Aspects of antimicrobial resistance in Australian swine-origin *Pasteurella multocida*

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Honours Thesis

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

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Abstract

Pasteurella multocida is a causative agent of many diseases in a broad range of hosts. It is particularly noted in pigs as a cause of pneumonia and atrophic rhinitis. As treatment and control of *P. multocida* infections has relied heavily on antimicrobials, surveillance of antimicrobial resistance is necessary to ensure that treatment remains effective. The aim of this study was to investigate the antimicrobial resistance (AMR) of *P. multocida* isolates from Australian pigs, the potential risk of AMR genes spreading from other Gram-negative bacteria and the possibility of using bacteriophages as an alternative treatment to *P. multocida* infections.

Antimicrobial susceptibility testing was performed on 273 *P. multocida* isolates collected from pig farms across Australia between 2014 and 2019. Resistance to tetracycline (22.7%), chlortetracycline (22%), florfenicol (0.7%) and ampicillin (0.4%) was identified. Examination of the transferability of AMR genes from ceftriaxone and ampicillin resistant *E. coli* isolates to *P. multocida* through plasmid-mediated conjugation revealed that AMR genes were not stably transferred from *E. coli* to *P. multocida*. Efforts to isolate bacteriophages with lytic activity against *P. multocida* from environmental samples were not successful, however the addition of mitomycin C to *P. multocida* strains resulted in lysis of cells and visible clearing of the bacterial culture for 5 of 7 isolates, indicating prophages were induced.

This study demonstrated that *P. multocida* infections in Australian swine can still be successfully treated with antimicrobials and that the risk of acquiring AMR genes from other highly resistant Gram-negatives is low. Despite low frequency of resistance to tested antimicrobials on-going surveillance and reducing antimicrobial usage should be a priority as *P. multocida* outbreaks can have significant impact on economic costs and animal welfare. Failure to isolate lytic bacteriophages from environmental sources indicates that phage therapy would be reliant on isolation of phage stocks from sources samples that would carry *P. multocida* specific phages such as nasopharyngeal wash samples from pigs.

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List of Abbreviations

°C	degree Celsius
mg	milligram
µL	microliter
AMR	Antimicrobial resistance
APVMA	Australian Pesticides and Veterinary Medicines Authority
AR	Atrophic Rhinitis
BHI	Brain heart infusion
CAMHB	Cation-adjusted Mueller Hinton broth
CFU	Colony forming units
CLSI	Clinical Laboratory Standards Institute
CRC	Cooperative research centre
CSFV	Classical swine fever virus
DNA	Deoxyribonucleic acid
ECOFF	Epidemiological cut-off
ETEC	Enterotoxigenic *Escherichia coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
g	Gravitational force equivalent
IAV	Influenza A virus
ICE	Integrative conjugative element
L	Litre
LB	Luria bertani
LPS	Lipopolysaccharides
MDR	Multi-drug resistant
MH	Mueller-Hinton

MIC	Minimum inhibitory concentration
mL	milliliter
MLST	Multilocus sequence typing
nm	Nanometre
OD	Optical density
OMP	Outer membrane protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMT	*Pasteurella multocida* toxin
PRDC	Porcine respiratory disease complex
PRCV	Porcine respiratory coronavirus
PRRSV	Porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus

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1. Introduction

Louis Pasteur is considered one of the fathers of immunology and his work contributed greatly to our understanding of the nature of disease and prevention of infection. In the late 19th century Pasteur isolated the organism responsible for fowl cholera and made the remarkable discovery that cultures of the organism became attenuated over time. Pasteur found that chickens inoculated with the attenuated strain survived, whereas those that were inoculated from fresh cultures perished. His observations showed that when attenuated, the organism could be used to elicit a protective immune response (1, 2). The bacteria he identified as the causative agent of fowl cholera was later named Pasteurella in his honour.

Pasteurella multocida is a ubiquitous bacterium and the cause of a number of diseases affecting a broad range of animals, including fowl cholera in poultry, haemorrhagic septicaemia in cattle and buffalo, atrophic rhinitis in swine, snuffles in rabbits and enzootic pneumonia (3). Infections caused by this bacterium are a global concern impacting on animal welfare and resulting in livestock production loss.

1.1 Characterisation of *P. multocida*

Pasteurella multocida is a nonmotile Gram-negative rod or coccobacillus with bipolar staining characteristic, and a member of the *Pasteurellaceae* family, which includes four subspecies: *P. multocida, P. septica*, *P. gallicida and P. tigris. Pasteurella multocida* is a facultative anaerobe that forms round, greyish, non-haemolytic colonies on blood agar with a characteristic sweet odour (Fig 1). Some pathogenic strains produce thick hyaluronic capsules resulting in mucoid colonies (4). Isolates grow well at 37°C on most enriched media although not on MacConkey agar (4). The bacterium is oxidase, indole, catalase and orthenine decarboxylase positive and most isolates will ferment sucrose, glucose and maltose (5).

(a) (b)

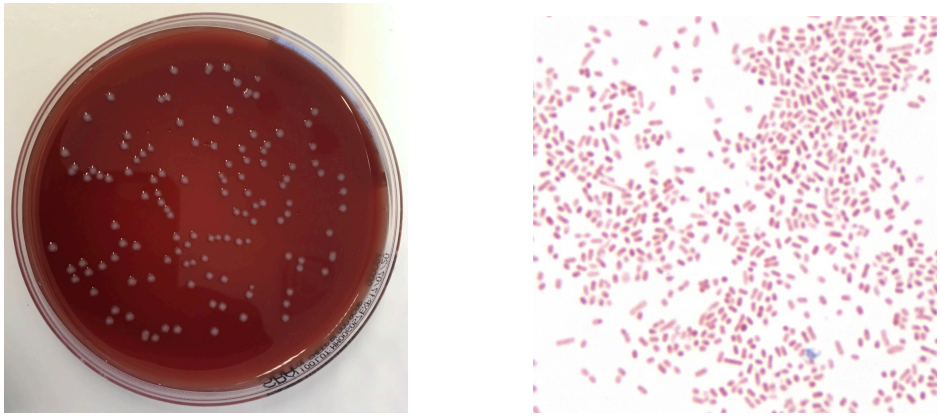

Figure 1. (a) *P. multocida* colonies *on agar* on blood agar; (b) Gram stain of *P. multocida* bacteria. Adapted from https://www.idimages.org/images/organismdetail/?imageid=1726&altimageid=122

Since its discovery, there have been a number of methods used to differentiate strains of *P. multocida,* resulting in difficulty in comparing data from different studies due to the lack of standardisation (6). The most commonly used method is capsular serotyping, with five serotypes identified based on the capsular polysaccharide (serotypes A, B, D, E and F) using an indirect haemagglutination test (5-7). Type A and B capsules are composed of hyaluronic acid, type D capsule contains heparin and type F capsule contains chondroitin. Serotypes A and D are most commonly isolated from clinical infections in swine (6, 8, 9).

Pasteurella mutocida strains can be further classified into 16 somatic serotypes (1-16) using a gel diffusion immune-precipitation method developed by Heddleston, based on their cell wall lipopolysaccharides (10). Capsular and somatic serotyping are often used in combination, with the isolate designated by capsular serogroup followed by the somatic serovar, (e.g. A:1) (11). This is the standard serotyping scheme, however, these methods are time consuming and can be problematic, particularly with the Heddleston method, due to a lack of consistency in results and isolates that cannot be typed (2, 11-13). *P. multocida* have also been classified by biovars, ribotyping and analysis of outer membrane proteins (OMPs), however serotyping has been the most common method used (14-19).

Molecular typing methods have been used in the classification of *P. multocida*, such as multilocus sequence typing (MLST), PCR, pulsed-field gel electrophoresis and whole genome sequencing. Multiplex capsular PCR (*cap* mPCR) has been developed to provide a rapid alternative to capsular serotyping (20) and multiplex liposaccharide PCR (LPS mPCR) has also been used differentiate *P. multocida* isolates into eight distinct LPS genotypes (11). MLST, which is generally considered the gold standard molecular typing method for bacterial pathogens, involves sequencing fragments from seven housekeeping genes to determine genetic relationships between isolates (15, 21, 22). Two separate MLST schemes have been developed for *P. multocida,* allowing for results to be easily compared between laboratories and archived; the Rural Industries Research & Development Corporation (RIRDC) scheme which investigates avian isolates, and the Multi-host scheme which covers isolates from a variety of animal hosts and was developed to examine the evolutionary relationships of isolates. There are 1,617 *P. multocida* isolates, 267 of which were collected from swine, available in the PubMLST database (https://pubmlst.org/pmultocida/, accessed 07/02/2021). Though it is highly effective in characterising bacterial isolates, MLST has a limited ability to differentiate isolates with a common ancestor, as they may have identical MLST types (23-25). Whole genome sequencing is being used with greater regularity in epidemiological investigations due to its high discriminatory power in differentiating bacterial strains. The first whole genome sequence of *P. multocida* was reported in 2001 (26) and currently the National Center for Biotechnology Information (NCBI) genome database has 288 *P. multocida* genome sequences from sourced from different hosts, 60 of which are from pigs (https://www.ncbi.nlm.nih.gov/genome/genomes/912, accessed 27-04-2021).

1.2 Pathogenesis

P. multocida has been isolated from a range of wild and domestic animals and can spread vertically from infected dams and horizontally via nose-to-nose contact, aerosols and potentially through fomites (6, 27, 28). High stocking density and poor ventilation therefore significantly increase the risk of transmission. There have also been studies supporting interspecies transmission. The OMP profiles

of bovine, ovine, porcine and avian *P. multocida* isolates were compared and determined that similar or identical strains were pathogenic in different species (15). Another study examined the genotypic diversity of *P. multocida* isolates from swine and poultry using MLST and determined that some sequence types were shared between the two species (13). Zoonotic infections can also occur, usually as a result of bites or scratches from cats and dogs and contact of skin lesions with upper respiratory mucosal secretions. These infections are typically localised and symptoms of infection include oedema, cellulitis and bloody or purulent exudate at the wound site (5). Extreme cases are rare but can result in bacteraemia, endocarditis and meningitis (5, 29).

1.2.1 Predisposing Factors

P. multocida are common commensals found in the upper respiratory tract and tonsils of wild and domestic animals. They often form part of the normal flora and may persist for long periods of time without evidence of disease, making it difficult to eliminate from herds. *P. multocida* is also an opportunistic pathogen and infections may develop following an infection by a primary pathogen or due to abnormal environmental conditions that impact the respiratory defences (3). If the respiratory defence mechanisms have been impaired, there is a higher chance of *P. multocida* infection.

The primary defence mechanisms of the respiratory system involved in the clearance of particles are the mucociliary apparatus and phagocytosis. The mucociliary apparatus is a clearance mechanism designed to catch foreign particles in the nasal mucosa and remove them from the respiratory tract. The structure of the nasal cavity and velocity of air causes turbulence of airflow resulting in particles larger than 10 µm forced onto the surface of the nasal mucosa (30). Particles become trapped in the mucous layer and ciliated epithelial cells move the particles towards the pharynx to be swallowed. Smaller particles may escape the nasal cavity but can become trapped in the tracheal or bronchial mucosa. Disruption of the mucociliary apparatus can exacerbate an existing disease or provide the opportunity for a secondary infection (31). Primary pathogens and poor environmental conditions,

such as dry air, dust, low temperature and ammonia can negatively impact the mucociliary clearance in swine, predisposing the host to *P. multocida* infection (32). *Mycoplasma hyopneumoniae* penetrates the respiratory mucosa and adheres to the ciliated epithelial cells, reducing the clearance by the mucociliary apparatus (33). *Bordetella bronchiseptica* also attaches to ciliated epithelial cells of the upper respiratory tract which prevents clearance and in addition produces toxins that cause moderate turbinate atrophy. Swine Influenza virus (SIV), Pseudorabies virus (PRV), and porcine respiratory coronavirus (PRCV) multiply in the epithelium of the conducting airway leading to necrosis and reducing the effectiveness of the immune response (34).

Macrophages are another key component of respiratory defence, removing foreign particles that escape the mucociliary apparatus and recruiting neutrophils (31). The mucocilary apparatus does not cover the alveoli, therefore alveolar macrophages are the primary defence against particles in the alveolus. *M. hyopneumoniae,* SIV, PRV, PRCV and Porcine reproductive and respiratory syndrome virus (PRRSV) have been shown to impair the function and reduce the number of pulmonary macrophages (34-36).

Poor environmental conditions can have an adverse effect on respiratory defences, exacerbating infections and increasing the severity of pulmonary lesions (37). Reduced air quality due to airborne pollutants such as gases, particulate matter and microorganisms can impair the mucociliary apparatus. Particulate matter or pollutants can irritate and inflame the respiratory epithelium and lessen mucociliary clearance (38). Gases such as ammonia, carbon dioxide and hydrogen sulphide are common in animal housing facilities and can cause health issues at high levels. Ammonia is highly water soluble and is readily absorbed by the mucous in the respiratory tract when inhaled; at high levels it can reduce clearance of inhaled bacteria by increasing the viscosity of the mucous (37, 39). Exposure to cold air can also cause increase mucous viscosity and decreased mucous clearance (31, 40). Dry air (humidity less than 50%) and high temperatures can lead to dehydration of mucous layer

and damage to the mucociliary apparatus. In addition, adverse environmental factors can provoke an acute or chronic stress response which can suppress the immune system. The release of cortisol following exposure to stressful conditions can decrease the proliferation of lymphocytes and reduce antibody production (41, 42).

1.2.2 Virulence Factors

Virulence factors are intrinsic characteristics cells that enable pathogens to colonise and invade host cells, suppress or evade the immune response, damage cells and obtain nutrients from the host. A number of *P. multocida* virulence factors have been identified, including the bacterial capsule, putative fimbriae and adhesins, liposaccharide molecules, nutrient acquisition mechanisms, enzymes and toxins.

The capsular structures of *P. multocida* have been shown to play a role in virulence, as encapsulated strains have a great ability to avoid innate host immune responses (43). Capsules are polysaccharide structures adherent to the cell wall and function to restrict access of molecules to the cell, facilitate adherence and interfere with phagocytosis (4). Several studies have demonstrated that acapsular strains of *P. multocida* are less virulent than encapsulated strains and this has been largely attributed to the antiphagocytic properties of the capsule (43, 44).

Analysis of the *P. multocida* genome has shown genes that encode for putative fimbriae and adhesion proteins, facilitating adhesion to the mucosa allowing *Pasteurella* to colonise and cause disease (26, 45). Outer membrane protein A (OmpA) of *P. multocida* has been shown to bind strongly to fibronectin, an extracellular matrix protein, allowing for adherence and colonisation (46). Type 4 fimbriae, which have been associated with adhesion in other bacteria, have been identified on serogroup A, B, D and F strains, though the importance of these fimbriae in the virulence of *P. multocida* has not yet been fully determined.

Lipopolysaccharide (LPS) molecules, or endotoxins, form part of the outer membrane of Gram-negative bacteria and are a determinant of virulence and stimulates the host immune response. The structural components of the LPS are the lipid A molecule that is responsible for endotoxic activity and inner and outer core oligosaccharides. The LPS of *P. multocida* is believed to have similar endotoxic effects as other Gram-negative bacteria and these effects are noticeable in buffalo infected with serotype B:2 and E:2 strains, where the animals will often develop haemorrhagic septicaemia (47). The clinical signs of haemorrhagic septicaemia such as increased temperature and increased serum TNFα, have been replicated in buffalo calves by injecting them with purified LPS taken from a type B:2 strain (48). The LPS is an important virulence determinant and a complete structure is required to proliferate and cause disease. A *P. multocida* mutant with a truncated LPS structure was compared to a highly virulent A:1 strain that causes fowl cholera, with the virulence highly attenuated in the mutant along with an increase in susceptibility to antimicrobial peptides (49).

