# A phenotypic and Genotypic Investigation of *Mycoplasma felis*

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**Abstract**

*Mycoplasma felis* is frequently isolated from cats showing signs of respiratory or ocular infection however its role as a pathogen is still undefined. Some evidence suggests that *M. felis* may be a pathogen in ocular infections however evidence for pathogenic involvement of respiratory isolates is far less definitive. Ocular clinical isolates were cultured to identify phenotype; antimicrobial susceptibility and haemolytic activity. The strains were then sequenced along with a reference strain using Next Generation Sequencing technology, to determine if phenotype could be correlated with genotype and identify any genetic potential for virulence.

Six antimicrobials were tested to identify antimicrobial resistance on a range of isolates and the reference strain NCTC 10160. Fluoroquinolones were the most effective; however, recommendations state these should be employed conservatively as a third line antimicrobial, due to their importance in human medicine. Tetracyclines, the first line of treatment in *M. felis* infection, were also effective with $MIC_{90}$s of 0.06 µg/mL and 1 µg/mL for doxycycline and oxytetracycline, respectively. Little variance was observed between isolates, with only macrolides showing decreased efficacy. The reference isolate NCTC 10160 (from 1967) demonstrated the same susceptibility as the clinical isolates (2008-2014) and so this was not considered to be novel or emerging resistance. Haemolytic activity was observed in all isolates.

The clinical isolates were sequenced using Next Generation Sequencing technology to establish if the observed phenotype correlated with a specific genotype. No antimicrobial resistance markers were identified. Upon alignment with a reference genome (*M. cynos* C142) several genes shared sequence homology with good coverage, including those involved in nutrient uptake and lipoprotein signalling; however, no definitive markers of virulence were identified.

The generated MIC data will be useful as currently there are no established breakpoints in animal mycoplasmas. The MIC data available is limited and previously has been generated from research projects such as this. Overall, the

pathogen status of *M. felis* is still unclear. No antimicrobial resistance markers or known virulence markers were identified in this study, although the genes used for alignments were not exhaustive and the highly mutable genome of mycoplasmas may mean divergent or novel resistance genes are present.

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**Author's Declaration**

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

# Chapter I

# Introduction

## 1.1 Introduction to Mycoplasmas

Controversy and contradiction have been at the forefront of mycoplasma research since the organisms were first described in 1889 (Frank, 1889). Issues surrounded first the name itself (Krass and Gardner, 1973), quickly followed by questions surrounding their classification, morphology and pathogenicity (Barile and Razin, 1979). These wall-less mollicutes, confirmed as the smallest known organisms capable of self-replication, and holding a minute, minimalist genome (Rottem, 2003, Carvalho et al., 2005) are curiosities in the field of microbiology.

The mycoplasmas seem to be ubiquitous, having been isolated from most creatures, including horses (Wood et al., 1997), fish (Kirchhoff and Rosengarten, 1984), turtles (Salinas et al., 2011), and even from plants and insects (Garnier et al., 2001). There does seem to be a degree of host specificity for each mycoplasma species, with typical associations having been identified - *Mycoplasma pneumoniae* in humans, *M. agassizii* in turtles (Salinas et al., 2011), *M. hyopneumoniae* in pigs (Ciprian et al., 1988), *M. gallisepticum* in poultry (Khalifa et al., 2013) and *M. felis* in cats (Cole et al., 1967).

The parasitic relationship between mycoplasmas and their host may be attributed to their minute genome size, which limits their capacity to synthesise the proteins and nucleic acids essential for growth and reproduction (Rottem, 2003, Carvalho et al., 2005). This limited biosynthesis also makes mycoplasma species fastidious and difficult to culture. Despite this, they have proven to be a common and dreaded pest amongst researchers using cell and tissue culture, with between 15-35% of established cell lines being infected with mycoplasmas, as reviewed by Drexler *et al.* (2002).

Symptoms of mycoplasmosis are variable depending on the particular mycoplasma species involved in infection, the host species, and the presence of any co-infections. The organisms are known to colonise epithelial and mucosal tissues throughout the body, therefore infection is frequently associated with

respiratory symptoms or conjunctivitis, although mastitis, neurosystemic and arthritic symptoms have been recorded.

More than 120 mycoplasma species have been isolated to date (Brown et al., 2011). Most have been identified as commensal organisms; however, some clear primary pathogens such as *Mycoplasma mycoides* subspecies *mycoides* SC (small colony), the aetiological agent of contagious bovine pleuropneumonia (CBPP)(Westberg et al., 2004), and *M. genitalium*, isolated in cases of human pelvic inflammatory disease (Blanchard and Bebear, 2011), have also been identified. Primary pathogens seem to be the focus of most research, possibly due to the high impact to public health and costs associated with livestock sickness. Other species have been utilised in molecular and cellular biology research, the lack of cell wall and minimal genome making them ideal models for research (Fraser et al., 1995, Rocha and Blanchard, 2002, Gibson et al., 2008).

### 1.2 Mycoplasma felis

This study will focus on *M. felis*, which was first isolated from a case of feline conjunctivitis in 1967. Early research attempted to determine the pathogenicity of *M. felis* as a respiratory and ocular pathogen, however evidence was inconsistent, with Koch's postulates being established in some studies (Cole et al., 1967) but not others (Switzer, 1964), with no definitive explanation for variance. Reviewing the research to date, it seems to be accepted that ocular isolates of *M. felis* are pathogenic, with multiple publications associating the isolation of *M. felis* with the clinical presentation of conjunctivitis but with no reports of isolation from healthy cats (Shewen et al., 1980, Haesebrouck et al., 1991b, Espínolaz and Lilenbaum, 1996, Hartmann et al., 2010). Isolation from respiratory samples remains inconclusive; with *M. felis* being isolated from cats presenting with respiratory signs, but also from clinically healthy cats (Tan et al., 1977, Haesebrouck et al., 1991b, Low et al., 2007, Veir et al., 2008, Kompare et al., 2013) suggesting the organism to be an opportunistic commensal. *Mycoplasma felis* has also proven to be highly antigenic with some studies describing seroconversion in almost all cats tested (Binns et al., 2000, Holst et al., 2010), which also suggests frequent exposure.

### 1.3 Gene Sequencing

The revolutionary discovery of the structure and function of DNA in 1953 (Watson and Crick, 1953) opened up many possibilities in genetic research and development. Within fifteen years, the dideoxy method of determining nucleotide sequences now known as "Sanger sequencing", (Sanger et al., 1977) was established and the first genome had been sequenced, proving that sequence generation and the subsequent genetic organisation it identified, could provide new and relevant insight for researchers. Sanger sequencing is the basis of many current sequencing technologies and was instrumental in the development of the discipline of bioinformatics, an essential advancement in sequencing analysis (McCallum and Smith, 1977, Dayhoff et al., 1981, Bilofsky et al., 1986, Pearson and Lipman, 1988, Altschul et al., 1990, Staden et al., 2000). Although further developed now, Sanger sequencing is not without issues; the limited size of DNA fragments which can be sequenced (~1000 bp) making it a time consuming and costly exercise; Sanger sequencing was employed to sequence the first full human genome – it took around ten years and cost almost 3 billion dollars (Venter et al., 2001).

Sequencing technologies and analysis tools and databases are now so advanced that genome sequencing is being utilised by researchers worldwide to sequence genomes from a multitude of organisms for purposes as widespread as investigating the genetic basis of antibiotic resistance in bacteria, to identifying genetic mutations that correlate with disease phenotypes in humans (Morozova and Marra, 2008, van El et al., 2013).

Next Generation Sequencing (NGS) technology provided a solution to the limitations of Sanger sequencing; namely scalability, speed and throughput by employing massively parallel sequencing, capable of processing millions of reactions simultaneously thus generating huge volumes of data (Illumina, 2012). Although numerous platforms are available from biotechnology developers such as Illumina and Roche, the basic principles remain the same; fragment the target genome, generate a DNA library and the analyser will provide the nucleotide sequence of the fragments.

This simplistic principle however, is not without qualifications. The quality of the NGS data generated may be affected by an array of things, with library

preparation being critical to obtaining good quality data. The concentration, size distribution, nucleotide composition and amplification of the template DNA may all influence the quality of data output. Given that DNA yield is variable depending on the quality of the sample and the organism from which it is obtained, kits have been developed that not only allow multiple samples to be sequenced simultaneously using indexes (multiplexing), but also accommodate picogram concentrations of DNA template where DNA input is limited.

Analysers may generate their own quality control data. For example the Illumina MiSeq, which was used in this study, provides a "Q Score" indicative of whether a nucleotide has been correctly identified during sequencing. It is acknowledged that sequence quality diminishes towards the end of a read; however technology is under constant development and this is always being improved, paired end reads being a good example. Many of the platforms are capable of generating paired end reads, working from either side of the fragments, thus enabling longer strands to be processed and increasing sequence quality.

### 1.4 Mycoplasma Gene Sequencing

In 1995 the second bacterial whole genome, and the first of the mycoplasma genomes, *M. genitalium,* was published (Fraser et al., 1995). *M. genitalium*, a pathogen of the human genital tract, was identified as the smallest living genome at that time, which made it the focus of researchers aiming to create a synthetic genome (Gibson et al., 2008). In 1996 the whole genome sequence of the phylogenetically close *M. pneumoniae,* a pathogen of the human respiratory tract, followed (Himmelreich et al., 1996). Now more than 60 mycoplasma whole genomes or scaffolds are available on the National Centre for Biotechnology Information (NCBI) database. Although initially mycoplasma sequencing was dominated by those species identified as human pathogens (Fraser et al., 1995, Himmelreich et al., 1996, Sasaki et al., 2002, Shu et al., 2011), advancements in speed and capacity, in conjunction with reduced costs, has allowed growth in the sequencing of mycoplasmas of agricultural and veterinary significance (Liu et al., 2011, Wise et al., 2011, Brown et al., 2012). This has facilitated the development of molecular techniques for detecting these

fastidious organisms as well as producing a mine of information on their genetic potential.

For the most researched species, the established primary pathogens, interactions with the host are well documented and have shown that despite their minimalist genome, mycoplasmas are capable pathogens. Within their minimalist genome, mycoplasmas possess lots of repetitive non-coding sequence alongside genes inferring only basic biosynthetic and metabolic capabilities (Fraser et al., 1995, Citti and Blanchard, 2013). However, mycoplasmas are very efficient in colonising and infecting their hosts. Through the production of adhesins, lipoproteins and nucleases, the mycoplasmas are capable of hijacking biochemical products, inducing apoptosis (Into et al., 2002), degrade host cell DNA (Minion et al., 1993, Paddenberg et al., 1998), and even altering gene expression in host cells (Feng et al., 1999). Minion *et al.* (1993) established that nuclease activity was essential for the growth and survival of mycoplasmas, likely providing a means of acquiring nucleic acid precursors they are unable to synthesise themselves. Mycoplasmas efficiently produce metabolites such as reactive oxygen species and peroxide thus creating a cytotoxic environment for host cells (Cole et al., 1968, Pilo et al., 2005, Sun et al., 2008).

**1.5 Pathogenicity of Mycoplasmas**

Nucleases and proteases produced by the mycoplasmas provide a means of harvesting host DNA and proteins for their own biosynthetic pathways (Minion et al., 1993, Paddenberg et al., 1996, Paddenberg et al., 1998). The inadvertent consequences involving depletion of host nutrients and generation of by-products such as hydrogen peroxide lead to cytotoxicity and ultimately may induce apoptosis (Somerson et al., 1965, Cole et al., 1968, Paddenberg et al., 1996, Paddenberg et al., 1998).

Although generally considered to be extracellular parasites, several species have now been identified in intracellular locations (Tully and Whitcomb, 1979, Baseman and Tully, 1997, Sasaki et al., 2002). As for any intracellular pathogen, this location affords a degree of protection from the host immune system and antimicrobial therapy, possibly contributing to the chronicity of mycoplasma infections (Tully and Whitcomb, 1979). For those species that remain

extracellular throughout infection, alternative methods to evade the host immune response have been documented.

## 1.6 Susceptibility to Antimicrobial Drugs (AMD)

Antimicrobial drugs may be classed in numerous ways – according to their chemical structure, mode of action or pharmacodynamic properties. They may also be described as either bactericidal, eliminating infectious bacteria, generally through interference with cell wall or nucleic acid synthesis, or bacteriostatic, inhibiting growth by interfering with cell physiology or inhibiting protein synthesis (Sykes and Papich, 2014a).

Ideally, most agents would have bactericidal properties; however bacteriostatic drugs are entirely capable of clearing infections with the aid of the host immune system. It must also be understood that distinguishing these two distinct groups is difficult in some cases, with some bacteriostatic agents demonstrating bactericidal properties at sufficiently high concentrations. Furthermore, the categorisation of whether an AMD is bacteriostatic or bactericidal may vary according to the target organism, the host, and the concentration of AMD available at the site of infection (Sykes and Papich, 2014b).

## 1.7 Anti-Mycoplasma Agents

### *1.7.1 Tetracyclines*

Tetracyclines take their name from their four hydrocarbon ring chemical structure and were derived originally from the Streptomycetes (Cotton, 2015). The tetracyclines reversibly bind to the 30S ribosomal subunit thus preventing new amino acids being added to developing chains and stalling protein synthesis (Neu and Gootz, 1996, Schwarz and Chaslus-Dancla, 2001). Defined as bacteriostatic and demonstrating broad spectrum activity, the more recently developed tetracyclines such as doxycycline, demonstrate a higher efficacy against susceptible bacteria, even when compared to earlier generations of tertracyclines (Morley et al., 2005).

### *1.7.2 Macrolides*

Macrolides take their name from the macrolide ring structure common to these chemicals, also produced by Streptomycetes (Bauman, 2011). The structure may

be altered by the attachment of various deoxy sugar components (Berg et al., 2007). These changes in structural chemistry provide a range of macrolide drugs with differing properties. Common to all macrolides however, is their mode of action. Macrolides are protein synthesis inhibitors which bind to the 50S ribosomal subunit, inhibiting the formation of new peptide chains. Typically, macrolides are bacteriostatic however they have been proven to have bactericidal properties when the intracellular concentration is sufficiently high (Wermuth, 2011). These AMDs, such as azithromycin and erythromycin, are broad spectrum antibiotics, active against both Gram-positive and Gram-negative bacteria (Neu and Gootz, 1996). Erythromycin, discovered in 1949 was the original macrolide (Wermuth, 2011), with many derivatives such as azithromycin and clarithromycin now in use.

### *1.7.3 Fluoroquinolones*

Fluoroquinolones also take their name from their chemical structure – a quinoline ring with a fluorine atom attached. These drugs are entirely synthetic, derived from the original quinolone; nalidixic acid (Emmerson and Jones, 2003). Since then, numerous quinolone drugs have been developed, and a hierarchy of first (nalidixic acid), second (ciprofloxacin) and third generation (pradofloxacin) compounds are available (Andersson and MacGowan, 2003). Newer drugs such as pradofloxacin are most effective; these broad spectrum AMDs have provided a relatively new and effective line of defence, and are predominantly bactericidal (Neu and Gootz, 1996). Fluoroquinolones interfere with DNA and protein synthesis by binding to topoisomerase II (DNA Gyrase) or topoisomerase IV, responsible for untangling and packaging DNA. These enzymes are critical to DNA replication, thus their inhibition leads to cell death. The drugs are able to cross freely into eukaryotic cells via porins to attain high intracellular concentrations which are ideal for single dose administration (Sykes and Papich, 2014b).

## 1.8 Antimicrobial Resistance (AMR)

Since their discovery early in the 20$^{th}$ century, AMDs have been used routinely in the prevention, treatment and control of infection in humans, animals and plants but also within food producing animals to promote growth (Schwarz and Chaslus-Dancla, 2001, CDC, 2014). Their efficiency in preventing and clearing

infections, compounded with the development of vaccines, allowed the near elimination of previously fatal infectious diseases, such as tuberculosis and syphilis, from the developed world (CDC, 1999). However, less than 100 years since the discovery of penicillin, it is apparent that there has been a degree of misuse, resulting in the decreasing susceptibility of microbes to AMDs.

AMR may be an inherent property of an organism – e.g. mycoplasmas lack a cell wall and therefore cannot be targeted by beta-lactams, or an acquired property e.g. tetracyclines for which more than forty microbial gene mutations have been identified which result in a resistant phenotype (van Hoek et al., 2011).

Microbes are evolving more rapidly than their hosts; however it is crucial to keep in mind that heterogeneity exists in all microbial populations, thus the prevalence of resistance is not uniform. Chemotherapy then does not induce the mutation conferring resistance, rather, it selects for it, thus allowing the flourishing and development of populations resistant AMDs (Neu and Gootz, 1996, Schwarz and Chaslus-Dancla, 2001, van Hoek et al., 2011). It should also be noted that host commensals, as well as the target organism are exposed to AMDs (Sykes and Papich, 2014a), causing selection of resistant commensal bacteria and possibly altering the normal flora (Neu and Gootz, 1996). This in turn creates a reservoir of resistance genes within the host microbiome, thus creating an environment ripe for horizontal gene transfer between any microbes within that host (Morley et al., 2005) or any other recipient which the microbe may be transmitted to, further fuelling the development of AMD resistance. This has resulted in acquired resistance mechanisms being accountable for most resistance problems in modern medicine (Schwarz and Chaslus-Dancla, 2001).

Various mechanisms have been identified which confer resistance to AMDs, and these will be discussed further below, specifically with regard to resistance to anti-mycoplasma agents. It should be noted that these resistance mechanisms are not exclusive, and in many bacteria the simultaneous possession of multiple resistance mechanisms is not uncommon (Neu and Gootz, 1996, van Hoek et al., 2011) and may result in multi-drug resistant strains of bacteria as has been seen in *Mycobacterium tuberculosis*.

Many strategies and guidelines have been made available, with the aim of reducing or slowing the development of antimicrobial resistance *(Edwards et al., 2004, Morley et al., 2005, Weese, 2006, CDC, 2014, Sykes and Papich, 2014a, Sykes and Papich, 2014b)*. All guidelines acknowledge that empirical treatment must be minimised, and that use of AMDs must be lessened in order to protect their efficacy. In order to achieve this, it is recommended that an evidence-based approach to AMD employment is required.

A case study published in 2001 also emphasises this need, reporting on 3 cases of human mycoplasma infections, initially treated empirically. Each of the three cases demonstrated resistance to combinations of conventional mycoplasma therapies, requiring the employment of a pleuromutilin drug, at the time not approved for human administration (Heilmann et al., 2001), to effectively treat the infections. It was noted that in 2 of the three cases, facilities for antimicrobial susceptibility testing were unavailable and for the third case, no susceptibility testing was done until the patient was found to be unresponsive to traditional therapy. The outcome was that two of the patients had significant hospital stays and the other patient died (Heilmann et al., 2001). Had susceptibility testing been available and utilised in the first instance, the appropriate antimicrobial therapy could have been employed much earlier, potentially improving patient prognosis.

