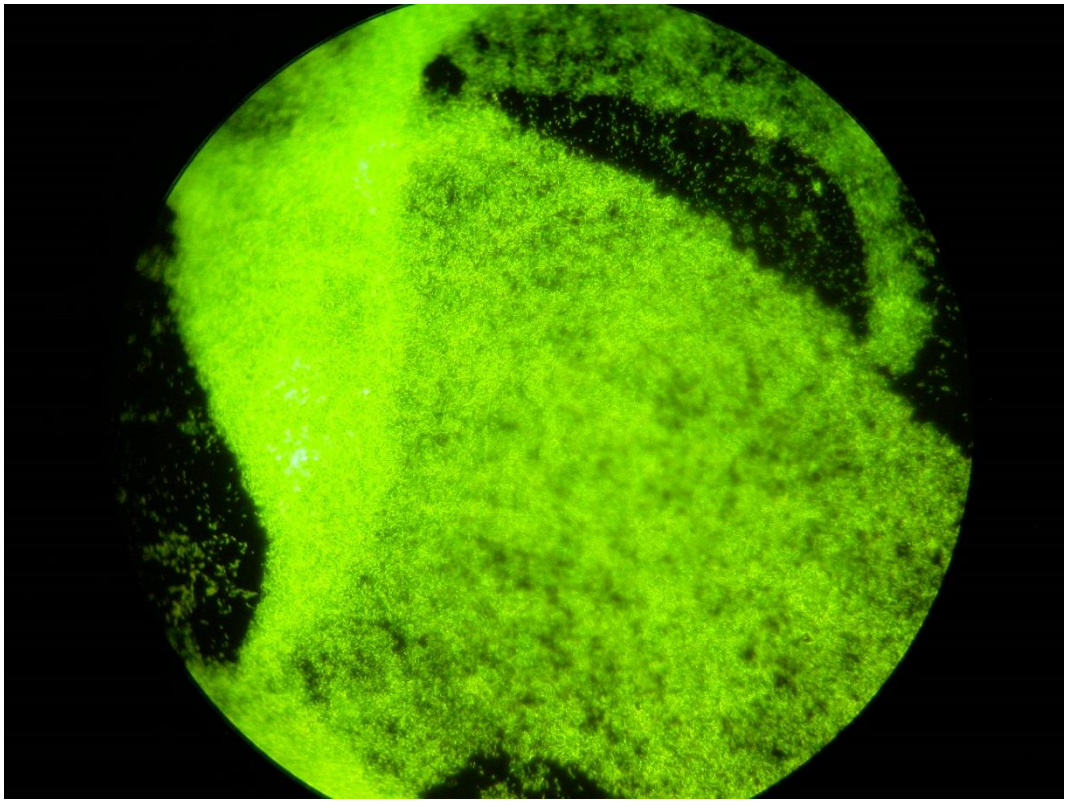
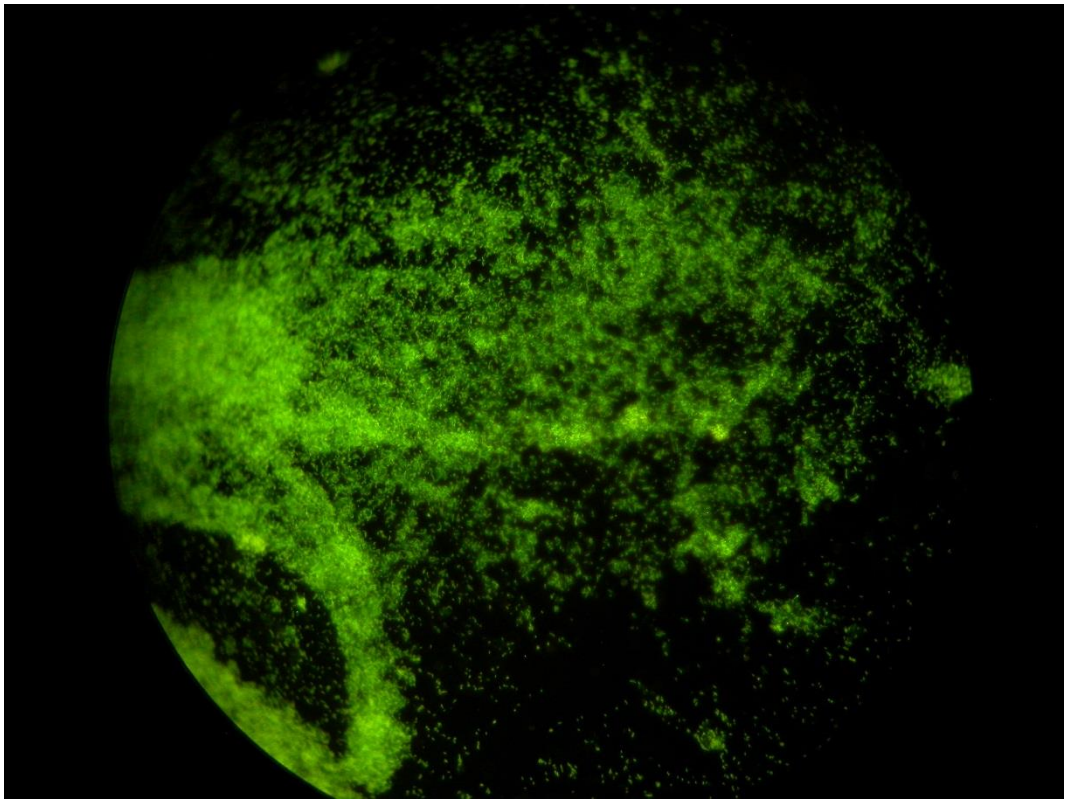


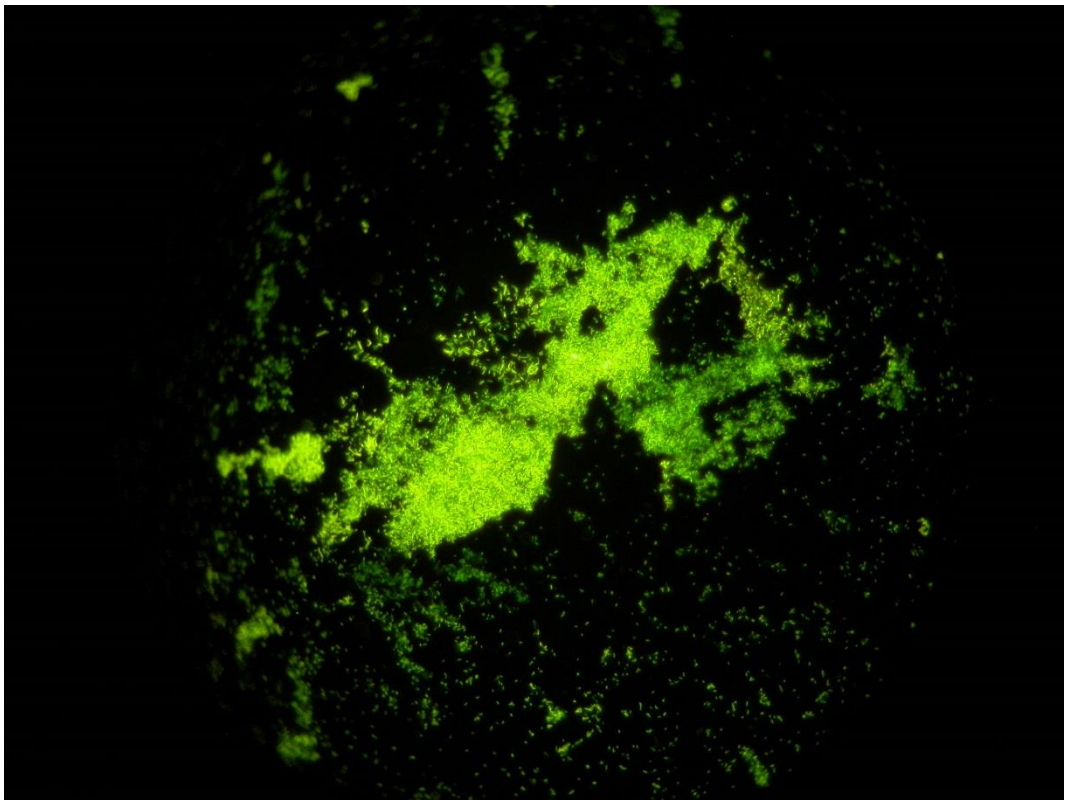
Figure 19. Photograph showing the different biofilm growth patterns of *M. avium* isolates across glass coverslips in BHI + 199 media. The isolates read left to right, 6, 8, 11, A1 (marked on plate as A), A2, (marked on the plate as C), A3 (marked on the plate as D).

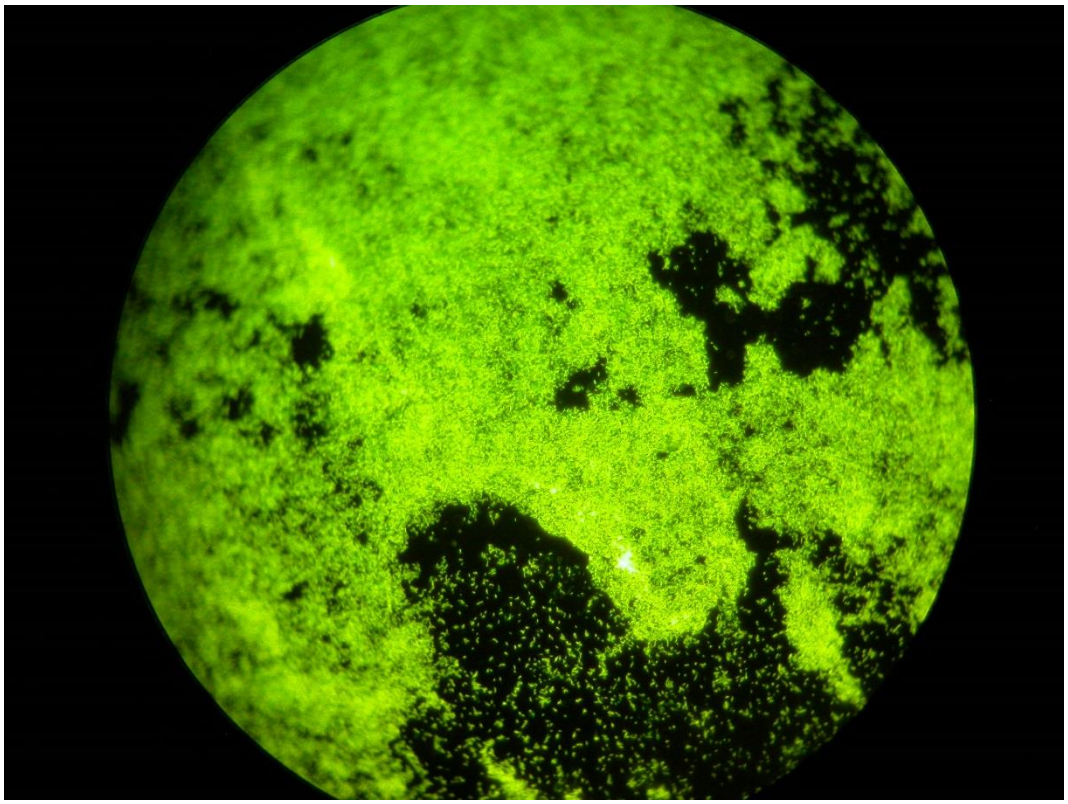
The phenotype differences of isolates were observed by auramine-O acid alcohol methylene blue staining. Isolate 6 was the most efficient biofilm producer (figure 20).











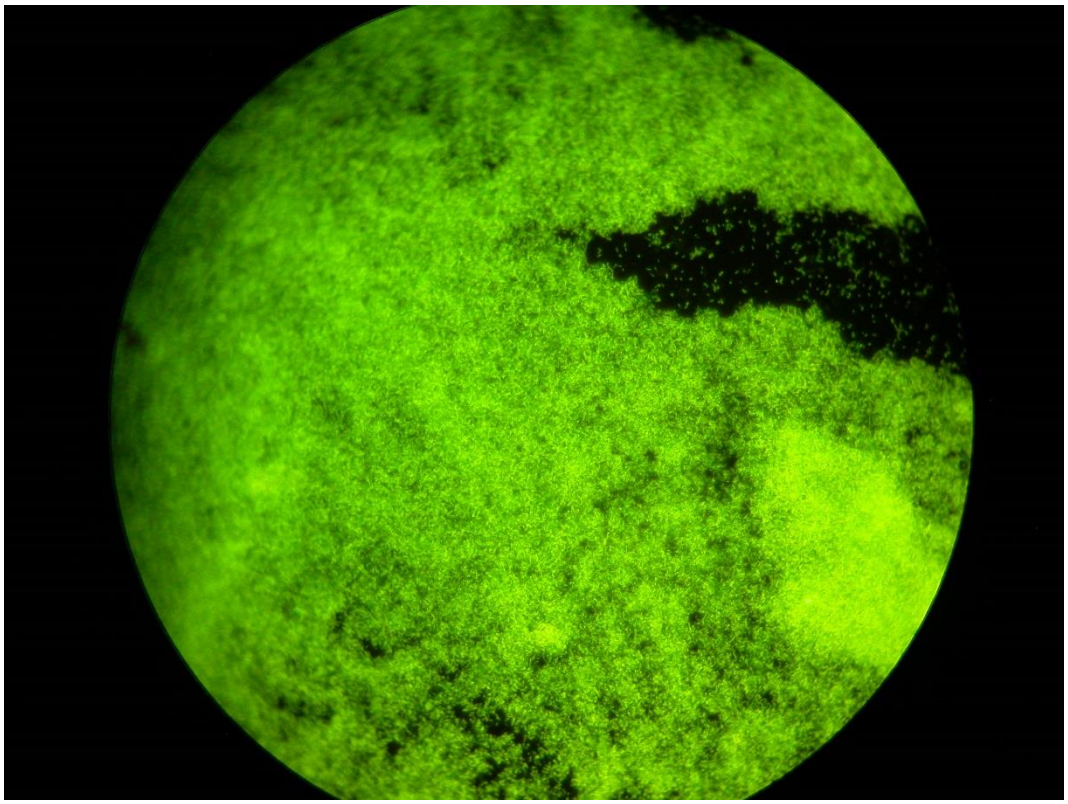


Figure 20. Six images showing isolate 6 biofilm under blue light after auramine-O, methylene blue staining. 40x magnification. Note prolific biofilm formation.

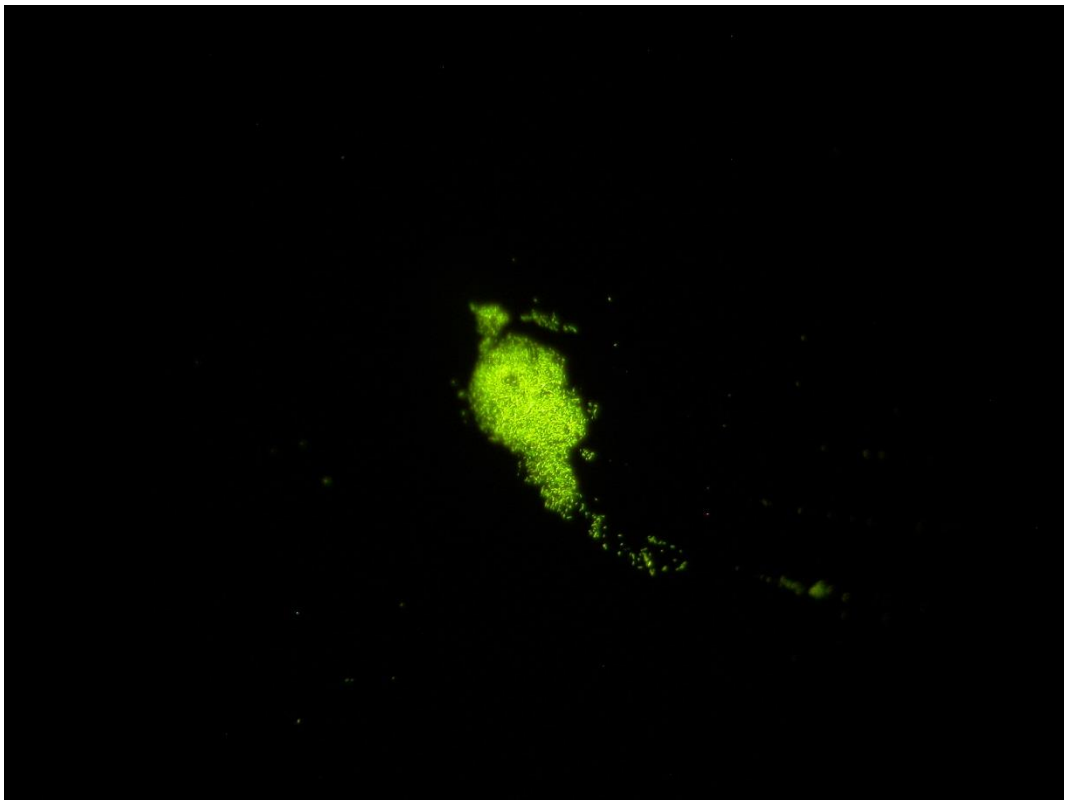


Figure 21. Isolate 8 under blue light after auramine-O, methylene blue staining. 40x magnification.

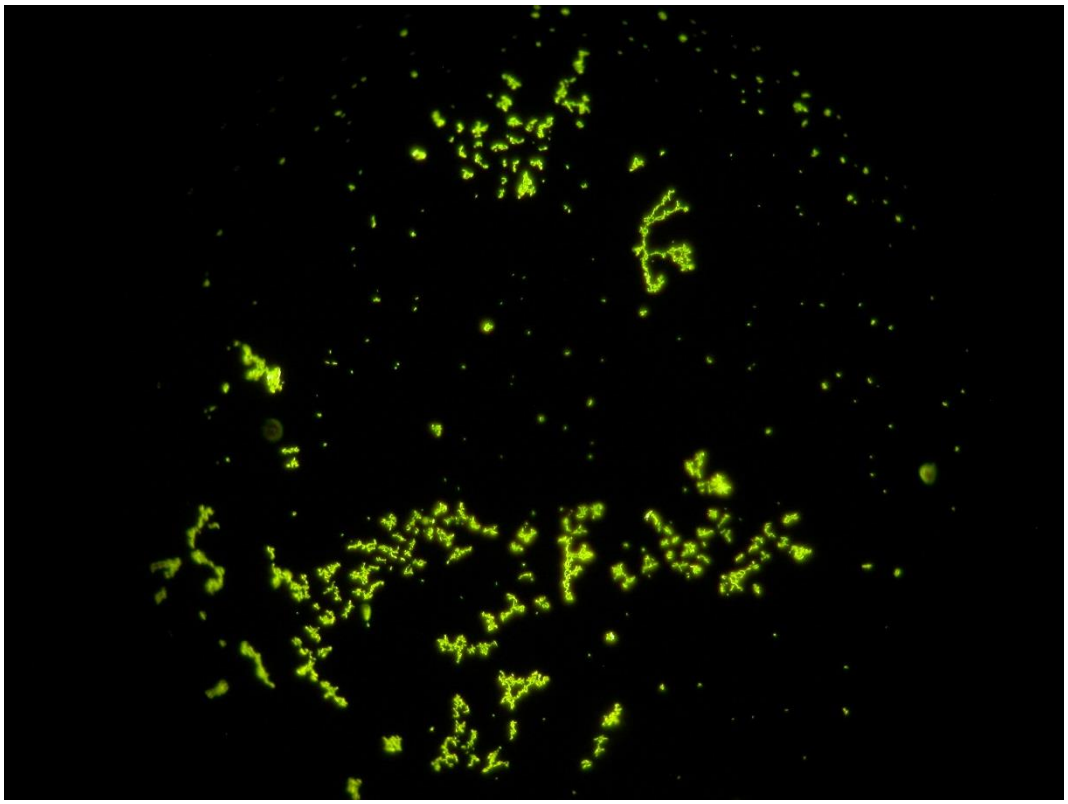


Figure 22. Isolate 11 under blue light after auramine-O, methylene blue staining. 100x magnification.