Iron is required by all bacteria to grow and with limited iron available from mammalian and avian hosts, bacteria have evolved mechanisms to acquire iron from the host and allow proliferation. The first complete genome sequence of *P. multocida*, Pm70, found more than 50 genes (2.5% of the genome) that potentially play a role in iron acquisition and metabolism (26). There is limited information on the importance of the different iron acquisition genes on virulence, but it is suggested that the presence of multiple iron acquisition systems may allow *P. multocida* to infect multiple hosts as the mechanism for acquiring iron can be a restricting factor in host specificity (5). Mutant *P. multocida* isolates with inactivated iron transport genes *ExbB*, *ExbD* and *TonB* genes, have displayed decreased virulence in mice, indicating they each play a role pathogenesis (50).

Enzymes are produced by *P. multocida* to promote virulence by enabling colonisation and degrading the extracellular matrix. Sialidases produced by *P. multocida* may play a role in nutrient acquisition by removing sialic acid from host membrane components and reducing effectiveness of mucin (51). A

study using mice demonstrated that sialic acid catabolism may not be necessary for infection, however, the uptake and transport of sialic acid may be an essential virulence factor (51). Studies have found that type B cultures taken from cases of haemorrhagic septicaemia produce hyaluronidase, which is relevant due to the highly virulent nature of this strain as it is commonly associated with haemorrhagic septicaemia in cattle and buffalo (52). However, the role and importance of hyaluronidase in the development of disease has not been determined.

Certain strains of *P. multocida* express a 146kDa cytotoxin, also known as *Pasteurella multocida* toxin (PMT). This toxin is encoded by the *toxA* gene which is located on a lysogenic prophage (53). It is responsible for the clinical signs of atrophic rhinitis in pigs and is mostly expressed by serotype D, though there are several reports of toxigenic serotype A. PMT acts intracellularly, deamidating the α-subunit of the G-protein and activating a number of signalling pathways, including mitogenic and anti-apoptotic signalling. Ultimately the toxin inhibits activity of the osteoblasts and allows for unrestrained proliferation of osteoclasts which results in bone resorption (6, 54-56). The presence of PMT can be determined through ELISAs using PMT-specific monoclonal antibodies (6).

1.2.3 Porcine Respiratory Disease Complex

Porcine respiratory disease complex (PRDC) is a multifactorial disease state and the term is used to describe pneumonia or generalised respiratory tract disease signs that develop as a result of a combination of events, such as pathogen colonisation, poor environmental conditions, and genetic factors (31, 34). PRDC is a common condition and can cause significant economic losses due to the increased cost of treatment, high morbidity and potential mortality. Clinical signs often seen in pigs include coughing, fever, nasal and ocular discharge, depression, anorexia, laboured breathing and cyanosis in severe cases (6).

Clinical respiratory disease commonly results from polymicrobial infection with viral and bacterial pathogens (57). These pathogens can be divided into primary infectious agents which subvert defence mechanisms and are capable of causing respiratory infections on their own and secondary infectious agents which are opportunistic and typically establish following infection of primary pathogens (8, 31, 34, 58). Infection of two or more pathogens is more commonly associated with serious disease than pathogens acting alone (31, 57). Primary viral pathogens commonly associated with PRDC include PRRSV (Betaarterivirus suid 1), Classical swine fever virus (CSFV), Pseudorabies virus (PRV), Influenza A virus (IAV), PRCV and Porcine circovirus type 2 (PCV2) (34). The most common primary bacterial agents are *Actinobacillus pleuropneumoniae* and *M. hyopneumoniae* (31). Potential secondary bacteria include: *P. multocida, Streptococcus suis*, *E. coli*, *B. bronchiseptica*, *Haemophilus parasuis*, *Arcanobacterium pyogenes, Actinobacillus suis* and *Salmonella cholerasuis* (31, 34, 57). The pathogens associated with PRDC may vary significantly between countries, regions and farms (31). Data on Australian causes is limited to a Pork-CRC report on the underlying causes of pleurisy in pigs from 46 Queensland farms which reported *S. suis* and *M. hyopneumoniae* as the most frequently isolated pathogens, *P. multocida* was also fairly common, present 24 of the 46 farms (59). Pneumonic pasteurellosis without signs of atrophic rhinitis is most often caused by nontoxigenic Capsular Type A strains (8, 58).

1.2.4 Atrophic Rhinitis

Atrophic rhinitis (AR) is a disease of pigs that is characterised by the atrophy of the nasal turbinates and in severe cases can lead to facial distortion. Clinical signs of atrophic rhinitis are sneezing, coughing and nasal discharge. Young pigs in intensive indoor production systems are typically the most severely affected (6). Economic loss from atrophic rhinitis is not due to significantly increased mortalities but the reduced weight gain in affected animals. The two main aetiological agents associated with AR are *B. bronchoseptica* and toxigenic strains of *P. multocida* (30). *B. bronchoseptica* by itself causes only mild turbinate atrophy but makes pigs susceptible to colonisation by *P. multocida* which leads to more

severe lesions (3, 60, 61). Toxigenic capsular type D strains of *P. multocida* are typically associated with AR, and to a lesser extent capsular type A (8, 60, 62).

1.3 Treatment and control

Treatment of *P. multocida* associated disease is heavily reliant on the use of antimicrobials. The use of antibiotics in the pig industry has benefitted production and animal welfare by preventing and treating disease and improving growth rates. The antimicrobials commonly used in the treatment of bacterial respiratory infections in swine include: ampicillin, penicillin and cephalosporins, co-trimoxazole, florfenicol, erythromycin, tilmicosin, enrofloxacin and tulathromycin and tetracyclines (63). In Australia, prescribed antibacterial agents need to be approved and registered for use by the Australian Pesticides and Veterinary Medicines Authority (APVMA). The antibacterial agents currently registered for use are listed in Table 1. In order to maintain the effectiveness of critically important antimicrobials in human medicine, APVMA registered antimicrobials are assigned an importance rating of 'High', 'Medium' or 'Low' based on their significance in treating or preventing human infections. Antimicrobials with a 'High' rating are critical for treating or preventing human infections and therefore use of these is food producing animals would be restricted. The 'Low' and 'Medium' ratings indicate that there are alternative treatment options available in treating human infections (64).

Fluoroquinolones are not registered by the APVMA for use in the treatment of pigs and are banned from use in any food producing animal, and ceftiofur (3rd generation cephalosporin) can only be used off-label on an individual animal basis (65, 66). However, the results of a national study of Australian pig farms in 2006 showed the use of ceftiofur in 25% of herds (66). Recommended treatments for *P. multocida* infections in Australia include: chlortetracycline, oxytetracycline, florfenicol, penicillin, amoxicillin and trimethoprim-sulfonamide (sulfadimidine/sulfadiazine/sulfadoxine) (65).

Table 1. Antimicrobials registered for use by APVMA adapted from Cutler (2020) (65)

Drug Class	**Antimicrobial**	**Route of administration**	**ASTAG importance rating**
Moderate spectrum penicillin	Amoxicillin	IM injection Oral in water or feed	Low
Narrow spectrum penicillin	Penethamate	IM injection	Low
	Penicillin (and salts)	IM injection	Low
Aminoglycoside	Apramycin	Oral in water	Medium
	Neomycin	IM injection Oral in water or feed	Low
Tetracycline	Chlortetracycline	Oral in water or feed	Low
	Oxytetracycline	IM injection Oral in feed	Low
Macrolide	Erythromycin	IM injection	Low
	Tilmicosin	Oral in water or feed	Low
	Tulathromycin	IM injection	Low
	Tylosin	IM injection Oral in water or feed	Low
Bambermycin	Flavophospholipol	Oral in feed	Low
Amphenicol	Florfenicol	IM injection Oral in water or feed	Low
Lincosamide	Lincomycin	Oral in water or feed	Medium
Aminocyticol	Lincomycin-spectinomycin	IM injection Oral in water or feed	Medium
Aminocyticol	Spectinomycin		Medium
Quinoxaline	Olaquindox	Oral in feed	Low
Ionophore	Salinomycin	Oral in feed	Low
Sulfonamide	Sulfadimidine		Low
Pleuromutilin	Tiamulin	Oral in water or feed	Low
DHRI + sulfonamide	Trimethoprim + sulfonamide (sulfadimidine/ sulfadiazine/ sulfadoxine)	IM injection Oral in water or feed	Medium

1.3.1 Antimicrobial Resistance

The use of antimicrobial drugs has greatly improved our control and treatment of infections, however, the rapid spread of antimicrobial resistance (AMR) has become a serious health concern worldwide. The emergence of AMR, particularly in zoonotic bacterial pathogens and commensals, has sparked

concern for the possibility of cross-species transmission of antimicrobial resistance. The increase in resistance in pathogens affecting humans has largely been linked to human antibiotic use, but it raised questions about the use of antibiotics in livestock, particularly when antibiotics that are considered critically important for human medicine have been used in food animals (67). Besides therapeutic treatment, antibiotics have commonly been given to animals as growth promotants and added to feed or water to prophylactically treat large numbers of animals. Inappropriate or overuse of antimicrobials increases the likelihood of antimicrobial resistance developing, undermining effective treatment options and resulting in greater economic costs and higher morbidity and mortality rates (67). As a result, there has been an increased effort to examine and reassess animal production practices both locally and globally.

Some bacteria may be inherently resistant to certain antimicrobials, others may acquire antimicrobial resistance genes (ARGs) through the horizontal transfer of DNA between bacteria via mobile genetic elements or vertically through clonal lineages. Mobile genetic elements, such as plasmids, transposons, integrons and integrative conjugative elements (ICEs), may be transferred under selection pressure between bacteria through the excessive use of antibiotics (68). The mechanisms of horizontal gene transfer amongst prokaryotes are conjugation, transduction and transformation, as shown in Fig. 1. Conjugation is a process in which a donor cell transfers genetic material to a recipient cell, usually through a pilus in Gram-negative bacteria. Transformation is the taking up foreign genetic material from the environment and integrating it into the genome. Transduction occurs when DNA from a donor cell is transferred to a recipient cell via a bacteriophage (69). Through these processes, bacteria may acquire a number of resistance mechanisms, including: enzyme encoding genes, efflux pumps to expel antimicrobials from the cell, genes that remove or modify the binding site for antimicrobials and mutations restricting access of antimicrobials to their target (70).

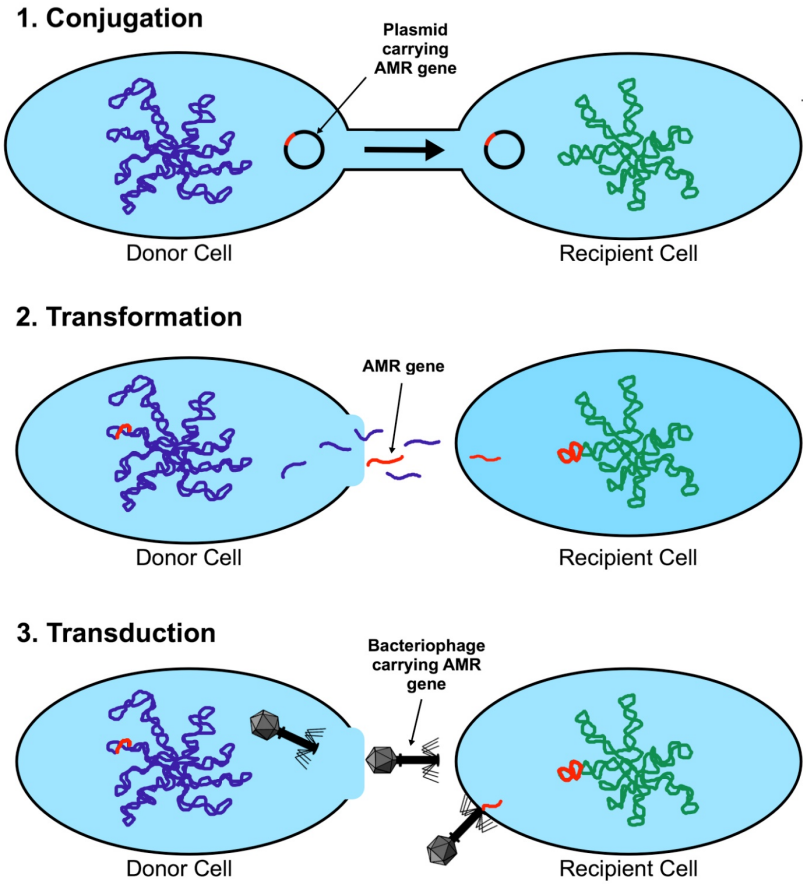

Figure 2. The mechanism of horizontal gene transfer. 1. Conjugation occurs when genetic material is transferred to another bacteria through direct contact. 2. Transformation occurs when a cell takes up foreign DNA from the environment. 3. Transduction is the process whereby DNA is transferred to a bacterium via a bacteriophage. Adapted from Furuya & Lowy, 2006 (71).

Antimicrobial resistance plasmids have been identified in several *P. multocida* isolates, including an Australian study which isolated a plasmid from a toxigenic type D *P. multocida* strain that is associated with atrophic rhinitis in pigs (72). A study of 13 β-lactam-resistant clinical *P. multocida* isolates showed that the bacteria carry several small (4-6kb) plasmids with 1-2 resistance genes. Furthermore, this study found that plasmids could be transferred horizontally through transconjugation with *E. coli* isolates, however, the plasmids were shown to be unstable in the *E. coli* isolates (73). ICEs are not commonly found in *P. multocida*, though they have been identified (74).

In order to limit the rise of AMR, it is important to determine which antimicrobials remain effective as treatment options. Susceptibility testing is used to determine the concentration of antimicrobial that

will inhibit the growth of bacteria and detect resistance within individual isolates. Techniques including disc diffusion and broth or agar dilution are commonly used to determine the in vitro susceptibility of bacteria. The disc diffusion method consists of placing antimicrobial saturated paper discs onto bacterial lawn plates and after incubation measuring the diameter of the zone of inhibition. The dilution methods involve incorporating different concentrations of antimicrobial agents into media and after incubating the test bacteria in that media, assessing the growth. The minimum inhibitory concentration (MIC) from dilution tests reflects the lowest concentration of antimicrobial agent that inhibits visible growth of the bacteria. The results of susceptibility tests are interpreted by using breakpoint values, which are zone diameter values and MICs that are used to categorise bacteria as susceptible, intermediate or resistant. The "susceptible" category indicates a microbial infection may be successfully treated with the recommended dosing regimen of an antimicrobial agent. The "intermediate" category, also referred to as "susceptible, increased exposure", indicates the infection may be successfully treated by adjusting the dosing regimen, or concentrating the antimicrobial agent at the site of infection. The "resistant" category indicates that the infection may not be inhibited by the antimicrobial agent and there is a high likelihood of therapeutic failure. Clinical breakpoints are set by committees that assess microbiological data, distribution outcomes from clinical studies, epidemiological cut offs, pharmacodynamics and pharmacokinetics (75). By using clinical breakpoints, laboratories can communicate to clinicians the probability of an antimicrobial successfully treating and infection.

Generating awareness of the need for judicious use of antimicrobials due to the consequences of indiscriminate usage and continued surveillance is necessary to combat the rise of antimicrobial resistance (76). Surveillance studies of antimicrobial resistance in *P. multocida* have reported low levels of resistance in Europe, North America and Australia, with very few cases of multi-drug resistance. Two studies collected pig isolates from respiratory infections in nine European countries from 2002-2006 and 2009-2012, with the highest levels of resistance to tetracycline (22.2% and 20.4%

respectively) followed by trimethoprim/sulfamethoxazole (3.5% and 5.3% respectively) (77, 78). Antimicrobial susceptibility studies from the United states and Canada showed similar results, with tetracycline having the highest level of resistance followed by very low levels of resistance to tilmicosin and penicillin (79, 80). An Australian study examined the antimicrobial resistance of 51 *P. multocida* isolates taken from pigs across Australia between 2002 and 2013, reporting 28% of the isolates were tetracycline resistant, 14% erythromycin resistant, 4% resistant to both ampicillin and penicillin and 2% resistant to florfenicol (63). However, given the small number of isolates tested this is unlikely to be representative of the entire Australian pig herd.