### 1.9 Antimicrobial Resistance in Mycoplasmas

Currently, in the treatment of confirmed *M. felis* infection, doxycycline is recommended as the first line of treatment (E Graham, personal communication). Although numerous studies have investigated the antimicrobial susceptibility of animal mycoplasmas (Taylor-Robinson and Bébéar, 1997, Vicca et al., 2004, Man et al., 2012), only one study has documented results for *M. felis* specifically (Kibeida, 2010). Using six field isolates, obtained from nasal flushes, and a range of AMDs including doxycycline, enrofloxacin and erythromycin, the $MIC_{90}$ values were recorded as 0.25 µg/mL, 0.031 µg/mL and 128 µg/mL respectively. Given the recommendations on prudent use of fluoroquinolones in veterinary medicine (Edwards et al., 2004, Weese, 2006), these data support doxycycline as a first line treatment.

The low susceptibility to erythromycin among *M. felis* isolates, demonstrated by Kibeida (2010) is echoed in other studies where macrolides are repeatedly least effective (Taylor-Robinson and Bébéar, 1997, Vicca et al., 2004, Man et al., 2012). One interesting study focussed on the emergence of resistance *in vitro* for mycoplasma strains *M. gallisepticum, M. iowae and M. synoviae* using selective culture and performing assays after each passage. This demonstrated that the most rapid emergence of resistance was to the macrolide erythromycin in all of the species tested (Gautier-Bouchardon et al., 2002). Resistance to oxytetracycline could not be produced in *M. gallisepticum* or *M. synoviae* after 10 culture passages and resistance to enrofloxacin was slow in all tested isolates.

Of other studies reviewed, one recorded decreased susceptibility to enrofloxacin in 59% (MIC range1 to >16 µg/mL) of *M. synoviae* field isolates tested (Lysnyansky et al., 2013) with other mycoplasmas; *M. hyopneumoniae, M. bovis and M. synoviae* giving $MIC_{90}$ values of 0.5 µg/mL, 4 µg/mL and 8 µg/mL for respectively (Vicca et al., 2004, Man et al., 2012, Lysnyansky et al., 2013). Man *et al.* (2012) also documented decreased susceptibility of *M. bovis* to doxycycline ($MIC_{90}$ of 32 µg/mL).

These studies indicate that AMR is emerging within the mycoplasmas, although susceptibilities appear to be inconsistent across species, they do seem to correlate with the level of exposure to specific AMDs, particularly within food production animals (Schwarz and Chaslus-Dancla, 2001, Morley et al., 2005, CDC, 2014, Wegener, 2012). Although multi-drug resistance only seems to be notable in *M. bovis*, the variance of susceptibilities between species is indicative that the emergence of resistance within any mycoplasma species is probable rather than possible when challenged by chemotherapeutic selection pressures.

### 1.10 Mechanisms of Resistance

The mycoplasmas are inherently resistant to all beta-lactam antimicrobials due to their lack of cell wall and therefore peptidoglycan target. For mycoplasma genomes which have been sequenced in their entirety, only minimal DNA repair genes have been documented (Rocha and Blanchard, 2002), this likely contributes to the high mutation rates which characterise mycoplasmas (Razin et al., 1998). This mutator phenotype created by reduced DNA repair mechanisms and increased mutation frequency confers an increased likelihood of acquired resistance. Acquisition of new genes has also been documented in mycoplasmas.

The main mechanisms of AMD resistance are summarised below:

- Production of enzymes
- Target modification
- Drug exclusion
- Ribosomal protection proteins

The production of enzymes such as esterases, allow the addition of new molecules to the drugs chemical structure, thus changing structure, function and action of the drug. This mechanism has been implicated in AMR to macrolides and also tetracyclines (Berg et al., 2007, Bauman, 2011).

Modifications such as the methylation of adenine nucleotide components of the 50S ribosomal subunit may lead to resistance to macrolides by decreasing the binding affinity of the target receptor (Neu and Gootz, 1996). Susceptibility to fluoroquinolones may also be affected by target modification; commonly chromosomal mutations which result in a conformational change in the A or B subunit of DNA gyrase, have been identified however may also be a result of increased drug efflux or reduced bacterial permeability or a combination of both (Neu and Gootz, 1996, Sykes and Papich, 2014b).

Active efflux, or energy dependent efflux, of AMDs also decreases the antimicrobial action of drugs such as macrolides which may be actively exported from cells. Tetracyclines are particularly vulnerable to this mechanism, and the *tetK* gene has been identified as mediating increased drug efflux within bacteria such as *Staphylococcus aureus* and *Enterococcus faecalis*, making them resistant

to all tetracyclines (van Hoek et al., 2011). Alternative mechanisms for drug exclusion involve the change in permeability of the bacterial cell wall, limiting the access of AMDs to target sites. Mutations within porins of the cell wall have also been identified as enabling resistance to drugs including macrolides, tetracyclines and fluoroquinolones, by reducing permeability and ultimately preventing AMDs reaching their target receptor.

Ribosomal protection proteins have been implicated in resistance to tetracyclines and macrolides. Within mycoplasmas, the predominant tetracycline resistance gene has been identified as *tetM* (Pich et al., 2006, Mardassi et al., 2012) which has been characterised as encoding a cytoplasmic protein capable of dislodging any tetracycline bound to its target site on the ribosome.

### 1.11 Minimum Inhibitory Concentration Testing

Susceptibility testing, or minimum inhibitory concentration (MIC) testing, is an effective tool used routinely in many laboratories to establish which AMDs are effective against which microbes. Its employment provides an *in vitro* measurement of how susceptible a microbe is to a particular drug by demonstrating the lowest concentration required to inhibit growth of a target organism (Andrews, 2001, Wiegand et al., 2008). The procedure employs one antimicrobial agent, at progressively lower concentrations, along with a standardised inoculum of the organism of interest, incubated for a defined period of time. Growth may be visualised by turbidity, a zone of inhibition or a colour change (using a pH indicator dye), indicating the concentration of the antimicrobial agent which prevents growth and reproduction of the organism.

The MIC value is between the concentration of antimicrobial in which the last growth is observed, and the next concentration, in which no growth is visible. This is of relevance whilst evaluating new antimicrobial agents but also in assessing antimicrobial resistance, and identifying if the resistance is inherent or acquired, through application of established breakpoints (Wiegand et al., 2008).

Well-standardised methods are essential in MIC testing as any variance in inoculum or antimicrobial concentration, pH, temperature, media composition or incubation period may result in significant variance in MIC values (European

Committee for Antimicrobial Susceptibility Testing of the European Society of Clinical and Infectious, 2003, Wiegand et al., 2008). It is also important to note that this test does not reflect the bactericidal effect of drugs or chemicals, with further culture being required to establish whether the organisms have been eliminated or if the original inoculum is still present but unable to grow (Andrews, 2001).

Depending upon the facilities of the testing laboratory and the target organism, various methods of MIC testing may be employed; agar dilution, agar diffusion or broth (micro) dilution.

### 1.12 MIC Testing of Mycoplasmas

Liquid dilutions in particular are preferable for mycoplasmas, which are slow growing. Liquid culture allows growth dependent changes to be observed, such as those induced by metabolites. Liquid culture has also proven to be more sensitive for slow growing organisms (WHO, 2014) and in slowing degradation of drugs (Heifets, 1991), this is particularly relevant within MIC testing of mycoplasmas as effective drugs, such as the tetracyclines, are considered to be unstable and degrade quickly (Wiegand et al., 2008). MIC testing in liquid culture within mycoplasma diagnostics is also beneficial as there is no requirement for a supplement to the media, as there is for solid mycoplasma media, thus reducing variances in media composition and improving standardisation. The employment of liquid microdilution also reduces the volume of reagents required and, as many antimicrobial agents may be set up on one plate, allows and easy and direct comparison of the antimicrobial activity of multiple agents in one test.

### 1.13 Project Summary

Currently, screening for *M. felis* is part of the routine diagnostic service at the Glasgow University Veterinary Diagnostic Service, and when isolated, whether from a respiratory or conjunctival sample, the infected cat is treated with antimicrobials. To date, only sparse antimicrobial breakpoints are available for mycoplasmas, with none available for *M. felis* specifically. Evidence suggests that *M. felis* is likely an ocular pathogen in cats, however when isolated from cats with respiratory tract disease, its pathogenic role is still unclear.

The first objective of this project is to examine antimicrobial susceptibility phenotypes amongst ocular clinical isolates and an *M. felis* reference strain in order to identify any emerging resistance and determine the most effective *in vitro* antimicrobials.

Secondly, within this project, the DNA sequence of a selection of clinical isolates and the reference strain will be obtained using Next Generation Sequencing Technology. Mycoplasmas identified as pathogens in humans and animals will be used as reference genomes in order to search for known markers of virulence in mycoplasmas and allow identification of any antimicrobial resistance genes.

In conclusion, this project aims to correlate observed phenotype with obtained genotype. This will allow evidence-based employment of antimicrobials and novel exploration of the *M. felis* genome and its potential for virulence which in future may be further investigated in cell infection studies.

## Chapter II

## Phenotypic Characterisation of *Mycoplasma felis* Clinical Isolates

### 2.1 Introduction

Although some studies have attempted to explore the pathogenicity of *M. felis*, the results have not been consistent or reproducible (Campbell et al., 1973, Shewen et al., 1980, Haesebrouck et al., 1991b, Wood et al., 1997).

This project aims to re-examine the pathogenic potential of *M. felis*, and establish whether virulence can be correlated with phenotypic markers. This chapter will record the phenotypic properties of *M. felis* clinical isolates including antibiograms and pattern of haemolysis. Examination of these markers will also provide immediate information regarding variance among different isolates and possibly identify emerging antimicrobial resistance (AMR).

These points are relevant in a clinical setting, where the straightforward identification of a phenotype of virulence will allow evidence-based recommendations to be made to veterinary practitioners regarding *M. felis* infection; firstly, is treatment required, and secondly, what is the most appropriate treatment.

#### *2.1.2 Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing using the MIC method is an effective tool used routinely in diagnostic laboratories to establish which AMDs are effective against which microbes. Liquid microdilutions are preferable for slow growing organisms such as mycoplasmas and, when set up on a 96-well microtitre plate, allows easy visualisation of results and comparison of antimicrobial susceptibilities. Within the Veterinary Diagnostic Services (VDS) laboratory of the University of Glasgow, antimicrobial susceptibility testing (discs) is utilised in routine practice for all samples received which are suspected to contain bacterial pathogens. However, susceptibility testing of mycoplasmas is not in place and due to their slow growing and fastidious nature, would be difficult to implement.

The method used in this study to establish MIC values of mycoplasmas in liquid culture will be based on a prior publication (Hannan, 2000).The liquid broth used

employs phenol red as a pH indicator – in the presence of *M. felis*, glucose is metabolised, producing acidic metabolites and causing the pH indicator to pale from red to yellow, indicative of growth. This colour change is used to identify the MIC value following 48-hour incubation under microaerophilic conditions.

MIC values for seven antimicrobials against clinical *M. felis* isolates will be determined; six of these have expected activity against *M. felis*: enrofloxacin, pradofloxacin, oxytetracycline, doxycycline, erythromycin and azithromycin. The ß-lactams are acknowledged to be bactericidal. By binding to and inhibiting penicillin binding proteins required for synthesis of the peptidoglycan layer of the bacterial cell wall, these drugs ultimately result in rupture of the bacterial cell wall (Sykes and Papich, 2014b). Due to their lack of cell wall, mycoplasmas are inherently resistant to ß-lactam antibiotics however for the purpose of this project; the ß-lactam ampicillin (AMP) was employed as a control.

Antimicrobial susceptibility testing allows the evaluation of new antimicrobials and also the assessment of antimicrobial resistance through the application of established breakpoints (Wiegand et al., 2008). Since acquired resistance has been demonstrated in numerous mycoplasmas (Roberts et al., 1985, Heilmann et al., 2001, Vicca et al., 2004, Pich et al., 2006, Man et al., 2012, Lysnyansky et al., 2013, Gautier-Bouchardon et al., 2014), susceptibility testing to aid directed therapy is increasingly important.

In 2011, the Clinical Laboratory Standards Institute (CLSI) published the first antimicrobial breakpoints for mycoplasmas, but for only three mycoplasma species, all implicated in human infection. To date, no mycoplasma breakpoints are available from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). The lack of defined breakpoints for animal mycoplasmas makes it challenging to provide a laboratory result that can confidently drive therapy. Throughout the literature, *in vitro* susceptibilities for mycoplasmas have been reported with various interpretations of MIC values (Whithear et al., 1983, Roberts et al., 1985, Taylor-Robinson and Bébéar, 1997, To et al., 2010, Schultz et al., 2012, Gautier-Bouchardon et al., 2014) therefore making it difficult to define any emerging resistance not only within this study, but in animal mycoplasmas in general.

### *2.1.3 Haemolysis*

Haemolytic activity represents the lysis of erythrocytes by a haemolysin, and may be observed during culture of an organism on blood agar. The colour of the agar beneath colonies is indicative of the type of haemolysis – with green being associated with α-haemolysis, a partial lysis of erythrocytes, and clearing of the agar beneath and surrounding the colonies, being indicative of ß-haemolysis, complete lysis of erythrocytes (Bauman, 2011). Cole *et al.* (1967) first characterised *M. felis* and described varying degrees of haemolytic activity on sheep, guinea pig, chicken, duck and horse blood agar. Although haemolytic activity is a useful tool in aiding the identification of organisms in culture *in vivo* haemolysis is unusual unless there is an underlying condition.

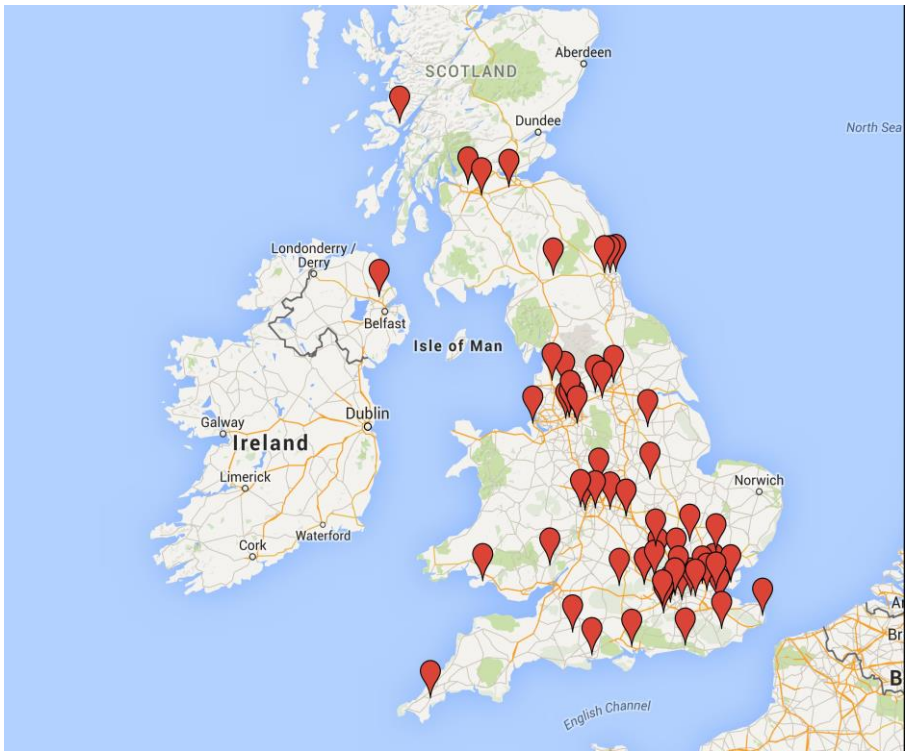
Research of haemolysin production in opportunistic pathogens such as *Staphylococcus aureus* and *Escherichia coli* has established that for those organisms possessing haemolysin markers, other virulence markers such as cytotoxic necrotising factor, were also identified. Thus, these organisms were determined to have higher potential virulence *in vivo* (Hughes et al., 1983, Cherifi et al., 1990, Cotar et al., 2010).

## 2.2 Materials and Methods

### *2.2.1 Mycoplasma Isolates*

Ocular swabs received by the University of Glasgow's Veterinary Diagnostics Service (VDS), for routine diagnostic testing were cultured in mycoplasma Liquid Broth (LB) and mycoplasma Agar (AG) (Mycoplasma Experience, Reigate, UK) as described below. Culture positive results were verified by *M. felis* species-specific real-time PCR, with original isolates frozen in cryovials (Thermo Fisher Scientific, Hemel Hempstead, UK) at -70°C. *Mycoplasma felis* strain 10160 (Cole et al., 1967) from the National Collection of Type Cultures (NCTC, Salisbury, UK) was used as a control strain throughout this study. Seventy-eight clinical isolates of *M. felis,* received between 2007 and 2014, from throughout the UK and Scandinavia, along with *M. felis* strain D8 (Wood et al., 1997) were available for this study.

**Figure 2.1 Origin of Mycoplasma Isolates Used in this Study**




Red pointers mark the origin of Mycoplasmas isolates used within this study. As demonstrated in the map, a variety of UK areas are represented. Two isolates from Scandinavia were also cultured for use (not shown).