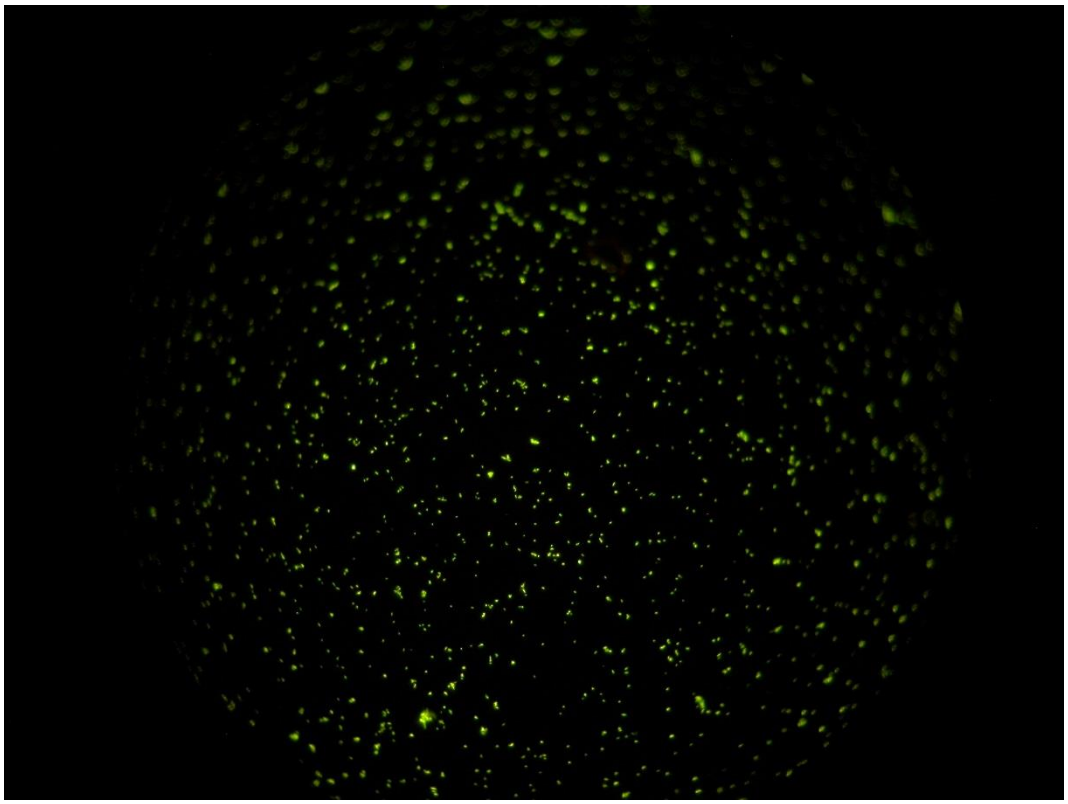


Figure 23. Isolate A1 under blue light after auramine-O, methylene blue staining. 40x magnification.

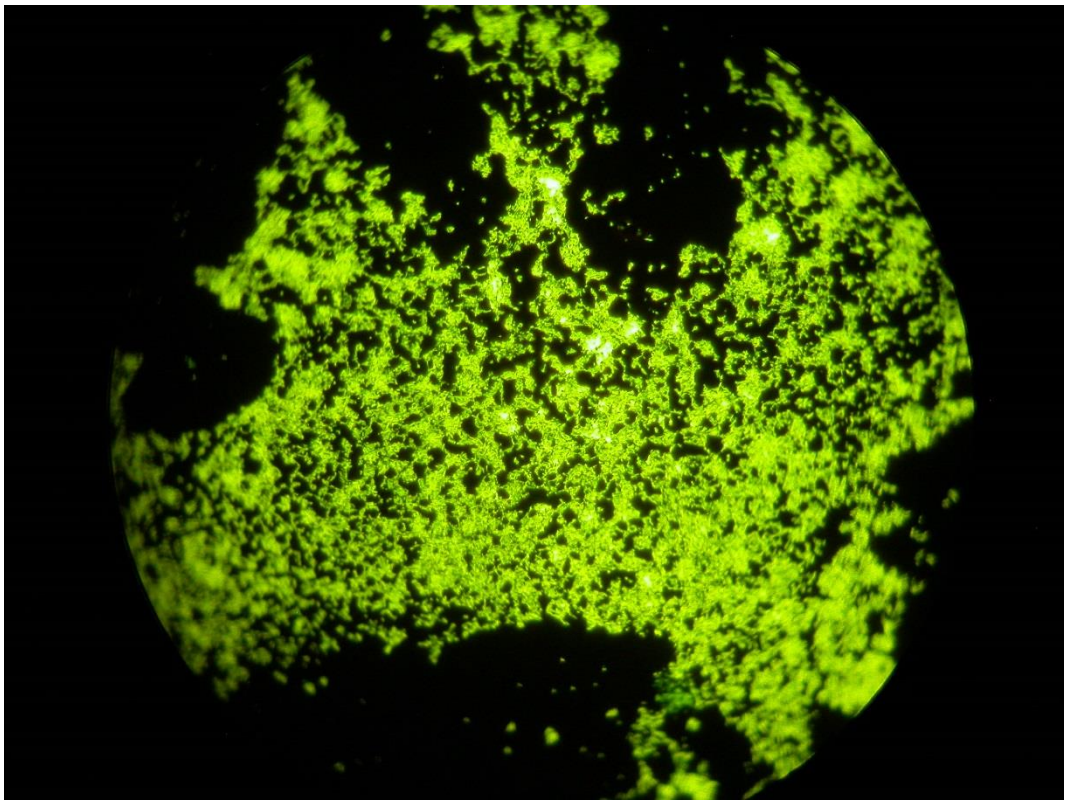


Figure 24. Isolate A2 under blue light after auramine-O, methylene blue staining. 40x magnification

Isolate differences were also determined by treating the isolates with 8µg/mL of cefoxitin. Isolate 6 proved the most susceptible to cefoxitin.

Table 9. Resulting ODs after MTT analysis on planktonic cells treated with 8µg/mL of cefoxitin. Isolates A1, A2 and A3 were all taken from the same patient at different time points in the infection.

Isolate	Planktonic Cells OD
6	0.054
8	0.089
11	0.158
A1	0.121
A2	0.102
A3	0.140

3.4 Antibiotic Biofilm Experiments

Biofilms were cultivated for four days at 37°C. Antibiotics were then added to mature biofilms and left to incubate overnight. Biofilms were then analysed the following day via the MTT protocol as outlined in the methods.

3.4.1 Biofilm Cells Increased Resistance to Cefoxitin

Initial results revealed the enhanced growth of *M. avium* populations after being treated with cefoxitin. This can be seen in table 10, where the ODs of planktonic cells were all lower, or of almost equal value as the untreated biofilm. However, the ODs between the control biofilm and the treated biofilm increased greatly across all isolates, except isolate 8.

Table 10. Resulting ODs after MTT analysis on biofilm cells. Biofilm cells were treated with 8μg/mL of cefoxitin. Control biofilm cells were not treated with cefoxitin. Isolates A1, A2 and A3 were all taken from the same patient at different time points in the infection.

Isolate	Treated Biofilm Cells OD	Control Biofilm Cells OD
6	0.578	0.201
8	0.106	0.105
11	0.427	0.176
A1	0.427	0.131
A2	0.340	0.142
A3	0.518	0.136

3.4.2 Increasing Antibiotic Concentration Against Mature Biofilms

Amoxicillin, ciprofloxacin and clarithromycin were most effective against mature biofilms (as seen in figure 25), however no antibiotic was able to completely eradicate the pre-existing biofilm. There was no significant reduction in biofilm formation with increasing antibiotic concentration (figure 25).

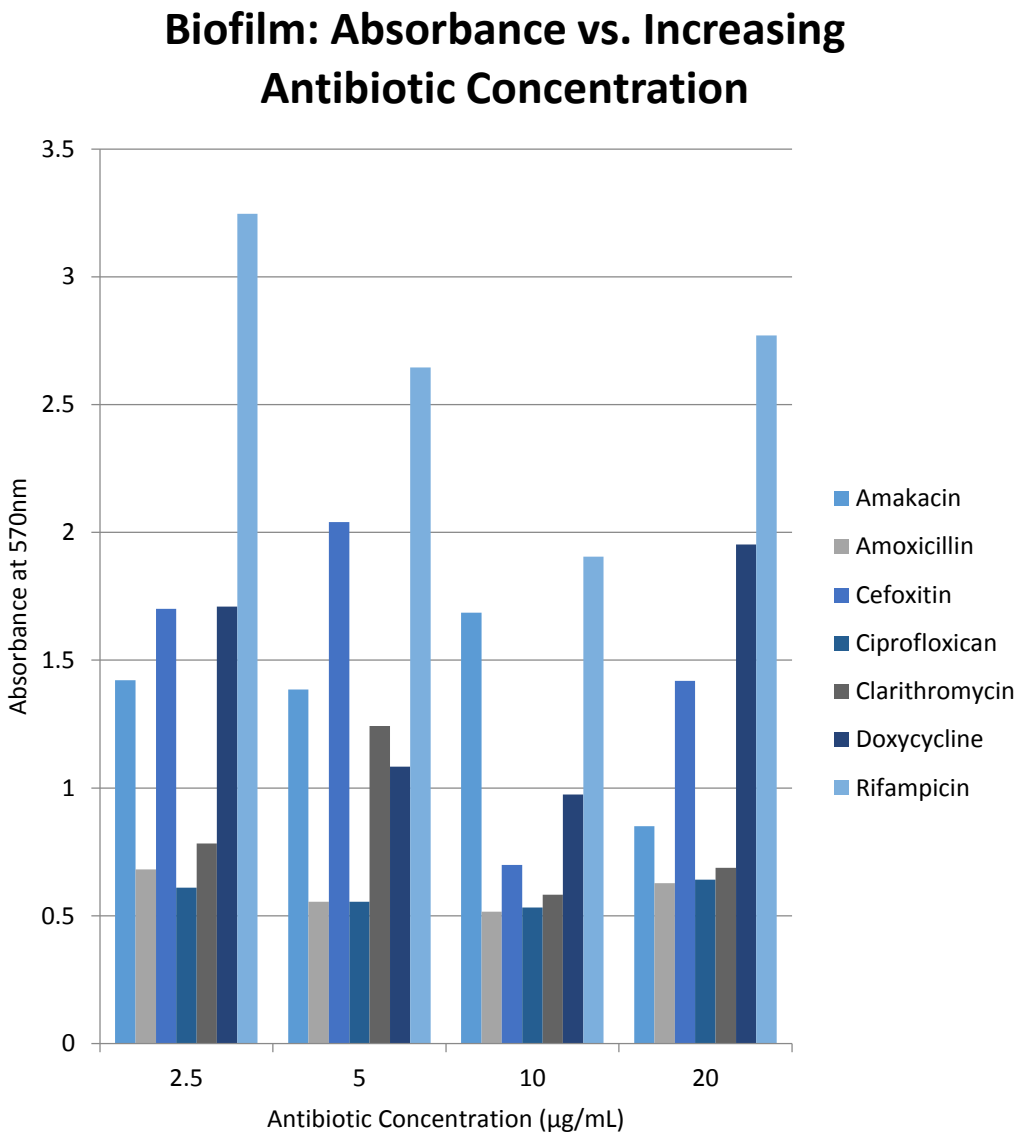


Figure 25. MTT ODs on seven day old mature *M. avium* isolate 7 biofilms treated with varying concentrations of antibiotics. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore, these absorbance values have been doubled to reflect this.

Antibiotic resistance variation was further explored between isolate biofilms. Rifampicin was chosen because of its apparent weaker antibiotic effect on *M. avium* biofilms and clarithromycin was chosen because of its strongest antibiotic effect on the biofilms. Across all isolates, increasing antibiotic concentration did not significantly decrease cell metabolism within a biofilm (figure 26 and figure 27).

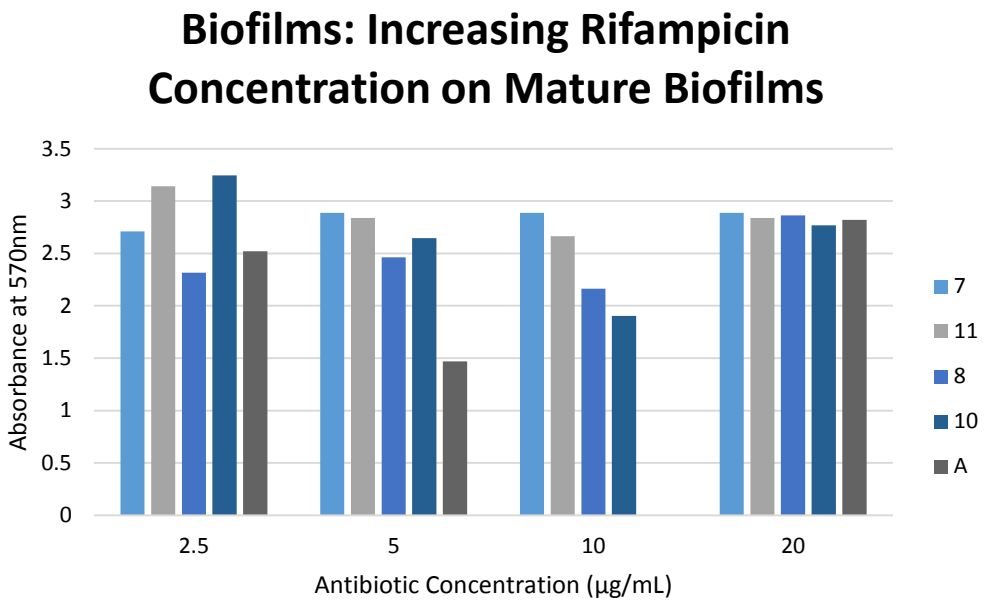


Figure 26. MTT ODs on five *M. avium* seven day old mature biofilms, treated with varying concentrations of rifampicin. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this.

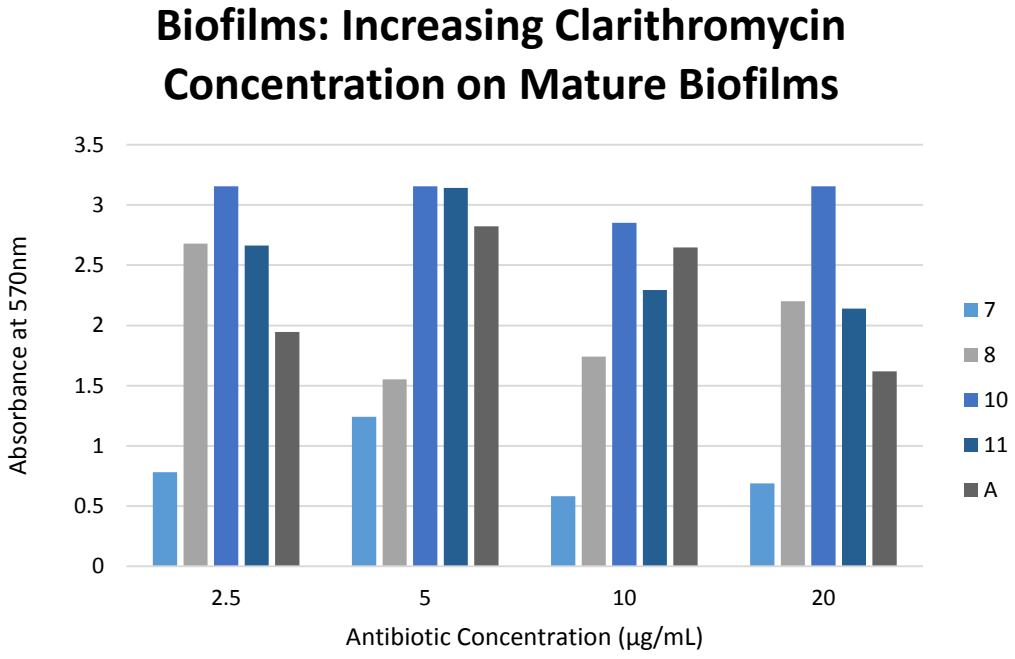


Figure 27. MTT ODs on five *M. avium* isolates, seven days old mature biofilms, treated with varying concentrations of clarithromycin. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this.

3.4.3 Antibiotic Synergy against Mature Biofilm

Isolate 1 through to 6 were tested for potential antibiotic synergy on *M. avium* biofilms (figure 28). Biofilms of non- *M. avium* isolates (isolates 3 and 4) were particularly susceptible to clarithromycin (2.5µg/mL) combined with ciprofloxacin (2.5µg/mL), as well as clarithromycin (2.5µg/mL) combined with doxycycline (2.5µg/mL). *M. avium* isolates ranged in their susceptibility to combination antibiotic therapy.

However, the average results from all four isolates showed that clarithromycin alone was the most effective at inhibiting biofilm metabolism (figure 29).

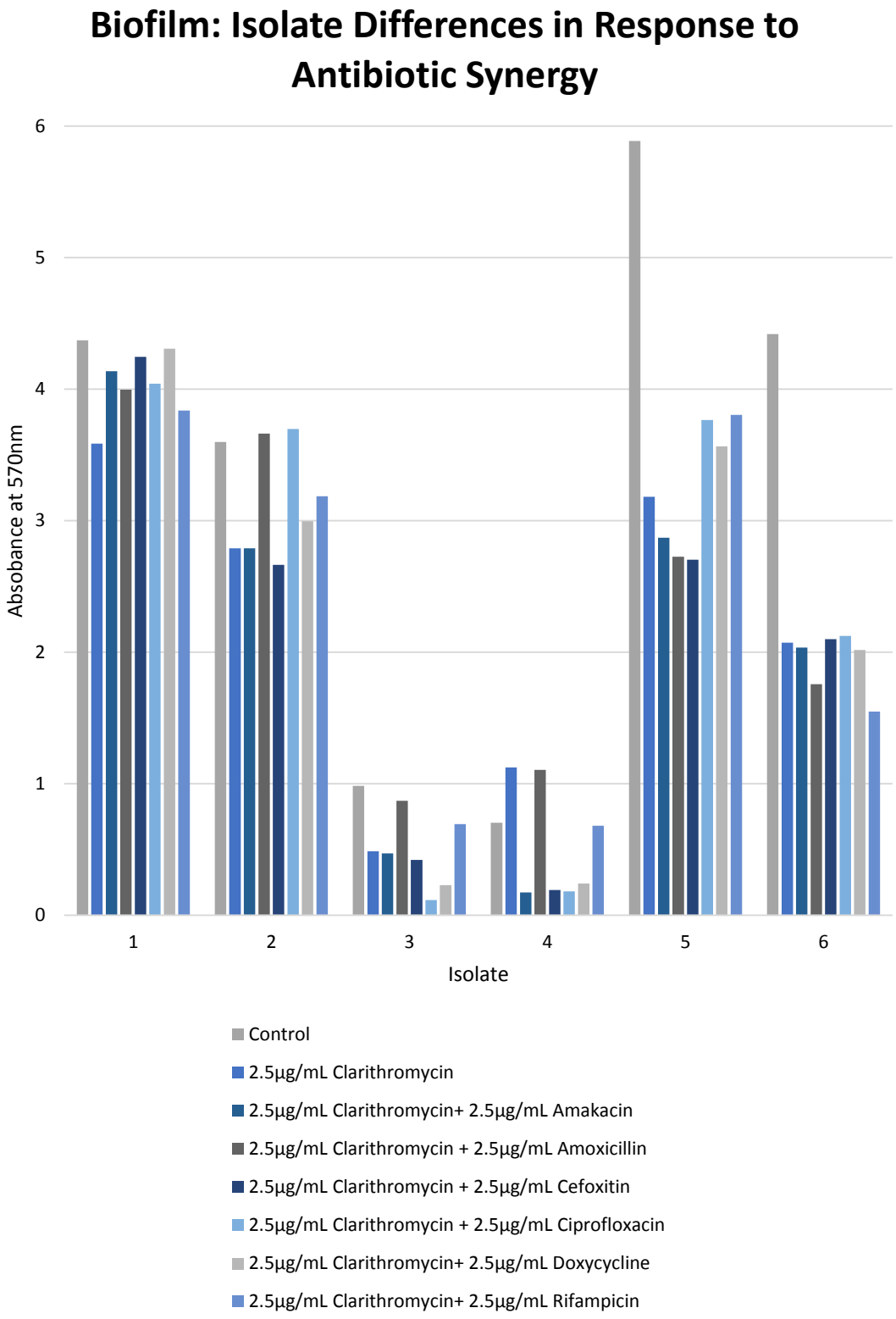


Figure 28. Comparison between MTT ODs on six, week old *M. avium* biofilms, after being treated with seven different antibiotics at 2.5µg/mL concentrations. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this.