Higher rates of resistance in *P. multocida* have been reported in Asia and the issue is particularly acute in China. A 2009 study examined 233 *P. multocida* isolates from pigs in China with clinical respiratory disease and reported resistance to lincomycin (96.6%), sulfamethazine (85.4%), amoxicillin (80.3%), clindamycin (80.3%), trimethoprim-sulfamethoxazole (74.2%), chlortetracycline (65.2%), tetracycline (58%), tilmicosin (28.3%), amikacin (14.2%), gentamycin (13.7%), kanamycin (12.8%), spectomycin (12%), erythromycin (6%) and chloramphenicol (2.6%). This study showed that 93.1% of the isolates from this study were multidrug resistant (81). It should be noted that the breakpoint values for certain antimicrobials used in the China study differ from those stated in Clinical Laboratory Standards Institute (CLSI) Performance Standards VET01-S3 (82). Notably, tetracycline resistance would increase to 99.4% using the VET01-S3 tetracycline breakpoint. In recent decades, China experienced an increase in production intensity in the agriculture industry. It is the world's largest consumer of antimicrobials in livestock and the unregulated use for prevention, treatment and growth promotion has led to its high rate of resistance (83, 84). In 2016, China implemented a national action plan to combat AMR which included aims to withdraw use of antibiotics as growth promoter and develop new antibiotics (85).

Studies from South Korea and Vietnam also reported significantly higher levels of resistance in *P. multocida* isolates taken from pigs. In South Korea, resistance was shown to oxytetracyline (63%), florfenicol (16.3), penicillin (9%), ampicillin (7.8%), trimethoprim-sulfamethoxazole (3%), enroflaxcin (2.4%) (18). From 2003 to 2013, South Korea has employed a national antimicrobial management program to monitor consumption and implement bans on particular antibiotics in animal feed. Despite this program, AMR rose in South Korea due to lack of legislation and imprudent use of antibiotics for treatment (86). In 2016, South Korea implemented a new 5-year strategy to address AMR. A study of *P. multocida* isolates from Vietnamese pigs has shown resistance to amoxicillin (75.9%), tetracycline (59%), kanamycin (15.7%), amikacin (15.7%), gentamicin (14.5%), ampicillin (9.6%), erythromycin (9.6%), chloramphenicol (4.8%) (87). A comparison of the antimicrobial resistance rates reported by different countries can be seen in Figure 3.

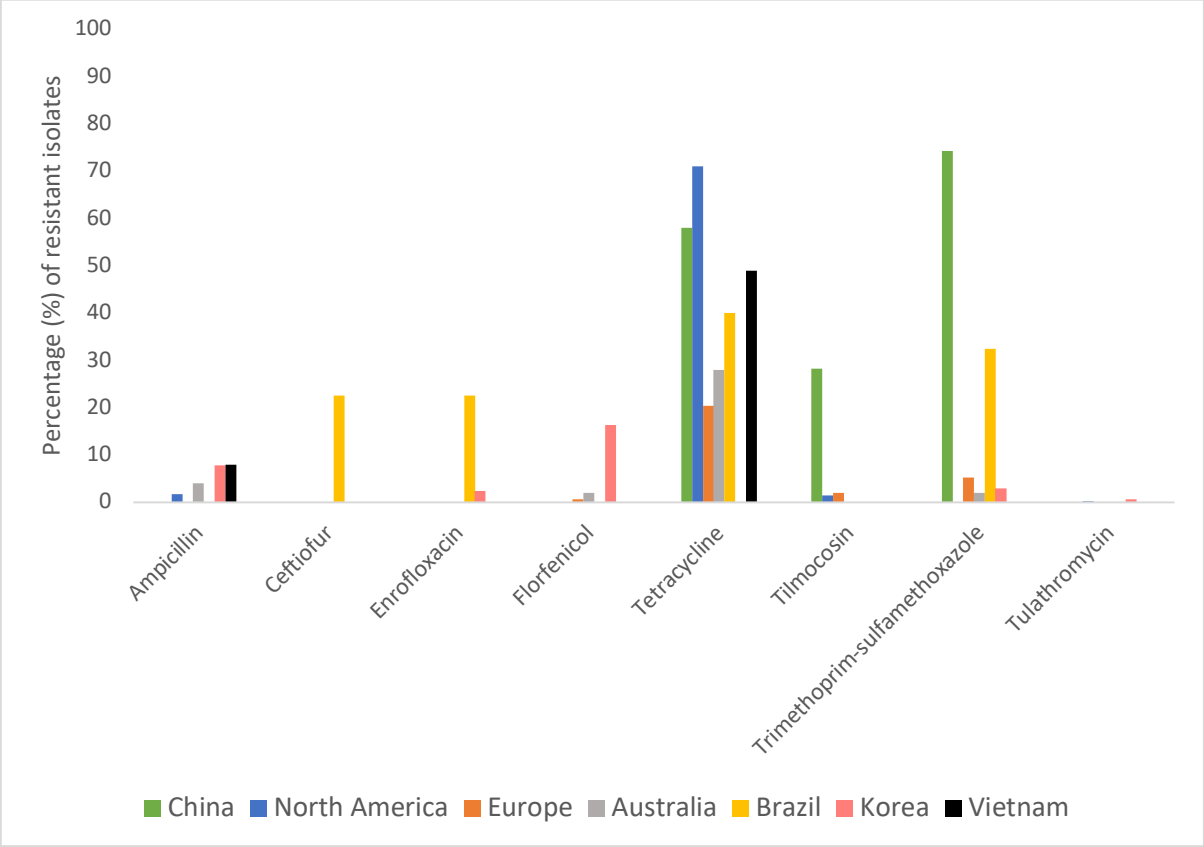

Figure 3. Comparison of the reported antimicrobial resistance rates in *P. multocida* isolates from swine in different countries. The different countries did not test each antimicrobial listed, those not tested

were recorded as 0% resistant isolates. Adapted from: Dayao (2014), El Garch (2016), Furian (2014), Kim (2019), Sweeney (2017), Tang (2009), Vu Khac (2019).

1.3.2 Alternatives to antibiotics

The rise of resistant pathogens in recent decades has led to an increased interest in the development of alternative treatment and prevention options. Vaccines have proven to be an effective measure against respiratory diseases and can decrease the severity and incidence of disease (31). Studies into potential *P. multocida* vaccines began over 100 years ago with Louis Pasteur determining that inoculation of chickens with an attenuated stain protected against virulent strains. Vaccines targeting *P. multocida* have been studied and are available, though few studies evaluate the effectiveness of vaccines in pigs. Killed vaccines, live attenuated vaccines, recombinant vaccines and subunit vaccines have been developed and examined with varying success (88). Killed whole cell vaccines have not proved to be greatly effective as they are not cross-protective against different strains and the immunity provided is often short lived (2). Live attenuated vaccines have been shown to provide better immunity than killed vaccines and provide potential cross-protection. A 2016 study of the protective efficacy of *P. multocida* for poultry showed that killed whole cell vaccines provided poor protection against strains with differing LPS structures, whereas live attenuated vaccines conferred strong protection regardless of LPS structure (89).

For pigs, protection against atrophic rhinitis has been achieved through vaccination with *B. bronchiseptica* and *P. multocida* bacterins or PMT toxoid (3). Atrophic rhinitis typically affects younger animals, so vaccinations are usually given to sows prior to farrowing to provide passive protection against PAR to offspring (6). Recombinant subunit PMT vaccines have been shown to elicit high levels of antibodies in pigs and in the colostrum produced by vaccinated sows (90, 91). Piglets from vaccinated sows, when challenged with *B. bronchoseptica* and *P. multocida*, will display greater weight gain and reduced turbinate atrophy compared to piglets from unvaccinated sows (90-93). Commercial

vaccines are available in North America, Asia and Europe but not in Australia. These vaccines are most effective when used in combination with good management practices and husbandry.

Another method used to manage bacterial infections is phage therapy. This method, which is re-emerging in the era of AMR, uses bacteria-infecting viruses called bacteriophages, or phages, which infect and multiply within bacteria and can cause cell lysis. Phages are ubiquitous organisms, found in all environments their bacterial host is found, with an estimated 10^{31} inhabiting the Earth. Phages are categorised by the international Committee on Taxonomy of Viruses (ICTV) according to nucleic acid (dsDNA, ssDNA or ssRNA) and morphology (tailed, polyhedral, filamentous or pleomorphic) (94). Over 96% of all phages are tailed, they form the order Caudovirales which is comprised of three families: *Siphoviridae* (non-contractile tails), *Myoviridae* (contractile tails) and *Podoviridae* (short tails). Tailed phages all contain linear dsDNA and consist of an elongated oricosahedral head and helical tail (95).

Phages typically have a narrow range of potential hosts, often infecting only particular species or strains. When phages recognise the specific receptor on the bacterial host cell, they adsorb to the cell and transfer genetic material into the cytoplasm (96). Once this occurs, either the lysogenic or lytic cycle will commence. In the lytic cycle, the phage DNA replicates within the host cell, proteins are synthesised and new virions are rapidly formed. The new phages lyse the host cell and are released (97). The lysogenic cycle involves the phage genome being into the genome of its host, a state referred to as 'prophage', and it reproduces through cell division. If the prophage is excised from the host genome then it may commence the lytic cycle. Phages are grouped to as either temperate or virulent according to their lifecycle. Temperate phages may initiate either the lytic or lysogenic cycle, whereas virulent phages do not enter the prophage state and can only reproduce through the lytic cycle (96). Virulent phages are typically preferred in phage therapy due to the rapid bactericidal effect and there is also reluctance to use temperate phages due to their ability to transfer genetic material, including virulence and antimicrobial resistance genes.

In the early twentieth century, promising research was conducted into phage therapy but this was curtailed with the introduction of antibiotics (98). While phage therapy is not a recent development, the desire to find alternatives to antibiotics has revived interest. There are many benefits to using phages to treat infections, for example: phages are host specific and unlikely to target normal flora, negative side effects are extremely rare, they are environmentally friendly and can be isolated from any environment in which bacteria is present (99). Phages therefore have the potential to provide a safe and economic alternative to antibiotics.

Disadvantages to phage therapy include the narrow host range and potential resistance to bacteriophages. Phages are only effective against specific bacteria and whilst this can be beneficial in reducing risk to normal microflora, it also limits the range of pathogens infected by the phages. Broad host range phages or phage cocktails containing multiple phages with a diverse host range may improve coverage of pathogens. There is also the potential for personalised therapy by isolating phages on-demand to treat specific bacterial strains. Bacteria are also capable of developing resistance to phages as they do with antibiotics. There are resistance mechanisms to prevent phages adsorbing to the host cell receptor, superinfection exclusion systems that prevent DNA entry into the host cell, restriction-modification systems which cleave foreign DNA introduced to the host cell and intracellular proteins that abort phage infections (100).

Numerous studies have been conducted into the use of phage therapy to control the spread of zoonotic pathogens and reduce the impact bacterial infections on livestock and production. Notable reports of phage therapy in pigs include treatment of ETEC diarrhoea in neonatal pigs. A 1983 study used a combination of two phages, P433/1 and P433/2 on piglets that were exposed to *E. coli* strain P433 (101). Of the 14 pigs used in this study, 7 were untreated and became seriously ill, resulting in the 4 deaths. The 7 piglets who received the phage therapy showed reduced duration of diarrhoea

and returned to normal health quickly. Another study evaluated the efficacy of 6 bacteriophages, GJ1-GJ6, individually and in combination for treatment of pigs infected with ETEC strain JG280. All phages individually demonstrated significant prophylactic activity, with the treated piglets experiencing shorter duration and severity of diarrhoea. The untreated piglets displayed a slight decrease in weight and the duration of diarrhoea was 1-2 days longer than the treated piglets (102).

The occurrence of *P. multocida* bacteriophages was first reported in 1956, however since then only a few studies have focused on *P. multocida* phages. In 2006, temperate phage F108 was isolated and characterised the through mitomycin C induction of a capsular type A *P. multocida* (103). Phage F108 is not virulent, however it was suggested as a potential tool for genetic manipulation as *P. multocida* cells do increase pathogenicity when lysogenic for F108 and the temperate phage was capable of performing generalised transduction. In 2017, the performance of two bacteriophage lysate vaccines was compared against a whole cell vaccine to treat chickens infected with different strains (A:1, A:3, A:4) of *P. multocida* (104). This study indicated that bacteriophage lysate vaccines provide greater cross-protectiveness than a whole cell vaccine as indicated by survival rate of chickens and antibody titres. As mentioned previously, whole cell vaccines do not provide adequate protection against strains with differing LPS structures, therefore it is not surprising that the whole cell vaccine performed poorly with different strains. However, the lysate vaccine was not compared to a live attenuated vaccine which has been previously shown to confer protection to different strains (89).

A virulent bacteriophage (PHB02) was isolated from wastewater on a swine farm in China (105). This bacteriophage was specific to capsular type, as it effectively lysed 30 out of 31 type A *P. multocida* strains but had no effect on capsular type D or F *P. multocida* strains or any other Gram-negative bacteria. Following on from this study, a lytic phage specific for capsular type D (PHB01) from wastewater on a pig farm (106). Host range tests indicated that this bacteriophage lysed 22 out of 37 type D isolates and showed no activity against other capsular types or bacterial species. Of the 37

capsular type D isolates, only non-toxigenic isolated were lysed, with four other non-toxigenic isolates and 11 toxigenic isolates showing resistance to the phage. In vivo tests were conducted by administering non-toxigenic Type D strains of *P. multocida* into mice and administering either PHB01 or PBS. Those administered PBS showed severe clinical signs and 80% died within 5 days, whereas those administered with PHB01 displayed milder signs of infection and had a 100% survival rate. This study indicates that phage therapy may be effective against non-toxigenic type A and type D strains, which would be beneficial on pig farms as these strains are typically responsible for pneumonic pasteurellosis.

1.4 Project Aim

Aims of this study

1) Determine the antimicrobial susceptibility data for a significant number of disease associated *P. multocida* isolates
2) Determine the transferability of plasmid backbones between Gram-negative bacteria
3) Isolate bacteriophages from different sources
4) Induce prophages and demonstrate lytic activity against heterogeneous strains

Hypotheses

1) *P. multocida* isolates will demonstrate low rates of antimicrobial resistance
2) Resistance to ceftriaxone and ampicillin will be transferred from *E. coli* to *P. multocida*
3) Lytic phages can be isolated from the environment for use in phage therapy
4) Mitomycin C will excise prophages from the bacterial chromosome of *P. multocida* isolates, inducing the prophage.

2. Methods

2.1 Bacterial Isolates

Pasteurella multocida isolates (n=292) were obtained from ACE laboratories, Bendigo, Victoria. Table 2 summarises isolates by year of isolation and body site, and a detailed list is presented in Appendix I. All available porcine *P. multocida* isolates collected between January 2014 and December 2019 were enrolled in the study, spanning 75 different farms across Australia. Prior to this study the *P. multocida* isolates were stored at -70℃ in 1ml of Luria Bertani (LB) Broth (BD Worldwide, USA) with 20% glycerol (Chem-supply, Australia). The isolates underwent two subcultures via streaking onto blood agar plates (Micromedia, Australia) and incubated at 37℃ overnight. The isolates were checked for possible contamination and isolates demonstrating mixed colonies were removed from the study (n=4).

Table 2. Summary of the year and site of isolation for each *P. multocida* isolate

Site of isolation	**2014**	**2015**	**2016**	**2017**	**2018**	**2019**	**Total for site of isolation**
Abdomen	0	1	0	0	2	1	4
Brainstem	0	0	0	1	1	0	2
Heart	0	1	8	0	0	0	9
Hock	0	0	0	0	2	0	2
Kidney	0	0	0	0	0	1	1
Lung	46	58	42	47	41	16	250
Gut	0	0	0	0	1	0	1
Nose	0	0	0	0	0	1	1
Tendon	0	0	0	0	1	0	1
Tonsils	0	0	0	0	1	0	1
Trachea	4	0	0	0	0	0	4
Spleen	0	0	0	0	1	0	1
Unknown	10	0	2	0	0	3	15
Total	60	60	52	48	50	22	292

2.1.1 MALDI-TOF Identification

Identification of the bacterial isolates was carried out using matrix assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF) typing (Bruker). A sample of each of the 288 isolates was applied to the MALDI target plate before adding 1 µl of 70% formic acid on to each isolate and finally overlaying the isolates with 1 µl of MALDI matrix solution. Identification was carried out using a Bruker MALDI biotyper as per manufacturer guidelines. Samples identified by MALDI-TOF as *P. multocida* that returned a log(score) value greater than 2.00 were accepted.