**Table 2.1 Details of *Mycoplasma felis* isolates studied during this project**

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| 10160 | 1967 | Cole et al. 1967 | - | - | - | - |
| D8 | 1997 | Wood et al. 1997 | - | - | - | - |
| 3 | Apr-08 | Ocular Swab | 7 m | BT39 | ocular discharge | - |
| 4 | Dec-09 | Conjunctival Swab | - | PA34 | conjunctivitis | - |
| 5 | Apr-08 | Conjunctival Swab | 11 m | G71 | r/c epiphora, purulent conjunctivitis, sneezing | - |
| 6 | May-08 | Conjunctival Swab | 4 y | EH54 | r/c discharge from eyes | - |
| 7 | Nov-08 | Conjunctival Swab | 1 y | M41 | Severe gingivitis and conj. | - |
| 8 | Nov-13 | Eye swab | 1 m | CA8 | - | - |
| 9 | Dec-13 | Eye swab | - | M26 | - | - |
| 10 | Dec-13 | Conjunctival Swab | 3 m | HX1 | Conj with mucopurulent discharge | Y |
| 11 | Apr-03 | Corneal Swab | - | BN1 | Corneal ulcer | Y |
| 12 | Jul-08 | Conjunctival Swab | 9 y 7 m | G61 | ulcer, leukosis, weight loss, anorexia | - |
| 13 | Sep-14 | Conjunctival Swab | 16 y 11 m | EH54 | corneal ulcer | - |
| 14 | Aug-14 | Conjunctival Swab | 11 m | SE9 | conj, green discharge, inflammed | - |
| 15 | Jun-08 | Conjunctival Swab | - | NW9 | - | - |
| 16 | Oct-13 | Conjunctival | 14 y | CM12 | Ulcer on eye | N |

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| | | Swab | | | | |
| 17 | Oct-13 | Eye swab | 2 m | LS8 | Conj. And chemosis | Y |
| 18 | May-08 | Conjunctival Swab | 1 y 8 m | CM7 | purulent conjunctivitis | - |
| 19 | Jul-08 | Conjunctival Swab | - | NP25 | conjunctivitis | - |
| 20 | Aug-08 | Conjunctival Swab | 1 y 2 m | BB1 | bilat conj ongoing. General malaise | - |
| 21 | Feb-14 | Conjunctival Swab | 13 y | SK8 | - | - |
| 22 | Apr-03 | Conjunctival Swab | 8 m | RM2 | Chronic conj. Recent resp symptoms | Y |
| 23 | May-08 | Conjunctival Swab | 12 w | SA2 | bilar conj, nasal discharge. | - |
| 24 | May-08 | Conjunctival Swab | - | NO | epiphora | - |
| 25 | May-08 | Conjunctival Swab | 7 m | NW10 | bilat severe conj with chemosis | - |
| 26 | May-08 | Conjunctival Swab | 3 y 8 m | NE29 | epiphor, severe conjunctivitis, worse L eye | - |
| 27 | May-08 | Conjunctival Swab | 1 y | SE4 | cat flu; had kittens 2 died pneumonia/pleurisy | - |
| 28 | Jun-08 | Conjunctival Swab | 8 y 7 m | G61 | acute unilat conj | - |
| 29 | Jun-08 | Conjunctival Swab | 3 y 6 m | DY8 | r/c sneeze, conj. Unilat ulcer | - |
| 30 | Jun-08 | Conjunctival | 9 m | HP23 | bilateral severe conjunctivitis | - |

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| | | Swab | | | | |
| 31 | May-14 | Conjunctival Swab | 11 m | B62 | - | - |
| 32 | Sep-08 | Conjunctival Swab | 3 m | SP8 | Conj. In group of kittens | Y |
| 33 | Sep-14 | Conjunctival Swab | 2 m | SS4 | conj | - |
| 34 | Jan-13 | Conjunctival Swab | 5 m | CM12 | Conj, swollen with puss | - |
| 35 | Aug-13 | Conjunctival Swab | - | LE14 | conj | - |
| 36 | Sep-13 | Conjunctival Swab | 2 m | OX12 | Conj, sneezing and pyrexia | - |
| 37 | Oct-13 | Conjunctival Swab | 3 m | CV22 | Recurrent conj. | Y |
| 38 | Oct-13 | Conjunctival Swab | 2 m | TR1 | Conj with discharge | - |
| 39 | Jan-14 | Conjunctival Swab | 1 y 8 m | CV22 | conj | - |
| 40 | Feb-14 | Conjunctival Swab | 4 y 8 m | RG9 | Severe conj, deep ulcer, blepharospasm | - |
| 41 | Apr-14 | Conjunctival Swab | 17 y | SG8 | conj | - |
| 42 | Jan-14 | Conjunctival Swab | 12 y 3 m | G61 | respiratory symptoms | - |
| 43 | May-14 | Conjunctival Swab | 11 m | M26 | Chronic conj. | - |

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| 44 | May-14 | Conjunctival Swab | 1 m | NE5 | conj | - |
| 45 | Jul-14 | Conjunctival Swab | 2 y | PR3 | severe conj | - |
| 46 | Jul-14 | Conjunctival Swab | 3 m | NE7 | conj | - |
| 47 | Feb-14 | Conjunctival Swab | 8 y | DK | conj and persistent sneezing | - |
| 48 | Nov-14 | Conjunctival Swab | 14 y 10 m | TW1 | - | - |
| NE01 | Mar-08 | Conjunctival Swab | 9 w | TN17 | bilat ulcerative KC, occasional sneezing | - |
| NE10 | Jan-13 | Conjunctival Swab | 1 y 5 m | RM15 | Severe conj. | - |
| NE11 | Mar-13 | Conjunctival Swab | 11 m | WS13 | Long term ocular discharge with conj and sneezing | - |
| NE12 | Sep-13 | Conjunctival Swab | 6 m | HP15 | Chronic conj | - |
| NE13 | Oct-13 | Conjunctival Swab | 20 y 5 m | SS17 | Chronic, recurring conj | - |
| NE14 | Nov-13 | Eye swab | 7 m | KT12 | Chronic conj | - |
| NE15 | Jan-14 | Conjunctival Swab | 5 m | BH11 | Blepharitis, epiphora, mucoid discharge | - |
| NE16 | Jan-14 | Conjunctival Swab | 7 m | CV5 | conj | - |
| NE17 | Mar-14 | Conjunctival Swab | 2 m | SS4 | - | - |

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| NE18 | Apr-14 | Eye swab | 9 m | GU1 | previous mycoplasma infection | - |
| NE19 | May-14 | Conjunctival Swab | 8 m | HD5 | corneal ulcer, FCV in household | - |
| NE02 | Nov-08 | Conjunctival Swab | 17 y | GU21 | Repeated URTI | - |
| NE20 | Jun-14 | Conjunctival Swab | 1 y 1 m | M44 | conj | - |
| NE21 | Aug-14 | Conjunctival Swab | 3 m | CH43 | persistent sneezing | - |
| NE22 | Aug-14 | Conjunctival Swab | 13 y 10 m | RG9 | watery mucopurulent discharge | - |
| NE23 | Sep-14 | Conjunctival Swab | - | M16 | conj | - |
| NE24 | Sep-14 | Conjunctival Swab | 5 m | DK | - | - |
| NE25 | Oct-14 | Conjunctival Swab | 17 y | AL4 | persistent sneezing | - |
| NE26 | Oct-14 | Conjunctival Swab | 4 m | PO16 | persistent sneezing | - |
| NE03 | Mar-13 | Conjunctival Swab | 6 m | DN22 | - | - |
| NE04 | Mar-14 | Conjunctival Swab | 7 y 3 m | TW1 | lymphoid rhinitis | - |
| NE05 | Apr-10 | Eye swab | 1 m | SM4 | recurrent eye problems | Y |
| NE06 | Aug-08 | Conjunctival Swab | 1 y 8m | B13 | pyrexia, sneezing, red and runny eye | - |
| NE07 | May-08 | Conjunctival | 2 m | ME5 | bilat conj and nasal discharge | - |

| Isolate ref | Date Rcvd | Origin | Age | Area | Presentation | Multicat Household |
|---|---|---|---|---|---|---|
| NE08 | May-08 | Swab Conjunctival Swab | 13 w | CT14 | sneezing, mild ocular discharge, mild nasal discharge post vacc | - |
| NE09 | Oct-08 | Conjunctival Swab | 3 m | MK7 | Flu, occular signs and sneezing | - |

### *2.2.2 Mycoplasma Media*

Mycoplasma Agar (AG):

The AG was prepared according to manufacturer's instructions (Mycoplasma Experience).Briefly, AG stored at 4°C, was melted in a boiling water bath then allowed to cool on the bench for 5 minutes. The mycoplasma supplement, stored at -20°C, was thawed in a 37°C water bath before transfer to a 56°C water bath with the cooled AG. The supplement and agar were kept in the 56°C water bath for approximately 20 minutes to allow temperatures to equilibrate. The mycoplasma supplement was poured into the agar and gently inverted to mix, avoiding bubbles, before being poured into 35 x 10 mm petri dishes (Falcon, Corning, USA) and allowed to set (approx. 30 minutes) before being refrigerated at 4°C for up to 2 weeks.

Mycoplasma Liquid Broth (LB):

The LB was prepared according to manufacturer's instructions (Mycoplasma Experience). Stored at -70°C, the LB was thawed in a 37°C water bath (approx. 1 hour). The media was then aliquoted, under aseptic conditions, in a laminar flow hood, in 2 mL volumes, into Vacuettes® (Greiner Bio-One, Kremsmuenster, Austria). Each prepared vacuette was stored at -20°C and thawed at room temperature as required. All prepared vacuettes were used within 2 weeks of preparation. This LB contains the pH indicator phenol red. The standard pH of the LB was found to be pH 7.2.

Preparation of Stock Mycoplasma Cultures

Each frozen isolate was thawed at room temperature and then inoculated into LB or AG as described below. The AG was streaked with a colony from the original *M. felis* clinical isolate or inoculated with 100 µL original liquid culture and allowed to dry before incubation. The LB was inoculated with 200 µL original liquid culture isolate, or an agar block known to contain colonies. The inoculated media were placed in a microaerophilic incubator (DW Scientific, West Yorkshire, UK) at 37°C under microaerophilic conditions (5% $O_2$, 5% $CO_2$, 5% $H_2$, 85% $N_2$), for 48 hours or until growth was observed. Liquid broth showing growth, indicated by the pH indicator within the media, was briefly vortexed then aliquoted into 1.5 mL cryovials (Thermo Fisher Scientific) in 200 µL volumes for storage. Visible colonies were cut out from agar in blocks using a sterile scalpel and placed in cryovials (Thermo Fisher Scientific) for storage at -

70°C.Non-inoculated and therefore negative LB and AG controls were included for each batch of isolates being cultured to ensure no contamination.

### *2.2.3 Viable Cell Count*

In order to ensure a standard concentration of inoculum for each MIC test and avoid spurious results, a viable cell count (VCC) was performed for the *M. felis* reference strain 10160, D8 and each *M. felis* clinical isolate according to previously published protocols (Hannan, 2000).

The VCC of strain 10160 was determined, in triplicate, for lag (freshly thawed, diluted 1:10 in LB and immediately tested for viability), lag +2 (thawed, diluted 1:10 in LB, and incubated for 2 hours before viability testing), and logarithmic growth cultures (thawed, diluted 1:5 and incubated for 48 hours prior to viability testing) in order to determine if there were identifiable differences in viability between growth phases and establish repeatability. The minimal variance in these results allowed the employment of lag +2 testing of clinical isolates which substantially reduced the time required for testing.

Isolates were tested in batches of eight, allowing one row per isolate on a 96-well plate. Ten-fold dilutions ($10^{0}$ to $10^{-9}$) of each culture were prepared in 1.5 µL microfuge tubes. The lowest concentration ($10^{-9}$) of each isolate was added to well 8 of the allocated row. From there, wells were inoculated from lowest to highest concentration, until well 1 of each row which held the highest concentration ($10^{-2}$). A sterility control (200 µL LB) was also added to well G11 to ensure sterility of the media. The 96-well plate was then sealed and incubated in a microaerophilic incubator, being checked for growth after 48 hours and again after 7 days. Growth across dilutions allowed colour changing units (ccu) to be calculated, which correlates well with colony forming units (cfu) traditionally used in enumerating bacterial cells (Stemke and Robertson, 1982).

A minimum concentration of $10^{4}$ccu/mL mycoplasma cultures was required for MIC testing; therefore any isolates with a viable count lower than this were concentrated by either adding a higher volume of inoculum to a lower volume of LB or by inoculating AG and incubating until colonies were visible and removing agar plugs into LB for further incubation. The AG plugs seemed to be most

effective in increasing concentration and so this was employed whenever possible.

### *2.2.4 Antimicrobial Preparation*

Antimicrobials as listed below were purchased in powder form and stored according to manufacturer's instructions until required (Table 2.2). Antimicrobial solutions were prepared in 2mL volumes. The calculation described by Hannan (2000) below, was used to generate a correction factor, taking account of purity and salt content, which in turn was used to calculate the volume of solvent required to ensure a starting concentration of 1000µg/mL.

*(mw of active base / total mw) x (purity / 100) = correction factor*

*Correction factor x weight of antimicrobial powder being used = volume of solvent required*

Azithromycin, erythromycin and enrofloxacin were dissolved in 100% ethanol, with all other antimicrobials being dissolved in deionised water ($dH_2O$). Solutions were adjusted to a pH of between 7.1-7.4 to ensure that only colour changes reflective of growth would be observed in LB. The solutions were then filtered, using a 10mL syringe (BD Plastipak, Madrid, Spain, and 0.20 µM pore filter (Sartorius, Surrey, UK) to ensure sterility. A concentration range (64 µg/mL to 0.03 µg/mL) was prepared for each antimicrobial solution by making two-fold dilutions in LB as described by Hannan (2000). Fresh solutions of azithromycin, erythromycin, doxycycline and oxytetracycline were prepared for each assay as previous publications noted them to be unstable (Hannan, 2000, Andrews, 2001, Wiegand et al., 2008). Stock solutions of ampicillin were stored in 200 µL aliquots at -20°C for up to 4 weeks (Hannan, 2000, Andrews, 2001); stock solution of pradofloxacin was stored in a glass vial, in the dark, at room temperature for up to 2 weeks (Wiegand et al., 2008); stock solution of enrofloxacin was stored in a glass vial wrapped in foil, in the dark, at room temperature. A previous publication found enrofloxacin to be stable for up to 56 days (Metry et al., 2012), however due to prohibitive costs; a 5 mL stock solution was retained for 5 months. The pH was monitored weekly, with adjustments being made as required. Efficacy was monitored using the MIC results of the reference isolate (NCTC 10160).

**Table 2.2 Properties of Antimicrobial Drugs Used in MIC Testing**

| Antimicrobial | Manufacturer | Solvent | Storage | Duration of Stability |
|---|---|---|---|---|
| Ampicillin[a] | Sigma-Aldrich Co. Ltd, UK | $dH_2O$[d] | -20°C | 30 days |
| Azithromycin | EDQM, France | 100% Ethanol | Dark, RT[b] | unstable |
| Doxycycline | Fischer BioReagents, UK | dH2O | Dark, RT | unstable |
| Enrofloxacin | EDQM, France | 100% Ethanol | Dark, RT | 5 months[c] |
| Erythromycin | EDQM, France | 100% Ethanol | Dark, RT | unstable |
| Oxytetracycline | EDQM, France | $dH_2O$ | Dark, RT | unstable |
| Pradofloxacin | Bayer Healthcare AG, Germany | $dH_2O$ | Dark, RT | 14 days |

[a] ampicillin was included in the study as a negative control
[b] RT: room temperature
[c] enrofloxacin was found to be stable after 5 months
[d] distilled water

### *2.2.5 Minimum Inhibitory Concentration (MIC) Testing*

Minimum inhibitory concentration testing was performed, as described by Hannan (2000). Isolates were tested in batches of four, together with the control strain 10160. To allow for preparation of five MIC plates, volume modifications to the original protocol were made as follows; 1200 µL of the first antimicrobial dilution (128 µg/mL) were required therefore 153.6 µL of each prepared antimicrobial solution was added to 1046.4 µL LB. Doubling dilutions were obtained using 600 µL antimicrobial dilutions into 600 µL fresh LB until a final concentration of 0.0625 µL/mL was achieved for each antimicrobial with the exception of ampicillin which was diluted to 0.5 µg/mL only. These antimicrobial dilutions were added to sterile 96-well plates in 100 µL volumes as represented in Appendix 1.

For each isolate MIC test, 9 mL inoculum was required. The VCC for each isolate was used to prepare an inoculum of $10^4$ ccu/mL of lag +2 growth phase culture which was then dispensed onto the MIC plate in 100 µL volumes.

Each 96-well plate included a VCC for the test isolate to ensure standardisation of the inoculum concentration. Solvent controls were included to ensure that the solvents used to dissolve the antimicrobials did not inhibit growth. An end-point control was prepared by adjusting the LB to pH 6.8, providing a colour indicator for growth. A sterility control (SC) (200 µL non-inoculated LB) and a growth control (GC) (200 µL inoculum) were also included. Control strain 10160 was also tested with each batch of clinical isolates to ensure consistent antimicrobial performance. To ensure a pure culture of each isolate was tested, AG was inoculated with 100 µL inoculum.

MIC plates were incubated in a microaerophilic incubator until the colour of the growth control well matched the colour in the end point control well. In experiments where the VCC was out-with the recommended range of $10^3$ to $10^5$ccu/mL(Hannan, 2000), the test was repeated. The AG for each isolate was checked at the same time as the MIC plate to ensure a pure *M. felis* culture. After all isolates had been tested, results were compared and isolates giving outlying results were repeat tested to ensure reproducibility.

### *2.2.6 Determination of Biostatic or Biocidal Activity*

Using the reference strain 10160, MIC testing was set up as described above. After 48 hours, 100 µL of culture was removed from each well showing no colour change, indicative of no growth, and inoculated onto AG. This further dilution of antimicrobial drug should allow growth of any viable mycoplasmas. Agar plates were examined after 48 hours incubation to check for growth, indicating the antimicrobial to be bacteriostatic or bactericidal for those showing no growth. This experiment was repeated in triplicate to ensure reproducibility.

### *2.2.7 Haemolysin Activity*

100 µL of each isolate was inoculated onto Sheep Blood Agar (SB) and incubated, as described above, for 48 hours before being examined for haemolysis. If no haemolysis was visible after this time the isolates were incubated for up to 7 days, being checked daily until haemolysis was visible.

## 2.3 Results

### *2.3.1 Mycoplasma Isolates*

Sixty of the 79 retrieved isolates were successfully cultured from original clinical isolates. Reference 10160 and strain D8 (Wood et al., 1997) were also cultured successfully, showing clear growth on AG and in LB after 48 hours incubation (Fig 2.2 and 2.3).

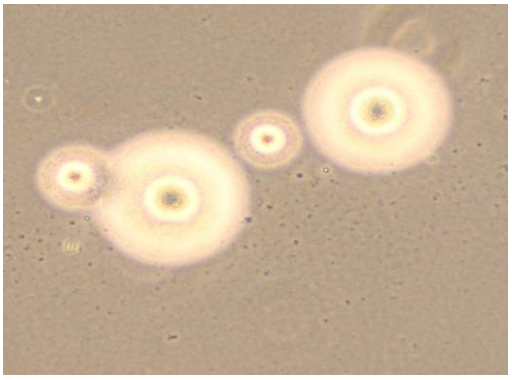


Figure 2.2 *M. felis* colonies (Olympus CKX41 x40) after 48 hours incubation (typically 0.15-0.3 µm in diameter)

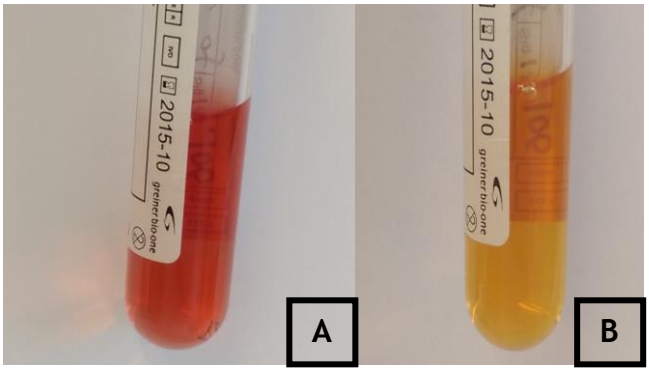



Figure 2.3 Preparation of stock culture
A. Freshly inoculated liquid broth
B. Liquid broth after 48 hour incubation

### *2.3.2 Viable Cell Count*

The comparison of results for lag, lag+2 and logarithmic growth phase viability counts of strain 10160 are shown in Table 2.3. Viable cell counts of $10^4$ ccu/mL or above were attained for 38 of the isolates immediately. Twenty-two isolates required concentration due to initial viable counts of $10^3$ccu/mL or less. Further concentration yielded 14 more isolates with concentrations of $10^4$ccu/mL or greater, giving a total of 51 clinical isolates, plus strain D8, with concentrations sufficient for MIC testing.

**Table 2.3 VCC results for different growth phase cultures of *M. felis* strain 10160**

| Growth Phase | Viable count (ccu/ml) | | |
|---|---|---|---|
| lag | $10^6$ | $10^7$ | $10^6$ |
| lag +2 | $10^6$ | $10^7$ | $10^7$ |
| log | $10^6$ | $10^6$ | $10^6$ |

Performed in triplicate, Viable Cell Count (VCC) results are shown above for *M. felis* reference strain 10160 in varying growth phases.