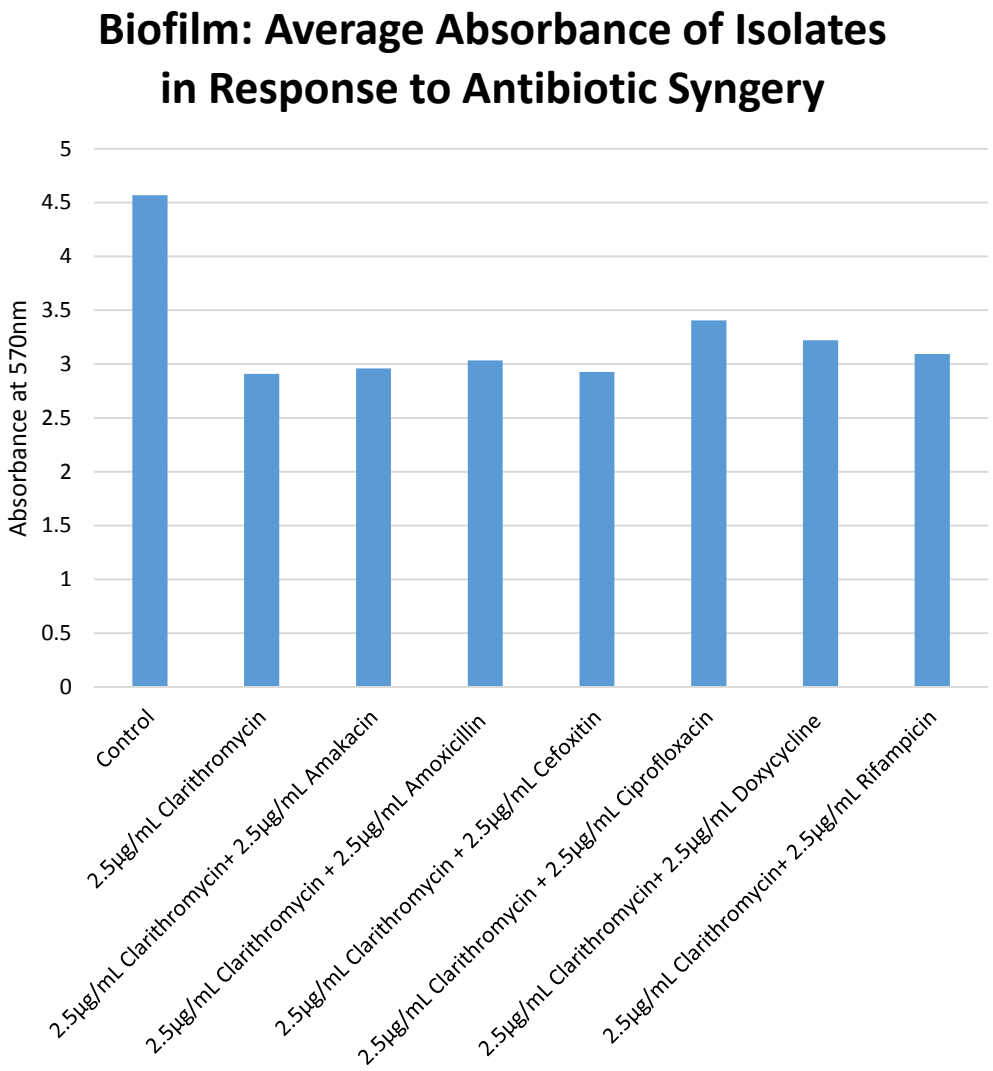


Figure 29. Average MTT ODs of six, week old *M. avium* biofilms, after being treated with seven different antibiotics at 2.5mg/mL concentrations. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore, these absorbance values have been doubled to reflect this.

The concentration of antibiotics were increased to 15µg/mL and both aspirin and ibuprofen at 3.6mg/mL were used against *M. avium* mature biofilms across all isolates. The average absorbance of all confirmed *M. avium* isolates showed that 15µg/mL clarithromycin was most effective antibiotic against *M. avium* biofilms agreeing with our planktonic results (figures 9, 10 and 11). However, the absorbance values were all significantly higher than the planktonic populations (figure 30). Doxycycline (at

15µg/mL) was the most ineffective antibiotic tested. No MICs for *M. avium* isolates were able to be determined due to the high resistance of all *M. avium* biofilms.

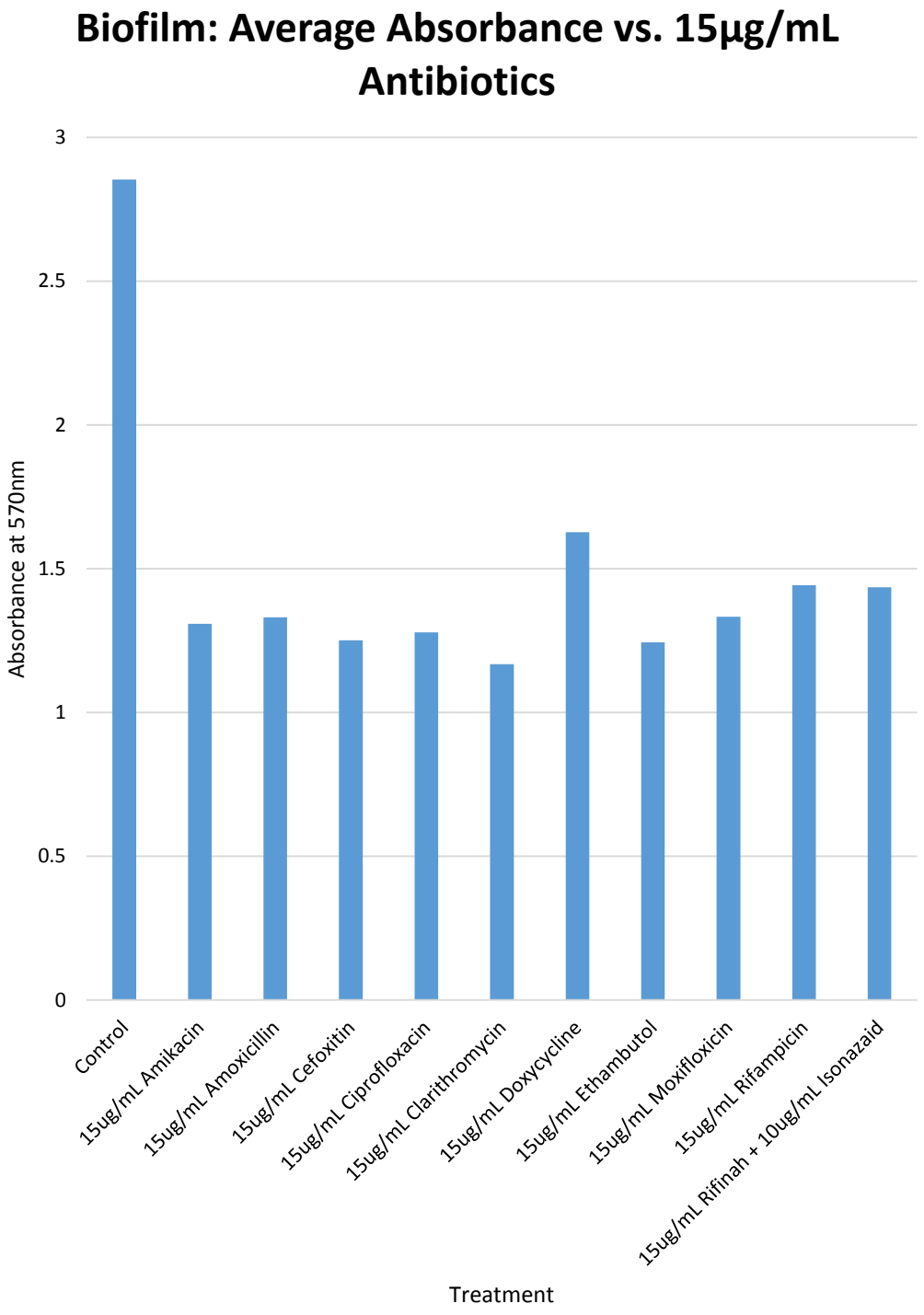


Figure 30. Average MTT ODs of 10 three day old mature *M. avium* biofilms treated with varying antibiotics at 15µg/mL. Raw MTT results for untreated control samples were diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this.

3.5 Biofilm Dispersing Agents

Three day old biofilms were treated with three biofilm dispersing agents, L-arginine (at 4mg/mL), 2,4-dinitrophenol (DNP) (at 10µg/mL) and Lipitor ™ (at 10µg/mL), in conjunction with three antibiotics at a concentrations between 2.5µg/mL and 5µg/mL (figure 31). The treated biofilms and planktonic cells were incubated over 48 hours, before an MTT analysis was carried out and the absorbance was measured at 570nm. No significant decrease of *M. avium* biofilms were observed. L-arginine was found to greatly increase biofilm formation even with the presence of antibiotics. DNP (at 10µg/mL) combined with 5µg/mL clarithromycin and 5µg/mL ciprofloxacin had the most effect on *M. avium* biofilms, however, this was not significant.

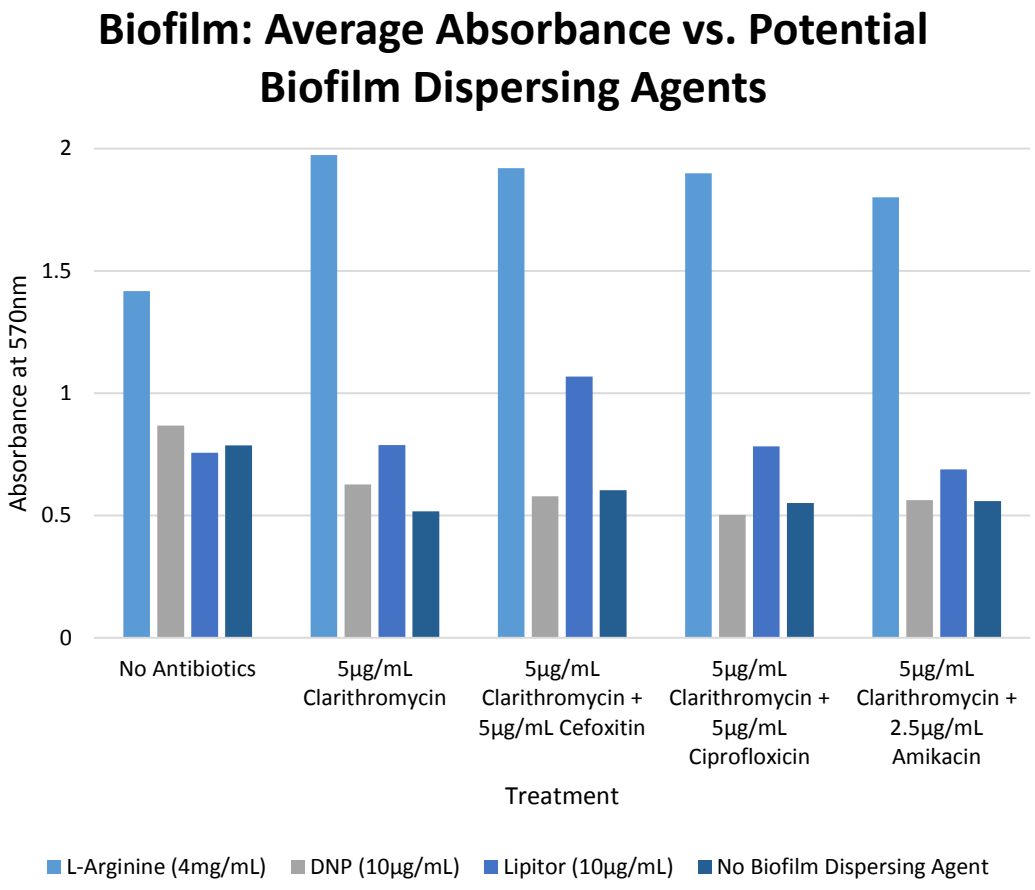


Figure 31. Average MTT ODs of 6, three day old, mature *M. avium* biofilms treated with varying antibiotics at 2.5-5µg/mL concentrations and potential biofilm dispersing agents.

This protocol was repeated with methylglyoxal (MGO) (at 10µg/mL), mannitol (at 40mM) and active manuka honey (at 0.2%) Again, no potential biofilm distributors significantly increased the effect of the antibiotics against mature biofilms (table 11).

Table 11. MTT ODs at 570nm, on four day old mature *M. avium* isolate 6 biofilms, treated with potential biofilm dispersing agents. Raw MTT results were diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this. Samples denoted with an asterisk show samples that had poor biofilm growth before treatment.

Treatment and Concentration	Control	MGO (10µg/mL)	Mannitol (40mM)	Lipitor (10µg/mL)	DNP (10µg/mL)	Active Manuka (0.2%)
0µg/mL	2.672	2.32	1.876	1.669	1.379	1.82
5µg/mL Clarithromycin	1.568	2.153	1.284	1.428	1.349	1.273
5µg/mL Clarithromycin + 5µg/mL Cefoxitin	1.699	2.053	1.053	1.145	1.086	1.134
5µg/mL Clarithromycin + 5µg/mL Ciprofloxacin	0.514*	0.798*	1.626	1.58	1.294	1.243

Aspirin (at a 4mg/mL concentration) combined with EDTA (at a 8mg/mL concentration) resulted in complete eradication of the *M. avium* biofilm as well as complete sterilization as seen in table 12 below (absorbance at 570nm =0.102).

Table 12. MTT ODs at 570nm, on four day old mature *M. avium* biofilms, treated with potential biofilm dispersing agents. Samples denoted with an asterisk show samples that had poor biofilm growth before treatment.

	Control	MGO (10µg/mL)	Mannitol (40mM)	Lipitor (10µg/mL)	DNP (10µg/mL)	Active Manuka (0.2%)	Aspirin (4mg/mL) + EDTA (8mg/mL)
Absorbance at 570nm	2.327	1.62	1.615	0.695*	2.044	1.97	0.102

3.6 Aspirin as a Potential *Mycobacterium avium* Biofilm Distributor

The drastic effects of aspirin on *M. avium* planktonic cells was not seen against all *M. avium* mature biofilm isolates (figure 32). Again, the isolates all showed a wide variety of susceptibility to antibiotic treatment. In contrast to all other isolates tested, isolate 1 was more inhibited by 5µg/mL Clarithromycin than aspirin (at a 1.8mg/mL concentration).

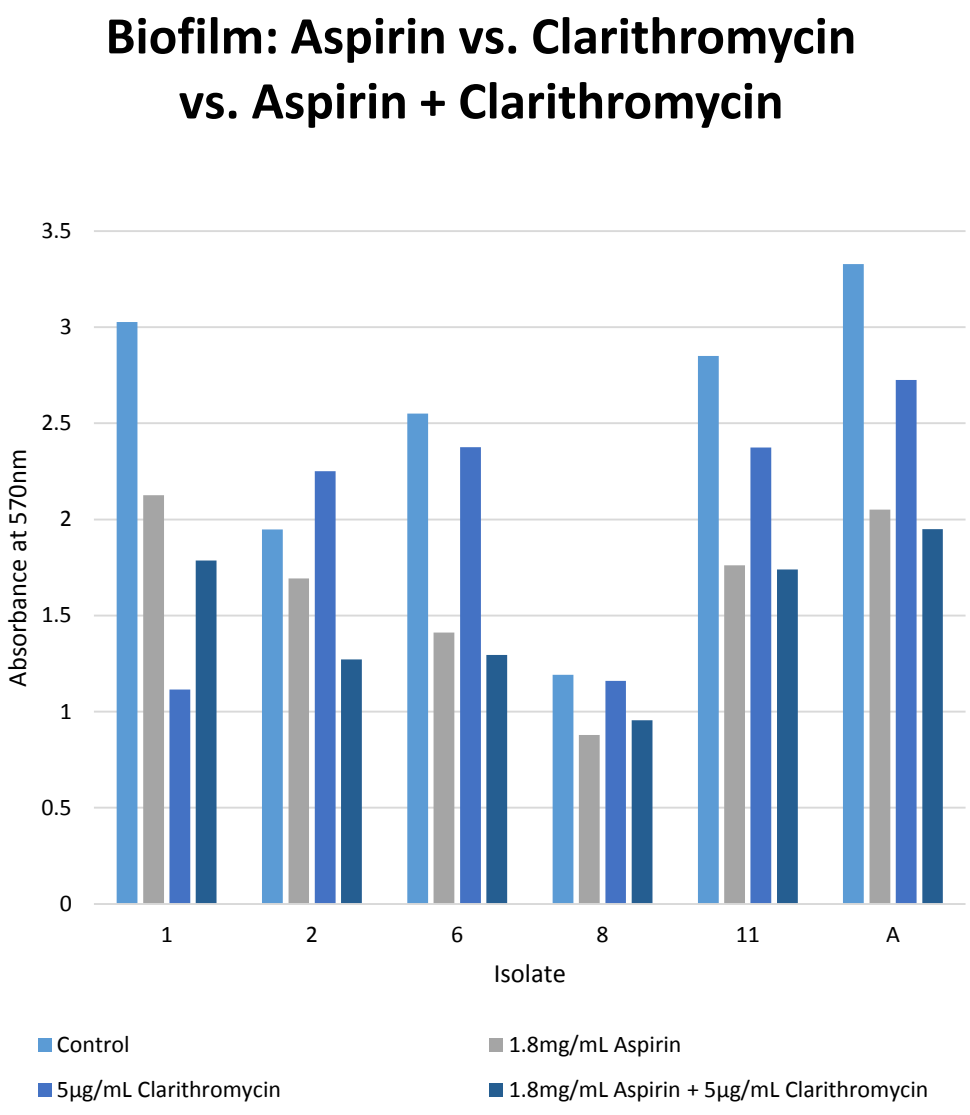


Figure 32. MTT ODs of six, week old *M. avium* biofilms, comparing aspirin at 1.8mg/mL concentrations to 5µg/mL clarithromycin concentrations, and showing their effectiveness combined. Raw MTT results were

diluted 1:2 to provide accurate readings, and therefore these absorbance values have been doubled to reflect this.

The averages from all ten confirmed *M. avium* isolates showed a decrease of absorbance value with increasing aspirin concentration (figure 33). Half of the maximum therapeutic concentration of aspirin (1.8mg/mL) achieved better cell inhibition than 5µg/mL clarithromycin. A concentration of 1.8mg/mL aspirin combined with 5µg/mL clarithromycin was more effective than the maximum therapeutic concentration (3.6mg/mL) of aspirin alone. However, 3.6mg/mL aspirin combined with 5µg/mL clarithromycin was the most effective, with average absorbance dropping from 2.681 (untreated control) to 1.307 (figure 33).

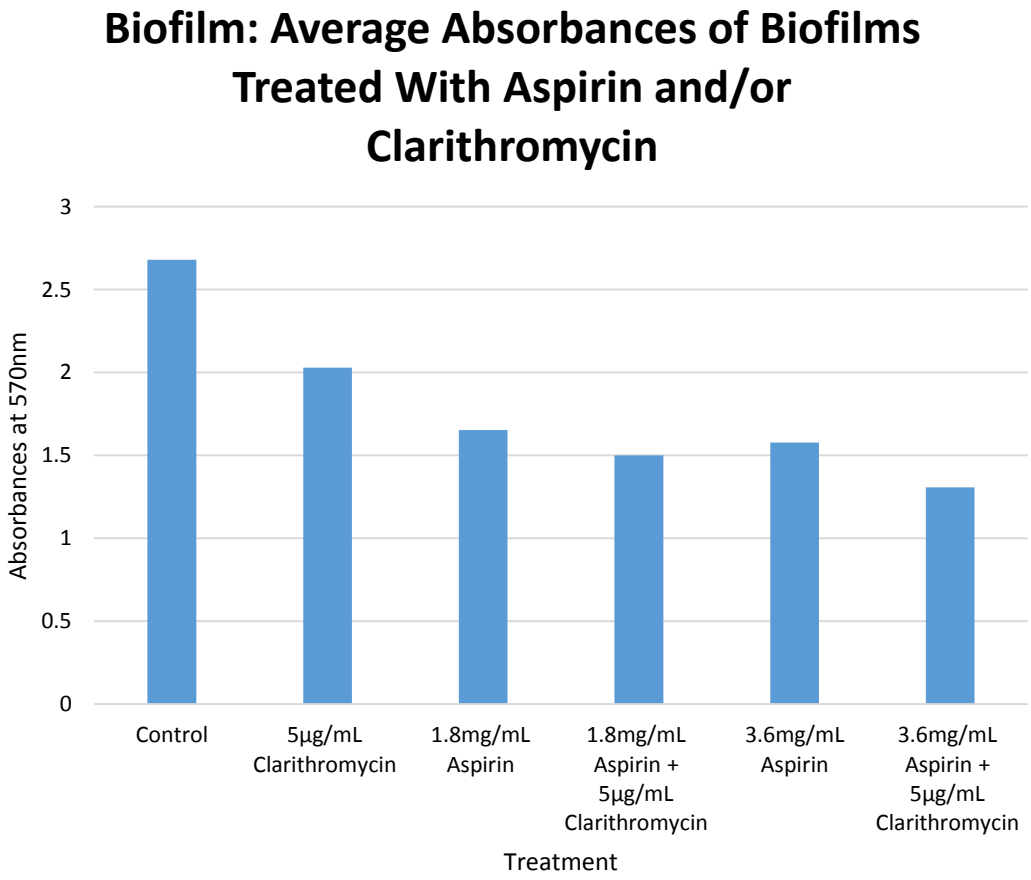


Figure 33. Average MTT ODs from 10, mature *M. avium* biofilms, comparing aspirin at 1.8mg/mL and 3.6mg/mL to 5µg/mL clarithromycin, and showing their effectiveness combined. Raw MTT results were

diluted 1:2 to provide accurate readings, and therefore, these absorbance values have been doubled to reflect this.