2.2 Bacterial Growth Optimisation

Antimicrobial susceptibility testing was performed according to Clinical Laboratory Standards Institute (CLSI) Performance Standards, M31-A3 (107). The guidelines recommended colony forming units/ml (CFU/ml) is 1.0×10^8 for susceptibility testing. A randomly selected *P. multocida* isolate was cultured overnight in four different growth media in order to determine which media could most consistently provide a CFU/ml of 1.0×10^8. The growth media used were brain heart infusion (BHI) (Thermo Fisher Scientific, Australia), Luria Bertani (LB), tryptic soy (TS) (Thermo Fisher Scientific, Australia) and cation-adjusted Mueller Hinton broth (CAMHB) (Thermo Fisher Scientific, Australia). In a Nunc™ 96 well polystyrene flat bottom microtitre plate (Thermo Fisher Scientific, Australia), 220 µl of each media was added in triplicate and mixed with a single colony and then grown overnight at 37℃. Bacterial cultures were adjusted to an absorbance of 0.7 at 620 nm (Tecan EVO 150) and plated onto blood agar. The agar plates were incubated overnight at 37℃ and then the colonies on each plate were counted to determine the CFU/ml. Of the four media trialled, LB was determined to provide the most consistent results, however, the CFU/ml was higher than required with an average of 3.5×10^8 CFU/ml. In order to achieve the optimum CFU/ml using LB, the absorbance would be adjusted. Follow up tests were conducted using the same method above, with a larger sample size (8 isolates) and absorbance set at 0.4, 0.55, and 0.7. The results indicated that the optimal absorbance was between 0.4 and 0.55 and therefore a further test was performed with absorbances of 0.42, 0.44 and 0.48.

2.3 Minimum Inhibitory Concentration (MIC) testing

All *P. multocida* isolates were subjected to antimicrobial susceptibility testing through broth microdilution according to CLSI Performance Standards, VET01-S3 (82). *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 were used as reference strains as recommended in CLSI VET01-S3 (82). *P. multocida* isolates, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were grown on blood agar plates overnight at 37℃. Bacterial cultures were harvested from blood agar plate using 1 µl sterile loops (Copan), suspended in 500 µl Luria Bertani broth and grown overnight at 37℃. Using a multichannel pipette (Thermo Fisher Scientific, Australia) 200 µl of each of the overnight culture was transferred to 96 well polystyrene flat bottom microtitre plates and the absorbance measured.

The broth microdilution was performed using ampicillin, cefoxitin, ceftiofur, chlortetracycline, ciprofloxacin, florfenicol, gamithromycin, gentamicin, neomycin, tetracycline, tilmicosin, trimethoprim/sulfamethoxazole, tulathromycin. All antibiotics were sourced from Sigma-Aldrich, Australia, with exception of gamithromycin which was obtained from BioAustralis, Australia. The antibiotics were added to 96-well plates using a modified robotic liquid handling system (Tecan Evo 150). The layout of the plates is outlined in Figure 4. The minimum inhibitory concentration (MIC) was determined from the lowest concentration of antibiotics required to inhibit bacterial growth. The MIC results were categorised as resistant, intermediate and susceptible using the criteria specified in CLSI performance standard, VET01-S3. Swine breakpoints were unavailable for gamithromycin and were substituted with cattle breakpoints. Breakpoints were not available for cefoxitin, ciprofloxacin, gentamicin, neomycin or trimethoprim/sulfamethoxazole. Epidemiological cut-off values (ECOFFs) provided by the European Committee on Antimicrobial Susceptibility Testing, were available for ciprofloxacin and gentamicin. ECOFFs separate bacterial populations into those with and without phenotypically detectable resistance. ECOFFs cannot predict clinical success of antimicrobials but can used in surveillance when clinical breakpoints haven't been determined.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**FOX** 32↓	16	16	**NEO** 64↓	**NEO** 0.25	2	**GEN** 16↓	16	2	16↓	**AMP** 8↓	**BLANK**
B	16	8	8	32	**TIL** 64↓	1	8	8	1	**TUL** 8	4	**BLANK**
C	8	4	4	16	32	0.5	4	4	0.5	**SXT** 4/76↓	2	**BLANK**
D	4	2	**GAM** 2	8	16	0.25	2	2	0.25	2/38	1	**BLANK**
E	2	1	**TET** 2↓	4	8	0.12	1	1	**XNL** 0.12	1/19	0.5	**POS**
F	1	0.5	1	2	4	0.062	0.5	**FFN** 0.5	**TUL** 128↓	0.5/9.5	**AMP** 0.25	**POS**
G	**FOX** 0.5	**CTC** 0.25	0.5	1	**TIL** 2	0.031	**GEN** 0.25	**XNL** 8↓	64	0.25/4.75	**BLANK**	**POS**
H	**CTC** 32↓	**GAM** 32↓	**TET** 0.25	0.5	**CIP** 4↓	**CIP** 0.015	**FFN** 32↓	4	32	**SXT** 0.12/2.38	**BLANK**	**POS**

Figure 4. Layout of the broth microdilution plates for susceptibility testing. Each antibiotic is labelled with the highest and lowest concentration (µg/ml). FOX – Cefoxitin; CTC – Chlortetracycline; GAM – Gamithromycin; TET – Tetracycline; NEO – Neomycin; TIL – Tilmicosin; CIP – Ciprofloxacin; GEN – Gentamicin; FFN – Florfenicol; XNL – Ceftiofur; TUL – Tulathromycin; SXT – Trimethoprim/Sulfamethoxazole; AMP – Ampicillin; POS – Positive control. The squares labelled BLANK and POS contain only the *P. multocida* culture and CAMHB.

2.4 Transconjugation

The transferability of AMR genes present on plasmid backbones from *E. coli* to *P. multocida* isolates was assessed using five tetracycline resistant *P. multocida* isolates and six tetracycline susceptible *E. coli* isolates carrying β-lactamase genes bla_{CMY-2} and bla_{CTXM} (Table 2). The *E. coli* isolates originated from different bird species on Penguin Island were reported to carry highly transferrable resistance genes and were stored at Murdoch University Antimicrobial Resistance and Infectious Diseases Laboratory at -80℃ (108). These samples were subcultured onto blood agar and incubated overnight at 37℃.

Table 3. List of *E. coli* and *P. multocida* isolates used for conjugation and the resistance characteristics

Isolate ID	**Species**	**AMR genes**	**AMR Phenotype**
130ESB	*E. coli*	bla_{CMY-2}, bla_{TEM-33}	AMP, CRO, CFT, FOX
157ESB	*E. coli*	$bla_{CTXM-15}$, qnrS	AMP, CRO, CFT, CIP
194ESB	*E. coli*	$bla_{CTX-M-27}$, strA, strB, sul2	AMP, CRO, CFT
219ESB	*E. coli*	$bla_{CTX-M-15}$	AMP, CRO, CFT
233ESB	*E. coli*	$bla_{CTX-M-14}$	AMP, CRO, CFT
288ESB	*E. coli*	$bla_{CTX-M-14}$	AMP, CRO, CFT
19120401	*P. multocida*	Not known	TET
19120559	*P. multocida*	Not known	TET, CTC
19120602	*P. multocida*	Not known	TET, CTC
19120412	*P. multocida*	Not known	TET, CTC
19120340	*P. multocida*	Not known	TET, CTC

AMP, ampicillin; CRO, ceftriaxone; CFT, ceftiofur; FOX, cefoxitin; CIP, ciprofloxacin; TET, tetracycline; CTC, chlortetracycline

Donor *E. coli* isolates and recipient *P. multocida* isolates were inoculated in LB broth (1mL) and incubated overnight at 37℃. In the initial experiments the cultured bacteria were combined at a donor : recipient (D:R) ratio of 1:2 and incubated at 37℃, following methods outlined by Mukerji.

(2020) (108). Subsequent experiments used ratios 1:1 and 1:5 and incubated the mixtures at 37℃ for 4 and 8 hours. After incubation, each of the mixtures and the wild-types were subcultured onto five BHI agar plates containing different antibiotics: 1) TET, tetracycline (5 µg/ml); 2) AMP, ampicillin (10 µg/ml); 3) CRO, ceftriaxone (2 µg/ml); 4) TET+AMP, tetracycline (5 µg/ml) and ampicillin (10 µg/ml); 5) TET+CRO, tetracycline (5 µg/ml) and ceftriaxone (2 µg/ml). The plates were incubated at 37℃ and examined for growth every 24 hours over a 72-hour period. Identification of bacterial growth was performed by MALDI-TOF typing. Bacterial colonies that formed on TET+AMP and TET+CRO plates were subcultured onto new antibiotic plates to assess whether resistance was maintained (Figure 5).

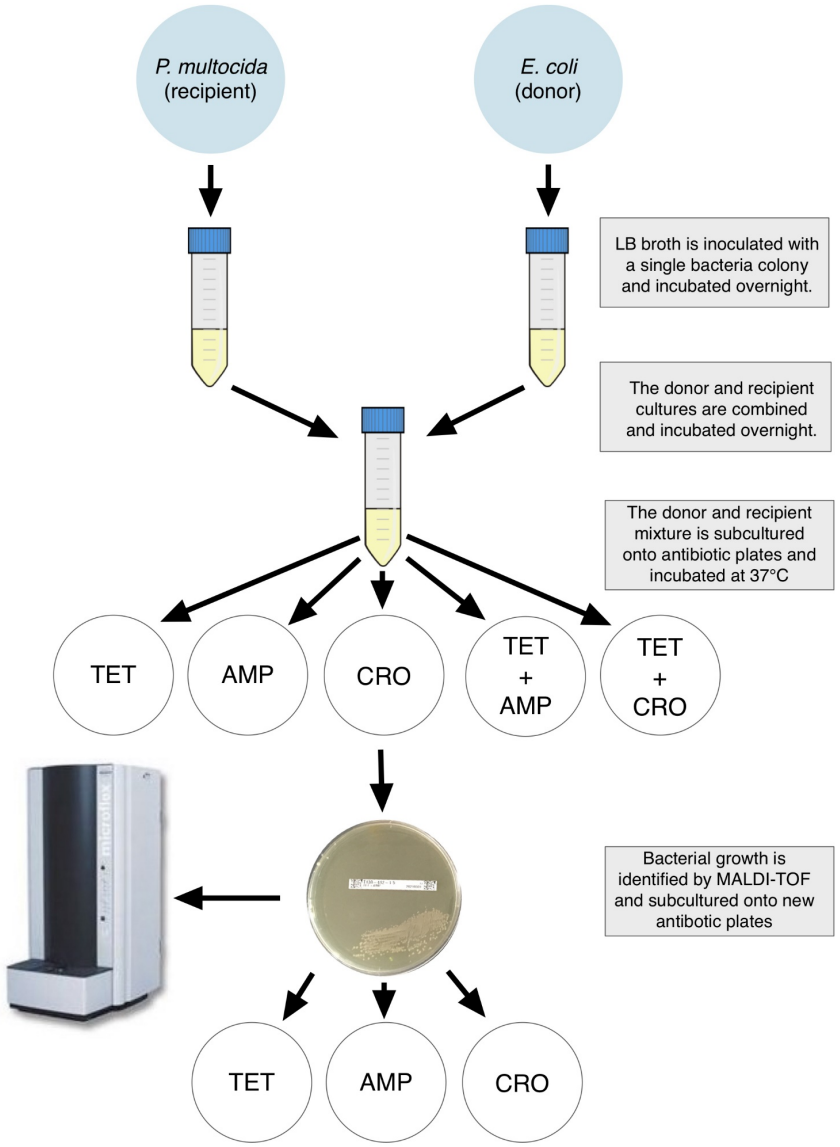

Figure 5. Workflow diagram for the transfer of AMR genes via conjugation. TET, tetracycline; AMP, ampicillin; CRO, ceftriaxone.

2.5 Isolation of lytic bacteriophages

2.5.1 Phage Enrichment

Swine faecal samples from the Murdoch University farm and from a New South Wales piggery, effluent samples from a local farm, water samples from the south west region of Western Australia and sewage samples from the Southern Metropolitan area were used in attempt to isolate phages. Faecal samples were suspended in SM buffer at a 1:10 ratio and mixed using a magnetic stirrer for 24 hours at 4°C. The effluent, water and sewage samples did not undergo this initial step. All samples were centrifuged at 4000 g for 10 minutes before being filtered through a 0.45 µm syringe driven membrane filter unit (Pall Corporation, USA). An equal volume of 2x LB broth and the filtrate were aliquoted into 50 ml falcon tubes. The solution was then inoculated with a single colony of *P. multocida* selected for isolation of phages and incubated at 37°C for 21 hours on an orbital shaker at 150 rpm. The culture was centrifuged at 4000 g for 10 minutes then filtered through a 0.22 µm membrane filter (Pall Corporation, USA). The collected lysate was used for phage isolation.

2.5.2 Phage isolation

Two methods of phage isolation were undertaken with the lysate. The first method involved the spot testing the lysates onto lawn plates of their host bacterial isolates (109). Bacterial isolates were taken from blood agar plates and suspended in 4 ml of CAMHB. The samples were then vortexed and 1 ml was dispensed onto BHI agar plates and rotated to ensure even coverage. The excess broth was removed, and the plates were allowed to dry before four, 20 µl drops of phage lysate were applied to the lawn plate. The plates were allowed to dry and then incubated for 24 hours at 37°C. Phage growth was indicated by the formation of phage plaques. The second method of phage isolation was the double-layer agar method (106). In 3 ml of molten soft TS agar containing 5% newborn calf serum, 100 µl of phage lysate and 300 µl bacterial broth was added. The soft agar was poured onto prepared TS agar plates containing 5% calf serum. The plates were incubated for 24 hours at 37°C and then

examined for plaques. This method was also repeated with BHI agar in place of TS agar containing newborn calf serum.

2.6 Induction of prophages using Mitomycin C

Mitomycin C was used to induce prophages in the chromosome *P. multocida* isolates. When the prophages are induced, viral replication commences causing bacterial cells to lyse. The first attempt at inducing prophages using Mitomycin-C was performed following the methods outlined by Campoy (103). Seven *P. multocida* isolates were subcultured onto blood agar and incubated overnight at 37°C. Falcon tubes containing 2 ml of BHI broth were inoculated with single colony of *P. multocida* taken from the first overnight culture and incubated overnight at 37°C. The bacterial broth was diluted 1/100 in fresh BHI broth and returned to the incubator for 2 hours. After 2 hours, 2970 µl of the bacterial broth was combined with 30 µl of 50 µg/ml Mitomycin-C (Sigma-Aldrich, Australia) and incubated at 37°C for 30 minutes. The mitomycin C treated culture was centrifuged at 4000x g for 10 minutes and the supernatant was removed. The pellet remaining in the base of the falcon tube was then resuspended in fresh BHI broth and incubated at 37°C for 2 hours. The culture was then centrifuged for a further 10 minutes, the supernatant was filtered through a 0.22 µm membrane filter and the lysate was tested for the presence of bacteriophages through the spot test method described above.