### *2.3.3 Enrofloxacin Efficacy*

Two pH adjustments were required for enrofloxacin, at day 27 and day 33 as the pH had become slightly acidic (from pH 7.2 to pH 6.8 and pH 6.7 respectively). The strain 10160 MIC results for enrofloxacin were as follows: 1/13 result of 0.25µg/mL (8%) and 12/13 results of 0.125µg/mL (92%). These results show no significant shift or deterioration in efficacy of the enrofloxacin stock solution over a 5 month period.

### *2.3.4 Minimum Inhibitory Concentration (MIC) Testing*

The MIC results of all clinical isolates and strain D8 are shown in Appendix 2. Of the isolates tested, 11 had viable counts out-with the required range, with a further 2 agar plates showing contamination after 48 hours culture. These isolates were purified where necessary and the VCC and MIC tests were repeated.

The antimicrobial control, ampicillin, was proven to be ineffective as expected, with an $MIC_{90}$ of >64 µg/mL for all clinical isolates and the reference strain tested. The most effective antimicrobial tested was the bactericidal fluoroquinolone, pradofloxacin, with $MIC_{90}$ of 0.03125 µg/mL shown to be effective at eliminating growth *in vitro*. Isolates were also highly susceptible to doxycycline with an $MIC_{90}$ of 0.0625 µg/mL, in comparison to oxytetracycline with an $MIC_{90}$ of 1 µg/mL. Most isolates appeared resistant to both macrolides with erythromycin being least effective, recording MIC values of ≥8 µg/mL for all isolates; 28 isolates (55%) showing MIC values of ≥64 µg/mL and azithromycin

demonstrating an $MIC_{90}$ of ≥32 µg/ml for clinical isolates and the reference strain.

MIC values for each antimicrobial were mostly consistent for each isolate tested; MIC distribution was within 3 dilutions for more than 90% of clinical isolates for each antimicrobial. Enrofloxacin exhibited the greatest range in antimicrobial activity among clinical isolates, ≤0.03 - 8 µg/mL. Six isolates appeared resistant to enrofloxacin, with MIC values of ≥ 2 µg/mL. All $MIC_{50}$ and $MIC_{90}$ values for clinical isolates are shown in Table 2.4. The $MIC_{50}$ and $MIC_{90}$ values for the reference strain can be seen in Table 2.5. When comparing the $MIC_{50}$ values of reference strain 10160 against the clinical isolates, the results for the fluoroquinolones were identical. For the macrolides and tetracyclines, a difference of only one dilution was observed. $MIC_{90}$ values were also consistent across the range of antimicrobials tested, with the exception of enrofloxacin for which the $MIC_{90}$ was 0.125 µg/mL for reference strain 10160 in comparison to the clinical isolates with an $MIC_{90}$ of 2 µg/mL.

Those isolates with MIC values showing non-conformance were selected for repeat testing. The reference strain results for enrofloxacin were highly consistent, with an MIC of 0.125 µg/mL for 12 of 13 runs, and one at 0.5 µg/mL. This is in contrast to the results from the isolates tested, demonstrating the greatest range in antimicrobial activity, reflected in the MIC range for enrofloxacin (0.03125 µg/mL to 8 µg/mL). Three isolates were retested as their initial MIC values for doxycycline were higher than expected (8 µg/mL, 1 µg/mL and 4 µg/mL), when compared to the $MIC_{90}$ (0.0625 µg/mL). Upon repeat testing, each doxycycline MIC value fell to within the consistent range and $MIC_{50}$ and $MIC_{90}$ values were adjusted accordingly for final results (Table 2.4).

**Table 2.4 MIC distribution for *M. felis* clinical isolates**

| | | Concentration Range (µg/mL) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Antimicrobial** | | **>64** | **64** | **32** | **16** | **8** | **4** | **2** | **1** | **0.5** | **0.25** | **0.125** | **0.0625** | **0.03125** | **$MIC_{50}$[a]** | **$MIC_{90}$[b]** |
| Azithromycin | Number of Isolates | 1 | 1 | 8 | 11 | 11 | 12 | 6 | 1 | | | | | | 8 | 32 |
| Doxycycline | | | | | | | | | | | | 5 | 11 | 35 | 0.03125 | 0.0625 |
| Enrofloxacin | | | | | | 1 | 2 | 3 | 1 | 1 | 3 | 30 | 3 | 7 | 0.125 | 2 |
| Erythromycin | | 5 | 23 | 12 | 7 | 4 | | | | | | | | | 64 | 64 |
| Oxytetracycline | | | | | | | | 2 | 21 | 20 | 3 | | 2 | 3 | 0.5 | 1 |
| Pradofloxacin | | | | | | | | | | | 2 | 2 | 3 | 44 | 0.03125 | 0.03125 |
| Ampicillin[c] | | 51 | | | | | | | | | | | | | >64 | >64 |

[a] Concentration required to inhibit growth in 50% of isolates (26 isolates)
[b] Concentration required to inhibit growth in 90% of isolates (46 isolates)
[c] Ampicillin was used as a negative control

**Table 2.5 MIC distribution for *M. felis* reference strain NCTC 10160**

| | | Concentration Range (µg/ml) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Antimicrobial** | | **>64** | **64** | **32** | **16** | **8** | **4** | **2** | **1** | **0.5** | **0.25** | **0.125** | **0.0625** | **0.03125** | **$MIC_{50}$** | **$MIC_{90}$** |
| Azithromycin | | | | 2 | 5 | 5 | 1 | | | | | | | | 16 | 32 |
| Doxycycline | | | | | | | | | | | | 3 | 6 | 4 | 0.0625 | 0.125 |
| Enrofloxacin | | | | | | | | | | 1 | | 12 | | | 0.125 | 0.125 |
| Erythromycin | | | | 10 | 3 | | | | | | | | | | 32 | 32 |
| Oxytetracycline | | | | | | | 1 | 5 | 7 | | | | | | 1 | 2 |
| Pradofloxacin | | | | | | | | | | | | | | 13 | <0.03125 | <0.03125 |
| Ampicillin | Number of Isolates | 13 | | | | | | | | | | | | | >64 | >64 |

[a] Concentration required to inhibit growth in 50% of isolates
[b] Concentration required to inhibit growth in 90% of isolates
[c] Ampicillin was used a negative control

### *2.3.5 Determination of Biostatic or Biocidal Activity*

Pradofloxacin and enrofloxacin were identified as bactericidal at the minimum concentration 0.03125µg/mL. Azithromycin and erythromycin were shown to be bactericidal at the highest concentration tested (64 µg/mL) but were otherwise bacteriostatic. All other antimicrobial drugs were demonstrated to be bacteriostatic, regardless of concentration.

### *2.3.6 Haemolysin Activity*

All isolates except one were cultured on SB and shown to be haemolytic, with 25 showing ß-haemolysis and 25 showing α-haemolysis. The isolate which could not be cultured on SB was cultured on AG and sub-cultured after 48 hours to horse blood agar where it was observed to be alpha haemolytic.

## 2.4 Discussion

Historically the first line of treatment of *M. felis,* and mycoplasmosis in general, is the tetracyclines (Kibeida, 2010). This group of antimicrobials have been well established in human and veterinary medicine. Resistance to tetracyclines has been acknowledged to be emerging amongst a variety of bacterial genera, including mycoplasmas, with numerous mutated genes identified in the mechanism of resistance (Taylor-Robinson and Bébéar, 1997, Schwarz and Chaslus-Dancla, 2001, Morley et al., 2005, Reller et al., 2009, Blanchard and Bebear, 2011, Schultz et al., 2012). One study found that *M. bovis* demonstrated an $MIC_{90}$ of 32 µg/mL for doxycycline (Man et al., 2012). This resistance to the tetracyclines was not reflected by other isolates tested by Man et al., with *M. pneumoniae, M. genitalium, M. homins, M. fermentans, M. hyosynoviae and M. hyopneumoniae* having $MIC_{90s}$ consistently below 2 µg/mL. Although still higher than the $MIC_{90}$ observed in this project (0.0625 µg/mL) this indicates that although emerging, resistance may be isolated to particular species of mycoplasma. The Kibeida (2010) study also supports this; the doxycycline $MIC_{90}$ for *M. felis* recorded in that study was 0.25 µg/mL, much closer to the $MIC_{90}$ observed in this project.

All clinical isolates and the reference strain used in this project showed highest susceptibility to pradofloxacin. Pradofloxacin is the only third generation quinolone currently approved for use with companion animals (Sykes and Papich, 2014b) and the guidelines promote its conservative use, recommending it is avoided as a first line of treatment, in order to minimise developing resistance (Morley et al., 2005, Weese, 2006). Enrofloxacin is employed in human and animal medicine, whereas pradofloxacin is licensed specifically for animals only (dogs and cats) (Health, 2013). Due to concerns regarding retinal toxicity, the use of enrofloxacin in the treatment of cats is now uncommon (Gelatt et al., 2001) yet, being licensed for use in human and animal medicine does suggest that enrofloxacin is likely more regularly employed than pradofloxacin, thus increasing the likelihood of emerging resistance to this drug. However, taking the outlying MICs into account, the enrofloxacin $MIC_{50}$ of 0.125µg/ml is still notably lower than the macrolides tested and slightly lower than oxytetracycline ($MIC_{50}$ 0.5µg/ml). Kibeida (2010) found the $MIC_{90}$ of enrofloxacin to be 0.031 µg/mL which is remarkably lower than the $MIC_{90}$ observed during this project (2

µg/mL). This apparently higher $MIC_{90}$ may be attributed to the inability to repeat test those isolates with outlying values. This is supported by the $MIC_{50}$ 0.125 µg/mL and that 78% (40 isolates) were inhibited at this concentration or below.

Although it would have been preferable to repeat test the isolates with outlying MIC results, numerous genes, and their subsequent mutations, have been identified in fluoroquinolone resistance. Therefore it may prove useful to check for the presence of those so far identified, once the sequencing information is available, to determine if mycoplasmas are developing resistance to this drug.

Of other studies reviewed, *Mycoplasma hominis, M. hyosynoviae, M. bovis* and *M. hyopneumoniae* were noted to have decreased susceptibility to macrolides, particularly erythromycin (Taylor-Robinson and Bébéar, 1997, Vicca et al., 2004, Man et al., 2012), with *M. bovis* having an $MIC_{90}$ of >128µg/mL (Taylor-Robinson and Bébéar, 1997), however *M. pneumoniae, M. genitalium and M. fermentans* showed MICs of less than 1µg/mL (Taylor-Robinson and Bébéar, 1997).

The decreased susceptibility to macrolides observed in this project echoes these previous studies and may reflect the long-standing use of erythromycin in veterinary and human medicine. Interestingly erythromycin, although requiring higher MICs, demonstrated bactericidal properties when reference strain 10160 was tested. The MIC was 32µg/mL which, when removed onto an agar plate, showed recovery and growth of *M. felis*. However, the higher concentration (64µg/mL) was also removed onto agar and showed no growth, indicative of bactericidal action. The macrolides are acknowledged to have bactericidal properties at high concentrations but for this difference to be observed over one dilution, particularly when susceptibility was apparently low, was unexpected.

It is important to note that although a range of MICs were recorded for each antimicrobial, these did not seem to represent emerging resistance over time. The clinical isolates spanned 6 years (2008-2014), with the D8 strain from 1997 and the 10160 reference strain from 1967. When the $MIC_{50}$ results of the reference strain 10160 are compared to the results of the clinical isolates, there is an increase of only one dilution concentration for the macrolides and tetracyclines, with the fluoroquinolone $MIC_{50}$s being identical.

A total of 51 *M. felis* isolates plus the 10160 reference strain were MIC tested using the liquid broth method previously published by Hannan (2000) and validated again recently by Kibeida (2010). The isolates demonstrated the fragile and fastidious nature characteristic of mycoplasmas, with 19 original isolates failing to recover from freeze storage and a further 8 isolates failing to reach sufficient concentrations for testing even after concentration. Interestingly, a number of the recovered isolates demonstrated notable growth after only 24 hours incubation, with some showing visible growth after only 16 hours, contradicting the expected slow growth of mycoplasmas. Of those which did reach the required VCC, initial results proved problematic, with numerous viable counts not being reproduced during the MIC. It was considered that the VCC should be performed in duplicate, and so 8 isolates were selected at random, removed from storage and processed for VCC. Although each VCC was performed with isolates processed identically, from the same frozen aliquot and same incubation vacuette, 4 of the 8 isolates tested in duplicate showed variance of one doubling dilution in viable counts. This demonstrated an inherent variability in growth and viability of the mycoplasmas. The mycoplasmas are known to be fragile so this variability may be attributable to the sometimes long-term storage affecting viability or that they were viable but not cultivable in the time frame allocated. Nevertheless, these data indicated that a margin of error should be accounted for when diluting inoculum for MIC testing. Subsequent VCCs were performed singly for each isolate. Inoculum dilutions were calculated to obtain a $10^4$ccu/ml concentration for MIC, allowing for a doubling dilution on either side which would still fall within the range for standardised inoculum recommended by Hannan (2000) which was verified in the VCC included when MIC testing.

While preparing VCCs and working with another research student also preparing MIC plates, it was observed that LB thawed on the bench and LB thawed in a 37°C water bath were subtly different in colour. Both bottles of LB were from the same lot and had been stored in the same conditions so the difference in colour was unusual. The LB thawed in the water bath was marginally darker red than the LB thawed on the bench. The pH of both bottles was tested, with no discernible difference. The dependence of colour changes in the media for MIC results required we reduce the likelihood of any colour change not indicative of

growth. Given this, it was considered that the water bath and the incubator temperature was equal, therefore if temperature could affect colour then all LB should subsequently thawed in a water bath.

## Chapter III

## Genetic Sequencing and Analysis of *Mycoplasma felis* Clinical Isolates

### 3.1 Introduction

Thus far, the pathogen status of *M. felis* is ambiguous. Mycoplasmas acknowledged to be pathogenic have been identified as expressing a variety of virulence factors, including lipoproteins, nucleases and adhesins (summarised in Table 3.2). Full genome sequencing of *M. felis,* identifying the presence of known mycoplasma virulence genes, would provide insight into its pathogenicity and potentially allow a clearer definition of its role as a pathogen in cats, allowing informed decisions to be made regarding treatment. Phenotypic data corresponding to antimicrobial resistance has been obtained during this study, providing the opportunity to potentially correlate *M. felis* phenotype with genotype as well as identify any emerging resistance; it would therefore be prudent to investigate the *M. felis* data for known antimicrobial resistance genes.

Mycoplasma lipoproteins are known to interact with host immune cells, via toll-like receptors (TLRs) or by acting as cytokine inducers for host monocytes and macrophages, ultimately having the potential to induce or prevent apoptosis (Into et al., 2002, Sasaki et al., 2002, Gerlic et al., 2007, Zuo et al., 2009). Lipoproteins, have been described as one of the most important factors in pathogenic activity (Feng et al., 1999, Zuo et al., 2009), consequently the identification of known lipoproteins in *M. felis* would be of great interest and beneficial in determining its status.

Nuclease activity has been identified in pathogenic mycoplasmas such as *M. pulmonis, M. hyorhinis, M. fermentans* and *M. penetrans* (Minion et al., 1993, Paddenberg et al., 1998, Chambaud et al., 2001). These nucleases may be integral or peripheral membrane proteins which mycoplasmas are capable of secreting. It has been suggested that mycoplasmas use these endogenous nucleases to procure precursors of DNA synthesis (Minion et al., 1993, Paddenberg et al., 1998). Although mycoplasma nucleases have been shown to be harmless to host cells under normal conditions, stressed cells allow these proteins to enter internucleosomal sites, thus enabling degradation of host DNA

(Paddenberg et al., 1998). Investigating the *M. felis* genome, to search for the genes encoding these proteins may allow insight into its pathogenicity.

Adhesins have been described in pathogenic mycoplasmas including *M. bovis* and *M. pneumoniae* with their roles in cytadherence and antigenic variation being well characterised (Dallo et al., 1996, Sachse et al., 1996, Chourasia et al., 2014). Adhesins are crucial for colonisation and infectivity and therefore virulence (Razin and Jacobs, 1992), thus their presence or absence in *M. felis* would be another step forward in defining its role.

Whilst exploring the potential pathogenicity of *M. felis,* it would be of benefit to veterinarians if any emerging antimicrobial resistance could be identified. Homologues of the *tetM* antibiotic resistance gene found in *Streptococcus* have also been identified in mycoplasmas (Roberts et al., 1985) indicating that they are capable of acquiring genes as well as being highly mutable (Razin et al., 1998, Blanchard and Bebear, 2011). Emerging fluoroquinolone resistance has also been identified in mycoplasmas, with researchers revealing a previously characterised mutation in the quinolone resistance determining region (QRDR) of the *ParC* gene, known to confer resistance (Lysnyansky et al., 2013).

Varying degrees of haemolytic activity have been observed in most mycoplasmas (Minion et al., 1993), with numerous haemolysin encoding genes having now been identified. Cole *et al*. identified haemolytic activity, describing peroxide as the major haemolysin within mycoplasmas (Cole et al., 1968). Given that haemolytic activity of *M. felis* has been observed during culture, it would be reasonable to search for the presence of a corresponding gene.

## 3.2 Materials and Methods

### *3.2.1 Mycoplasma felis DNA Extraction*

All clinical isolates which demonstrated a viable cell count of $10^3$ ccu/ml or greater in earlier testing (52 plus reference isolate), were selected for extraction of nucleic acids. *Mycoplasma felis* genomic DNA was extracted from 3 mL of logarithmic growth phase liquid broth cultures, using the QIAamp DNA Mini Kit as per manufacturer's instructions. For each isolate being extracted, a purity plate was set up from the same liquid broth culture in order to identify any possible contamination. Mycoplasma DNA is fragile in nature therefore the protocol was modified, firstly to process 3 mL instead of 1 mL of culture to increase DNA yield, and also all steps were performed on ice and centrifugation at 4°C, in order to inhibit endogenous nucleases. The proteinase K incubation time was also reduced to 10 minutes. Elution volumes were reduced to 50 µL to concentrate mycoplasma DNA within each extract and DNA extracts were stored at -20°C. Sufficient DNA for sequencing was obtained from 50 clinical isolates and the reference strain 10160. The sequencing kit selected was able to multiplex only 48 samples, therefore 3 clinical isolates which had shown little overall phenotypic difference were selected to be omitted from sequencing in order to allow space for the reference strain and 47 clinical isolates.

### *3.2.2 DNA Template Preparation for Next Generation Sequencing*

#### *3.2.2.1 Quantification*

Extracted mycoplasma DNA was quantified using a Qubit®fluorometer (Life Technologies, Paisley, UK). The Qubit allows fluorometric quantification of double stranded (dsDNA) by detecting the signal from an intercalating fluorophore, only emitted when bound to the target dsDNA. This allows an accurate measure of the concentration of dsDNA available which is essential for Next Generation Sequencing (NGS). Briefly, the equipment was calibrated using the supplied standards as per manufacturer's instructions. 20 µL of mycoplasma extract was added to an optical tube containing 180 µL of dilution buffer, prepared according to manufacturer's instructions (1 µL Quant-iT reagent plus 199 µL Quant-iT buffer per mycoplasma extract). The mixture was then briefly vortexed, taking care to avoid bubbles before being read on the fluorometer.