The concentration of antibiotics used was increased to 15µg/mL, to try and replicate earlier kill results of aspirin. Ibuprofen was also investigated as a potential *M. avium* biofilm dispersing agent, and was shown to be the more effective against *M. avium* biofilms at the same concentration than aspirin (figure 34). Aspirin combined with ibuprofen (both at a concentration of 3.6mg/mL) were the most effective against mature biofilms (absorbance decreasing from 2.769, in the control, to 0.937). The addition of 15µg/mL did not improve the effectiveness of aspirin with ibuprofen.

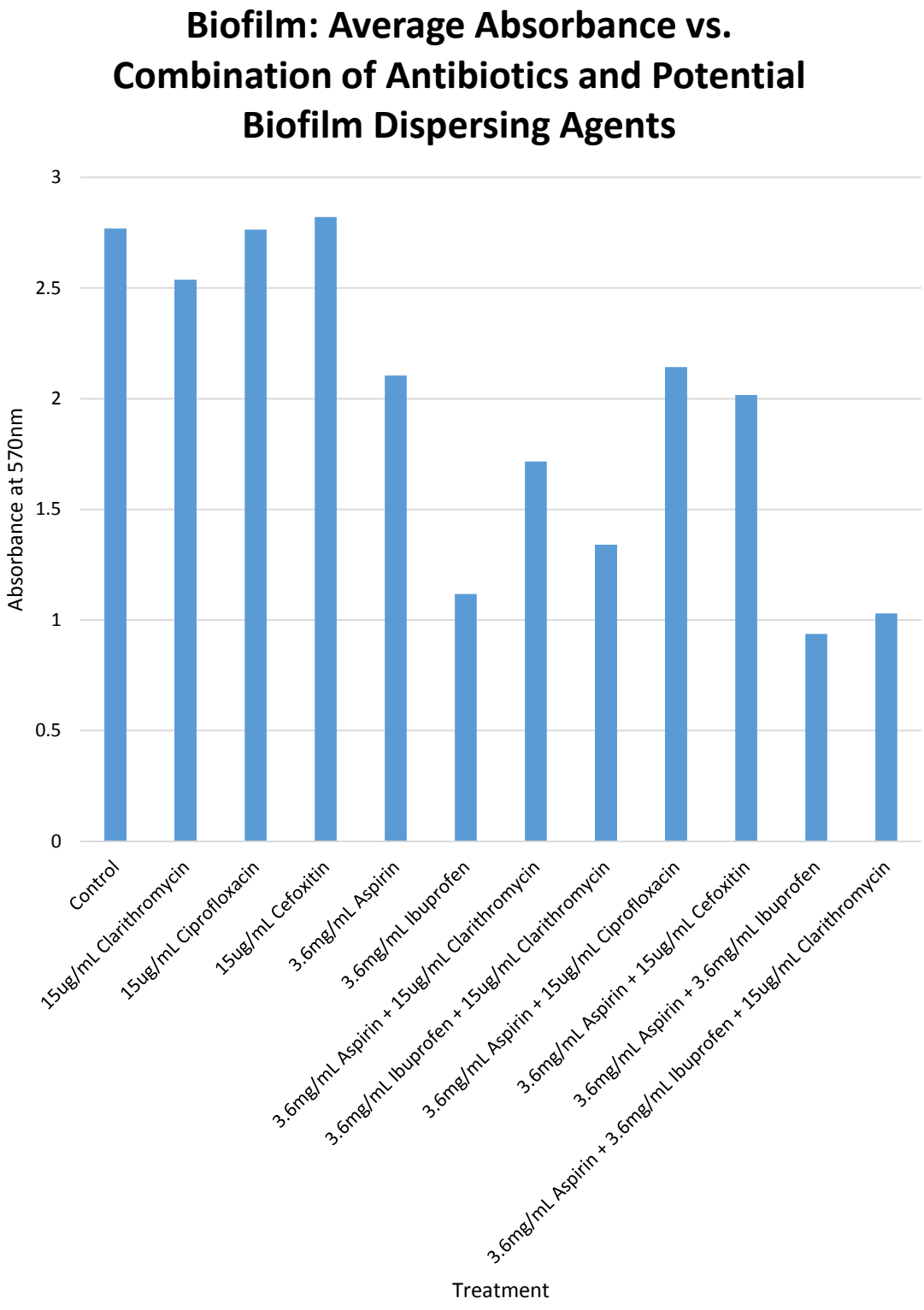


Figure 34. Average MTT ODs of three to four day old mature *M. avium* biofilms treated with 3.6mg/mL aspirin and/or 3.6mg/mL ibuprofen with/without 15µg/mL antibiotics. Raw MTT results were diluted 1:2 in order to be accurately read on the spectrophotometer, however, data displayed has been doubled to reflect this.

More experiments into potential biofilm distributors, such as N-acetyl-L-cysteine and ibuprofen, were conducted using isolates 1, 6 and 11 as they offered the most diverse range of biofilm phenotype and drug resistance. Against isolate 1 a concentration of 3.6mg/mL aspirin was the most effective (absorbance value of 0.857) and was not enhanced by the addition of 15µg/mL clarithromycin or 4mg/mg N-acetyl-L-cysteine. However, both isolate 8 and 11 mature biofilms showed susceptibility to aspirin (3.6mg/mL) in combination with N-acetyl-L-cysteine (at a concentration of 4mg/mL). This susceptibility increased further when 15µg/mL of clarithromycin was added (figure 35).

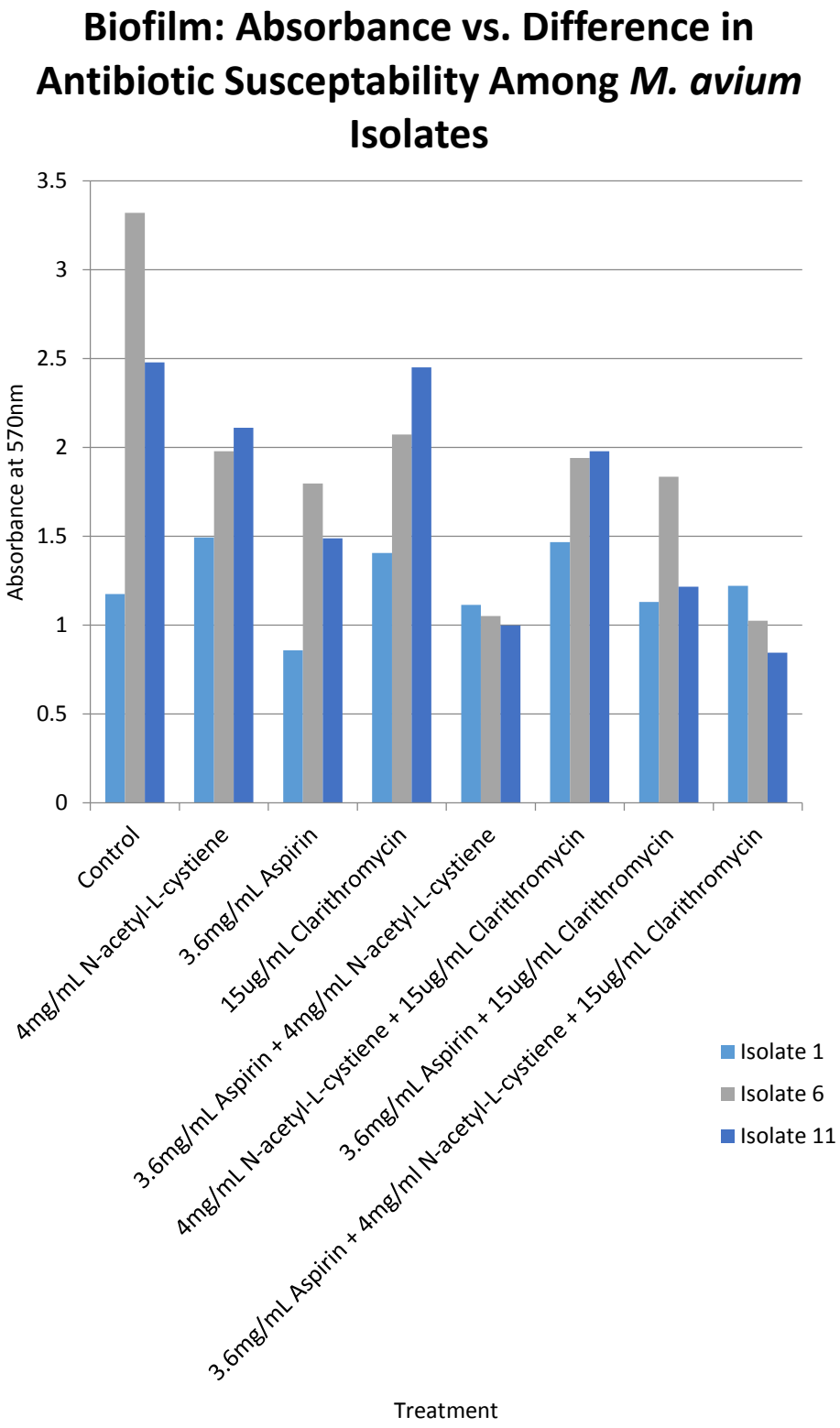


Figure 35. Four day old mature *M. avium* biofilms treated with antibiotics and potential biofilm dispersing agents.

3.7 Longevity of Aspirin on *Mycobacterium avium*

To analyse whether the effect seen with aspirin on *M. avium* was bactericidal or bacteriostatic an experiment was carried out into the longevity of aspirin. Aspirin remained effective over a 5 day period as seen in table 13.

Table 13. Growth of untreated isolate 6 on BHI + 199 agar plates in comparison to aspirin treated isolate 6.

Day	Growth on plates spread with aspirin treated isolate	Growth on control plates spread with untreated isolate
1	No	Yes
2	No	Yes
3	No	Yes
4	No	Yes
5	No	Yes

3.8 Acidity of Aspirin

The acidity of the stock 100mg/mL aspirin solution was measured, to determine if an acidic environment was causing the decrease in biofilm growth via lower absorbance values. Aspirin did cause a decrease in pH of the growth media (table 14). Therefore 100mg/mL aspirin stock was made in EtOH and adjusted with NaOH to pH 6.5. A 1/100 1M ACES Buffer was added to the BHI + 199 media to resist any pH change with the addition of aspirin.

Table 14. pH of BHI + 199 media and aspirin.

Solution	pH
BHI + 199 media	6.5
BHI + 199 media + 4µL aspirin of 100mg/mL stock aspirin solution	6.65
BHI + 199 media + 40µL of 100mg/mL stock aspirin solution	5.18
BHI + 199 media + 1/100 1M ACES Buffer + 40µL of 100mg/mL stock aspirin solution	6.53

3.9 SEM Images

Isolate 6 was chosen for SEM imaging, as it had previously shown to be an efficient biofilm producer under auramine-o, methylene blue staining (figure 20).

The untreated control sample showed aggregation of cells, and little cellular damage, as seen in figure 36. MTT analysis of replicate samples showed the untreated control had an absorbance value of 2.727 at 570nm.

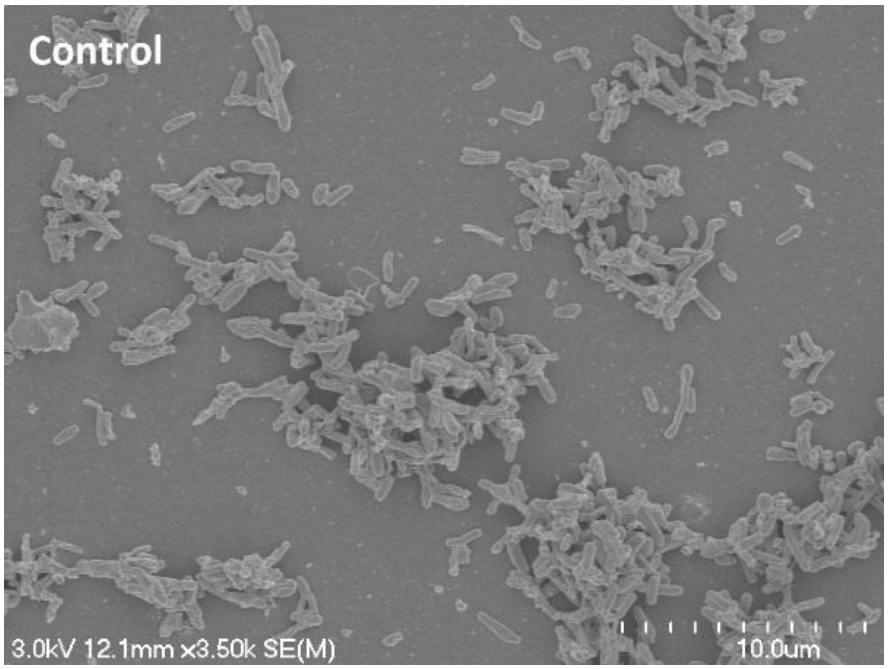


Figure 36. SEM image showing untreated *M. avium* bacterial cells, displaying normal biofilm growth. MTT OD reading of 2.727 at 570nm

All samples had sufficient biofilm growth, and pore channels could clearly be seen, as shown below in figure 37.

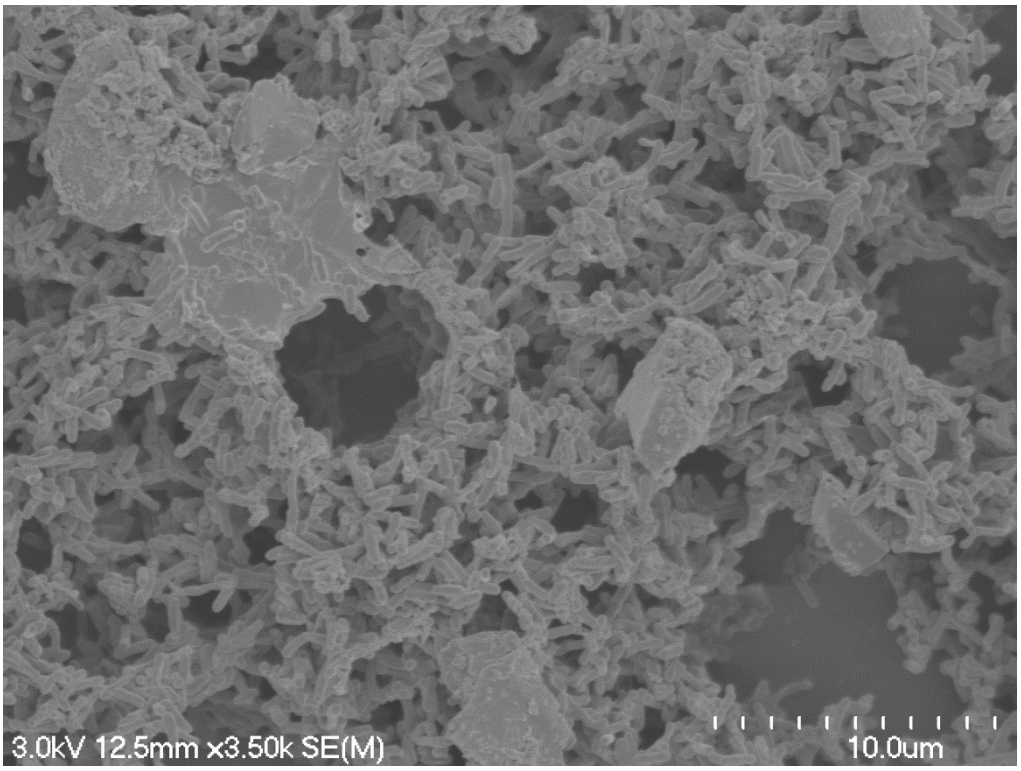


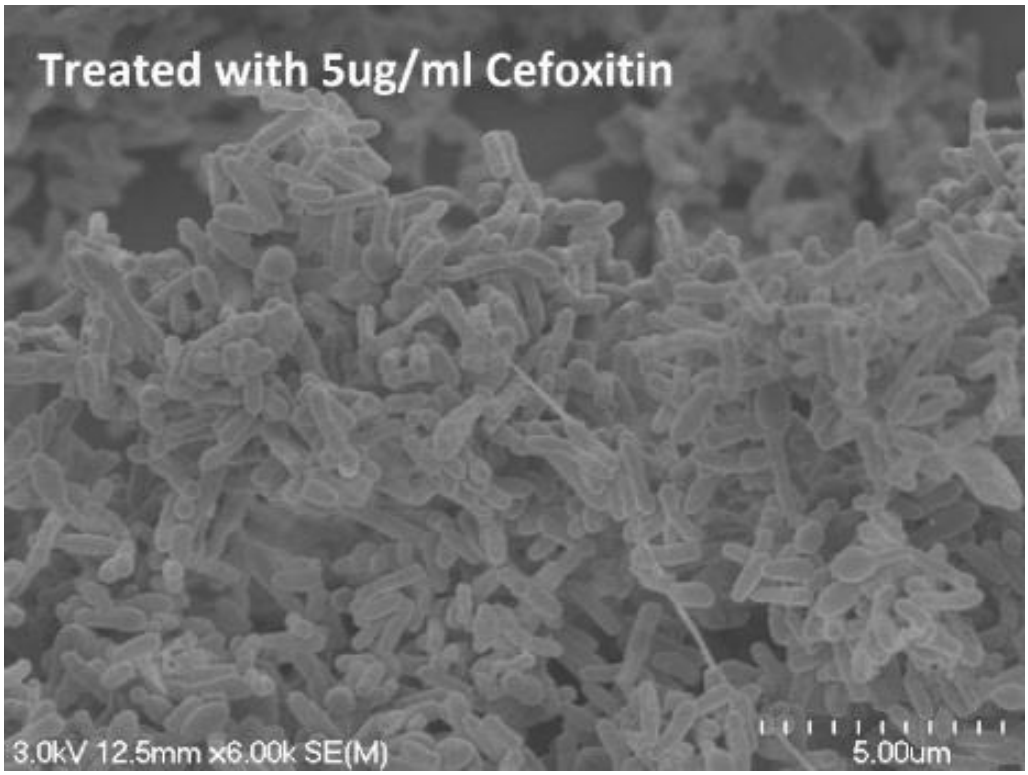
Figure 37. Untreated control *M. avium* biofilm, showing dynamic three-dimensional shape with pore channel. MTT OD reading of 2.727 at 570nm.

Comparison of the control (figure 36) to the images below (figure 38), the addition of 4mg/mL aspirin shows cellular damage to the bacterial cells. The cell walls appear flaky and wrinkled with the actual cells seeming distorted and dimpled in comparison to the control (as seen below) .This was also replicated in MTT analysis, where the addition of aspirin decreased absorbance value to 1.045 at 570nm

Figure 38. *M. avium* mature biofilm treated with 4mg/mL aspirin. MTT OD reading of 1.045 at 570nm.

Treatment with 5µg/mL clarithromycin, cefoxitin and ciprofloxacin showed apparent wrinkling and cracking of the cellular surface. Samples treated with 5µg/mL clarithromycin showed increased aggregation of cells (figure 39).

Figure 39. *M. avium* mature biofilm treated with 5µg/mL clarithromycin. MTT OD reading of 1.659 at 570nm.