2.6.1 Monitoring bacterial growth in microplate reader

The second attempt at inducing bacteriophages using Mitomycin-C was performed following the methods outlined by Pullinger (2003) and involved monitoring the growth rate of bacteria in a microplate reader before and after the addition of mitomycin C (53). Initially, the bacterial growth was measured in a microplate reader to assess the growth rate of bacteria at different concentrations in growth media. The seven isolates used in the first attempt were added to 2 ml of BHI broth and grown overnight at 37°C. The overnight culture was subcultured at ratios of 1:10 and 1:100 in BHI broth on a 96 well polystyrene flat bottom microtitre plate. The plate was incubated at 37°C in a microplate

reader (Tecan Spark) and shaken at 270 rpm. The growth of the bacteria was measured at an OD of 620nm until the absorbance of each isolate reached 0.3. The 1:10 dilution had a faster growth rate and so it was used for subsequent experiments.

On a 96-well plate 175 µl of BHI broth and 19.5 µl of bacterial culture was added to eight columns and the plate placed in the microplate reader and incubated at 37°C for 4.5 hours while shaking. After 4.5 hours, each *P. multocida* isolate had an absorbance reading ≥ 0.3 at OD_{620} and column 1-6 received varying concentrations of mitomycin C, with column 7 and 8 serving as controls (Figure 6.). The plate was returned to the microplate reader and absorbance was read every 10 minutes for 21 hours. The induction of lysogenic phages was determined by the visible clearing and the optical densities read at 620nm.

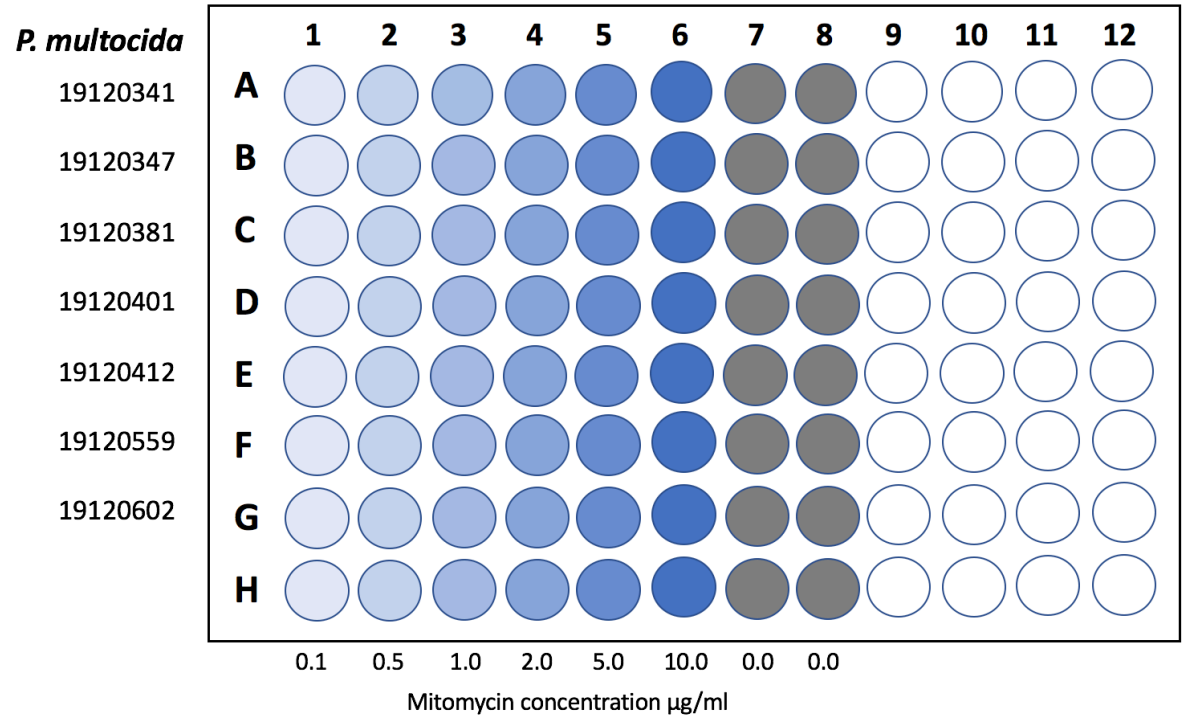

Figure 6. A schematic diagram of bacteriophage induction plates for monitoring bacteria growth at different concentrations of mitomycin C.

This experiment was repeated using concentrations of mitomycin C at 0.05, 0.1, 0.25 and 0.5 µg/ml. Following 21 hours of growth in the microplate reader, wells that showed a decrease in bacterial growth were removed from the plate had the culture removed using a pipette. and this was transferred to Eppendorf tubes. Eppendorf tubes were centrifuged at 10,000 g for 10 minutes and four 20 µl drops of lysate were spot tested onto bacterial lawn plates, as described above. The plates were incubated overnight at 37°C and observed for phage plaques.

3. Results

3.1 MALDI-TOF Identification

Based on Bruker MALDI Biotyper analysis, 286/288 isolates were confirmed to be *P. multocida*, with a log(score) value ≥ 2.00. Resulting in a final number of 286 isolates enrolled into the study.

3.2 Bacterial Growth Optimisation for Susceptibility Testing

After the blood agar plates were incubated, the colonies on each plate were counted, the average number of colonies was 141 ± 21.52 from the plates with BHIB media, 155 ± 1.73 from the LB plates, 124.33 ± 23.03 from the TS plates and 61 ± 17.35 from the CAMHB plates. To achieve target CFU of 1.0×10^8 the optimal colony count should be 45-46. Of the four different growth media (BHIB, CAMHB, LB, TS), CAMHB was the closest to achieving the target CFU/ml of 1.0×10^8, however there was variation between the three replicates. Though the CFU/ml was higher than required, LB had the most consistent results (Table 1) and follow up tests used LB as the growth media. The average colony count of 8 *P. multocida* isolates was calculated at absorbances of 0.4, 0.42, 0.44, 0.48, 0.55, and 0.7, results are shown in Table 4. It was determined that in order to achieve a CFU/ml of 1.0×10^8 using LB, the absorbance should be 0.46.

Table 4. Colony count and CFU/ml of a *P. multocida* isolate in different media

Isolate number	Media	Colony Whole	CFU/mL
19120377	BHIB	142	3.2E+08
19120377	BHIB	119	2.7E+08
19120377	BHIB	162	3.6E+08
19120377	LB	154	3.5E+08
19120377	LB	154	3.5E+08
19120377	LB	157	3.5E+08
19120377	TS	102	2.3E+08
19120377	TS	123	2.8E+08
19120377	TS	148	3.3E+08
19120377	CAMHB	57	1.3E+08
19120377	CAMHB	80	1.8E+08
19120377	CAMHB	46	1.0E+08

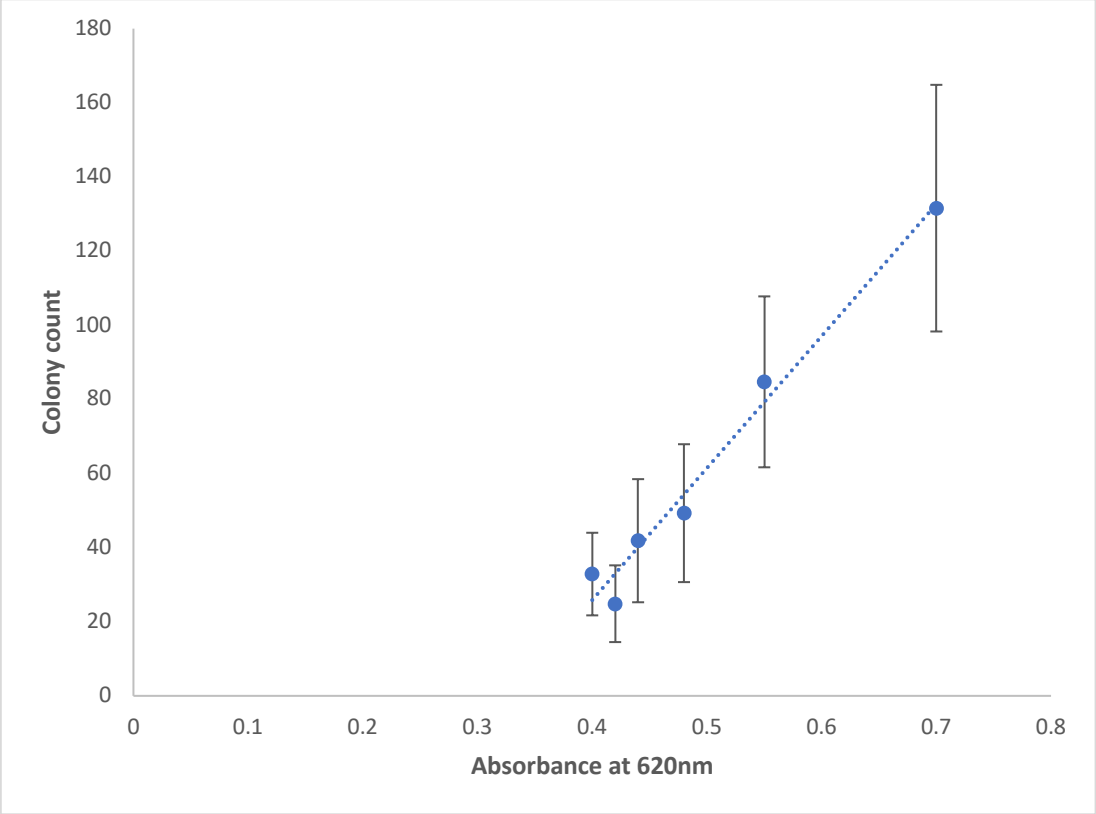

Figure 7. The average colony count of eight *P. multocida* isolates grown in LB media after the absorbances were adjusted. The blue markers indicate the averages and the errors indicate the standard deviation.

3.3 Minimum Inhibitory Concentration

The results of MIC testing are presented in Table 5., isolates are determined to be clinically resistant to an antimicrobial if they exceed its breakpoint. Due to lack of growth in control wells, thirteen isolates were removed, leaving a total of 273 isolates. Of the 273 isolates, 121 (44.3%) were susceptible to all antibiotics tested. Seventy-nine isolates (28.9%) were classified as intermediate and 73 (26.7%) were determined to be resistant to at least one antimicrobial. Of the resistant isolates, 95.9% were resistant to either tetracycline or chlortetracycline, 2.7% to florfenicol and 1.4% to ampicillin.

Breakpoints were not available for cefoxitin, ciprofloxacin, gentamicin, neomycin or trimethoprim/sulfamethoxazole, so it cannot be determined if they were clinically resistant. ECOFF values for ciprofloxacin and gentamicin are 0.064 µg/ml and 8 µg/ml, respectively. No isolate had a MIC that exceeded the ECOFF values for these antimicrobials, so it is likely that they are all susceptible. Multidrug resistance phenotype (resistance to three or more antimicrobial classes) was not detected among the *P. multocida* classified as clinically resistant.

Table 5. Minimum inhibitory concentration distribution for *Pasteurella multocida*

Antimicrobial	0.015	0.03	0.063	0.125	0.25	0.5	1	2	4	8	16	32	64	128	Resistant (%)
Ampicillin					270	1	1				1				0.4
Cefoxitin						273									N/A
Ceftiofur				273											0
Chlortetracycline					72	53	88	29	22	9					22
Ciprofloxacin	273														N/A
Florfenicol						271				1		1			0.7
Gamithromycin								273							0
Gentamicin					14	60	167	32							N/A
Neomycin					31	54	76	78	6				7	19	N/A
Tetracycline					147	45	19	14	48						22.7
Tilmocosin								248	9	7	9				0
Trimethoprim/Sulfamethoxazole				270				1	1	1					N/A
Tulathromycin										273					0

Number of *P. multocida* isolates at the corresponding minimum inhibitory concentration (μg/ml) of antimicrobial. Shaded areas indicate the range of dilutions evaluated. Vertical lines indicate resistance breakpoints for ampicillin, ceftiofur, chlortetracycline, gamithromycin, florfenicol, tetracycline, tilmocosin and tulathromycin.

3.4 Transconjugation

The wildtype *E. coli* grew on the plates containing only ampicillin and ceftriaxone but did not grow on plates containing tetracycline. The wildtype *P. multocida* isolates grew on the plates containing only tetracycline. The transconjugant plates displayed limited growth over the 72-hour period on the TET+AMP and TET+CRO plates. Initial experiments using the 1:2 (D:R) ratio and incubation period of 4 hours had limited success. After 24 h, neither the TET+AMP or TET+CRO plates had any bacterial growth. All TET+AMP plates had colonies after 48 hours, however only 2 of the TET+CRO plates had grown colonies after 72 hours. All plates with bacterial growth at the end of the 72-hour period were confirmed to be *P. multocida* by MALDI-TOF typing.

The experiment was repeated using different donor-recipient ratios (1:1, 1:5) and increasing the duration of mixing to 8 hours. This produced similar results as the previous experiment, however, twice the number of TET+CRO plates had grown colonies by the end of the 72h period. The transconjugant colonies were subcultured onto blood agar and incubated overnight at 37°C before being subcultured onto new TET+AMP and TET+CRO plates and returned to the incubator. After 48 hours, there was no bacterial growth on the plates. The experiment was repeated but returned similar results to the previous attempts. However, in this attempt there was one plate that displayed significant growth after 24 hours. The bacteria from this plate were identified as *E. coli* by MALDI-TOF and subcultured onto a BHI plate containing only tetracycline. After 24 h, there was bacterial growth on the TET plate and once again this was identified as *E. coli* by MALDI-TOF.

3.5 Bacteriophage Isolation

The attempts to isolate bacteriophages from faecal, effluent, water and sewage samples were unsuccessful. Neither the spot test method nor the double layer agar method resulted in phage plaques. The initial attempt to induce the lytic cycle of potential lysogenic phages by adding mitomycin

C to *P. multocida* culture were similarly unsuccessful as determined by the absence of visible clearing and lack of plaques present on the lawn plates after the spot test.

The growth of the *P. multocida* isolates was monitored in a microplate reader before and after the introduction of mitomycin C and is shown below in Figures 2- 8. The isolates were incubated while shaking for 4.5 hours before the mitomycin C was added (when absorbance at 620nm reached between 0.2-0.5). For all the isolates, when mitomycin concentrations of 2, 5 and 10 µg/ml were added, the bacteria ceased growing and the absorbance remained the same. At lower mitomycin C concentrations, the cultures continued to grow, with absorbance peaking between 0.6-0.96. The concentrations of mitomycin C that proved most successful in clearing the bacterial cultures were 0.05 µg/ml, 0.1 µg/ml and 0.25 µg/ml. After the addition of these mitomycin concentrations, isolates 19120340, 19120412 and 19120559 displayed rapid decline in absorbance after 2.5 hours, indicative of bacterial lysis due to lysogenic phages entering the lytic cycle (Fig. 2, 6 and 7). Isolates 19120347 and 19120381 also visibly cleared at the lower mitomycin concentrations over a longer duration of time.

The lysate was pipetted onto lawn plates to isolate phages. After overnight incubation, no phage plaques had formed.