#### *3.2.2.2 Tapestation*

The Agilent 2200 TapeStation (Agilent Technologies UK Ltd, Edinburgh, UK) separates, images and analyses fluorescently labelled dsDNA (35-1000 bp) within a closed electrophoresis system (Padmanaban et al., 2013), using a ScreenTape instead of a gel, and ultimately providing an electropherogram as well as a traditional gel image to size and quantify DNA fragments.

TapeStation consumables were removed from refrigeration at 4°C and allowed to come to room temperature. 3 µL of High Sensitivity D1K Ladder was added to the first optical tube and capped. This ladder provides a control for both the validity of the ScreenTape and the electrophoresis, as fragments sizes are known. 2 µL of each isolate extract was added to separate tubes with 2 µL of sample buffer, capped and briefly vortexed to mix. All samples were centrifuged briefly before being loaded onto the TapeStation instrument and initiating electrophoresis via computer software. The electropherogram generated was used to check the quality of the extracted mycoplasma DNA by quantifying the number and size of fragments within the tested extract.

### *3.2.3 Optimisation of DNA Shearing and Size Selection*

Fragmentation of DNA is required to ensure the length is compatible for sequence library generation and subsequent clustering on the desired sequencing instrument. The Covaris™ S220 Focused-ultrasonicator™ (Life Technologies, Renfrew, UK) fragments DNA by hydrodynamic shearing, in which the sample is exposed to short bursts of sonication. This mechanical shearing of DNA is very reliable, allowing control of the average shear size and making it ideal for NGS. In this case 300-500 bp, was the optimal size range to match the Illumina MiSeq platform used in this study.

AMPure XP Beads (Beckman Coulter Inc., High Wycombe, UK) utilise an optimised buffer (polyethylene glycol and sodium chloride) and magnetic beads selectively bind negatively charged DNA fragments. These beads are used routinely in molecular biology laboratories to purify PCR amplicons by binding to larger DNA fragments and allowing excess primers, nucleotides and salts to be removed in the supernatant. An elution step then releases the bound DNA. The concentration of these beads may be varied in order to selectively bind DNA

fragments of particular sizes, allowing size selection, and thus the fine-tuning of DNA fragment size range for sequencing.

To optimise the sonication and AMPure bead clean up (detailed below), several sonication treatment times were tested to identify the optimal fragment size range of sheared mycoplasma DNA. Isolates were run on the TapeStation to determine which sonication time and bead concentration yielded the best DNA fragment size range for DNA library preparation.

#### *3.2.3.1 DNA Fragmentation (Shearing) by Focused Ultrasonication*

50 µL of each mycoplasma extract was added to a microTUBE (Covaris, Brighton, UK) and capped. Each microTUBE was then loaded onto the Covaris™ S220 and sonicated for 75 seconds with the following parameters: peak incident power – 175 W; duty power - 5%; number of cycles per burst -200. Following sonication, isolates were stored at 4°C until clean up.

#### *3.2.3.2 Use of AMPure XP Beads for Size Selection*

Following sonication, all mycoplasma extracts were mixed with 0.7x AMPure XP beads in order to remove fragment sizes smaller than 300 bp. Numerous protocols were available, each with slight variations in recommended concentrations, therefore optimisation was required. The following method was the most effective protocol for recovery of mycoplasma DNA. Beads were allowed to come to room temperature (RT) for 30 mins. 50 µL of each mycoplasma extract was dispensed into separate wells of a 96-well plate and 35 µL of AMPure beads added to each extract before mixing slowly 10 times with gentle pipetting. After 5 mins incubation at RT the plate was moved to a magnet till the solution cleared and the beads had formed a pellet before removing the supernatant. The beads were allowed to dry for 2 mins at RT. Each pellet was then flooded with 100 µL of 70% ethanol (freshly prepared) and drained using a pipette and the process repeated. The plate was removed from the magnet and the pellet resuspended in 50 µL of 10 mM Tris by pipetting gently and incubating at room temperature, for 2 mins, before putting the plate back onto the magnet. The beads were allowed to form a pellet and once completely clear, the eluate, with the selected size fragments in suspension, was then transferred to a fresh well before quantification and library preparation.

Following optimisation, all of the mycoplasma extracts to be sequenced were sonicated and size selected using the optimised protocol. A number of these size selected extracts were subsequently electrophoresed on the TapeStation to ensure the size selection procedure had indeed been optimal and consistent.

### *3.2.4 DNA Library Preparation*

The ThruPLEX®DNA-seq Kit (Rubicon Genomics, Cambridge) was selected as it fitted a number of criteria desired for our library preparation method. It was able to process as little as 50 pg of template DNA which was necessary because of the low DNA yield obtained from our Mycoplasma isolates as well as generating good diversity of libraries obtained when compared with other leading commercial brands, whilst also allowing the efficient and simultaneous sequencing of 48 isolates. In addition, indexing reagents and the ThruPLEX® stem-loop adaptors were already pre-dispensed in a 48-well format to allow a three-step, single-tube reaction, which reduced manipulations and as a result meant less scope for contamination.

The mycoplasma DNA templates were prepared, and library synthesis was completed according to manufacturer's instructions. The Rubicon kit uses ThruPLEX® technology, based on stem-loop adaptors (Fig 3.1). The optimal number of cycles for amplification was determined as eight, using the DNA concentration obtained prior to library preparation to inform the estimation process as detailed in the manufacturer's instructions.

The amplified DNA was purified using AMPure beads, as described above, to remove primers and adaptor dimers prior to sequencing. In accordance with guidelines from Illumina (Essex, UK) a DNA concentration of 4 nM was required for denaturation prior to sequencing. Library quantitation is essential to allow efficient multiplex sequencing and optimal cluster density for the flow cell. The concentration of the pooled library was calculated by a combination of the concentration measured by Qubit and the average insert size by Tapestation using the following formula:

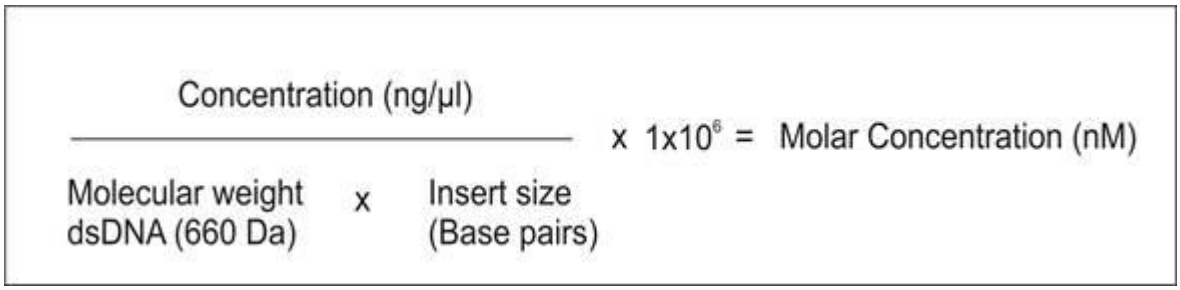



**Figure 3.1 Calculation of Library Molar Concentration**

The amount of DNA (ng/ul) was obtained for each isolate extract using the Qubit, with peak size being obtained using the TapeStation. These concentrations were recorded for each extract in order that they could be pooled for multiplex sequencing. At this stage one clinical isolate was removed from the panel for sequencing as the concentration was too low. The remaining 46 isolates plus reference strain 10160 were sufficient to continue.

The 47 remaining template libraries were then diluted, using the supplied hybridization buffer, to a concentration of 4 nM to ensure equal concentrations of each template. The 4 nM libraries were then denatured using a fresh NaOH solution before all samples were pooled for multiplex sequencing. The pooled DNA was again tested on the Tapestation and Qubit to ensure the correct concentration and fragment size were achieved.

For improved quality control during sequencing, the pooled library was diluted and the internal control, PhiX, added at 1% before commencing the sequence run as recommended by Illumina. The PhiX genome is well defined and diverse, with balanced nucleotides. Given that Illumina has optimised cluster detection for balanced representation of adenine, thymine, guanine and cytosine nucleotides, the addition of PhiX allows a more diverse set of clusters. This is of particular importance for mycoplasma sequencing as they are known to possess low-GC content and therefore the cluster diversity provided by PhiX becomes crucial. Sequencing was carried out on the MiSeq Illumina) platform, generating 150 bp paired-end reads.

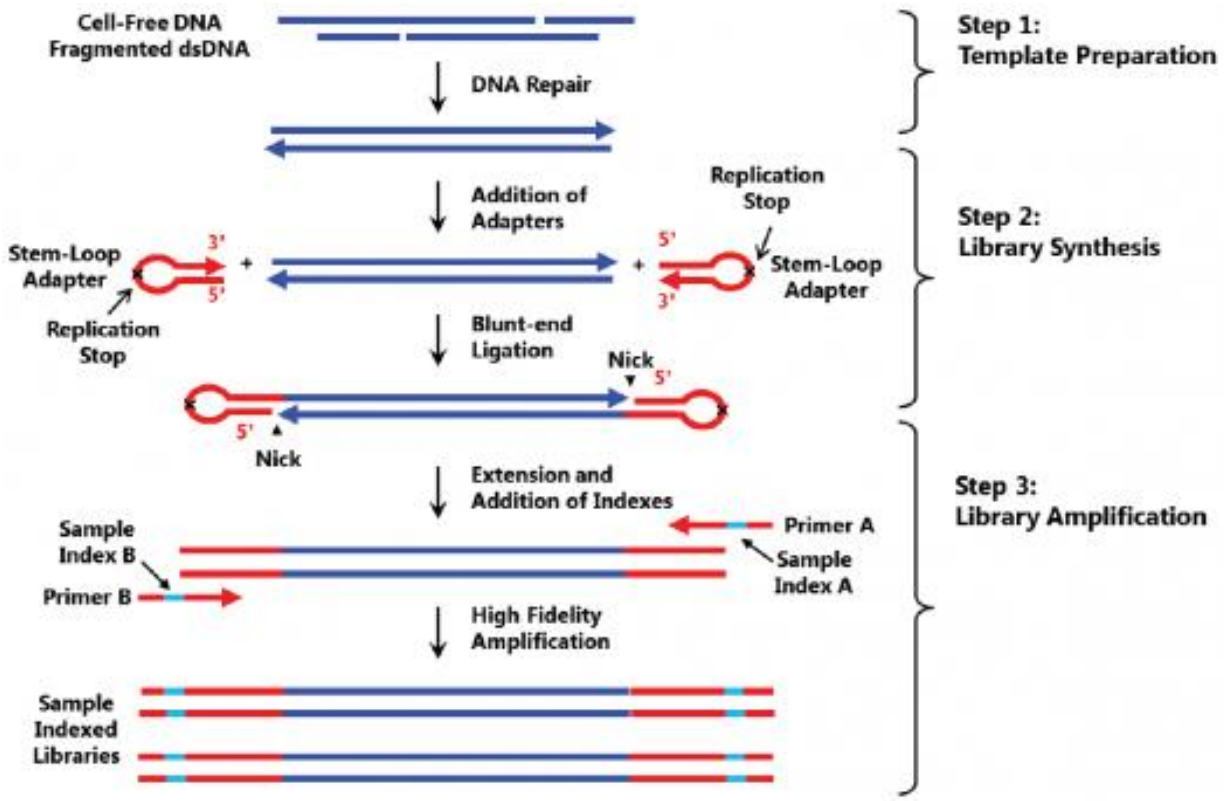



**Figure 3.2 ThruPLEX DNA-seq Technology** (Rubicon, 2012)

Initially, the DNA template is repaired to provide blunt ends with which the stem-loop adaptors can ligate. The adaptors 5' ends are blocked, leaving a gap at the 3' end. They cannot ligate to each other, and lack the single strand tail typical of Y adaptors, therefore reducing background ligations. The template DNA is extended and then amplified, at which point Illumina compatible indexes are added. The final library product for NGS has sequencing primers, index tags and P5/P7 termini on either side of the isolate DNA. The P5 and P7 termini are defined sequences, complimentary to sequences on the surface of the flow cell, allowing the termini and attached library fragment to bind to the flow cell for sequencing.

### *3.2.5 Next Generation Sequencing*

The MiSeq Illumina NGS platform is capable of producing paired reads of 300 bp through parallel sequencing of the fragmented DNA pool. The capability of multiplex sequencing, using indexed samples (Fig 3.2 and Fig 3.4), reduces time and cost, and in this case, allows the simultaneous sequencing of 47 M. felis isolates. The pooled DNA was briefly centrifuged before being added to the MiSeq reagent cartridge (Figure 3.3) and loaded into the MiSeq and the Rubicon index sequences added to the software setup as per manufacturer's instructions.

**Figure 3.3 MiSeq and Consumables**

This single use reagent cartridge is pre-filled with sequencing reagents. The prepared sample libraries are added to the highlighted aperture while the other apertures allow access to separate reagents required during the process. The cartridge is then loaded onto the MiSeq, shown on the right. *Image obtained from Illumina Inc.*

Unique 8 nucleotide dual indexes are attached to each library during preparation, allowing the libraries to be pooled and loaded in the same flow cell. Libraries are then sequenced in one MiSeq run, with all generated sequences being exported in a single data file. The index sequences are then sorted using Illumina software to create different file for each template.

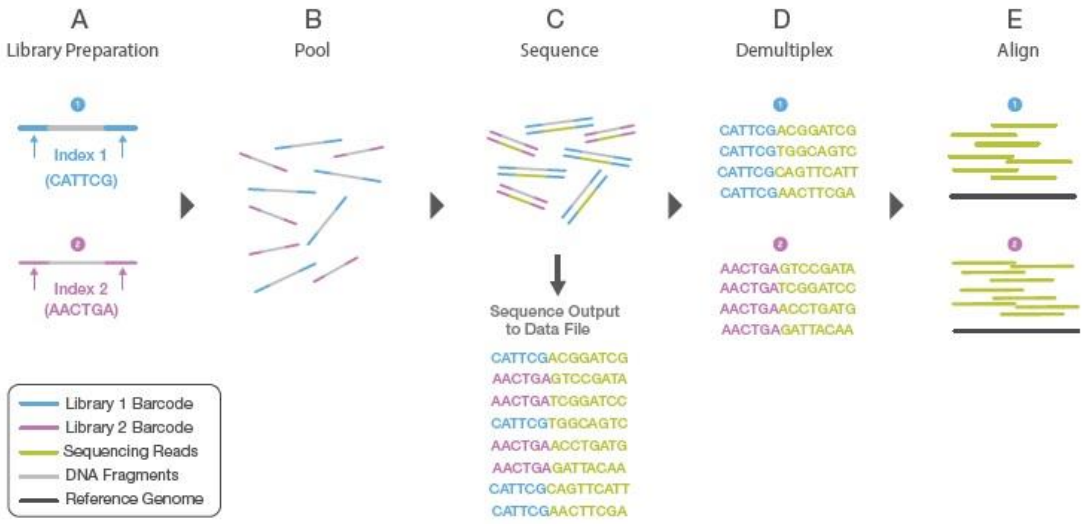



**Figure 3.4 Overview of Library Multiplexing/Demultiplexing for Analysis** (Illumina, 2016)

### *3.2.6 Mycoplasma felis Sequence Analysis*

#### *3.2.6.1 Pre Analysis Data Trimming*

The sequence data for 46 clinical isolates, and the reference strain 10160, were trimmed of adapters and subsequently further trimmed of 25 bp on each end, using CLC Genomics Workbench 7.5.1 software (CLC), to improve overall sequence quality of the usable data set (Fig 3.7). This read trimming step is crucial in NGS analyses as contamination of the data with adaptor or primer sequences may result in alignment errors or an increased number of unaligned reads in downstream analyses.

#### *3.2.6.2 De novo Assembly*

The purpose of *de* novo assembly is ultimately to produce a novel genome sequence. Through the assembly of short sequence reads, larger contiguous sequences (contigs) are produced which may then be further assembled to create scaffolds. Using the CLC software standard parameters for *de novo* assembly, contigs were generated, initially for the mycoplasma reference strain 10160 and then for a further 12 of the 46 isolates (selected according to number of reads generated in sequencing, ensuring a wide range – Table 3.3). For best results, a low number of contigs with a high depth of coverage is required.

Standard CLC *de novo* assembly parameters allow this to be measured, firstly by reporting the number and length of contigs generated (excluding those <200 bp), but also providing the option of mapping reads back to the newly generated contigs, thus allowing depth of coverage to be determined.

Unfortunately, *de novo* assembly failed to provide a reference scaffold; upon examination of the generated contig sequences, much of the data appeared to be non-coding, low complexity sequence with poor depth of coverage. With the time involved in attempting to *de novo* assemble these for 12 isolates and the reference isolate; it was clear that not all planned analyses could be done for each of the 47 isolates. The 12 isolates selected for *de novo* assembly, along with the reference strain 10160 were therefore used for all further analyses.

#### *3.2.6.3 Alignments Using the Basic Local Alignment Search Tool (BLAST)*

Newly generated reads may be investigated using BLAST databases which catalogue whole genomes, scaffold genomes and individual gene sequences previously sequenced and published. Using BLAST, the newly generated reads can be aligned, or mapped, to existing genetic sequences. Generally using a reference sequence, these alignments allow comparison and identification of variation. A selection of the largest contigs generated for reference strain 10160 were run using the Basic Local Alignment Search Tool (BLAST) to the NCBI nucleotide (blastn) database however results were inconsistent and we were again unsure if the contigs were accurate.

The top contigs for each of the 12 clinical isolates were run in BLAST, however remained inconsistent. These results left us unsure if the contigs were accurate enough to pursue further analyses at this stage. Using the trimmed reads themselves, without assembly, the BLAST hits became more consistent with 10 of the 13 selected isolates returning the same organism – *Mycoplasma cynos* as most homologous. The remaining two isolates (both of which had a very low number of reads available) returned *Mycoplasma canis* as the top result with *Mycoplasma cynos* being second. At this stage, *Mycoplasma cynos* was selected as a reference genome.

#### *3.2.6.4 Alignments Using Mycoplasma cynos as a Reference Genome*

Trimmed reads for each isolate were used for all subsequent alignments and BLAST analyses. The *M. cynos* genome identified as similar in BLAST was then imported to CLC for alignments with the reference strain 10160 and all 46 clinical isolates.

#### *3.2.6.5 Other Alignments*

A selection of mycoplasma whole genome sequences (WGS) and individual gene sequences were imported from BLAST to CLC for alignment and comparison. The WGS were selected as they share the same phylogenetic grouping (fermentans) as *M. felis* (Brown et al., 1995) or because they showed high similarity in BLAST.

To identify any emerging resistance or virulence factors in the *M. felis* isolates, individual genes were selected which were well characterised in other organisms. These included known antimicrobial resistance genes, genes inferring haemolytic activity and those of known mycoplasma lipoproteins, nucleases and adhesins proven to increase virulence in other mycoplasmas. All imported files (Table 3.1 and 3.2) were then aligned for comparison with the *M. felis* reference strain 10160 and clinical isolate reads.