Treated with 5ug/ml Cefoxitin
3.0kV 12.5mm x6.00k SE(M)
5.00um


Treated with 5ug/ml Cefoxitin
3.0kV 12.5mm x13.0k SE(M)
4.00um

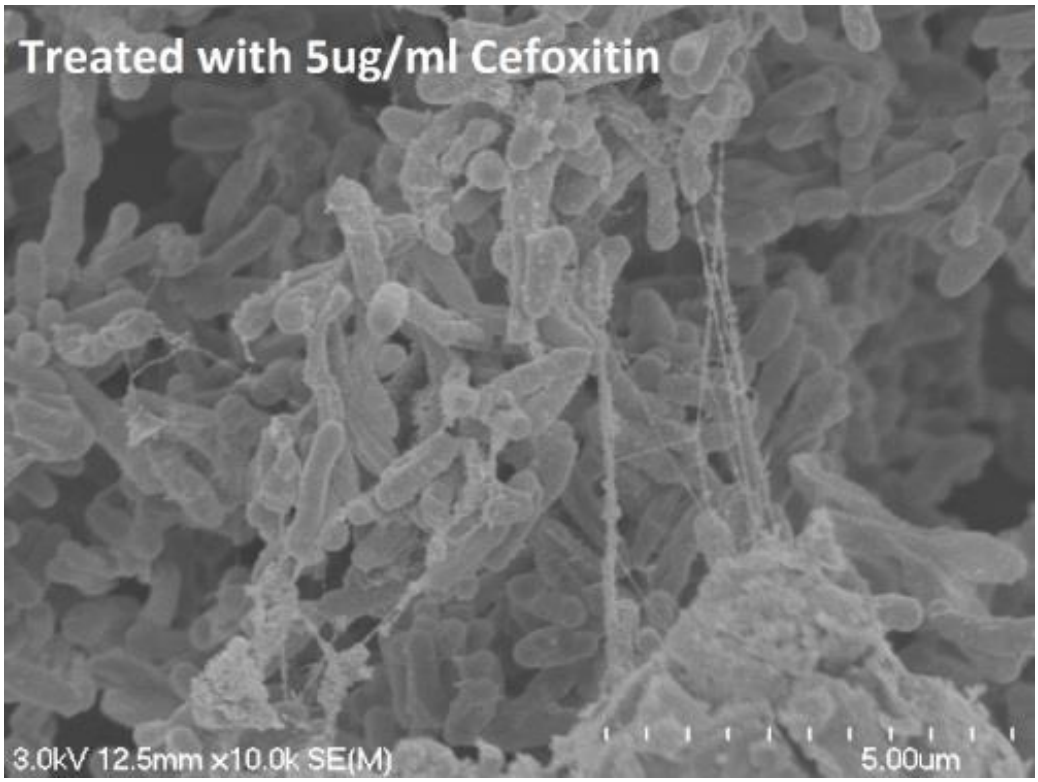
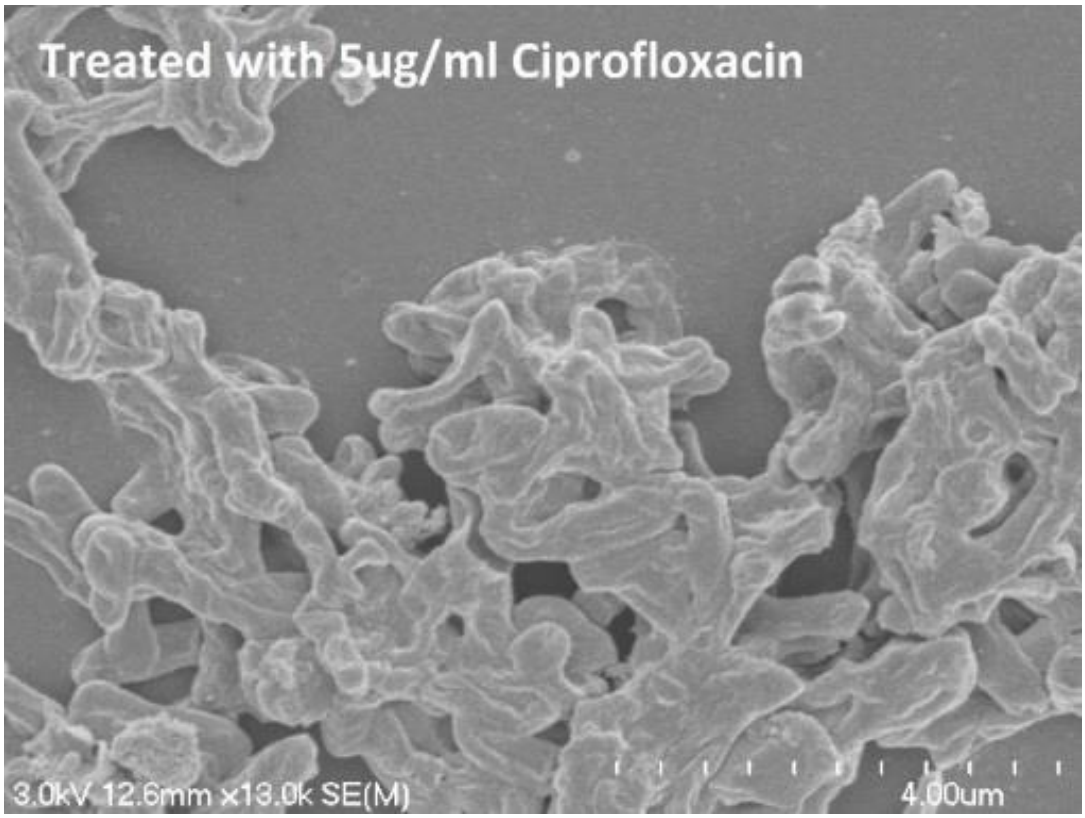


Figure 40. Three SEM images showing *M. avium* mature biofilm treated with 5µg/mL cefoxitin. Note the formation of "fibres" between the cells. MTT OD reading of 1.944 at 570nm.



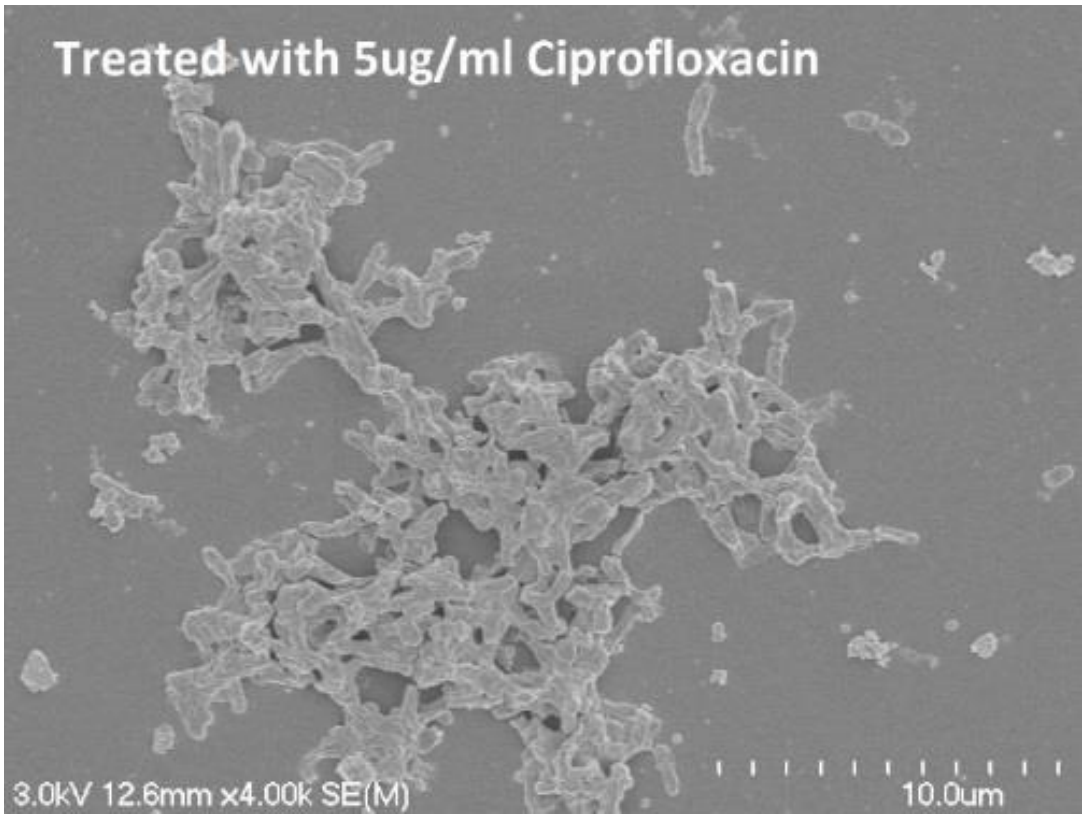


Figure 41. Two SEM images showing *M. avium* mature biofilm treated with 5µg/mL ciprofloxacin. Note the aggregation between the cells and development of a coating over the cells. MTT OD reading of 2.219 at 570nm.

The treatment of *M. avium* biofilms with DMSO shows the same cell aggregation was seen as samples treated with 5µg/mL ciprofloxacin (figures 41 and 42). This aggregation increased when antibiotics were also added to the sample (figures 43, 44 and 45). DMSO did not significantly affect *M. avium* biofilm, nor have a synergistic effect with antibiotics.

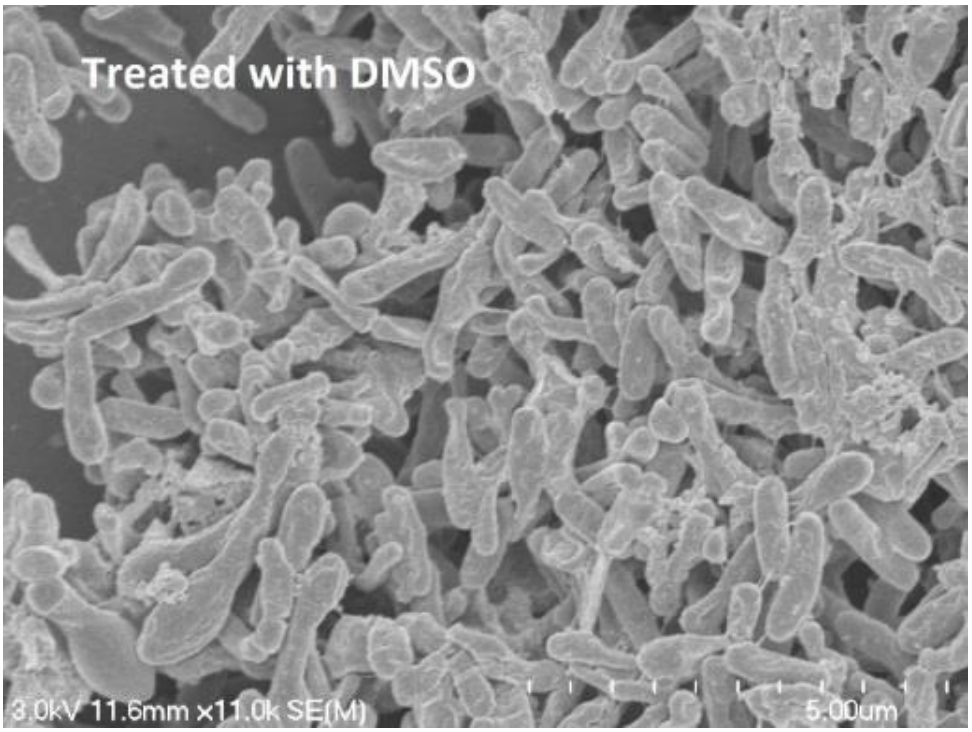


Figure 42. *M. avium* mature biofilm treated with 40µL/mL DMSO. Note the aggregation between the cells and slight development of a coating over the cells. MTT OD reading of 2.428 at 570nm.

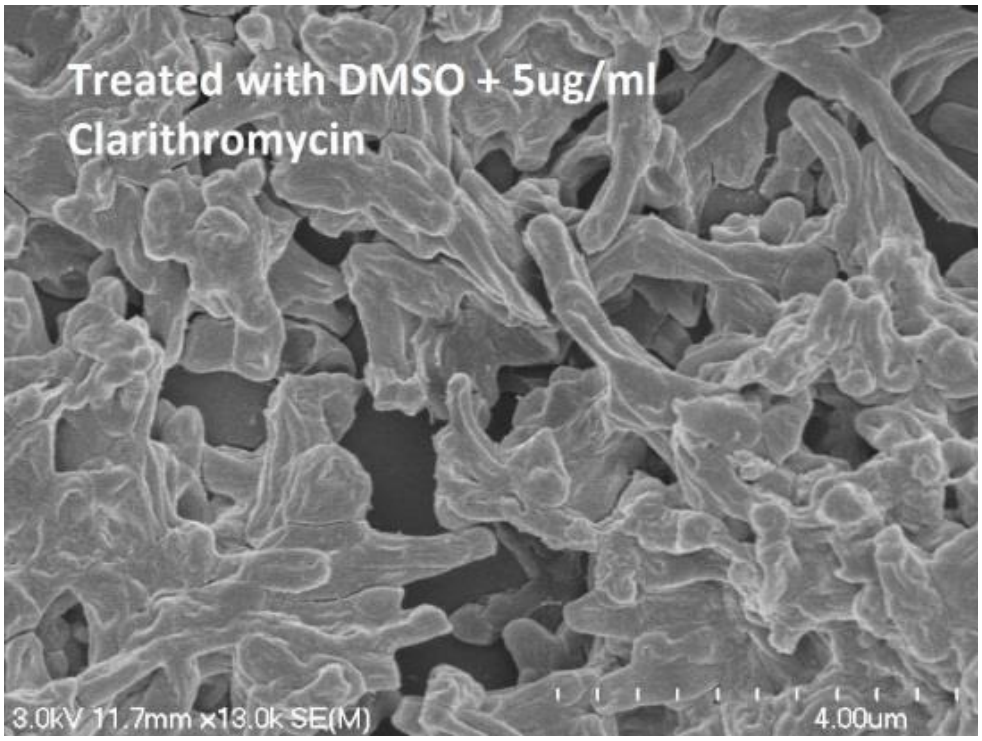


Figure 43. *M. avium* mature biofilm treated with 40µL/mL DMSO and 5µg/mL clarithromycin. Note the increased aggregation between the cells and development of a coating over the cells. MTT OD reading of 2.127 at 570nm.

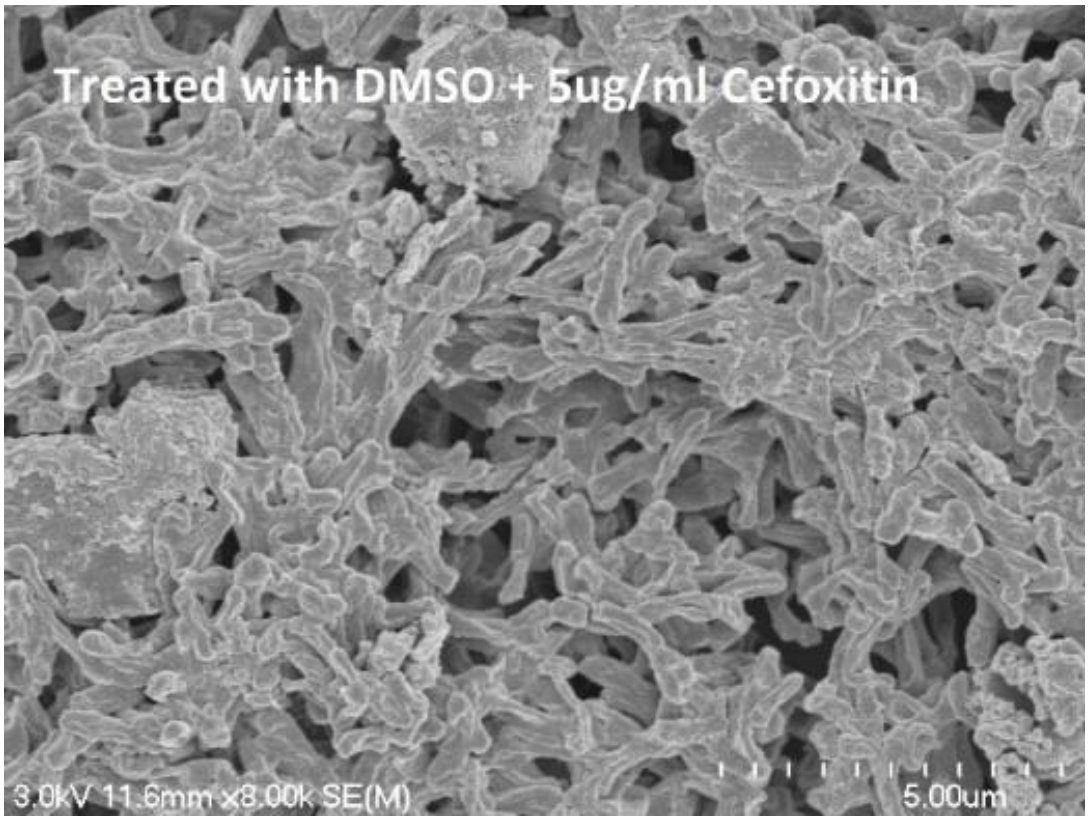


Figure 44. *M. avium* mature biofilm treated with 40µL/mL DMSO and 5µg/mL cefoxitin. Note the increased aggregation between the cells and development of a coating over the cells. MTT OD reading of 2.002 at 570nm.

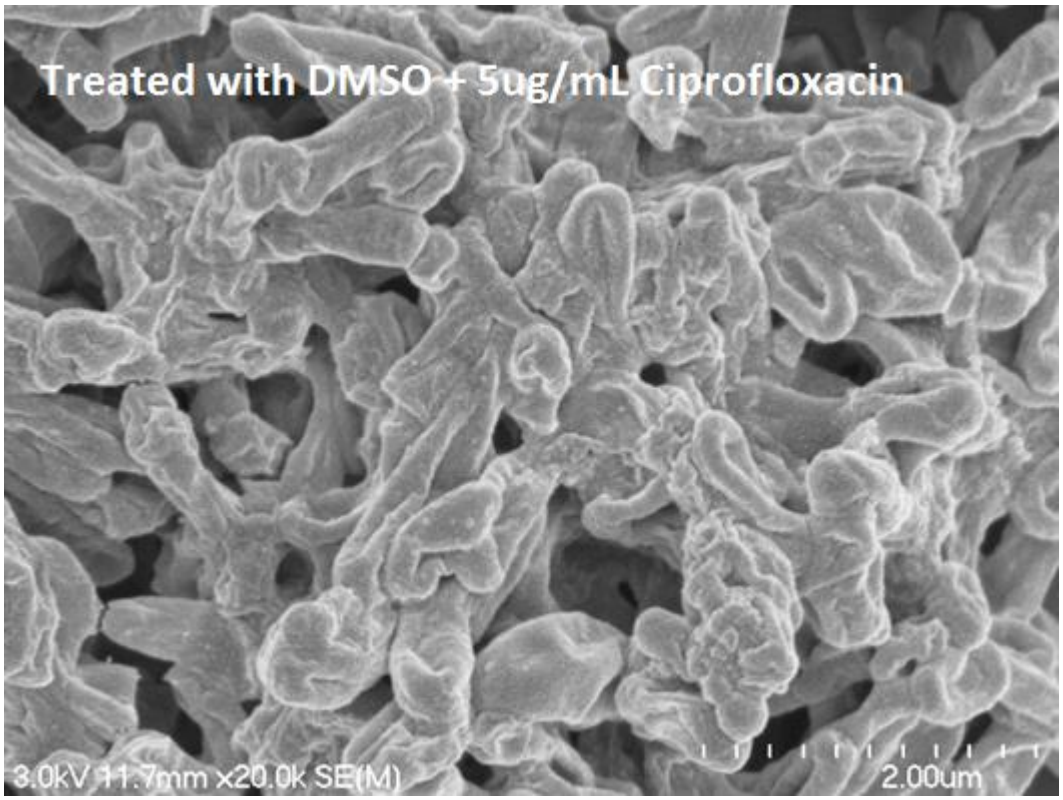


Figure 45. *M. avium* mature biofilm treated with 40µL/mL DMSO and 5µg/mL ciprofloxacin. Note the increased aggregation between the cells and development of a coating over the cells. MTT OD reading of 2.525 at 570nm.

In contrast to the samples treated with antibiotics only (figure 39, 40 and 41), when 4mg/mL aspirin was also added to the 5µg/mL clarithromycin, reduced cell aggregation and increased cellular damage was observed to a greater extent than with 4mg/mL aspirin alone. The damaged cells were extremely distorted in shape, in comparison to the healthy, plump cylindrical control cells. The damaged cell were wrinkled and flaky with indented cell walls as seen in the image below (figure 46). This damage was also observed in the MTT OD reading which decreased from 2.727 in the untreated control to the 0.081.

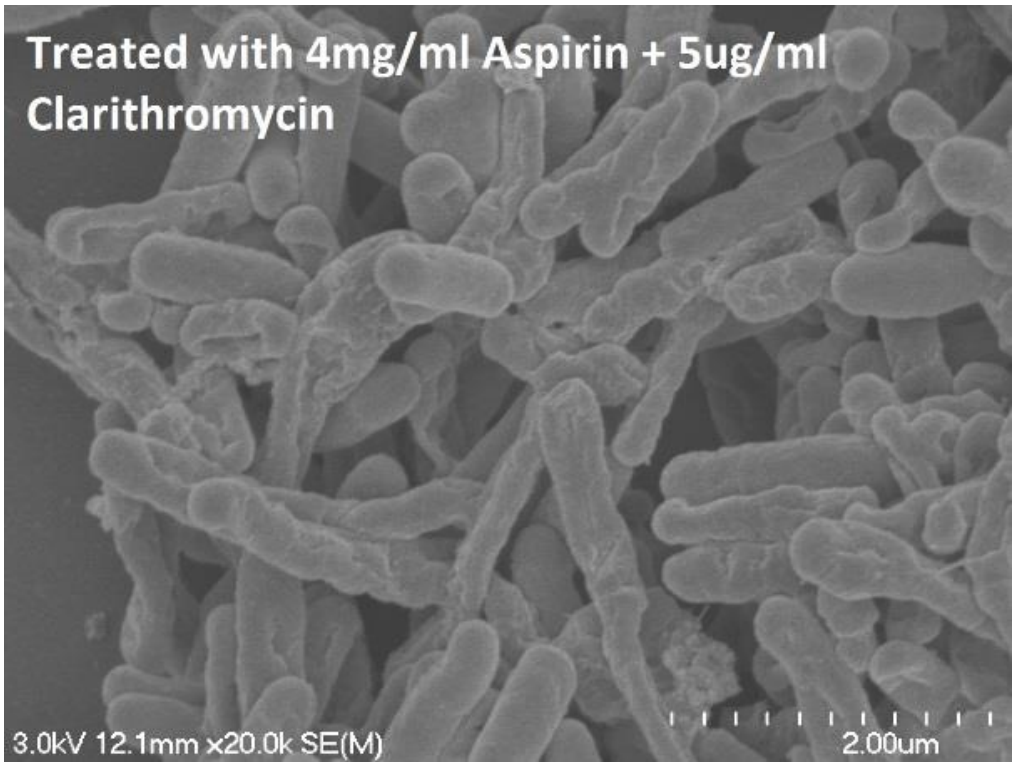


Figure 46. Biofilm sample treated with 4mg/mL aspirin and 5µg/mL clarithromycin. MTT OD reading of 0.081 at 570nm.