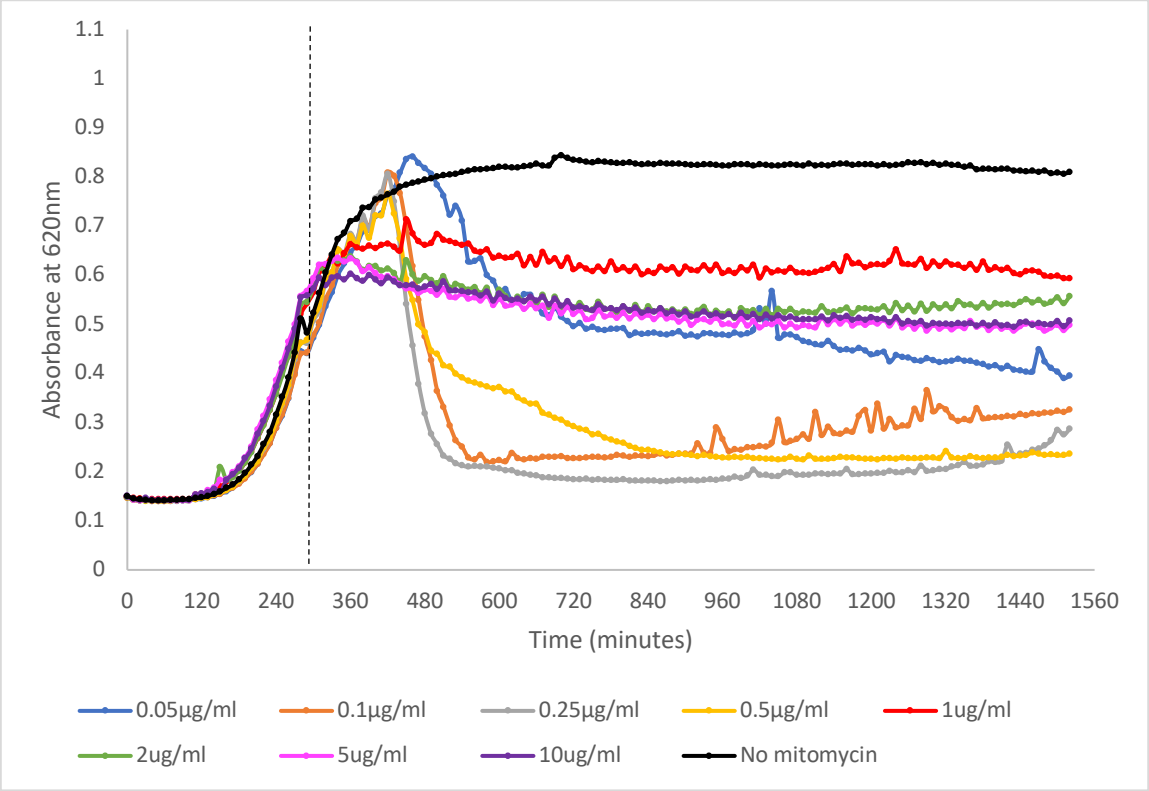

Figure 8. A620 of *P. multocida* isolate 19120340 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

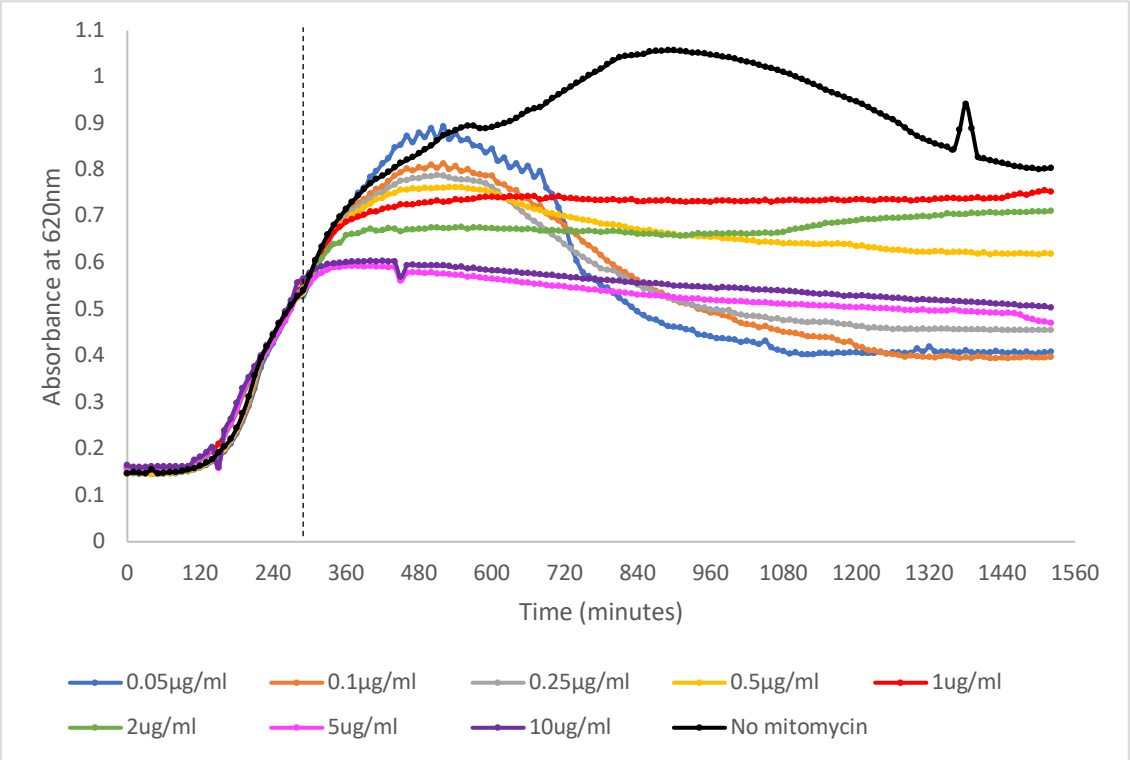

Figure 9. A620 of *P. multocida* isolate 19120347 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

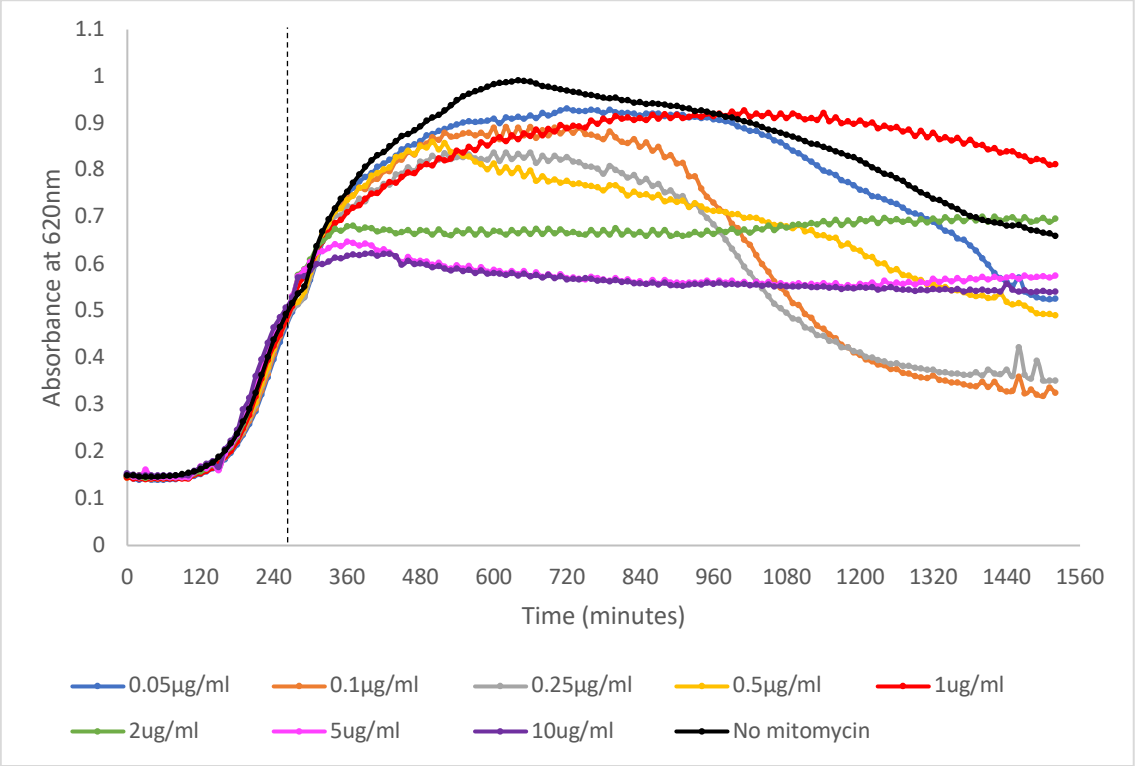

Figure 10. A620 of *P. multocida* isolate 19120381 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

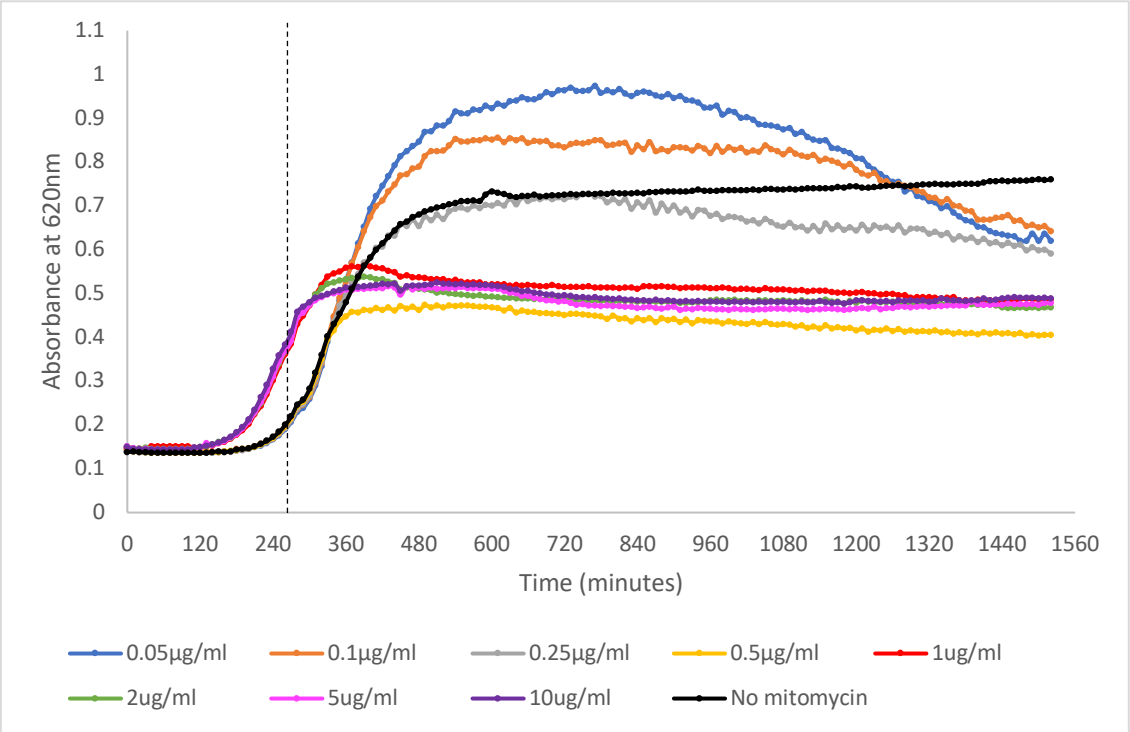

Figure 11. A620 of *P. multocida* isolate 19120401 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

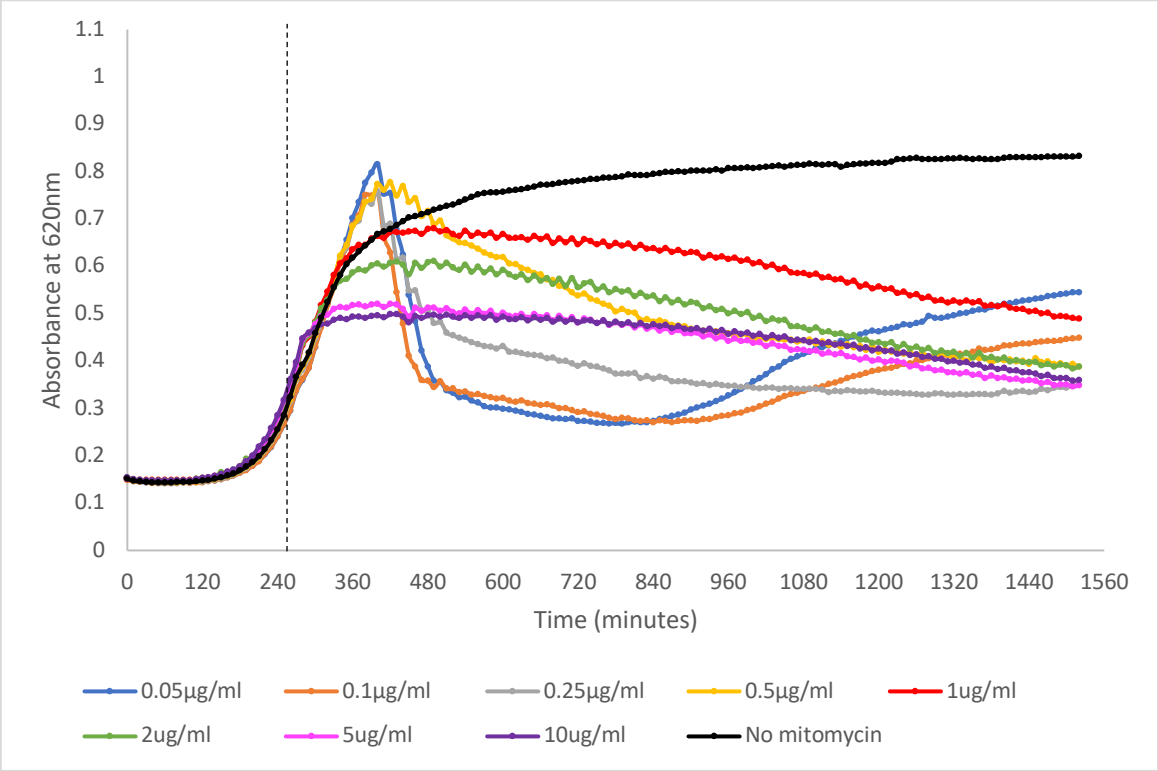

Figure 12. A620 of *P. multocida* isolate 19120412 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

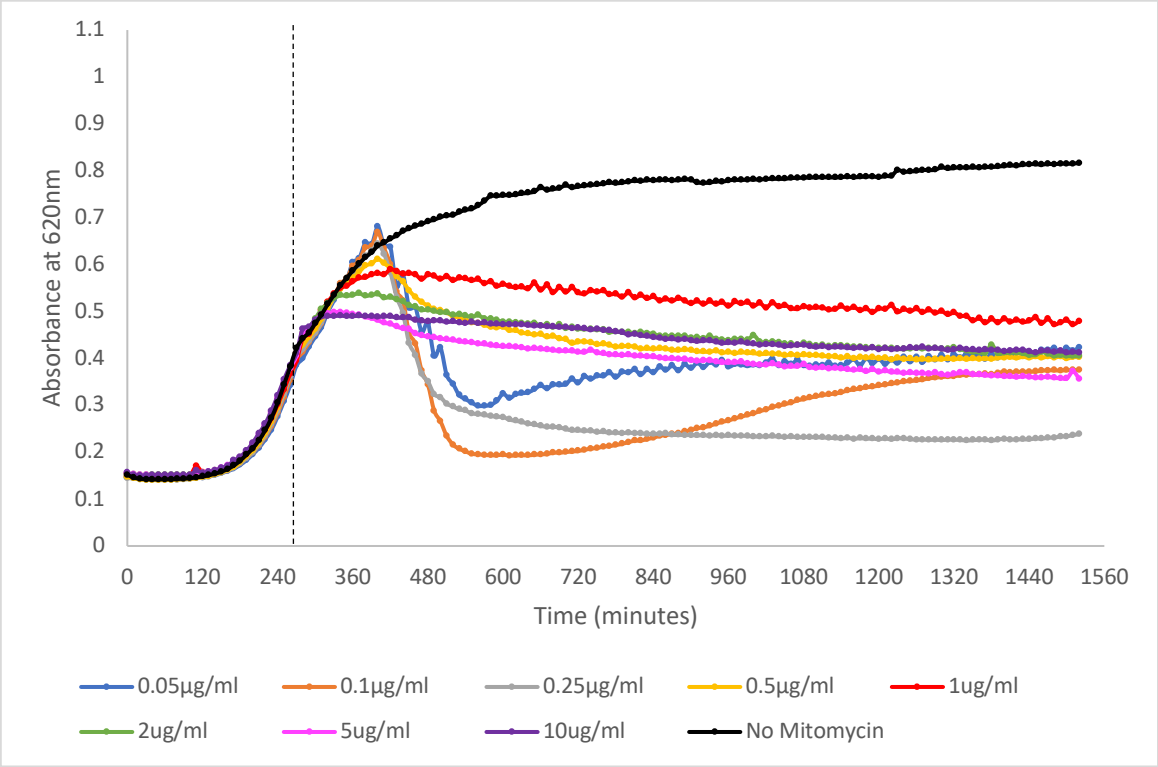

Figure 13. A620 of *P. multocida* isolate 19120559 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