Since isolate 3 returned the largest number of reads, after alignment with the *M. cynos* whole genome, the unmapped reads were then exported to a FASTA file to be run on BLAST in order to determine if these reads would correspond to any other organism.

**Table 3.1 Mycoplasma whole genome sequences within the *M. fermentans* phylogenetic grouping, which were aligned against trimmed reads from the *M. felis* clinical isolates**

| Mycoplasma Genome | Accession Number |
|---|---|
| *M. cynos C142* | HF559394 |
| *M. fermentans M64* | CP002458.1 |
| *M. felis ATCC23391(partial)* | KL370829 |
| *M. bovis NM2012* | CP011348 |
| *M. pneumoniae M129* | NC_000912 |
| *M. pulmonis UAB CTIP* | AL445566 |
| *M. agalactiae PG2* | NC_009497 |
| *M. hyopneumoniae 232* | AE017332 |
| *M. mycoidesstr PG1* | NC_005364 |
| *M. synoviae* | NZ_ARQH01000042 |
| *M. gallisepticum* | CP006916 |
| *M. genitalium* | CP003770 |

**Table 3.2 Well-characterised virulence genes, aligned with trimmed reads from the *M. felis* reference strain and clinical isolates.**

| Organism | Gene/Protein | Description | Accession No. |
|---|---|---|---|
| *M. cynos* | hapA | haemagglutinin (cytadherence) | KJ997962 |
| *Escherischia coli* | hha | haemolysin | AF294319 |
| *Staphylococcus aureus* | Hla | alpha haemolysin | KM019673 |
| *M. cynos* | hlyA, B, C, D | haemolysin | HF559394 |
| *U. ureaplasma* | hlyC | haemolysin | NC_002162 |
| *M. pneumoniae* M129 | hmw1 | cytadherence accessory protein | NC_000912 |
| *M. pneumoniae* M129 | hmw2 | cytadherence accessory protein | NC_000912 |
| *M. pneumoniae* M129 | hmw3 | cytadherence accessory protein | NC_000912 |
| *M. putrefaciens* | LppB | transport protein | EU429511 |
| *M. genitalium* | TlyC | haemolysin | CP003770 |
| *M. hyopneumoniae* | nuc | exonuclease | AE017332 |
| *M. agalactiae* | P80 | membrane lipoprotein | X95628 |
| *M. pulmonis* | Mnu | membrane nuclease | U38841 |
| *M. gallisepticum* | MslA | nuclease co-operation | CP001872 |
| *M. hyopneumoniae* | MnuA | nuclease | AE017332 |
| *M. hyopneumoniae* | MnuA | nuclease | NC_007295 |
| *M. penetrans* | MYPE4380 | nuclease | NC_004432 |
| *M. pulmonis* | MYPU_6940 | membrane nuclease | AL445565 |
| *M. pulmonis* | MYPU_6930 | membrane nuclease, lipoprotein | AL445565 |
| *M. pulmonis* | MYPU_1390 | nuclease, lipoprotein | AL445565 |
| *M. hominis* | OppB, C, D, F | cytadherence, ATPase activity | X99740 |
| *M. mycoides* | OppA, B, C | transmembrane permease | KM410303 |

| Organism | Gene/Protein | Description | Accession No. |
|---|---|---|---|
| *M. pneumoniae* | orf6 | cytadherence | Z33397 |
| *M. pneumoniae* | P1 | cytadherence | AF290000 |
| *M. pneumoniae* | P1 | cytadherence | JN048896 |
| *M. pneumoniae* | P1 | cytadherence | AB024618 |
| *M. pneumoniae* | P1 | cytadherence | AF286371 |
| *M. agalactiae* | P80 | membrane lipoprotein | NC_009497 |
| *Enterococcus faecium* | parC | fluoroquinolone resistance | LN624829 |
| *M. gallisepticum* | parC | fluoroquinolone resistance | AF372652 |
| *M. synoviae* | parC | fluoroquinolone resistance | AY819792 |
| *M. felis* (strain CO) | rpoB | transcription | DQ234670 |
| *Streptococcus pneumoniae* | tetM | tetracycline resistance | AY466395 |
| *M. gallisepticum* | tetM | tetracycline resistance | GQ424446 |
| *M. hyopneumoniae* | TlyA | haemolysin | AF326115 |
| *M. felis (strain CO)* | tuf | elongation factor | FJ896397 |
| *M. bovis* | VspE, A, F, J, B, K, L, M, N, O | Lipoprotein | AF396970 |
| *M. bovis* | VspE, C, F | Lipoprotein | AF396972 |
| *M. gallisepticum* | VlhA | haemagglutinin | EU284138 |

## 3.3 Results

### *3.3.1 Mycoplasma felis DNA Extraction*

Following optimisation of the manual extraction process, to increase DNA available for harvest and ensure inhibition of endogenous nucleases, the DNA yield was improved. Sufficient DNA was obtained from 50 clinical isolates plus the reference strain 10160.

### *3.3.2 DNA Template Preparation for Next Generation Sequencing*

The DNA yield obtained from the *Mycoplasma felis* samples ranged from 0.6 – 1.7 ng/µL.

### *3.3.3 Optimisation of DNA Shearing and Size Selection*

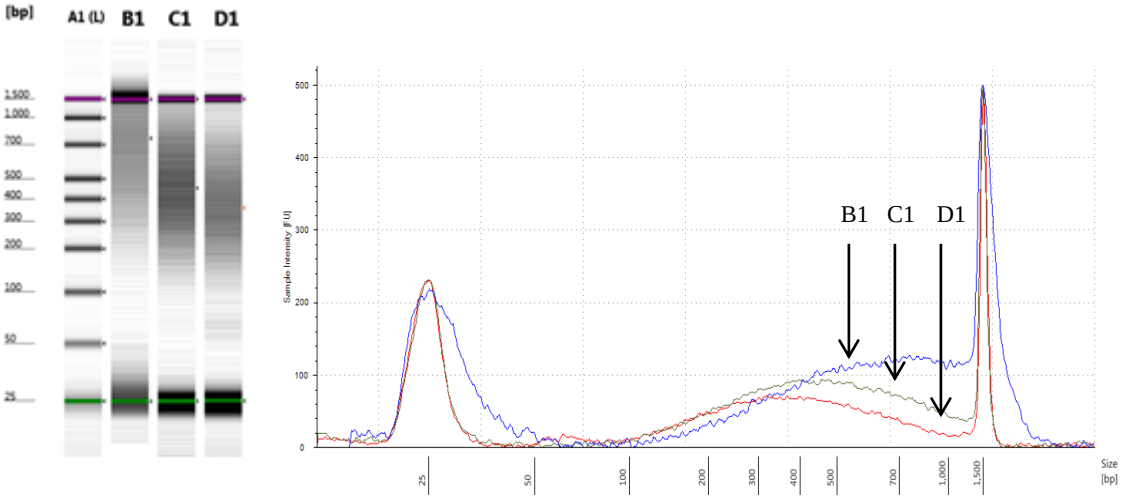



**Figure 3.5 Traditional gel image and electropherogram comparing various sonication times**

Traditional gel image and electropherogram comparing various sonication times to quantify the number and size of fragments within the tested mycoplasma extract in order to determine optimal time to retrieve the desired fragment sizes for NGS.

A1 - High sensitivity D1K Ladder, B1 - 35 sec sonication of mycoplasma isolate, C1 - 45 sec sonication of mycoplasma isolate, D1 - 60 sec

The smear at the lower size range of each lane in the gel image is indicative of some degradation. Mycoplasmas are very fragile and therefore this observation is not entirely unexpected. The high molecular weight band at the top of each gel lane is indicative that substantial larger fragments are still present. The electropherogram mirrors this result, showing a left shift in the central peak with increasing sonication time. This demonstrates only a minimal increase in the number of smaller fragments but showing only a minimal reduction in the number of large fragments, leaving a significant number still too large for NGS. Therefore extended sonication times were tested.

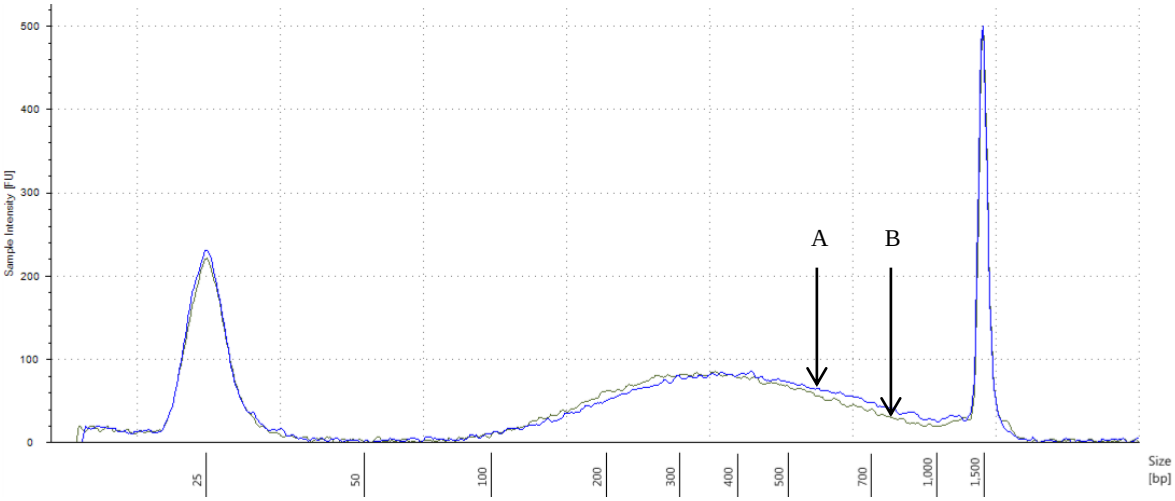



**Figure 3.6 Electropherogram comparing 60 second and 75 second sonication time of mycoplasma extract**
A -60 sec sonication of mycoplasma isolate, B -75 sec sonication of mycoplasma isolate

The electropherogram shows a subtle left shift at 75 seconds sonication with a slight increase in the number of fragments between 100 and 300 bp in size. However, it also shows a slightly reduced quantity of fragments of 1000-1500 bp. A peak remains indicating that larger fragments are still present. Given that the optimal fragment size for the selected sequencing kit was 300-500 bp, 75 seconds sonication was chosen and applied for all extracts to be sequenced.

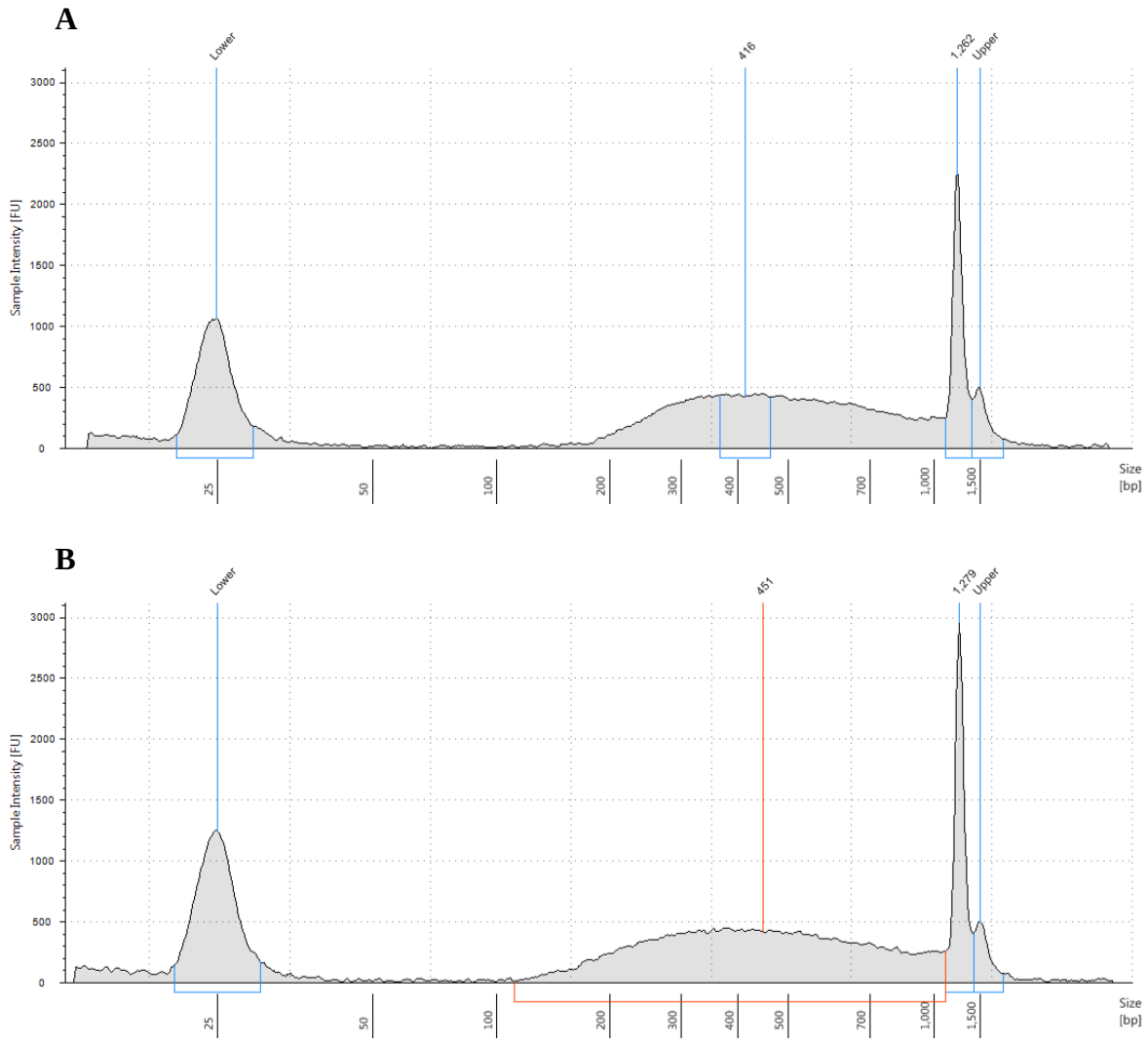



**Figure 3.7 Electropherograms showing comparison of mycoplasma extracts sonicated for 75 seconds and cleaned up using 0.7x (A) and 0.8x (B) AMPure bead concentrations.**

This comparison shows peak size to be similar for both AMPure bead concentrations, however distribution is more compact using the 0.7x concentration, with markedly fewer fragments of <200 bp which would be more suited to NGS. The large peak at >1000 bp indicates large fragments are still present. Isolates were also run on a genomic DNA (gDNA) tape to investigate the significance of the large peak prior to NGS.

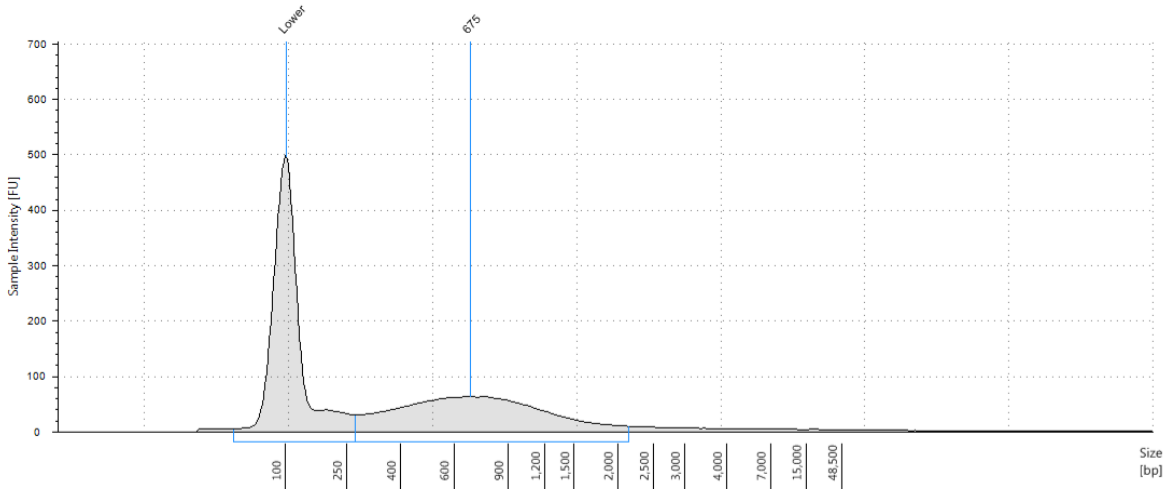



**Figure 3.8 Electropherogram using gDNA tape on mycoplasma DNA sonicated for 75 seconds to identify larger DNA fragments unsuitable for sequencing**

This electropherogram shows that the large peak observed using the high sensitivity tape represents a minimal number of fragments >1000 bp, with distribution peaking around 675 bp size fragments. The peaks towards the smaller fragment end of each electropherogram represent smaller fragments unsuitable for NGS.

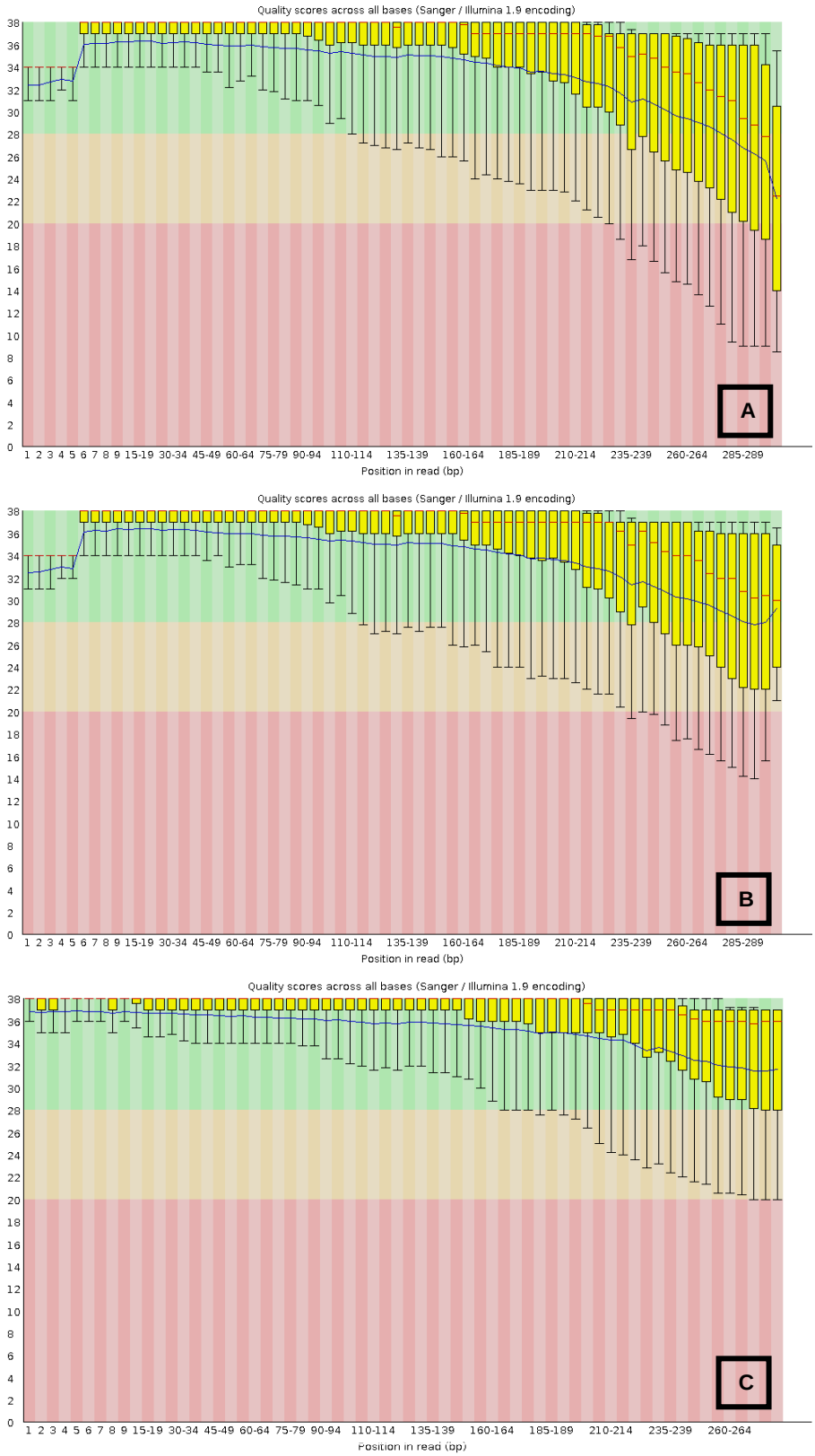
Using 1.8 x AMPure beads for size selection as previously described, also allows a "reverse clean-up" in which larger fragments (>1000 bp), recognised as being problematic in MiSeq sequencing, can be removed prior to NGS.