EDTA (at a concentration of 8mg/mL) produced the sample formation of "fibres" between cells, as did 5µg/mL cefoxitin (figure 47). The addition of antibiotics to EDTA increased the formation of the "fibres" between *M. avium* cells within the biofilm as seen below (figures 48, 49 and 50).

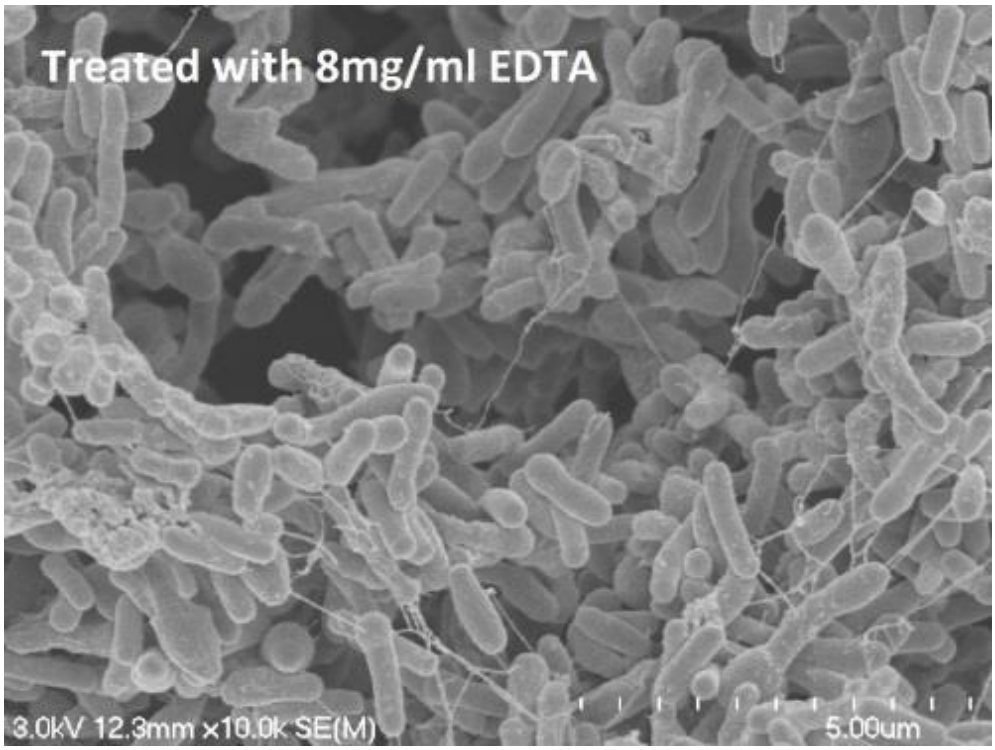


Figure 47. *M. avium* biofilm sample treated with 8mg/mL EDTA. MTT OD reading of 2.622 at 570nm. Note the fibre-like formation between the cells.

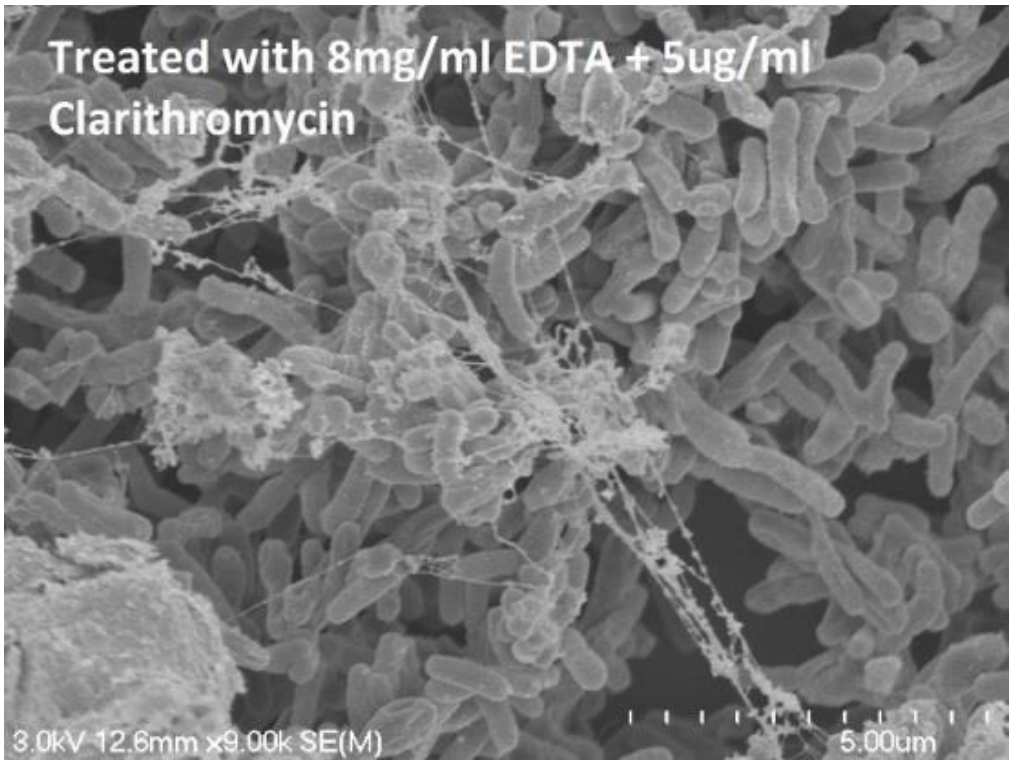
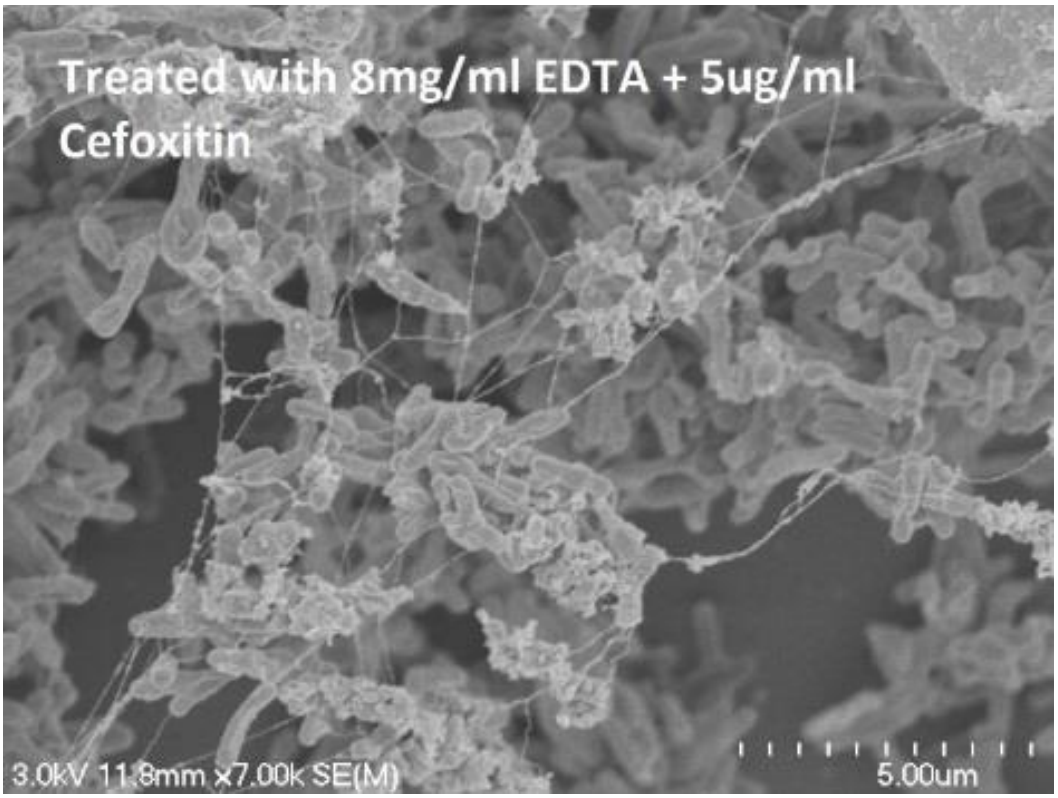


Figure 48. *M. avium* biofilm sample treated with 8mg/mL EDTA and 5µg/mL clarithromycin. MTT OD reading of 1.747 at 570nm. Note the fibre-like formation between the cells.



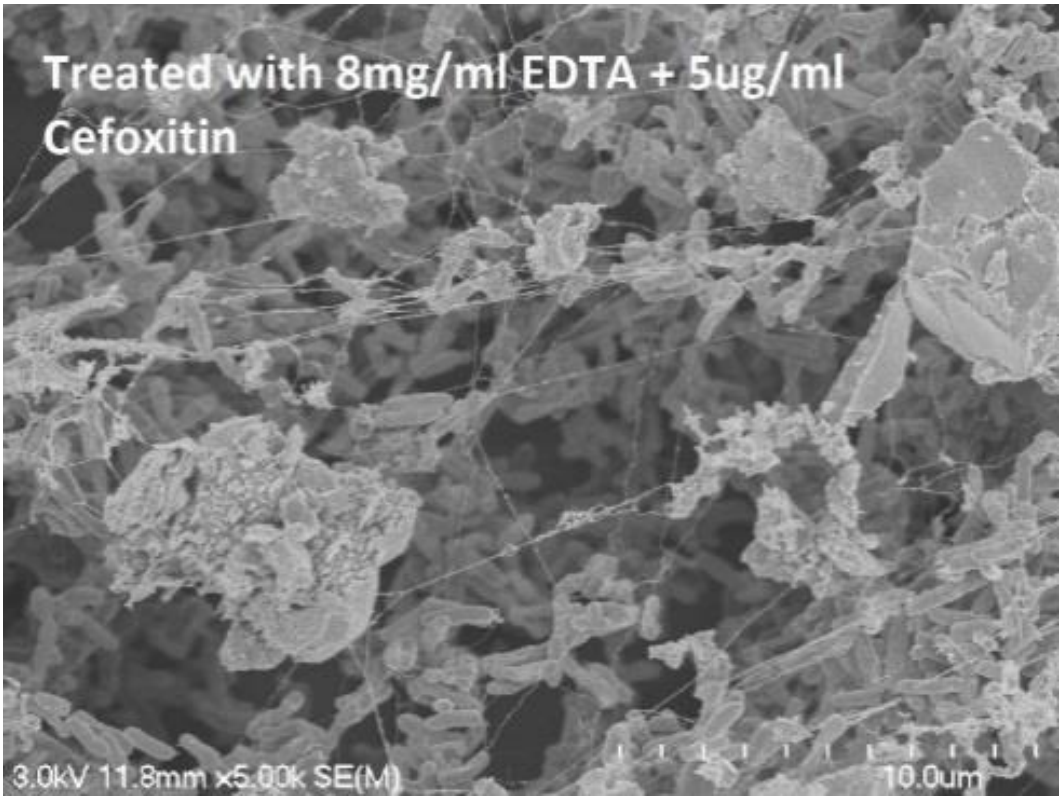
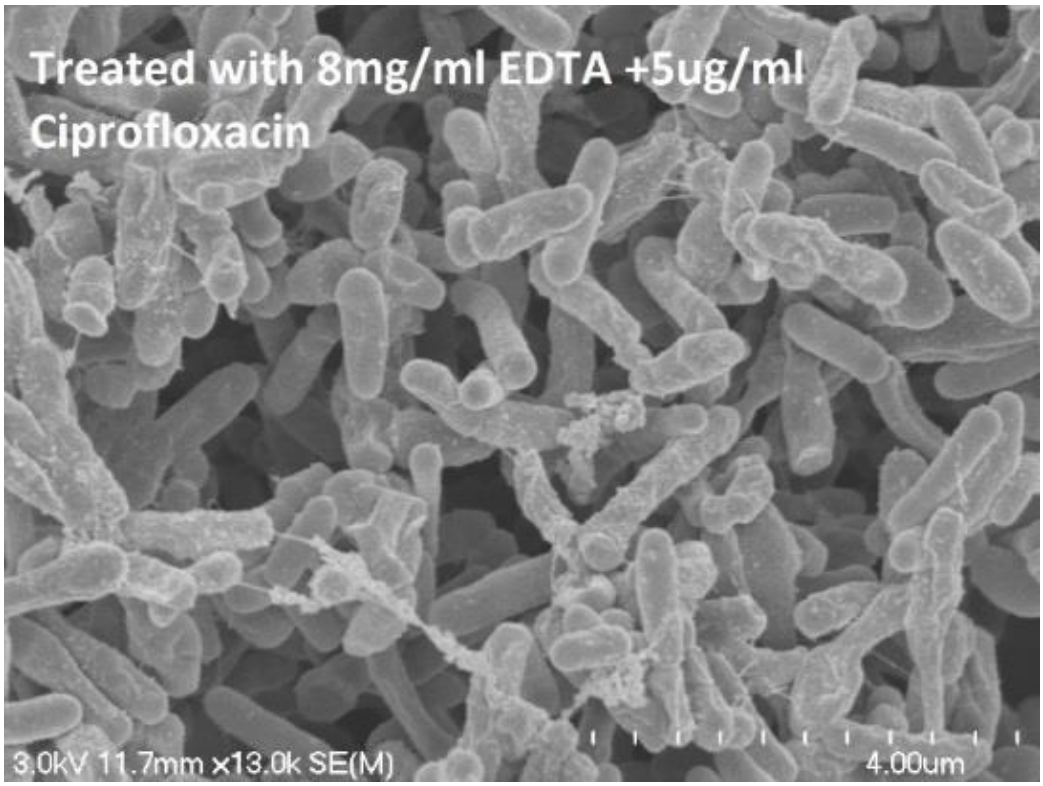


Figure 49. *M. avium* biofilm sample treated with 8mg/mL EDTA and 5µg/mL cefoxitin. MTT OD reading of 1.656 at 570nm. Note the fibre-like formation between the cells.



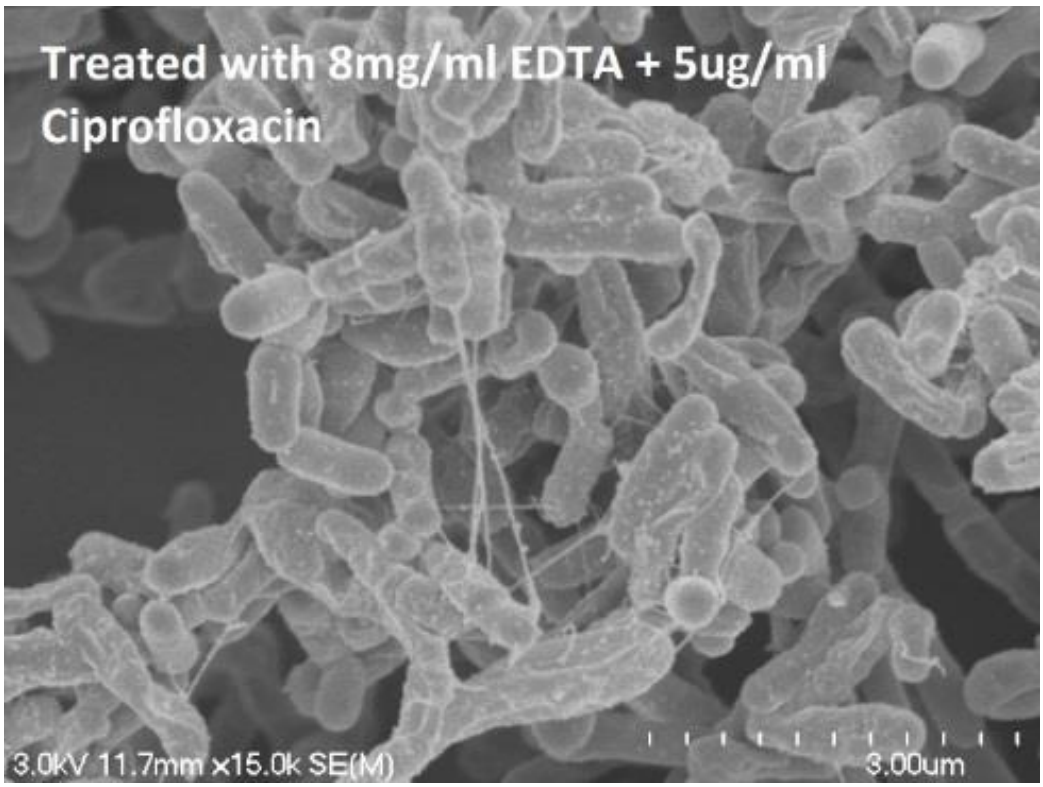
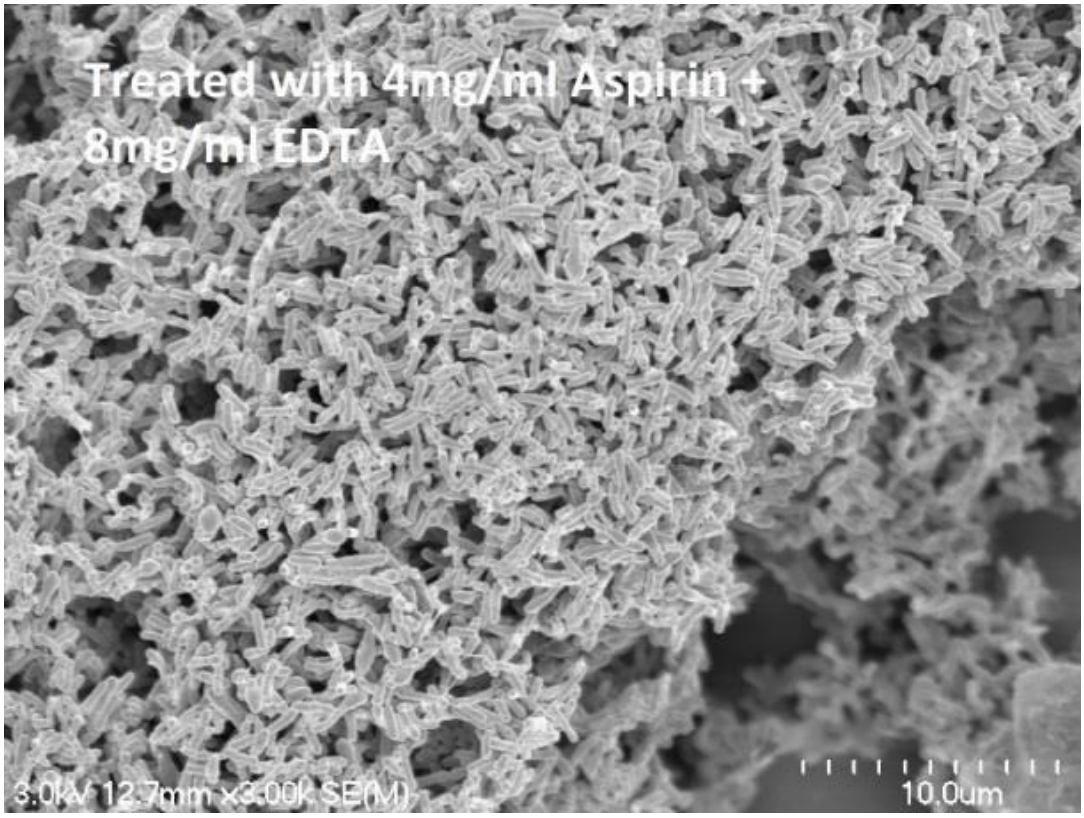


Figure 50. *M. avium* biofilm sample treated with 8mg/mL EDTA and 5µg/mL ciprofloxicin. MTT OD reading of 1.524 at 570nm. Note the fibre-like formation between the cells.

In contrast to these images, figure 51 shows the biofilm sample treated with 4mg/mL aspirin as well as 8mg/mL of EDTA showed no “fibres” forming between the individual cells, as seen in the previous figures 48, 49, and 50. However, although the cells remain intact as seen in figure 51 below, the MTT analysis shows complete sterilization of the mature *M. avium* biofilm (OD reading of 0.037). The sterilization of mature *M. avium* biofilms with 4mg/mL aspirin and 8mg/mL EDTA is replicated with the addition of 5µg/mL of clarithromycin, cefoxitin or ciprofloxacin (MTT OD absorbance values of 0.055, 0.022 and 0.004 respectively).