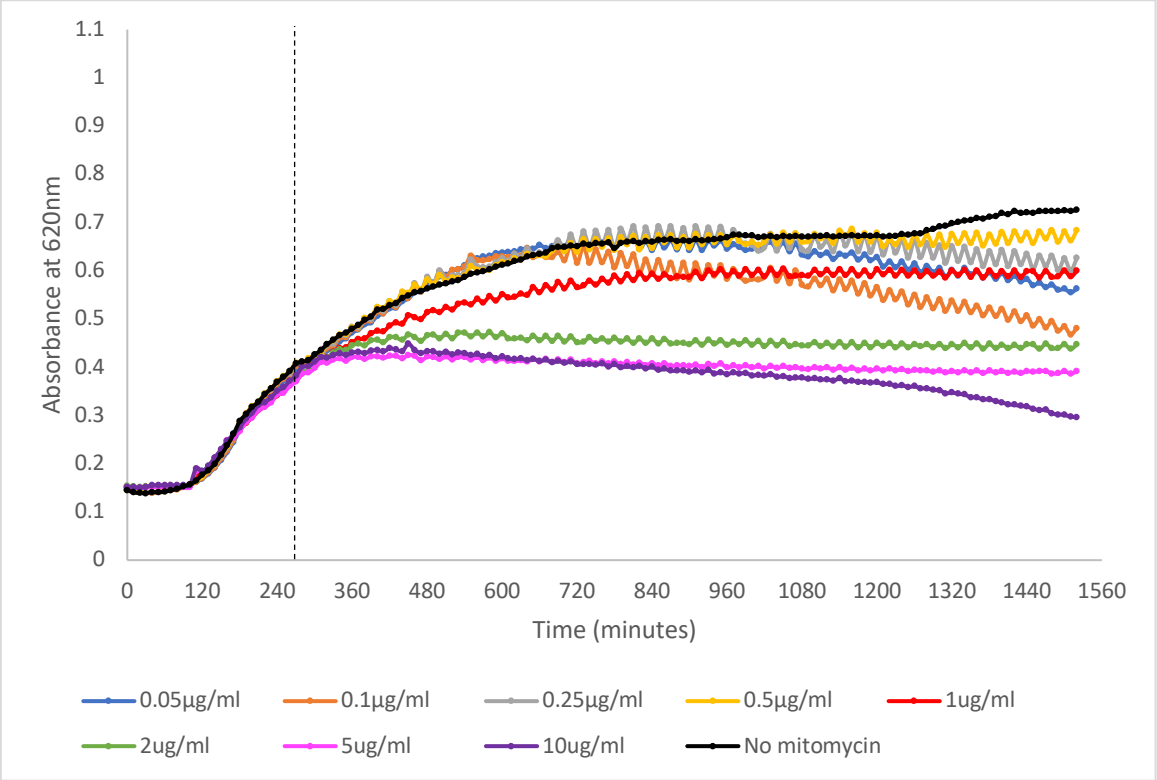

Figure 14. A620 of *P. multocida* isolate 19120602 over 25.5 hours. Each line represents a different concentration of mitomycin-C added to the bacterial broth. The black dashed line indicates when mitomycin-C was added.

4. Discussion

Antimicrobial resistance is a global health challenge that reduces the options for effective prevention and treatment of infections caused by microbes. As a result of emergence and spread of resistant pathogens, diseases that result from bacterial infections are prolonged due to unsuccessful treatment, and there is an increased risk of higher mortality rates. In addition to impacting the welfare of humans and animals, there are substantial economic consequences to the increasing prevalence of AMR (110, 111).

Surveillance of AMR in bacterial pathogens is necessary to ensure available therapies remain effective in treating infections and to track changes in microbial populations. In Australia, there has been limited, although increasing levels of on-going surveillance of animal pathogens. The MIC results from this study have shown relatively low AMR rates in *P. multocida* isolates. The highest level of resistance was to tetracycline with 22.7% of isolates resistant and 22% for chlortetracycline. This is consistent with previous surveillance studies in the Europe, North America and Australia, with only one other Australian study providing antimicrobial susceptibility data for *P. multocida* isolates from pigs (63). Two isolates were resistant to florfenicol and one to ampicillin. Resistance to these antimicrobials has previously been reported in *P. multocida* isolates from Australia, Europe, Korea, North America and Vietnam, however the resistance rates are typically low (18, 63, 78, 80, 87). Antimicrobials of lower importance to human health, such as tetracyclines, penicillins and sulfonamides, are predominantly used to treat bacterial infections in Australian piggeries, therefore it is not unusual to see higher rates resistance to these antimicrobials (66).

A common issue when reporting on antimicrobial susceptibility is many antimicrobials do not have breakpoint data available, limiting effective reporting on the susceptibility of each drug. There were three antimicrobials used in this study that did not have breakpoint or ECOFF values, cefoxitin, neomycin, trimethoprim/sulfamethoxazole. Cefoxitin is categorised of medium importance and is not

registered for use in Australian animals, and all isolates in this study were susceptible to the lowest concentration of cefoxitin tested. Trimethoprim-sulfamethoxazole is also not registered for use in animals, however, other trimethoprim/sulfonamide combinations can be used and have the potential to select for cross resistance to antimicrobials used in humans (65). Of the 273 isolates, 270 were susceptible to the lowest concentration of trimethoprim/sulfamethoxazole that was tested. The other 3 isolates had a MIC of ≥ 1/19 μg/ml. Three surveillance studies have used the resistant breakpoint ≥ 4/76 μg/ml for trimethoprim/sulfamethoxazole, based on the breakpoint values for *Haemophilus influenzae* and *A. pleuropneumoniae* (18, 63, 78). Consequently, if we used this breakpoint for this study, two isolates would be considered resistant. There was greater variance in the MIC results with neomycin, which is commonly used in Australian piggeries to treat enterotoxigenic or enterotoxaemic *E. coli* and *Salmonella* infections in pigs (65, 66). The concentration range tested for neomycin was 0.25 to 64 μg/ml, and 9.5% of the isolates had a minimum inhibitory concentration of ≥64 μg/ml. It is possible that these isolates are resistant to neomycin however due to the lack of available breakpoints or ECOFF values it could not be definitively stated.

None of the isolates tested were multi-drug resistant and they were susceptible to all critically important antimicrobials. The MIC results in this study are similar to those seen in North America, Europe and Australia. On-going monitoring of susceptibility will enable the industry to recognise if resistance spreads and determine if the current treatment options remain effective.

Understanding how bacteria acquire resistance allows us to determine the probability of resistance spreading. Horizontal gene transfer has played a significant role in the evolution and diversification of microbes and allowed for the proliferation of AMR genes in bacteria. Conjugation is a fundamental mechanism for disseminating AMR and virulence genes and this study evaluated the potential for transferral of resistance genes between *E. coli* and *P. multocida* through conjugation. The *E. coli* isolates used in this study have previously demonstrated plasmid-mediated transfer of antimicrobial

resistance genes between different *E. coli* strains (108). The growth of *P. multocida* on AMP and CRO agar plates after mixing with *E. coli* indicates that resistance encoded mobile genetic elements were transferred between the Gram-negative bacteria. However, when the transconjugants were subcultured onto new antibiotic plates, they failed to grow after 72 hours of incubation. This could suggest that the transferred resistance between *E. coli* and *P. multocida* was unstable or the acquired DNA was not functional in the recipient genome.

It is possible that resistance was not transferred to *P. multocida* and that the limited bacterial growth on the TET+AMP and TET+CRO agar plates was a result of the antimicrobials degrading during incubation. The stability of antimicrobials can be affected by temperature, light, pH and the growth medium (112). Assuming that plasmid-mediated conjugation did occur, there are a number of possible explanations for loss of plasmids after transfer (113-115). Maintaining plasmids imposes a fitness costs on the host cells, due to production of proteins and use of cellular machinery and materials (113-116). However, if there is a selective advantage conferred by plasmids, such as the presence of antibiotics, plasmids will typically be maintained despite the metabolic burden (117-119). Plasmids may also be lost if they fail to segregate into a daughter cell during cell division. Plasmids occur in one or more copies in each host cell and the probability of plasmid being lost during segregation is dependent on the copy number of plasmids, a low copy number meaning there is a greater probability of being lost. (117, 119).

Plasmids may face barriers to replication and establishment in unrelated hosts. The recipient cell may have an active DNA restriction system that recognises and destroys foreign DNA (120). Plasmids that have adapted to overcome those barriers have a broader host range. Plasmids of the group IncP-1 typically have a broad host range and are capable of transferring and replicating in virtually all Gram-negative bacteria. The backbone of these plasmids encodes essential genes for effective replication, maintenance and stable transference (120, 121). Other plasmids have a narrower host range, for

example, F-Plasmid of *E. coli* cannot replicate effectively in *Pseudomonas* bacteria due to failure of the replication protein RepE to form a stable complex with the replication enzyme DnaB helicase in *Pseudomonas* (122).

The transconjugation experiment did result in a single case in which tetracycline resistance transferred from *P. multocida* to *E. coli*, either on a plasmid or via an ICE. This transconjugant was identified as *E. coli* via MALDI-TOF and grew well when subcultured onto agar plates with 5 µg/ml tetracycline. The experiment was repeated twice but failed to produce another tetracycline susceptible *E. coli* transconjugant. A previous study has attempted to mobilise the *P. multocida* plasmid pB1000 in an *E. coli* isolate that carried IncP conjugation machinery, however, while the plasmid is highly stable in *P. multocida*, it proved unstable in *E. coli* and the transferred resistance was not maintained (73).

The results of the transconjugation experiments demonstrate that AMR genes present on mobile genetic elements within *E. coli* were not easily transferred to *P. multocida,* providing some indication that the risk horizontal spread of AMR genes from other more resistant bacteria is low. Antimicrobial resistance in *P. multocida* isn't a critical health threat at this current time, nonetheless, minimising antimicrobial use reduces risk of increasing AMR rates. In recent years, there has been a surge of interest for different treatment options, such as phage therapy, to serve as an alternative or supplement to antimicrobial therapy.

Phages can be found in any environment that bacteria exist, however, the attempts to isolate bacteriophages against *P. multocida* from different environments outside of the swine respiratory tract was not successful. A potential explanation for this could be that the environmental sources used were not ideal and isolation of phages may be more likely from a source with a higher concentration of the target bacterium (123). The environmental sources used in the study were swine faecal samples, water samples, effluent and sewage and as *P. multocida* colonises in the upper

respiratory tract, efforts to isolate phages may have been more successful with the use of lung samples or nasopharyngeal washes. However, a virulent phage that demonstrated lytic activity against capsular type A *P. multocida* has previously been isolated from sewage water collected on a swine farm (105). Success in Isolation of phages is also highly dependent on the bacterial species. A 2015 study demonstrated that efforts required to isolate phages from different environmental sources varied greatly depending on the species. The study compared bacteria, noting that phages targeting *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Salmonella* were more readily isolated than phages for *Staphylococcus aureus, Acinetobacter baumannii, Enterococcus faecium and Enterococcus faecalis* (124). There are very few resources available that detail the isolation of phages for *P. multocida* from environmental sources and the results from this study indicate that isolating lytic phages on-demand may not be feasible.

Though lytic phages are preferred for use in phage therapy, temperate phages also have the potential to reduce pathogenicity of bacteria. Temperate phages can be engineered to affect the fitness of the bacterial cells or resensitise resistant bacteria to antimicrobials (125). Temperate phages incorporate into the bacterial genome as prophages and replicate during cell division. Prophages can be induced to enter the lytic cycle through exposure to external stressors such as certain antibiotics, reactive oxygen species, UV-C radiation, changes in nutrients available to the cell and DNA damaging agents (126). In order to induce prophages within the genome of *P. multocida*, seven isolates were exposed to Mitomycin-C, a cytotoxic drug commonly used as a cancer treatment, with potent antibacterial properties. Mitomycin C is an antineoplastic agent that through alkylation can form crosslinks between complementary DNA strands resulting in the inhibition DNA synthesis (127).

The first attempt to induce prophages and demonstrate lytic activity by adding 0.5 µg/ml of Mitomycin-C to bacterial broth for 30 minutes was unsuccessful. Using the microplate reader in the second attempt, visible clearing and declining absorbance demonstrated that Mitomycin-C at

concentrations of 0.05 µg/ml, 0.1 µg/ml and 0.25 µg/ml was optimum in inducing prophages. At higher concentrations of Mitomycin C, the bacteria ceased growing however there was no decline in absorbance, likely due to the toxicity of mitomycin C. Lytic activity was observed in five of the seven isolates, indicating that these strains potentially harbour temperate phages. Isolates 19120401 and 19120602 did not demonstrate a decline in bacterial growth. Clearance of the bacteria can occur at different rates and to varying degrees following prophage induction (128, 129). Of the five isolates, three (19120340, 19120412 and 19120559) rapidly declined in absorbance within of 3 hours mitomycin being introduced, whereas the other two isolates reduced to the same degree but over a longer period of time (14 – 16 hours). It should be noted, in the three cases where rapid cell lysis occurred, bacterial growth gradually recommenced which could indicate a return to lysogeny.

Though visible clearing did occur, the spot test failed to produce and phage plaques. This result does not appear to be unusual, a study of *P. multocida* bacteriophages performed spot tests using 29 phage lysates and reported visible signs of cells lysis from only 38% of the lysates (128). It is possible that the dilution effect on mitomycin of applying the phage lysate to the plate took it below an active concentration, or that cell growth on the plate surface was faster than phage lysis. Further research with a larger sample size may be required to isolate bacteriophages using this method.

There were some limitations in this study that could be addressed, including the lack of breakpoint values for certain antimicrobials. Future research into conjugation between *P. multocida* and other bacteria could optimise the methods used, as there are a number of factors can impact on conjugation rate, such as the donor-recipient ratio, the growth phase of the bacteria, the mating time and the mating method (130). In regard to bacteriophages, using alternative environmental sources, or fresh respiratory tract tissues from abattoir samples for the enrichment of bacteriophages may increase success in isolating phage for *P. multocida* and further study will be required to prove prophage

induction took place, as mitomycin C is also capable of inducing bacteriocins, which are antimicrobial peptides produced by certain bacteria (79).

5. Conclusion

P. multocida isolates from Australian swine do not currently have high levels of resistance and the recommended antimicrobial treatments should remain effective in controlling disease outbreaks. Furthermore, *P. multocida* appears refractory to obtaining AMR genes from *E. coli,* a ubiquitous gatherer of mobile resistance elements, indicating there is a low risk of *P. multocida* rapidly acquiring resistance from other species. However, as *P. multocida* infections can cause serious respiratory diseases, impacting on both the welfare of the animals and the economic cost, ongoing surveillance of antimicrobial susceptibility is necessary to effectively manage and provide timely response to outbreaks of resistant bacteria. In addition to continued monitoring of AMR in pathogenic bacteria, alternative methods of controlling outbreaks need to be available if the current situation worsens. This study demonstrated there may be difficulties in the isolating phages against *P. multocida* through conventional methods. The addition of mitomycin C resulted in the lysis of bacterial cells for 5 of the 7 isolates tested, however, successful isolation of phages may require a larger sample size. To determine the feasibility of phage therapy as an alternative treatment option for *P. multocida* infections, further research could be conducted into the use of alternative source samples for phage enrichment, such as nasopharyngeal washes or lung samples and increasing the number of bacterial isolates used for the isolation of temperate phages.