### *3.3.4 Mycoplasma felis Sequence Analysis*

#### *3.3.4.1 Pre-Analysis Data Trimming*

Figure 3.9 demonstrates the improvement of sequence quality scores after trimming of Illumina adaptors and an additional 25 bp trim of reads prior to analysis.

**Figure 3.9 FastQC per Base Sequence Quality Report**




A: raw data quality scores, prior to adapter trimming
B: quality scores after adapter trimming (sequences of less than 80 bp were removed entirely)
C: quality scores for paired sequence after trimming, size filtering and an additional 25 bp trim on both ends

**Table 3.3 Number of trimmed reads generated by NGS for each of the clinical isolates selected for alignment to whole genome references and genes of interest**

| Isolate | Number of reads generated | Number of reads aligned to *M. cynos* C142 whole genome | Percentage of reads aligned to *M. cynos* C142 whole genome |
|---|---|---|---|
| 10160 | 315,368 | 53,013 | 16.81 |
| 15 | 382,298 | 62,702 | 16.4 |
| 21 | 239,500 | 40,920 | 17.09 |
| 24 | 186,718 | 28,737 | 15.39 |
| 32 | 720,200 | 134,485 | 18.67 |
| 3 | 7,445,148 | 1,337,951 | 17.97 |
| 42 | 462,906 | 71,008 | 15.34 |
| 46 | 293,742 | 46,434 | 15.81 |
| 4 | 2,751,950 | 500,486 | 18.19 |
| 5 | 1,148,860 | 198,946 | 17.32 |
| 6 | 1,684,144 | 314,522 | 18.68 |
| 8 | 539,708 | 36,699 | 6.8 |
| 9 | 322,862 | 49,954 | 15.47 |

#### *3.3.4.2 De novo assembly*

The *de novo* assembly of reference strain 10160 and the selected clinical isolates generated contigs averaging between 1368 and 4425 bp, however these contigs generated inconsistent results on BLAST and on close examination, much of the sequence was noted to be repetitive or non-coding with little depth of coverage. Unfortunately, lack of confidence in the accuracy of the contigs being generated in *de novo* assembly, and the time constraints of the project, left us unable to pursue further assemblies and their subsequent analyses at this stage.

#### *3.3.4.3 Alignments Using BLAST*

After inconsistent BLAST hits to the NCBI database were obtained from the generated contigs, trimmed original sequence reads were analysed with BLAST to the NCBI database and used for all subsequent analyses. The trimmed reads returned consistent BLAST hits and allowed the selection of a reference genome. *Mycoplasma cynos* C142 (accession number HF559394) was the most common match using trimmed sequence reads generated from the *Mycoplasma felis* clinical isolates and reference strain.

#### *3.3.4.4 Alignments Using M. cynos as a Reference Genome*

Trimmed reads, from each clinical isolate in addition to the reference strain 10160, were aligned in CLC to the *M. cynos* C142 whole genome which has been well characterised and annotated in NCBI. Twelve *M. felis* isolates, demonstrating varying degrees of coverage (Figure 3.10), plus the reference isolate 10160 were selected for further analysis.

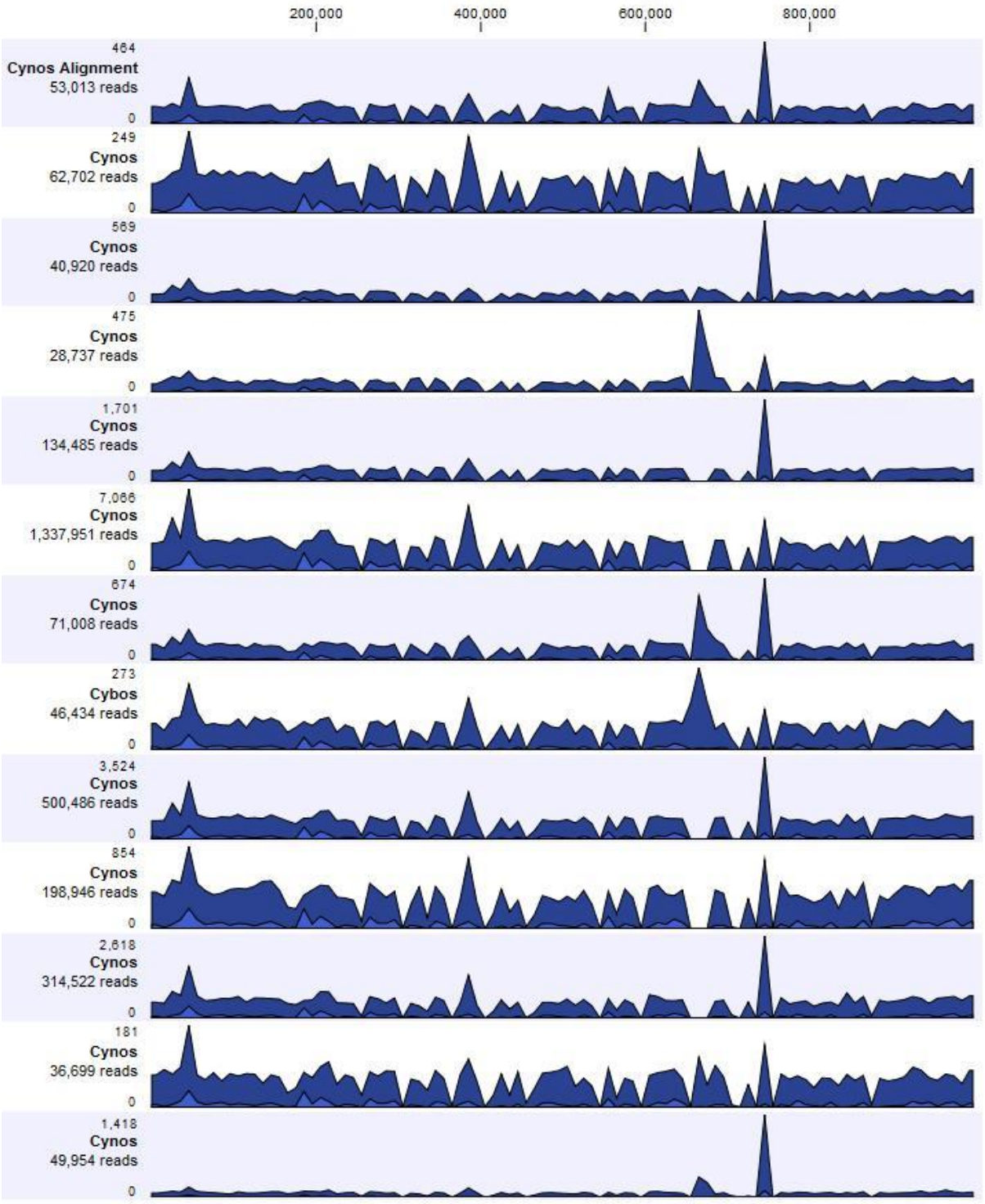



**Figure 3.10 Representation of coverage of twelve *Mycoplasma felis* isolates and reference strain 10160 aligned to *Mycoplasma cynos* whole genome using CLC Genomics Workbench 7.5.1.**

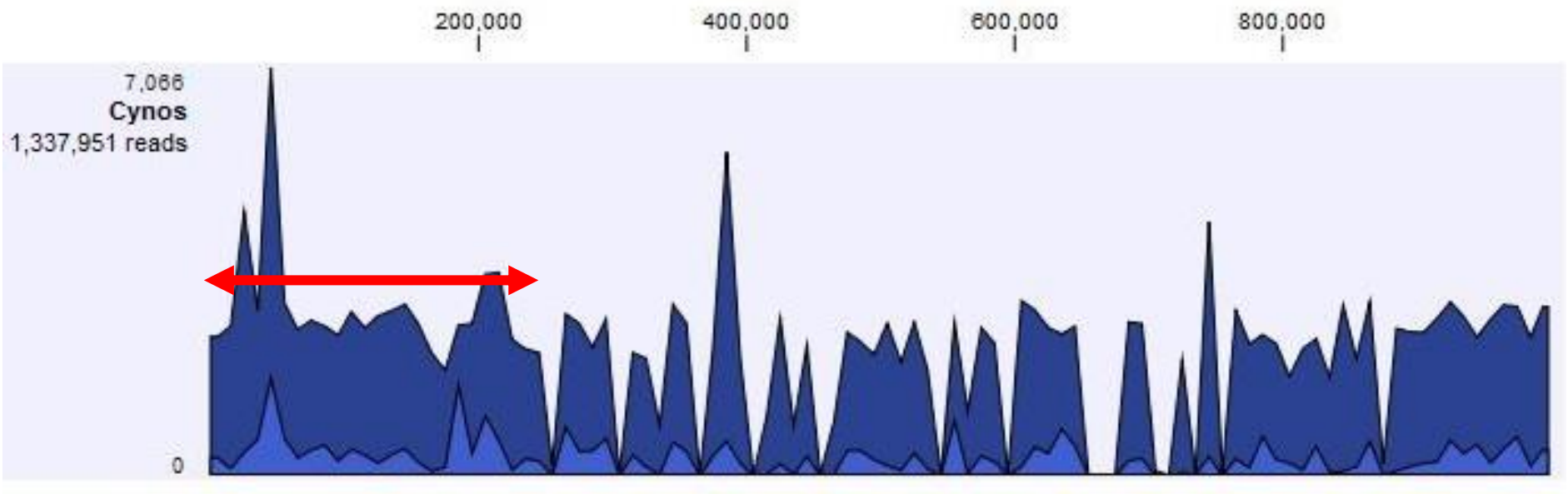



**Figure 3.11 Isolate 3 aligned to *M. cynos* whole genome, image represents alignment to whole genome, arrowed section shown in Figure 3.12 at higher magnification.**

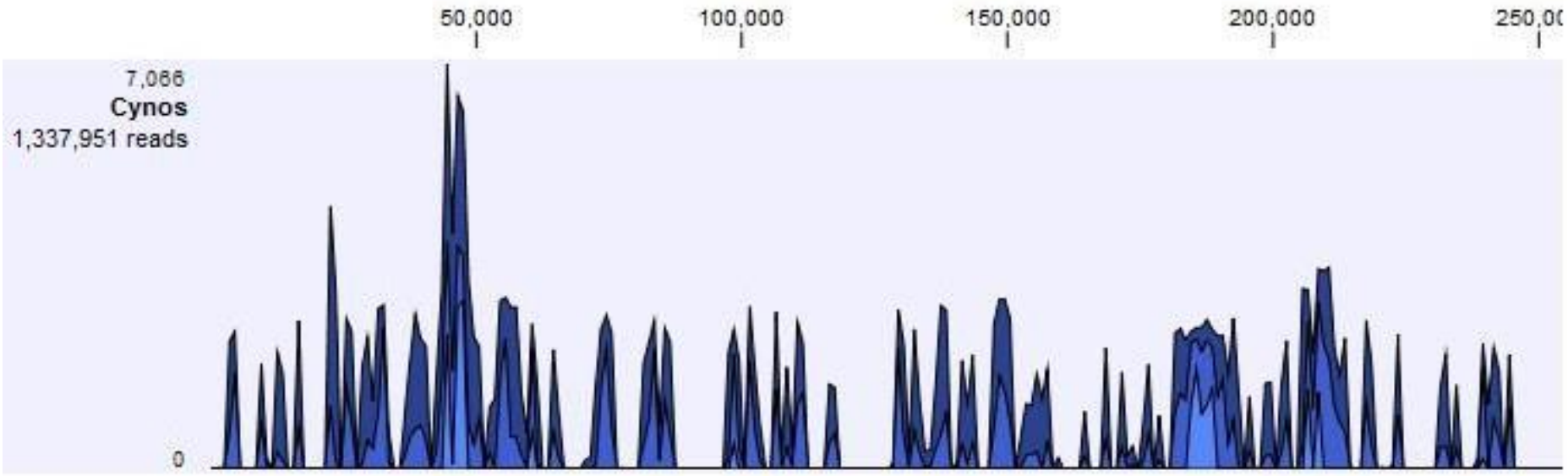



**Figure 3.12 Isolate 3 aligned to *M. cynos* whole genome, image represents alignment between bases 1 and 250,000 of *M. cynos* (highlighted on Figure 3.11)**

#### *3.3.4.5 Other Alignments*

Unmapped reads from isolate 3 were run on BLAST to identify if genetic sequence from any other organisms may be present, explaining why they did not map to *M. cynos*. Unfortunately, using BLAST standard parameters for a nucleotide megablast, no hits were returned, with "no significant similarity" being found.

Table 3.1 shows all mycoplasma whole genomes which were downloaded from NCBI for alignment, with the selected *M. felis* data, in CLC Genomic Workbench 7.5.1. These whole genome alignments demonstrated sparse and varied coverage from the *M. felis* reads. For example; *M. felis* reference strain 10160 demonstrated mapping of between 2.8% (*M. mycoides* strain PG1) and 6.83% (*M. fermentans*) of reads to each mycoplasma whole genome tested, including an *M. felis* scaffold genome (Accession number KL370829), to which 3.24% of the 10160 reads were mapped. *M. cynos* was significantly better with 16.8% of reference strain 10160 reads mapping to the *M. cynos* C142 genome, however this still leaves a substantial number of reads unmapped, thus preventing interpretation.

Table 3.2 displays the individual genes of interest that were selected from NCBI for alignment to the *M. felis* sequence reads. None of the genes of interest were shown to align convincingly with any of the *M. felis* isolates, again showing sparse coverage. Upon investigation, the aligned areas appeared to be mostly repetitive, non-coding sequence.

**Table 3.4 *Mycoplasma cynos* proteins and their genome locations; proteins aligned (partial or complete) with *M. felis* clinical isolates and reference strain 10160 are listed in bold.**

| Protein | Start Location | End Location |
|---|---|---|
| Lipoprotein | 67434 | 69851 |
| **ABC transporter permease** | **70370** | **73426** |
| **ABC transporter ATP-binding protein** | **143200** | **145137** |
| ABC transporter ATP-binding protein | 145146 | 146936 |
| Permease | 191064 | 191285 |
| Lipoprotein signal peptidase | 214681 | 215328 |
| **Lipoprotein signal peptidase** | **215403** | **216692** |
| **Peptide ABC transporter permease** | **269957** | **271117** |
| **Peptide ABC transporter permease** | **271118** | **272425** |
| **ABC transporter permease** | **288110** | **289141** |
| **ABC transporter permease** | **289141** | **290172** |
| Lipoprotein | 391576 | 394650 |
| Lipase | 408928 | 415338 |
| Nuclease, enda/nucm family | 477694 | 478431 |
| Uncharacterised lipoprotein MCAP_0231 | 622506 | 624263 |
| Lipoprotein | 626746 | 627855 |
| Intergrase | 727225 | 728148 |
| DNA/RNA nuclease | 802044 | 802622 |
| Orfg1 | 838478 | 839020 |
| Nuclease | 852690 | 854018 |
| **Peptidase M17** | **922571** | **923950** |
| Membrane nuclease (Mnua) | 967642 | 968136 |
| P60-like protein | 972628 | 973872 |

Some regions of interest corresponding to the proteins as shown in Table 3.4 (in bold) were identified following alignment of the sequenced isolates to the *M. cynos* genome. The majority of these alignments correspond to transporter permease proteins which are integral components of the cell membrane, facilitating the movement of molecules in and out of the cell. Peptidase M17 refers to a leucyl aminopeptidase (LAP), a member of the M17 metallo-peptidase family of proteins (Bhosale et al., 2010). *Mycoplasma felis* was also identified as having sequence homology with a lipoprotein signal peptidase annotated in *M. cynos*.

### 3.4 Discussion

In sequencing the *M. felis* genome, there were two purposes: to determine if any antimicrobial resistance, phenotypically observed in earlier experimental work, could be correlated with known antimicrobial resistance genotypes; and also to use NGS technology as a means of screening the available clinical isolates for genes known to increase virulence in other mycoplasmas including those associated with immunomodulation and adhesion in host cells.

The optimisation of mycoplasma template for input and the library preparations themselves involved significant time at the bench leaving limited time for detailed analyses of the generated data sets. The results described here are therefore preliminary and more thorough analyses of the data may enable additional conclusions to be drawn.

Ideally, for accurate and workable NGS data to be obtained, the DNA library should be representative of the sample, with evenly distributed reads across the entire genome. Currently, the whole genome for *M. felis* is unavailable therefore it was considered that *de novo* assembly would be of benefit, even if it was only achieved for the reference strain, it would allow that scaffold to be used as a reference for the clinical isolates along with any future work. The *de novo* assembly of the *M. felis* genome was unsuccessful and contigs obtained through *de novo* assembly in CLC when run on BLAST returned numerous (non-mycoplasma) bacterial hits and only minimal mycoplasma hits, with very sparse alignments. Accurate *de novo* assembly requires reasonable overlap between the generated reads from a given sample depending on the software parameters used. The high GC content and volume of repetitive non-coding sequence in mycoplasmas (Rocha and Blanchard, 2002, Shu et al., 2011) may also havehindered accurate *de novo* assembly. For the *de novo* assembly of the *M. cynos* C142 genome, a combination of sequencing technologies were required in order to allow generation of a scaffold (Walker et al., 2013), indicating that the difficulty experienced in our study is not unusual.

Fragment size distribution was shown to be within range using the TapeStation, but showed a marked level of degradation, indicated by a lower size range of fragments unsuitable for NGS. It would be interesting to sequence these

fragments and determine if, together with the larger fragments, they provide a more whole representation of the *M. felis* genome.