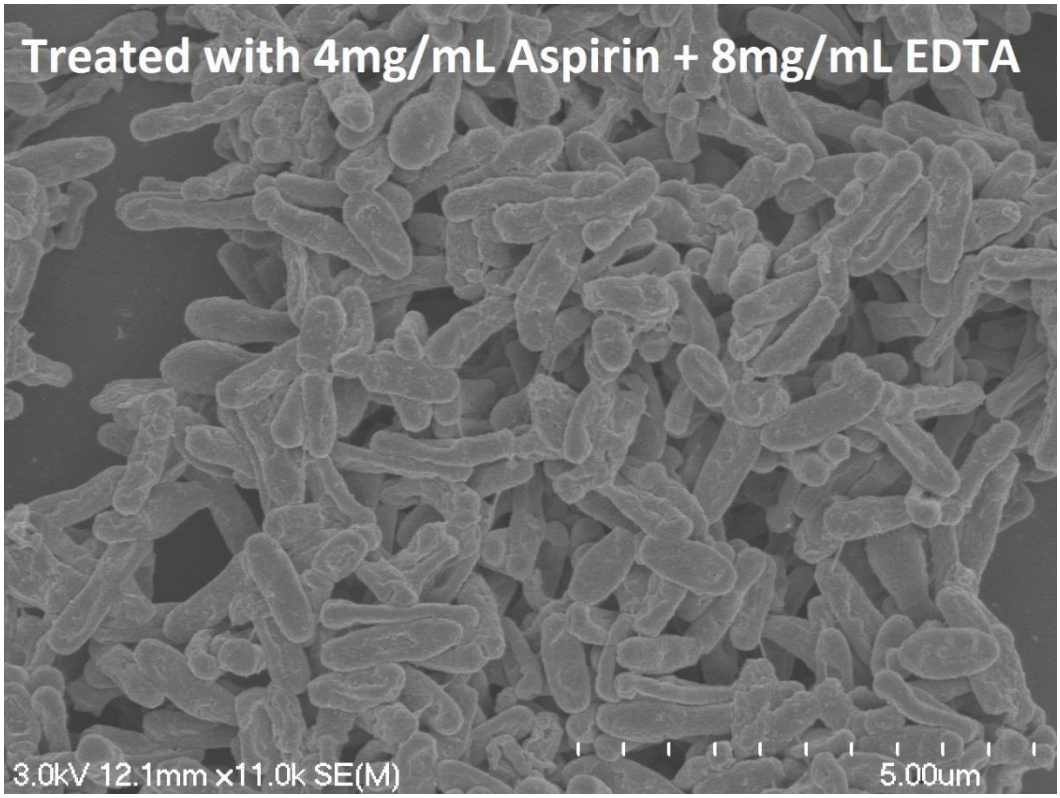


Figure 51. Two SEM images showing *M. avium* biofilm samples treated with 4mg/mL aspirin and 8mg/mL EDTA. MTT OD reading of 0.037 at 570nm. Note the lack of fibre-like formation between the cells.

This sterilization was not observed in the sample which had a decreased concentration of aspirin (0.4mg/mL), and effect of aspirin on the *M. avium* biofilm was reduced. MTT OD absorbance values increased from 0.037 in the 4mg/mL aspirin and 8mg/mL EDTA sample to 1.746 in the 0.4mg/mL aspirin and 8mg/mL EDTA sample. This shows that aspirin, not EDTA, is the main effector on mature *M. avium* biofilm metabolism.

3.10 qRT-PCR

Furthermore, RNA from planktonic, biofilm and biofilm treated with aspirin was isolated, and used for downstream analysis.

3.10.1 RNA Quality

RNA quality was measured on Thermo Fischer Scientific ™ Nanodrop 2000™ Spectrophotometer. Total RNA quality was suitable for downstream applications, as shown in table 15 below.

Table 15. Total RNA quality and concentration after RNA extraction was performed on the three isolate 6 samples.

Sample	260/280	230/260	Concentration ng/µL
Planktonic	2.03	1.75	66.1
Biofilm	2.03	1.91	72.7
Aspirin-treated Biofilm	2.02	1.9	115.8

3.10.2 qRT-PCR Results

All three total RNA samples were run on Corbett Life Science Roto-Gene 6000, with four primer sets (Gtf, MAI, Tmpt1 and Tmpt2). Tmpt1 showed the most significant down regulation compared to the other primer sets, however the MAI primer sets that were used as a housekeeping gene was not suitable (as seen below in the differences in cycle number shown in table 16 and in figure 52).

Table 16. qRT-PCR cycle Results. Results denoted with an asterisk show significant increase in copy number.

Primer Sets	Sample Cycle Repeat at 80°C		
	Planktonic	Biofilm	Aspirin
MAI	25.35	26.37	25.15
Gtf	25.35	25.07	23.3
Tmpt1	15.58	15.58	12.31*
Tmpt2	17.76	18.23	15.27

These PCR products were then run out onto an agarose gel, using the gel-electrophoresis and gel imaging protocols outlined in chapter 2 section 2.7 and seen below in figure 52.

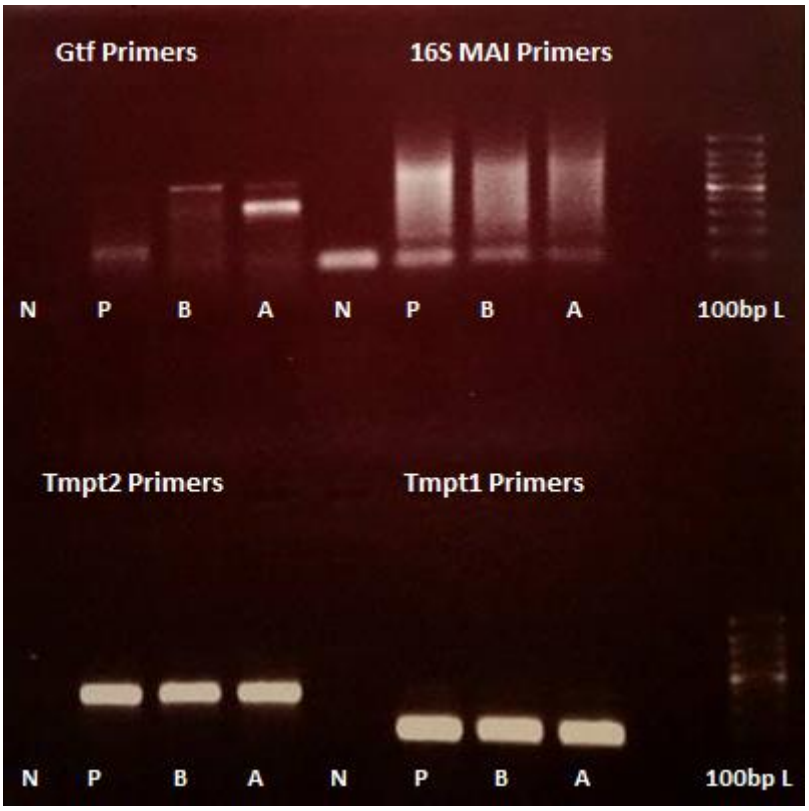


Figure 52. Gel electrophoresis photo showing the resulting products of four primer sets after qRT-PCR had been performed from isolated RNA. N stands for the negative control; P denotes RNA isolated from planktonic cells; B stands for RNA isolated from biofilms; A denotes the RNA isolated from biofilms treated with aspirin.

Chapter Four

4. Discussion

The main objective of this study was to explore the antibiotic resistance of *M. avium* isolates obtained from the Waikato Hospital and to determine any synergy between antibiotics and potential biofilm dispersing agents. This is important due to the nature of MAC infections, and their potential to cause chronic illness, and required intensive and prolonged antibiotic treatment (Thoen *et al.,* 1981; Ingen *et al.,* 2012). Strains identified from the hospital isolate samples were either *M. avium* subsp. *hominissuis* or *M. avium* subsp. *avium* (as seen in figure 8).

M. avium is slow growing (Starkova *et al.,* 2013) which aids in the difficulty of successfully eradicating it from a human host. The slow metabolism provides a natural defence mechanism with antibiotics often targeting sites of cell replication and growth, such as DNA replication, or cell wall synthesis (Takayama & Kilburn, 1989: Mikusovà *et al.,* 1995; Bosne-David *et al.,* 2000; McNabe *et al.,* 2011). This combined with the factor of added antibiotic resistance acquired from biofilm formation makes *M. avium* a difficult pathogen to eliminate.

This study showed that aspirin, compared to all other antibiotics and potential biofilm dispersing agents, had a detrimental effect on the growth and biofilm forming capabilities of the *M. avium* isolates. In comparison to the antibiotics tested, concentrations of aspirin showed increased killing capability in both planktonic, newly formed and mature (up to three week old) biofilms.

4.1 Improved Cultivation of *Mycobacterium avium*

Previous studies conducted on *M. avium* have reported culture times up to several weeks (Wu *et al.,* 2009; Do *et al.,* 2014), therefore it was important that we discovered a media that promoted rapid biofilm formation of *M. avium* isolates. BHI + Middlebrook 7H9 media gave the best planktonic growth results for *M. avium*, agreeing with previous studies. Middlebrook 7H9 + Tween80 media however, showed prolific biofilm formation despite only having a 0.001 OD absorbance value at 570nm from the MTT analysis (compared to Middlebrook 7H9 alone with and OD absorbance value of 1.036 at 570nm). This biofilm growth could be due to biofilms more readily forming under stressful, nutrient poor conditions, hence why the OD value was so low yet mature biofilms were observed under auramine-O methylene blue stain. This agrees with previous research which suggests that biofilm formation occurs under stressful, nutrient limited conditions (Geier *et al.,* 2008; McNabe *et al.,* 2011). Auramine-o methylene blue double stain was used due to its properties of staining acid fast bacteria, such as *Mycobacterium* species, providing a secondary check to the identification PCR, showing that the bacteria observed were of *Mycobacterium* species. Although, due to the addition of Tween80, which has been shown to increase antibiotic efficiency in gram-positive bacteria, it was unable to be used in following MIC and synergy experiments with antibiotics. Therefore, BHI + 199 media was used instead as it gave the same biofilm and planktonic growth rates as BHI + Middlebrook 7H9 media but was more readily available. In future studies BHI + 199 media should be used for rapid *M. avium* cultivation.

Effective cultivation was needed to provide consistent, repeatable biofilm phenotypes for experimentation to be conducted on. Due to these results from crystal-violet staining, glass coverslips were used over polystyrene to promote biofilm growth in 24 well plates. This method was extremely successful and produced replicable results and

allowed efficient processing of high volumes of samples for MTT analysis, staining and SEM imaging.

However, biofilms are formed in nature under nutrient-poor conditions and in mixed species communities (Thoen *et al.,* 1981; Flores *et al.,* 2005). The rapid formation of *M. avium* biofilms we were able to achieve does not fully represent MAC biofilms found in patients, however, it may provide a more accurate model of pulmonary MAC infections than planktonically grown *M. avium.*

M. avium isolates grew faster on BHI+ 199 + 5% sterile horse serum agar plates compared to other agar medium, with colonies forming within 7 days. However, for sufficient growth, two week incubation was needed, which was similar to previous methods.

4.2 Isolate Differences

McNabe *et al.* (2011) found direct phenotypic differences in the cultivation of *M. avium* subsp. *hominissuis* isolates. Our findings also show clear phenotypic and susceptibility differences among the ten confirmed *M. avium* isolates, which has also been reported in previous studies (Freeman *et al.,* 2006; Ingen *et al.,* 2012). Using PCR methods from Tran *et al.* (2014) all isolates except 9, were able to be further defined into subspecies. For isolates 2, 5, 6, 7, 10, 11 and A, primer sets identified by Tran *et al.* (2014) that target the IS*901* and IS*900* regions of *M. avium* would be able to further identify the isolates as either *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *hominissuis*. Isolates are unlikely to be *M. avium* subsp. *paratuberculosis* as mycobactin was not included in the growth media. However, no similarities in antibiotic susceptibility were observed between isolates that were identified as being of the same subspecies- in either planktonic or biofilm phenotype. Biofilm phenotype was also independent of subspecies, shown in figure 19, isolate 8 (identified as *M. avium* subsp. *avium* via subspecies PCR)

shows similar biofilm growth as isolate 11 (identified as *M. avium* subsp. *paratuberculosis* or *M. avium* subsp. *hominissuis* via subspecies PCR) on glass coverslips. SAUTYPE PCR confirmed genetic difference between all of the isolates, even among isolates of the same subtype. This potentially gives an explanation for the differences in observed phenotype and antibiotic susceptibility. Isolate 1 was observed to grow in small micro-colonies in broth media and on the glass coverslip, never forming cohesive biofilms. This may explain why the dramatic effects of aspirin and other biofilm dispersing agents appeared insignificant against isolate 1. Isolates that appeared phenotypically similar, such as 2 and 7, showed some similar antibiotic susceptibility results, although generally the range in antibiotic susceptibility between isolates was extremely diverse and often differed in the most effective combination of antibiotics, and even concentrations. For example, what was strong against one isolate was weak against another (as seen in figures 26, 27, 32 and 35). Isolate 7 mature biofilms showed susceptibility to 10µg/mL clarithromycin, but not 5µg/mL. Isolate A mature biofilms showed susceptibility to 5µg/mL rifampicin, but not 5µg/mL clarithromycin, which conflicts with the average results (figure 30), which identify clarithromycin as the overall most effective antibiotic. Unfortunately this isolate diversity contributes to the difficulty of MAC infection treatment. Better methods for identification of *M. avium* sub-species and even genetic markers for isolate differences would greatly improve treatment by tailoring the therapy to specific subspecies. The idea of genetically tailored treatment has been discussed significantly in recent literature, however, the financial and resource practicality of using genetic testing in hospital diagnostics restricts its usefulness.

4.3 *Mycobacterium avium* MICs

One of this studies aims was to identify MICs and potential antibiotic resistant strains of *M. avium* hospital isolates. Our findings show that clarithromycin was the most effective antibiotic against planktonic and biofilm *M. avium* populations, which agrees with previous research (Heifets, 1988; Inagaki *et al.,* 2011). However, conversely to previous research, all of the isolates had MICs >15µg/mL to clarithromycin even in planktonic phenotype. Previous studies have identified highly resistant isolates to have >16µg/mL MICs to clarithromycin, although only a small percentage of their study size was highly resistant whereas all of the isolates tested in our research were (Meier *et al*., 1994; Nash & Inderlied, 1995; Wallace Jr *et al*., 1996; Park *et al.,* 2009; Inagaki *et al.,* 2011). This high number of resistant isolates agrees with our first hypothesis and could potentially be due to the nature of how the isolates were obtained. Isolates were collected at the Waikato Hospital from patients with chronic MAC infections. If samples were collected after a patient had previous, unsuccessful antibiotic therapy, antibiotic resistance from persister cells within the biofilm could have conferred their resistance to the isolate and thus increasing the antibiotic resistance to commonly used antibiotics. This could account for the level of MICs shown especially MICs >128µg/mL. History of when the isolates were obtained from patients during the course of treatment would be useful for investigating this theory.

The method of using MTT analysis for determining bacterial growth and MICs has been used in previous studies (Mosmann, 1983; Mohammadzadeh *et al.,* 2006; Primm & Franzblau, 2007; Singh *et al.,* 2011; Trafny *et al.,* 2013) and is widely accepted as a valid measure. In this study MTT analysis was used due to the quick result acquisition time frame as well as the ease of practically being able to observe the OD of samples. The method of MTT analysis relies on actively metabolising cells taking up the yellow, water soluble MTT and reducing it to an insoluble purple formazan crystal. The formazan can

then be dissolved in a solubilising solution which kills the cells allowing samples to be safely handled. The OD of the dissolved purple colouration can be measured on a spectrophotometer and used as a measure of cell population and growth via the level of metabolism (Mosmann, 1983; Mohammadzadeh *et al.,* 2006; Primm & Franzblau, 2007; Singh *et al.,* 2011; Trafny *et al.,* 2013).

Consistently, *M. avium* biofilms achieved higher absorbance values indicating more growth and therefore were more resistant to antibiotic treatment. No MICs could be determined for any antibiotics tested on *M. avium* biofilms. This agrees with our second hypothesis and previous research on increased resistance to antibiotics when the cells are in biofilm phenotype (Falkinham, 2007; McNabe *et al.,* 2011). However, the increase in antibiotic resistance due to biofilm phenotype alone suggests that the resistance is physical and not genetically induced antibiotic resistance, such as what was identified in some true clarithromycin resistant strains of *M. avium.* This could also be suggested from our research showing a plateau effect of antibiotics on mature biofilms (figure 25, 26 and 27). This agrees with Falkinham's (2007) earlier research on *M. avium* target grown in catheters. All antibiotics require the ability to physically reach their action site within the bacterial cell in order to have an antibacterial effect. It is possible that the physical barrier created by the *M. avium* biofilm prevents the antibiotics reaching their targets, thus allowing more internal *M. avium* cells to grow unhindered.

Interestingly, the addition of antibiotics at higher concentrations increased planktonically grown cells metabolism and biofilm formation. Doxycycline was investigated for antibiotic effectiveness as well as potential biofilm disrupting properties. However, the absorbance for planktonic cells grown with 25µg/mL doxycycline was higher than the untreated control both being above 2.0 OD absorbance values at 570nm. This shows that doxycycline is ineffective in the treatment of *M. avium*

potentially even increasing the growth of *M. avium* populations. Ingen *et al.* (2012) reported that *Mycobacteria* combat tetracyclines, such as doxycycline and rifampicin via drug efflux pumps, potentially explaining the increased growth of *M. avium* and apparent lack of effect of rifampicin, Rifinah™ and doxycycline (figure 10). Our findings also show a plateau effect of antibiotics on *M. avium* planktonic and biofilm phenotypes, which has not been shown for *M. avium* in other research (figures 9, 25, 26 and 27). Concentrations of antibiotics 5µg/mL to 20µg/mL showed insignificant differences in their ability to inhibit *M. avium* growth (figure 9 and 25). Furthermore the addition of 8mg/mL cefoxitin to *M. avium* biofilms showed an increase across in MTT OD all isolates, compared to untreated control biofilms (table 10). This increase in *M. avium* biofilm growth after being treated with high doses of antibiotics agrees with previous research which also observed increased biofilm formation with the addition of sub inhibitory antimicrobials (McNabe *et al.,* 2011). McNabe *et al.* (2011) suggest that this is a response to competition for nutrients in bacterial communities, such as biofilms. Bacterial cells will synthesise and release antimicrobial compounds to gain an advantage by killing surrounding competing cells. The increase in biofilm formation in the presence of sub inhibitory antimicrobials suggests that the *M. avium* cells are using this mechanism to combat the external chemical attack.

Furthermore, McNabe *et al.* (2011) showed that *M. avium* cells that gained increased resistance in biofilm phenotype retained this resistance to antibiotics when dispersed into planktonic populations, which was not explored during our research. Clinically, this could be detrimental in the treatment of *M. avium* pulmonary infections where sub inhibitory concentrations of antibiotics would only further increase biofilm formation, thus exacerbating existing MAC infections. This further highlights the need for biofilm dispersing agents to be used in conjunction with antibiotics for successful treatment.

4.4 Antibiotic Synergy

No secondary antibiotics were found to have significant synergistic effect on planktonically grown *M. avium* with clarithromycin, often clarithromycin alone was more effective than in combination with other antibiotics (figure 30, table 11). The addition of a tetracycline, doxycycline at 25µg/mL showed no antibiotic synergy with any other of the antibiotics used in this research.

Although no significant antibiotic synergy was found during this study, the need to maintain multiple-drug therapy is vital, in order to prevent antibiotic resistance developing.

4.5 *Mycobacterium avium* Biofilm Genes

It is well known that *M. avium* forms biofilms in nature, and several studies have identified genes within the *M. avium* genome that are responsible for biofilm production. Several of these genes are involved in GPL biosynthesis, such as *guaB2* and *gtf*, where GPL has been identified as important for initial establishment and adherence of early biofilms (McNabe *et al.,* 2011). Our findings show that in established biofilms, *gtf* doesn't play such an important role in maintaining the biofilm phenotype. McNabe *et al.* (2011) showed that expression of *guaB2* and *gtf* was greater when sub inhibitory antibiotics were added. Further research needs to be conducted into determining if *gtf* and *Guab2* are also important for the dispersal of *M. avium* biofilms, not just formation. Tmpt1 and tmpt2, which are primers targeting a hypothetical SAM- dependent methyltransferase gene showed minimal change between samples. Tmpt1 showed a significant increase in the total RNA of aspirin treated *M. avium* sample, amplifying almost three cycles ahead of the biofilm and planktonic *M. avium* samples (table 16). However, the tmpt2 primer set, which was supposed to target the same gene, showed

variation across all three samples. This could be due to our protocol or primer design. Further testing would need to be carried out in order to confirm these results, and no conclusive judgements could be made from our findings. MAI primers, which target the 16S-23S rRNA, ITS region, were used as a housekeeping gene for the qRT-PCR experiments. However, the cycle numbers were not even for every sample (table 16 and figure 52) indicating that it was not a good housekeeping gene. In future experiments more appropriate housekeeping genes should be investigated.