6. References

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Appendix I

AMRID ID	Farm Code	Site of isolation	Date of isolation
19120332	38	Not known	20/1/14
19120333	38	Not known	20/1/14
19120334	38	Not known	20/1/14
19120335	38	Not known	20/1/14
19120336	38	Not known	20/1/14
19120337	38	Not known	20/1/14
19120338	38	Not known	20/1/14
19120339	38	Not known	20/1/14
19120340	4	Fresh lung	24/1/14
19120341	16	Lung swab	30/1/14
19120342	16	Lung swab	30/1/14
19120343	35	Fresh Lung	30/1/14
19120344	16	Lung swab	3/2/14
19120345	16	Lung swab	4/2/14
19120346	16	Lung swab	4/2/14
19120347	10	Fresh Lung	11/2/14
19120348	10	Tracheal swab	11/2/14
19120349	21	Lung swab	17/2/14
19120350	21	Lung swab	17/2/14
19120351	21	Lung swab	17/2/14
19120352	21	Lung swab	17/2/14
19120353	21	Lung swab	17/2/14
19120354	10	Tracheal swab	21/2/14
19120355	10	Lung swab	21/2/14
19120356	21	Fresh Lung	24/2/14
19120357	18	Fresh Lung	11/3/14
19120358	21	Lung swab	18/3/14
19120359	6	Lung swab	21/3/14
19120360	21	Lung swab	1/4/14
19120361	21	Lung swab	6/5/14

19120362	21	Lung swab	6/5/14
19120363	21	Lung swab	6/5/14
19120364	4	Fresh lung	12/5/14
19120365	21	Lung swab	11/7/14
19120366	10	Lung swab	28/7/14
19120367	10	Tracheal swab	28/7/14
19120368	10	Tracheal swab	28/7/14
19120369	10	Lung swab	28/7/14
19120370	21	Lung swab	18/8/14
19120371	21	Lung swab	18/8/14
19120372	21	Lung swab	18/8/14
19120373	21	Lung swab	18/8/14
19120374	21	Lung swab	18/8/14
19120375	21	Lung swab	18/8/14
19120376	4	Fresh lung	22/8/14
19120377	74	Fresh Lung	10/9/14
19120378	74	Fresh Lung	10/9/14
19120379	74	Fresh Lung	10/9/14
19120380	74	Fresh Lung	10/9/14
19120381	20	Lung swab	22/9/14
19120382	23	Fresh Lung	13/10/14
19120383	23	Fresh Lung	13/10/14
19120384	21	Lung swab	2/12/14
19120385	21	Lung swab	2/12/14
19120386	21	Lung swab	2/12/14
19120387	21	Lung swab	2/12/14
19120388	35	Lung swab	18/12/14
19120389	54	Not known	19/12/14
19120390	54	Not known	19/12/14
19120391	21	Lung swab	24/12/14
19120392	38	Fresh Lung	2/1/15
19120393	38	Fresh Lung	2/1/15
19120394	38	Fresh Lung	2/1/15

19120395	38	Fresh Lung	2/1/15
19120396	56	Fresh Lung	19/1/15
19120397	56	Fresh Lung	19/1/15
19120398	56	Fresh Lung	19/1/15
19120399	35	Fresh Lung	30/1/15
19120400	28	Fresh lung	10/2/15
19120401	39	Fresh Lung	17/2/15
19120402	39	Fresh Lung	17/2/15
19120403	4	Fresh lung	19/2/15
19120404	4	Fresh Lung & Heart	19/2/15
19120405	4	Fresh Lung	19/2/15
19120406	4	Fresh Lung	19/2/15
19120407	21	Lung swab	23/2/15
19120408	21	Lung swab	23/2/15
19120409	64	Lung swab	21/3/15
19120410	64	Lung swab	26/3/15
19120411	14	Lung swab	14/4/15
19120412	14	Lung swab	14/4/15
19120413	4	Fresh Lung	27/4/15
19120414	4	Fresh Lung	27/4/15
19120415	4	Fresh Lung	27/4/15
19120416	4	Fresh Lung	30/4/15
19120417	4	Fresh Lung	30/4/15
19120418	4	Fresh Lung	30/4/15
19120419	4	Fresh Lung	30/4/15
19120420	21	Lung swab	5/5/15
19120421	21	Lung swab	5/5/15
19120422	4	Fresh Lung	6/5/15
19120423	37	Fresh Lung	25/5/15
19120424	39	Fresh Lung	2/6/15
19120425	39	Fresh Lung	2/6/15
19120426	4	Fresh Lung	3/6/15
19120427	4	Fresh Lung	3/6/15

19120428	41	Fresh Lung	10/6/15
19120429	4	Fresh Lung	29/6/15
19120430	21	Fresh Lung	6/7/15
19120431	21	Fresh Lung	6/7/15
19120432	21	Fresh Lung	6/7/15
19120433	21	Fresh Lung	6/7/15
19120434	21	Fresh Lung	6/7/15
19120435	21	Fresh Lung	6/7/15
19120436	21	Fresh Lung	6/7/15
19120437	21	Fresh Lung	6/7/15
19120438	20	Abdominal swab	7/7/15
19120439	45	Fresh Lung	24/7/15
19120440	55	Fresh Lung	24/7/15
19120441	55	Fresh Lung	24/7/15
19120442	21	Lung swab	24/7/15
19120443	4	Fresh Lung	3/9/15
19120444	47	Fresh Lung	11/9/15
19120445	29	Fresh Lung	30/10/15
19120446	21	Lung swab	19/11/15
19120447	55	Fresh Lung	20/11/15
19120448	55	Fresh Lung	20/11/15
19120449	21	Lung swab	1/12/15
19120450	12	Lung swab	7/12/15
19120451	10	Lung swab	18/12/15
19120452	21	Fresh Lung	2/2/16
19120453	21	Fresh Lung	2/2/16
19120454	17	Fresh Lung	11/2/16
19120455	35	Fresh Lung	22/2/16
19120456	21	Lung swab	22/2/16
19120457	21	Lung swab	22/2/16
19120458	21	Lung swab	3/4/16
19120459	21	Heart	9/5/16
19120460	21	Heart	9/5/16

19120461	21	Heart	12/5/16
19120462	21	Heart	13/5/16
19120463	21	Heart	14/5/16
19120464	68	Heart	19/5/16
19120465	68	Pericardial swab	19/5/16
19120466	17	Fresh Lung	20/5/16
19120467	17	Fresh Lung	23/5/16
19120468	26	Fresh Lung	15/6/16
19120469	4	Fresh Lung	30/6/16
19120470	69	Not known	11/7/16
19120471	69	Not known	11/7/16
19120472	30	Fresh Lung	8/8/16
19120473	30	Fresh Lung	8/8/16
19120474	32	Fresh Lung	18/8/16
19120475	2	Fresh Lung	22/8/16
19120476	41	Fresh Lung	22/8/16
19120477	30	Fresh lung	12/9/16
19120478	27	Lung swab	14/10/16
19120479	27	Lung swab	14/10/16
19120480	59	Fresh Lung	16/10/16
19120481	2	Fresh lung	24/10/16
19120482	7	Fresh lung	24/10/16
19120483	7	Fresh lung	24/10/16
19120484	7	Fresh lung	24/10/16
19120485	50	Fresh lung	27/10/16
19120486	7	Fresh lung	7/11/16
19120487	7	Fresh lung	7/11/16
19120488	7	Fresh lung	7/11/16
19120489	35	Fresh Lung	14/11/16
19120490	35	Fresh Lung	14/11/16
19120491	35	Fresh Lung	14/11/16
19120492	3	Lung swab	15/11/16
19120493	3	Lung swab	15/11/16

19120494	67	Fresh Lung	21/11/16
19120495	21	Lung swab	24/11/16
19120496	57	Fresh Lung	1/12/16
19120497	51	Heart + Lung	6/12/16
19120498	51	Heart swab	6/12/16
19120499	51	Lung swab	6/12/16
19120500	25	Fresh Lung	28/12/16
19120501	40	Fresh Lung	28/12/16
19120502	25	Fresh Lung	29/12/16
19120503	8	Fresh lung	29/12/16
19120504	21	Fresh Lung	24/1/17
19120505	73	Fresh Lung	27/1/17
19120506	73	Fresh Lung	27/1/17
19120507	73	Fresh Lung	27/1/17
19120508	73	Fresh Lung	27/1/17
19120509	62	Fresh Lung	7/3/17
19120510	5	Fresh lung	13/4/17
19120511	5	Fresh lung	13/4/17
19120512	21	Lung swab	3/5/17
19120513	17	Fresh Lung	2/6/17
19120514	36	Lung Swab	14/6/17
19120515	36	Lung Swab	14/6/17
19120516	36	Lung Swab	14/6/17
19120517	42	Lung swab	20/6/17
19120518	22	Fresh Lung	27/6/17
19120519	35	Fresh Lung	27/6/17
19120520	35	Fresh Lung	27/6/17
19120521	2	Fresh lung	3/7/17
19120522	2	Fresh lung	3/7/17
19120523	61	Brainstem swab	15/8/17
19120524	61	Lung swab	15/8/17
19120525	16	Lung swab	23/8/17
19120526	16	Lung swab	23/8/17

19120527	16	Lung swab	23/8/17
19120528	53	Fresh Lung	23/8/17
19120529	53	Fresh Lung	23/8/17
19120530	17	Fresh lung	23/8/17
19120531	71	Fresh lung	17/9/17
19120532	21	Lung swab	19/9/17
19120533	21	Lung swab	19/9/17
19120534	21	Lung swab	10/10/17
19120535	21	Lung swab	16/10/17
19120536	21	Lung swab	16/10/17
19120537	21	Lung swab	16/10/17
19120538	21	Lung swab	16/10/17
19120539	30	Fresh Lung	17/10/17
19120540	65	Fresh Lung	20/10/17
19120541	2	Lung abscess	31/10/17
19120542	2	Fresh lung	31/10/17
19120543	49	Fresh Lung	14/11/17
19120544	49	Fresh Lung	14/11/17
19120545	68	Fresh Lung	14/11/17
19120546	30	Fresh Lung	7/12/17
19120547	30	Fresh Lung	7/12/17
19120548	2	Fresh lung	7/12/17
19120549	2	Fresh lung	7/12/17
19120550	68	Fresh Lung	22/12/17
19120551	21	Lung swab	22/12/17
19120552	30	Fresh Lung	4/1/18
19120553	21	Fresh Lung	1/2/18
19120554	2	Fresh lung	13/2/18
19120555	21	Lung swab	14/2/18
19120556	60	Fresh Lung	23/2/18
19120557	72	Fresh Lung	28/2/18
19120558	72	Fresh Lung	28/2/18
19120559	72	Brainstem swab	28/2/18

19120560	46	Spleen	28/2/18
19120561	21	Lung swab	28/2/18
19120562	21	Lung swab	28/2/18
19120563	21	Lung swab	28/2/18
19120564	44	Fresh lung	2/3/18
19120565	44	Abdominal swab	2/3/18
19120566	44	Fresh Lung	2/3/18
19120567	44	Lung swab	2/3/18
19120568	13	Lung swab	20/3/18
19120569	13	Fresh lung	20/3/18
19120570	13	Lung swab	20/3/18
19120571	9	Lung swab	2/4/18
19120572	66	Lung swab	11/4/18
19120573	45	Abdominal swab	11/4/18
19120574	15	Lung swab	11/4/18
19120575	11	Lung swab	3/5/18
19120576	11	Lung swab	3/5/18
19120577	11	Lung swab	3/5/18
19120578	56	Fresh Lung	18/5/18
19120579	56	Fresh Lung	18/5/18
19120580	71	Fresh Lung	25/5/18
19120581	24	Fresh Lung	6/6/18
19120582	24	Fresh Lung	6/6/18
19120583	48	Fresh Lung	15/6/18
19120584	34	Fresh Lung	15/6/18
19120585	34	Hock bursa	15/6/18
19120586	34	Lung swab	20/6/18
19120587	34	Fresh Lung	21/6/18
19120588	43	Lung swab	1/8/18
19120589	63	Fresh Lung	30/8/18
19120590	63	Fresh Lung	30/8/18
19120591	19	Fresh Lung	7/9/18
19120592	34	Gut abscess	11/9/18

19120593	34	Fresh lung	11/9/18
19120594	34	Tendon sheath swab	13/9/18
19120595	34	Hock swab	15/9/18
19120596	1	Fresh tonsil	15/9/18
19120597	19	Fresh Lung	19/10/18
19120598	19	Fresh Lung	19/10/18
19120599	25	Fresh Lung	19/10/18
19120600	33	Fresh Lung	27/11/18
19120601	21	Fresh Lung	6/12/18
19120602	58	Fresh Lung	4/1/19
19120603	30	Fresh Lung	13/1/19
19120604	30	Fresh Lung	13/1/19
19120605	30	Fresh Lung	24/1/19
19120606	44	Fresh Lung	29/1/19
19120607	44	Fresh Lung	29/1/19
19120608	44	Fresh Lung	29/1/19
19120609	52	Kidney + Lung	29/1/19
19120610	33	Fresh Lung	25/2/19
19120611	56	Lung swab	7/3/19
19120612	56	Lung swab	7/3/19
19120613	56	Lung swab	7/3/19
19120614	56	Lung swab	7/3/19
19120615	31	Fresh Lung	16/3/19
19120616	31	Lung swab	16/3/19
19120617	17	Nasal swab	29/3/19
19120618	44	Mediastinal lymph node + Lung	26/4/19
19120619	44	Abdominal swab	26/4/19
19120620	44	Mediastinal lymph node + Lung	26/4/19
19120935	Not known	Not known	17/12/19
19120936	Not known	Not known	17/12/19
19120937	Not known	Not known	17/12/19

Appendix II

Reagents

5% sheep blood agar	Micromedia, Australia
Ampicillin	Sigma-Aldrich, Australia
BBL™ Agar, Grade A	BD Worldwide, USA
Brain Heart Infusion broth	Thermo Fisher Scientific, Australia
Meuller Hinton broth	Thermo Fisher Scientific, Australia
Cefoxitin	Sigma-Aldrich, Australia
Ceftiofur	Sigma-Aldrich, Australia
Chlortetracyline	Sigma-Aldrich, Australia
Ciprofloxacin	Sigma-Aldrich, Australia
Difco™ Luria Bertani Broth	BD Worldwide, USA
Distilled water	
Florfenicol	Sigma-Aldrich, Australia
Gamithromycin	BioAustralis, Australia
Gelatine	Ajax Finechem, Australia
Gentamicin	Sigma-Aldrich, Australia
Glycerol	Chem-Supply
Luria Bertani Broth	
Magnesium sulfate	Univar
Mitomycin-C	Sigma-Aldrich
Mueller-Hinton Broth	Thermo Fisher Scientific, Australia
Neomycin	Sigma-Aldrich, Australia
Sodium chloride	Sigma-Aldrich, Australia
Sulfamethoxazole	Sigma-Aldrich, Australia
Tetracycline	Sigma-Aldrich, Australia
Tilmicosin	Sigma-Aldrich, Australia
Trimethoprim	Sigma-Aldrich, Australia
Trizma-Hydrochloride	Sigma-Aldrich, Australia
Tryptic Soy Broth	Thermo Fisher Scientific, Australia
Tulathromycin	Sigma-Aldrich, Australia

Equipment and materials

0.22 µm filter unit	Pall Corporation, USA
0.45 µm filter unit	Pall Corporation, USA
1 µl loops	Copan
1.5 mL Eppendorf tubes	Hurst Scientific
10 µl loops	Copan
15 mL FALCON tubes	Thermo Fisher Scientific, Australia
50 mL FALCON tubes	Thermo Fisher Scientific, Australia
Nunc™ 96-well polystyrene flat bottom microtitre plate	Thermo Fisher Scientific, Australia
96-well, 2 ml polypropylene deep well plate	Corning Life Sciences, USA
Centrifuge	Thermo Fisher Scientific, Australia
Eutech™ pH 700 meter	Thermo Fisher Scientific, Australia
MALDI-TOF biotyper	Bruker
Microplate reader	Tecan
Multi-channel pipette, pipette, pipette tips	Thermo Fisher Scientific, Australia
Petri dishes	Thermo Fisher Scientific, Australia
Robotic liquid handling system, EVO 150	Tecan
Syringe, without needle	Terumo, Australia
Vortex	Edwards, Australia