Although the DNA concentration was determined for each of the isolates and for the pooled isolates prior to sequencing, this unfortunately gives no indication regarding the quality or diversity of the DNA, or if it is capable of clustering on the flow cell during NGS. Robin *et al.* highlighted that the quantification techniques used in this project could be comparable to quantitative PCR, however their study used libraries proven to be concentrated and homogenous {Robin, 2016 #694} which cannot be said for the mycoplasma libraries used in this study. A droplet digital PCR machine is available within the laboratory and would use only a minimal amount of the DNA eluate, however during the practical work, time and cost were factors and this machine was not utilised. Given that one mycoplasma extract generated at least 3 times more reads than the others in this study (Table 3.3), it seems the droplet digital PCR is something which should have been prioritised.

Mapping to a reference genome allows differentiation between real sequences and possible variants to those of errors in assembly, thus providing insight on mapping quality. However, if no reference genome is available, or the generated sequence is not in the reference genome, possibly due to genome insertions or rearrangements in the template DNA, then no alignment will be made. It is acknowledged that mycoplasmas are highly mutable and contain substantial amounts of low complexity sequences. Carvalho *et al.* investigated mycoplasma genomes, hypothesising that a minimal genome would require only minimal DNA repair. It was actually identified that mycoplasmas lack many of the typical mismatch repair enzymes, their primary repair mechanism being nucleotide excision repairs. The result is an increase in DNA replication errors and thus, mutagenesis (Carvalho et al., 2005), indicating alignments may well prove difficult.

Interestingly, *M. cynos* was a much closer match to the generated reads in BLAST than other, phylogenetically closer, mycoplasmas within the fermentans group. *Mycoplasma cynos* has been identified as a respiratory pathogen in dogs (Walker et al., 2013), therefore, as it demonstrated the closest sequence homology to the *M. felis* isolates, it wouldn't be unreasonable to expect *M. felis* to be

pathogenic in cats. However, only 16.8% of the 10160 reference isolate reads aligned to the *M. cynos* whole genome. The unmapped reads from isolate 3 disappointingly returned no BLAST matches, however within the BLAST website; it does explain that results like this are common for low complexity queries, with filters within the program which mask low complexity regions. It would be interesting to take time adjusting these filters within BLAST in order to run a more thorough analysis.

The depth of coverage, of isolates aligned to the *M. cynos* whole genome (Fig 3.8), was notably variable; this may be explained by the variable number of reads generated for each isolate, which in turn may be explained by the quality of the original samples. The process of DNA extraction required lengthy optimisation after very low yields of DNA were obtained. The optimisation enabled sufficient DNA to be harvested for NGS; however, the yield was still low (0.6 – 1.7 ng/μL) and required a library preparation kit designed for low input DNA templates (Rubicon Genomics).

Although such kits are available, starting template concentration is one of the most influential factors in successful library preparation; too few copies of the template sequence yields a low complexity library. Amplification whilst useful, will only amplify the available DNA, therefore starting with a low complexity library will only produce more of the same limited fragments (Song et al., 2013, van der Walt, 2015, Vincent et al., 2016). Amplification bias has also been identified as a potential problem wherein any differences in synthesis efficiency, through the cyclic nature of PCR, are multiplied exponentially, thus creating an uneven representation of sequences (van der Walt, 2015).

For this reason, large research and development companies such as KapaBiosystems (Cape Town, South Africa) recommend optimising the protocol provided with any commercially available library preparation kit, in order to ensure the most efficient conversion of DNA template to library fragments thereby maintaining library complexity (van der Walt, 2015). For this project, the library preparation was carried out in accordance with the manufacturer's instructions, with no specific optimisation. It would be interesting to know if other researchers have worked with the Rubicon kit used here, and if they observed any difference in coverage following optimisation.

The *M. felis* data showed some homology with a selection of *M. cynos* genes including those encoding ABC transporter permeases, ATP binding proteins and a lipoprotein signal peptidase. These proteins have been identified in a variety of mycoplasma species, with some specificity in target molecules, indicating they are well conserved and likely essential to mycoplasma survival. Some studies have identified these proteins in the uptake of iron bound to transferrin or chelates, flagging them as potential virulence factor (Sanders et al., 1994, Li et al., 2011). The alignment of trimmed *M. felis* reads to the peptidase M17 region of the *M. cynos* genome indicates the presence of this gene, or a gene which shares significant sequence homology, within the *M. felis* genome.

Of particular interest in the alignment was the identification of a lipoprotein signal peptidase. These signal peptidases behave as proteases catalysing the cleavage of prolipoproteins to release signal peptides. There was no indication of which prolipoprotein this protease corresponds to. Further analysis would be useful as lipoproteins have been identified as an important marker of virulence in other mycoplasmas, aiding in colonisation and host immunomodulation, (Razin et al., 1998, Into et al., 2002, Sasaki et al., 2002, Zuo et al., 2009) thus their identification in *M. felis* would allow further molecular characterisation.

Peptidase M17 are cytoplasmic exopeptidases which have been well characterised in other prokaryotic and eukaryotic organisms in which they have been identified and have been established as cell maintenance enzymes, hydrolysing leucine substrates, having proteolytic properties and a role in DNA binding (Mitchell et al., 2014). Their exact role in cellular metabolism is still undefined and within mycoplasma research has not gone further than identifying the presence of the encoding gene.

A resistant phenotype was not observed during antimicrobial susceptibility testing, and so identifying genetic markers of resistance was disappointingly undirected. It is worth noting; however, that phenotype was recorded only after one passage. It has been demonstrated that resistance may be induced *in vitro* (Gautier-Bouchardon et al., 2002), therefore a selection of resistance genes, identified in other mycoplasmas, were used as references for alignments with the generated *M. felis* data in order to identify the presence of resistance genes in *M. felis* (clinical isolates and reference strain 10160) which may not have

been expressed at the time of culture. None of the selected resistance genes were identified, either within the *M. felis* clinical isolates, or the reference strain, supporting the earlier phenotype data of no antimicrobial resistance ( emerging or otherwise) in the *M. felis* genome. Given the mutability of mycoplasmas and their potential for recombination, it would be prudent to further explore this area.

## Chapter IV

## Discussion

The aim of this study was to incorporate microbiological investigations with cell-based infection studies and NGS technology to determine if *M. felis* should be treated when isolated from cats. By investigating antimicrobial susceptibility patterns and observation of a cell-line infected with *M. felis*, a phenotype would be available which could be further studied using a genotypic assessment, in order to correlate phenotype and genotype as well as identify any genetic potential for virulence. Time did not allow for the entire project to be completed, with the infection studies being sacrificed in order to complete the antimicrobial susceptibility testing and gene analysis. This was unfortunate as the cell-based data could have given further insight into *M. felis* interactions with host cells, potentially identifying virulence and pathogenicity factors.

The macrolides (erythromycin and azithromycin) proved to be least effective across all isolates. Interestingly, of the macrolide resistance genes selected as references, *M. felis* aligned with none of them;however, the list was not exhaustive. Given that the isolates spanned over forty years and all had similar susceptibility to the macrolides, this can't be described as novel or emerging, indicating it's not an evolutionary or acquired trait, but something inherent.

Other mycoplasma susceptibility studies have demonstrated that macrolides are effective *in vitro,* with significantly lower MIC values than those obtained in this research (Whithear et al., 1983, Schultz et al., 2012). It would be interesting to investigate the cause of this decreased susceptibility in *M. felis*; using a larger selection of macrolide resistance genes for alignment may be of benefit, and previous studies have described inducing resistance to erythromycin in as little as 2-3 passages (Gautier-Bouchardon et al., 2002). Repeating this experiment with the *M. felis* isolates could perhaps create a selected population for sequencing and analysis.

During this research, it should be noted that only ocular isolates were recovered for investigation; however, during sequence analysis and the search for an appropriate reference genome for alignments, the genome with closest homology in BLAST was that of *M. cynos,* a pathogen associated with canine infectious respiratory disease (Chalker et al., 2004).

Numerous studies have shown that *M. felis* may be present in the respiratory tract of clinically healthy cats as well as those showing signs of respiratory infection (Spradbrow et al., 1970, Foster et al., 1998, Binns et al., 2000, Chandler and Lappin, 2002, Bannasch and Foley, 2005, Helps et al., 2005, Johnson et al., 2005, Holst et al., 2010). Thus, defining *M. felis* as a pathogen of the respiratory tract has proven troublesome.

There is evidence that mycoplasma infection increases susceptibility to further infection (Ciprian et al., 1988) however, if *M. felis* is a commensal organism of the feline respiratory tract, admittedly likely with the potential to become an opportunistic pathogen, then it is worth considering which organism created the opportunity – which organism is the primary pathogen? Potentially, feline respiratory viruses such as feline herpesvirus (FHV) and feline calicivirus (FCV) may be the primary or secondary pathogens. Of the ocular isolates utilised in this study, co-infections with FHV and FCV were identified on original examination of the specimens, indicating they are capable of co-infecting with *M. felis*.

The infection study would have allowed the detection of cytopathic effect during mycoplasma infection, which would have allowed us to determine if *M. felis* interferes with host defences, which would certainly predispose to further infection. Having this information would provide further important evidence on directing therapy.

Observation of the mycoplasmas in cell culture may also have provided further guidance for the genetic analyses – perhaps identifying if *M. felis* is one of the mycoplasmas capable of becoming intracellular and thus directing a search for attachment organelle and associated proteins. It would also have allowed comparison of isolates; throughout this study, little variance in results has been observed, with antimicrobial susceptibilities being similar and no resistance demonstrated, and genetic analyses only varying in the number of reads generated and available for alignments. During culture, growth of numerous isolates was surprisingly faster than expected, even for isolates with identical VCCs, and haemolytic activity was varied with some isolates requiring different media for growth and observation. Perhaps in cell culture it would have been

easier to examine these qualities further and elucidate if they affect pathogenicity.

The minimal genome of mycoplasmas is well documented, with numerous studies attempting to elucidate the minimal gene complement and establish the essential genes (Fraser et al., 1995, Rocha and Blanchard, 2002, Pich et al., 2006, Gibson et al., 2008, Shu et al., 2012). A pan-genome analysis by Liu et al. (2012) estimated that across the mycoplasma genus, more than 8000 genes exist. Across the twenty species and strains of mycoplasmas examined, only 196 genes were shared by all, with around half of those dedicated to genetic processes such as translation or ribosomal structure. In the same study, three strains of *M. hyopneumoniae* were shownare 95% of genes (Liu et al., 2012).

There are multiple areas which could benefit from further investigation. One priority would be to complete the de novo assembly of the *M. felis* genome/s; however, an accurate full genome *de novo* assembly of such a size, in itself is a massive undertaking. This could constitute a separate bioinformatics project.

There are scaffolds available on NCBI for *M. felis* (NCBI Reference Sequence NZ_JNKA00000000.1) and this could be indicative that a whole genome assembly may be forthcoming or at least could be used to help generate an accurate assembly. To undertake such a project perhaps further depth of sequence would be advantageous and also to combine the various isolates' sequence to try and achieve a representative data set that will match existing scaffolds available.

Cole (1967) described the isolation and culture of *M. felis* from the eye of a cat with severe conjunctivitis. The isolate was then used to successfully infect the conjunctiva of kittens before being isolated again and proven to be identical to the original isolate, thus defining Koch's postulates. This is noteworthy; however, kittens lack a fully developed immune system, leaving them more susceptible to, and less able to clear infections. Numerous studies have investigated the conjunctival flora of cats (Shewen et al., 1980, Haesebrouck et al., 1991b, Haesebrouck et al., 1991a, Espínolaz and Lilenbaum, 1996, Hartmann et al., 2010), and reported that *M. felis* was isolated only in cases of symptomatic conjunctivitis. This suggests that *M. felis* could be considered an ocular pathogen and that treatment of such infections may be justified.

Within this study no definitive virulence factors could be identified. Of the selection of virulence genes identified in mycoplasmas, *M. felis* appears to possess none of them. In fact, several whole genomes of the mycoplasmas established as primary pathogens, were used for alignment in this study, including *M. fermentans, M. bovis, M. pneumoniae* and *M. genitalium* with *M. felis* failing to align reasonably with any of them. It would be prudent to acknowledge the limitations of the gene analysis in this study – the virulence genes selected for examination were not exhaustive, and given the highly mutable genome of mycoplasmas, it would be of benefit to further investigate this area in order to definitively identify the pathogen status of *M. felis*.

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Appendix 1. Antimicrobial Dilutions Set Up for MIC Testing

| | Concentration (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 64 | 32 | 16 | 8 | 4 | 2 | 1 | 0.5 | 0.25 | 0.125 | 0.0625 | 0.03125 |
| AMPICILLIN<br>AZITHROMYCIN<br>DOXYCYCLINE<br>ENROFLOXACIN<br>ERYTHROMYCIN<br>OXYTETRACYCLINE<br>PRADOFLOXACIN | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 128µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 68µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 32µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 16µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 8µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 4µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 2µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 1µl/ml antimicrobial | | EPC[a] 200µl LB | GC[b] 200µl | |
| | | | | | | | | | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 0.5µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 0.25µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 0.125µl/ml antimicrobial | 100µl inoculum ($10^3$-$10^5$ ccu/ml) plus 100µl 0.0.6125µl/ml antimicrobial |
| VIABLE CELL COUNT (ccu/mL) | -2 | -3 | -4 | -5 | -6 | -7 | -8 | -9 | | SC[c] 200µl LB | SOL 1[d] 20µl $dH_2O$ 180µl inoculum | SOL 2[e] 20µl 100% EtOH 180µl inoculum |

[a] End Point Control – liquid browth adjusted to pH 6.8 thus creating a colour change to which growth could be matched
[b] Growth Control – undiluted inoculum added to liquid browth to indicate viable organisms and rate of growth
[c] Sterility Control – liquid broth incubated alone to ensure no contamination of growth media
[d,e] Solvents used to dissolve antimicrobials (dH2O and 100% Ethanol) were added to inoculum to ensure they did not inhibit growth

Appendix 2. MIC Results for all Clinical Isolates and Strain D8

| | MINIMUM INHIBITORY CONCENTRATION | | | | | | |
|---|---|---|---|---|---|---|---|
| Sample | AMP | AZI | DOXY | ENRO | ERY | OTC | PRADO |
| 3 | n/a | 8 | 8 | 4 | 64 | 2 | 0.0625 |
| 4 | n/a | 4 | 4 | 8 | 64 | 1 | 0.0625 |
| 5 | n/a | 8 | 0.03125 | 0.125 | >64 | 0.0625 | 0.0625 |
| 6 | n/a | 4 | 1 | 1 | 16 | 1 | 0.03125 |
| 7 | n/a | 16 | 0.03125 | 2 | 32 | 0.5 | 0.25 |
| 8 | n/a | 1 | 0.0625 | 0.125 | 32 | 0.0625 | 0.03125 |
| 9 | n/a | 2 | 0.0625 | 4 | 32 | 1 | 0.25 |
| 10 | n/a | 32 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 11 | N/A | 32 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| 12 | n/a | 16 | 0.03125 | 2 | 64 | 1 | 0.125 |
| 13 | n/a | 4 | 0.0625 | 0.0125 | 32 | 0.03125 | 0.03125 |
| 14 | n/a | 4 | 0.03125 | 0.0125 | 16 | 0.03125 | 0.03125 |
| 15 | n/a | 64 | 0.0625 | 0.0625 | >64 | 1 | 0.03125 |
| 16 | n/a | 4 | 0.03125 | 0.125 | 32 | 0.5 | 0.03125 |
| 17 | n/a | 16 | 0.03125 | 0.125 | 16 | 0.5 | 0.03125 |
| 18 | N/A | 16 | 0.03125 | 0.03125 | 64 | 0.5 | 0.03125 |

MINIMUM INHIBITORY CONCENTRATION

| Sample | AMP | AZI | DOXY | ENRO | ERY | OTC | PRADO |
|---|---|---|---|---|---|---|---|
| 19 | n/a | 8 | 0.03125 | 0.0625 | 8 | 1 | 0.03125 |
| 20 | N/A | 2 | 0.03125 | 0.125 | 32 | 0.5 | 0.03125 |
| 21 | n/a | 32 | 0.0625 | 0.0625 | >64 | 1 | 0.03125 |
| 22 | n/a | 32 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 23 | n/a | 8 | 0.125 | 0.03125 | 32 | 0.5 | 0.03125 |
| 24 | n/a | 4 | 0.0625 | 0.125 | 16 | 0.25 | 0.03125 |
| 25 | N/A | 4 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 26 | n/a | 2 | 0.03125 | 0.25 | 32 | 1 | 0.03125 |
| 27 | N/A | 16 | 0.03125 | 0.125 | 64 | 0.25 | 0.03125 |
| 28 | N/A | 32 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 29 | n/a | 16 | 0.0625 | 0.125 | 32 | 1 | 0.03125 |
| 30 | n/a | 4 | 0.03125 | 0.03125 | 8 | 1 | 0.03125 |
| 31 | n/a | 8 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| 32 | n/a | 32 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| 33 | n/a | 16 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| 34 | n/a | 4 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 35 | n/a | 4 | 0.0125 | 0.25 | 16 | 1 | 0.03125 |
| 36 | n/a | 2 | 0.03125 | 0.03125 | 32 | 0.5 | 0.03125 |
| 37 | n/a | 8 | 0.03125 | 0.03125 | 64 | 0.5 | 0.03125 |

MINIMUM INHIBITORY CONCENTRATION

| Sample | AMP | AZI | DOXY | ENRO | ERY | OTC | PRADO |
|---|---|---|---|---|---|---|---|
| 38 | n/a | 16 | 0.03125 | 0.03125 | 64 | 0.5 | 0.03125 |
| 39 | n/a | >64 | 0.0625 | 0.125 | >64 | 0.5 | 0.03125 |
| 40 | n/a | 32 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 41 | n/a | 4 | 0.03125 | 0.5 | 8 | 1 | 0.125 |
| 42 | n/a | 8 | 0.03125 | 0.0125 | 16 | 0.5 | 0.03125 |
| 43 | n/a | 8 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| 44 | n/a | 16 | 0.03125 | 0.03125 | 32 | 0.25 | 0.03125 |
| 45 | n/a | 2 | 0.03125 | 0.0125 | 8 | 0.03125 | 0.03125 |
| 46 | n/a | 16 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| 47 | n/a | 8 | 0.125 | 0.125 | 64 | 2 | 0.3125 |
| 48 | n/a | 4 | 0.0625 | 0.125 | 16 | 0.5 | 0.03125 |
| D8 (KR) | n/a | 8 | 0.03125 | 0.25 | 64 | 1 | 0.03125 |
| NE01 | N/A | 8 | 0.03125 | 0.125 | 64 | 0.5 | 0.03125 |
| NE02 | n/a | 2 | 0.0625 | 2 | 32 | 1 | 0.03125 |
| NE03 | n/a | 16 | 0.03125 | 0.125 | 64 | 1 | 0.03125 |
| NE04 | n/a | 32 | 0.125 | 0.125 | >64 | 1 | 0.03125 |