4.6 *Mycobacterium avium* Biofilm Dispersing Agents

Previous research has shown that *M. avium* pulmonary infection depends on the ability of the *M. avium* isolate to form biofilms (Freeman *et al*., 2006; Yamazaki *et al.,* 2006). Clarithromycin at sub-inhibitory concentrations has been shown to effectively prevent the formation of biofilms (Carter *et al.,* 2003), however, no research into the dispersal of established *M. avium* biofilms has been conducted. With patients showing MAC infection it is highly likely that establishment of biofilms within the lungs has already occurred. Thus any treatment to prevent the formation of biofilms, while necessary to prevent reinfection, do not aid in the initial eradication of the established biofilm.

One aim of this study was to identify any biofilm dispersing agents that were effective on *M. avium* biofilms. Our findings show that previously identified potential biofilm dispersing agents; mannitol, N-acetyl-L-cysteine, active manuka honey, methylglyoxal (MGO), Lipitor®, 2,4-dinitrophenol (DNP) and EDTA; had no significant effect on dispersing *M. avium* biofilms or increasing their susceptibility to antibiotics. L-arginine had an adverse effect, greatly increasing biofilm formation in *M. avium* (figure 31). Conversely to previous findings, MGO (10µg/mL) had no effect on established *M. avium* biofilms, even with the addition of 5µg/mL clarithromycin, cefoxitin or ciprofloxacin. The MTT absorbance value of 0.798 in table 11, was most likely due to clearly observable

insufficient biofilm growth before the antibiotics and potential biofilm-dispersing agents were added. DNP, which uncouples energy producing pathways, had an effect on mature *M. avium* biofilms, approximately halving the MTT absorbance value (absorbance at 570nm 2.672 in untreated control decreasing to 1.379 with 10µg/mL DNP alone). This inhibiting effect was not significantly increased with the addition of antibiotics (table 11). The statin Lipitor™ also had weaker but similar pattern of effects as DNP. N-acetyl-cysteine (at a concentration of 4mg/mL) had adverse effects on planktonic cells of isolate 1 increasing the MTT OD at 570nm from 0.85 in the untreated control to 1.331. Mannitol was reported to reduce the antibiotic susceptibility of persister cells within biofilms. However, mannitol had no effect on already established biofilms. Potentially mannitol would have a role in eliminating persister cells after the biofilm is dispersed but more research would need to be conducted on *M. avium* persister cells.

The failure of all the previously identified potential biofilm dispersers tested in this research could be due to the limitations of fully exploring the necessary concentrations for activity against *M. avium.* However, due to previous studies that have also investigated the effect of identified biofilm disperses on less common bacteria, these failings could be due to the complexity of biofilm formation and QS, particularly between species.

Both aspirin and ibuprofen are non-steroidal, anti-inflammatory drugs, known to be non-selective COX inhibitors. Previous research showed that ibuprofen caused a decrease biofilm formation in *E. coli* (Naves *et al.,* 2010) which agrees with our findings on *M. avium*. Mature *M. avium* biofilms decreased with the addition of 3.6mg/mL concentrations of ibuprofen. Ibuprofen alone was also able to cause a decrease in planktonic *M. avium* cell population metabolism by half (figure 13), being more effective

than high (15µg/mL) concentrations of clarithromycin. Interestingly, a concentration of 3.6mg/mL ibuprofen combined with a concentration of 3.6mg/mL aspirin had a weaker effect on planktonic *M. avium* cell populations than either of these compounds alone. Ibuprofen was shown to sterilize *M. avium* planktonic cells at a concentration of 1mg/mL via TTC methods, however, FDA/EtBr showed that it was not as effective as 3.6mg/mL aspirin. The combination of ibuprofen (at 1mg/mL) with 16µg/mL of amikacin, amoxicillin, cefoxitin, moxifloxacin, rifampicin and Rifinah™ weakened the effect caused by ibuprofen alone.

It should be noted that the most effective form of biofilm dispersal was from physical disruption. However, this is not always practical in the treatment of patients and chemical methods needed to be explored.

4.7 The Effect of Aspirin on *Mycobacterium avium*

Aspirin is a non-steroidal anti-inflammatory drug, with salicylate as the active component (Price *et al.,* 2000). Salicylate produces a wide range of responses in bacteria, dependent on the species, such as affecting the production of virulence factors, decreasing antibiotic resistance or causing the initiation of multiple-antibiotic resistant phenotypes. In the incidence of increased antibiotic resistance, it is suggested that salicylate alters membrane protein synthesis, leading to increased resistance in normally susceptible strains. In *E. coli* this has been linked to the decrease in membrane porins and an increase in multi-drug efflux pump synthesis (Price *et al.,* 2000). *S. aureus* also showed increased fluroquinolone resistance (such as ciprofloxacin and moxifloxacin) with the addition of salicylate. However, research has also shown an increased susceptibility to aminoglycosides in both *E. coli* and *Klebsiella pneumoniae* (*K.*

pneumoniae), when grown with increased concentrations of salicylate (Price *et al.,* 2000).

However, in species of the same genus as *M. avium* ten genes in *M. tuberculosis* have been identified in mycobactin production (*mbtA to mbtJ*), with *mbtI* being identified as crucial for salicylate biosynthesis (Quadri *et al.,* 1998). *M. tuberculosis* produces siderophores such as mycobactin, which is used in the acquisition of iron. Iron is especially important in pathogens, often a limiting nutrient (Jurado, 1997) and is also implicated as being crucial for biofilm formation of many bacterial species, including *M. avium* (Islam *et al.,* 2012). Price *et al.* (2000) surmised that the inhibition of siderophore synthesis reduced *M. tuberculosis* pathogenicity. Previous research also showed increased resistance to isoniazid and rifampicin in *M. tuberculosis,* in the presence of salicylate (Schaller *et al.,* 2002). Denkin *et al.* (2005) showed that in *M. tuberculosis,* salicylate repressed gene expression of fatty acid synthesis as well as genes involved in transcription and translation, however gene expression of a benzoquinone methyltransferase was induced by salicylate (Sun *et al.,* 2001).

In contrast to these previous findings, our research show that planktonic *M. avium* cell populations are able to be completely sterilized with 4mg/mL aspirin, as seen with TCC indicator and FDA/EtBr staining (table 6 and figure 15). A concentration of 3.6mg/mL aspirin was more effective at causing cell death than 16µg/mL of any antibiotics tested in this study (figure 15). Furthermore, aspirin (at a concentration of 4mg/mL) in combination with EDTA (at a concentration of 8mg/mL) was able to completely eradicate three week old mature *M. avium* biofilms. This was slightly more effective than aspirin (4mg/mL) alone, and thus the EDTA while not a main antagonist of biofilms, did produce a synergistic effect with aspirin against *M. avium*. This agrees with the previous results from Al-Bakri *et al.* (2009) who also showed complete eradication of

established *P. aeruginosa* biofilms when 4mg/mL concentration of aspirin and 8mg/mL EDTA were added. *P. aeruginosa* is also a ubiquitous opportunistic human pathogen, capable of forming biofilms in the lungs of patients which can be fatal. However, unlike *M. avium, P. aeruginosa* is gram-negative, suggesting the possibility that the aspirin/EDTA combination acts upon a different pathway than affecting cell membrane structures.

Our findings suggest that aspirin is working at a concentration dependent manner. Aspirins effect on *M. avium* planktonic and mature biofilm cell populations showed a decreasing effect with decreasing concentration (figure 14 and 33). This is also replicated when comparing the effect of 0.4mg/mL aspirin with 8mg/mL EDTA to 4mg/mL aspirin with 8mg/mL EDTA. The effectiveness of aspirin at a 1:10 concentration also showed a dramatic reduction. Interestingly aspirin (at a concentration of 3.6mg/mL) alone, or combined with 15µg/mL clarithromycin caused significant decrease in the OD of planktonic *M. avium* isolates (figure 12). Conversely when 4mg/mL N-acetyl-L-cysteine was combined with aspirin (at a concentration of 3.6mg/mL) it hindered the inhibiting effect of aspirin on *M. avium* cells. Previous research has suggested several theories for reduced antibiotic susceptibility in biofilm phenotype. Firstly, that antibiotics fail to penetrate the biofilm, or that the nutrient depleted environment causes a massive reduction in bacterial metabolism and growth, reducing their susceptibility to antimicrobials. Lastly, studies have shown that biofilms matrixes of *P. aeruginosa* bind and inhibit antibiotics (Carter *et al.* 2006). Due to the observed concentration dependent manner in which aspirin works against *M. avium* planktonic and biofilm cells (table 4 and 5, figure 14), aspirin must be able to either; penetrate the biofilm or be actively taken up by *M. avium* cells. If aspirin was being inhibited, then a plateau effect seen with the antibiotics (figure 25, 26 and 27) should have also been observed for aspirin.

To insure that the decrease in pH was not effecting the experimental results, the aspirin stock was pH balanced with NaOH and HCl, to a pH of 6.5. A 1:100 1M dilution of ACES buffer was added to the BHI + 199 media to control any pH changes caused by the potential biofilm dispersing agents (table 14). Even though these steps were taken to eliminate the potential acidic effects on biofilms, aspirin dissolved in DMSO performed the same as aspirin dissolved in EtOH at pH 6.5. This suggests that acidity does not play a part in the eradication or sterilization of *M. avium* planktonic and biofilm cell populations. The longevity of aspirin was determined to cause permanent cell death via table 13. Isolate samples treated with a concentration of 4mg/mL aspirin and spread on BHI+ 199+ 5% horse serum agar plates after five days, did not grow and produce colonies. Although this time period was not long enough to show if aspirin is bacteriostatic or bactericidal, combined with the observed data from the FDA/EtBr staining after treatment with aspirin (figure 15), the high percentage of red fluorescing cells suggests that aspirin causes cell death, rather than having bacteriostatic action.

Aspirin also induced a slight effect on *gtf* gene expression, as shown in table 16. However difficulty around selecting an appropriate housekeeping gene for *M. avium* qRT-PCR experiments, means that future testing needs to be completed before these results can be interpreted.

4.8 SEM Imaging

Scanning Electron Microscopy gave insight into the structure of *M. avium* biofilms, accurately showing their dynamic nature, with clearly visible 3D structure and channels through the biofilm.

Techniques other than the ethanol dehydration-critical point drying technique used to prepare the *M. avium* biofilms for SME imaging have been identified as more effective at

observing biofilms. This study was limited by the techniques available, and as a result, the biofilm formation might not accurately be represented. Fratsei *et al.* (2004) report that samples prepared with the ethanol dehydration- critical point drying method, tend to strip away the outside matrix of biofilms, and accurately represent individual cells. Therefore the cellular damage seen was accurately represented but the true biofilm formation cannot be accounted for using these images. The presence of a biofilm surrounding the samples treated with 5µg/mL ciprofloxacin, DMSO and 5µg/mL clarithromycin, DMSO and 5µg/mL cefoxitin and DMSO with 5µg/mL ciprofloxacin could mean that the biofilm formation was at such an extent, that the ethanol dehydration-critical point drying technique did not remove the entire biofilm. Since this aggregation was not observed to such an extent in the control cells, it is possible that the slight stresses caused by the addition of sub MIC antibiotics and DMSO enhanced the biofilm formation of *M. avium* cells.

Unfortunately, we are unable to determine if aspirin prevents formation of the extracellular matrix when compared to SEM images of DMSO combined with antibiotics particularly. However clear cellular damage when treated with 4mg/mL is observed by the wrinkled, flaky and distorted cells. Cefoxitin (5µg/mL) and EDTA combined with any antibiotics produced the formation of fibres between the cells. This could potentially be the formation of *M. avium* biofilms. However when EDTA was combined with aspirin there was a clear lack of fibre-like formation between the cells.

It is important to consider the possibility of artefacts when studying SEM images. Artefacts are phenomena's that are produced during the preparation stage of SEM imaging. It is not yet possible to identify artefacts from observable results and is important to keep in mind when analysing SEM image results.

4.9 Limitations with this Study

The small sample number of confirmed *M. avium* isolates limits the value of the obtained MICs for planktonically and biofilm grown isolates. A larger sample size would reduce error and increase the statistical significance of these findings. All isolates were obtained from a specific location (the Waikato region) and came from clinical sources, thus all isolates were obtained from patients with predominantly chronic MAC infections. No *M. avium* isolates from environmental sources were obtained.

Susceptibility and genetic difference between isolates was not fully explored due to limited time and funding. MICs for isolates grown on agar could not be determined due to practicality and time restraints. Antibiotic synergy was also not able to be fully explored with antibiotic synergy titrations, however, these findings have highlighted the limited effect of currently used antibiotics on existing mature *M. avium* biofilms in particular. Isolate 6 was used for its ability to form substantial biofilms, however, future studies should include a wide range of *M. avium* isolates to determine the universal effects of aspirin on *M. avium.*

For more accurate results, the experiment investigating the longevity of aspirin should be extended to at least 14 days. Unfortunately due to time constraints and attention to other experiments, this could not be achieved in this study.

Further investigation into the transcriptome of *M. avium* biofilms could not be completed, due to time restraints. Therefore the mechanisms behind aspirin activity against *M. avium* could not be sufficiently determined.

Furthermore, the *in vitro* data obtained in this study cannot be correlated to potential *in vivo* results. The variety of body metabolism processes, and unknown site of action of aspirin and ibuprofen on *M. avium* or *M. avium* biofilms means that these findings may not corroborate with *in vivo* results.

Chapter Five

5. Future Recommendations

Future treatment of this disease needs to focus on the eradication of the biofilm in the patient. Physical disruption via physiotherapy and use of biofilm dispersing agents would improve the action of antibiotics and the host immune system in killing *M. avium* infection.

Biofilm preparation for SEM imaging should be prepared in a variety of techniques to better preserve the biofilm and reduce bias caused by certain techniques, such as critical-point drying (Fratesi *et al.,* 2004), which was used in this study. This would ensure more accurate imaging of *M. avium* biofilms as they exist in nature, and give more insight into their physical development and structure.

Further investigation into potential synergistic effects between antibiotics and biofilm dispersing agents against mature biofilms is needed.

Investigation into the physiological pathways that aspirin and ibuprofen affect, by the use of transcriptomics would help identify potential new targets for the development of antibiofilm drugs. Better housekeeping genes need to be identified for the use in future qRT-PCR experimentation.

Although aspirin shows potential *in vitro*, it's effectiveness against MAC infections *in vivo* needs to be determined.

Chapter Six

6. Conclusion

This research provides evidence that aspirin and ibuprofen are both effective against planktonic and existing biofilm formation. Aspirin and EDTA combined was able to eradicate and sterilize mature (three week old) *M. avium* biofilms. These results are important due to the high level of antibiotic resistance found in both planktonic and biofilm *M. avium* isolates.

Substances that were found to effect biofilms of other bacterial species, such as mannitol, N-acetyl-L-cysteine, active manuka honey, methylglyoxal, Lipitor®, 2,4-dinitrophenol and EDTA, had no significant effect on *M. avium* biofilms, with some of these substances causing an increase in biofilm formation. None of these substances showed synergistic effects when combined with antibiotics.

Chapter Seven

7. References

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Appendix One

Buffers and Solutions

1X PBS – phosphate buffered saline pH 7-7.4

8g NaCl

0.25g KCl

0.2g KH_2PO_4

1.15g Na_2HPO_4

Adjust with NaOH or HCl to pH 7-7.4

Make up to 1L with sterile mQH_2O.

0.1% DEPC treated water - diethyl pyrocarbonate

2mL DEPC

Make up with 2L mQH_2O,

Mix overnight using magnetic stirrer

Autoclave

2,3,5 Triphenyl Tetrazolium Chloride (TTC)

0.5g powder into 10mL sterile mQH_2O = Stock solution of 50mg/mL

Filter steralise through a 0.22µm filter

Store at -20°C in darkness

6X agarose gel loading buffer

3mL Glycerol

25mg Bromophenol Blue

20µl Xylene Cyanole

Make up to 10mL with sterile mQH2O

PEG – polyethyleneglycol potassium hydroxide pH 13.3-13.5

27mL PEG 200

465µl 2M KOH

Adjust with NaOH or HCl to pH 13.3-13.5

Make up to 50mL with sterile mQH2O

5mg/mL MTT Solution

0.05g MTT

Make up to 10mL with sterile PBS pH 7.2

20% SDS 50% DMF MTT Solubilizing Solution pH 4.7

10g SDS

25mL DMF

Make up to 50mL with sterile mQH2O

Adjust with HCl or NaOH to pH 4.7

BHI + 199 + ACES Buffer Media Recipe

6mL Glycerol

7.4g Brain Heart Infusion Media

1.92g 199 Media

Adjust with NaOH or HCl to pH 6.5

Make up to 200mL sterile mQH2O

Autoclave

Columbia Media

6mL Glycerol

7.4g Brain Heart Infusion Media

8.8g Columbia Media

Adjust with NaOH or HCl to pH 6.5

Make up to 200mL sterile mQH2O

Autoclave

Middlebrook 7H9 Media

6mL Glycerol

7.4g Brain Heart Infusion Media

1g Middlebrook 7H9 Media

Adjust with NaOH or HCl to pH 6.5

Make up to 200mL sterile mQH2O

Autoclave

Acid Alcohol Methylene Blue Counter Stain

0.2g Methylene Blue

0.5mL Concentrated HCl

74mL 95% EtOH

26mL sterile mQH2O

Appendix Two

Antibiotic Calculations

Amoxicillin

0.1g + 10mL sterile mQH2O = Stock solution of 10mg/mL

0.1mL of 10mg/mL Amoxicillin stock + 0.9mL sterile mQH2O = 1mg/mL Amoxicillin

Clarithromycin

0.1g + 10mL sterile mQH2O = Stock solution of 10mg/mL

0.1mL of 10mg/mL Clarithromycin stock + 0.9mL sterile mQH2O = 1mg/mL Clarithromycin

Doxycycline

0.1g + 10mL sterile mQH2O = Stock solution of 10mg/mL

0.1mL of 10mg/mL Doxycycline stock + 0.9mL sterile mQH2O = 1mg/mL Doxycycline

Cefoxitin

0.1g + 10mL sterile mQH2O = Stock solution of 10mg/mL

0.1mL of 10mg/mL Cefoxitin stock + 0.9mL sterile mQH2O = 1mg/mL Cefoxitin

Amikacin

500mg + 2.5mL sterile mQH2O = Stock solution of 250mg/mL

0.4mL of 250mg/mL Amikacin stock + 0.96mL sterile mQH2O = 10mg/mL Amikacin

0.1mL of 10mg/mL Amikacin stock + 0.9mL sterile mQH2O = 1mg/mL Amikacin

Ciprofloxacin

200mg/mL + 100mL sterile mQH2O = Stock solution of 2mg/mL

0.5mL of 2mg/mL Ciprofloxacin stock + 0.5mL sterile mQH2O = 1mg/mL Ciprofloxacin

Ethambutol

One 400mg tablet + 8mL sterile mQH2O = Stock solution of 50mg/mL

0.2mL of 10mg/mL Ethambutol stock + 0.8mL sterile mQH2O = 1mg/mL Ethambutol

Rifinah ™

Six 150mg/mL tablets + 9mL sterile DMSO = Stock solution of 100mg/mL

0.1mL of 10mg/mL Rifinah™ stock + 0.9mL sterile mQH2O = 1mg/mL Rifinah™

Moxifloxacin

One 400mg tablet + 8mL sterile DMSO = Stock solution of 50mg/mL

0.2mL of 10mg/mL Moxifloxacin stock + 0.8mL sterile mQH2O = 1mg/mL Moxifloxacin

Appendix Three

Biofilm Dispersing Agents Recipes

Aspirin (Acetylsalicylic Acid)

1.0g + 10mL sterile EtOH = Stock solution of 100mg/mL

Adjust pH to 6.5 with NaOH or HCl

EDTA

1.0g + 10mL sterile mQH2O = Stock solution of 100mg/mL

Ibuprofen

1.0g + 10mL sterile mQH2O = Stock solution of 100mg/mL

Mannitol

1.8217g +10mL sterile mQH2O = Stock solution of 1M mannitol

Manuka Honey

2.0g +10mL sterile mQH2O = Stock solution of 20% manuka honey

N- acetyl-L-cysteine

1.0g +10mL sterile mQH2O = Stock solution of 100mg/mL