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Antimicrobial Resistance Surveillance of Bovine Respiratory Disease Pathogens

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Executive summary

Bovine respiratory disease (BRD) is the most important infectious disease affecting feedlot cattle and the main indicator for therapeutic use of antimicrobials in the feedlot industry. BRD costs the Australian feedlot industry > $40 million annually. Initiated by a complex of viruses, secondary bacterial infection results in pneumonia causing high mortality if untreated. The main bacterial causes of BRD are *Mannheimia haemolytica, Pasteurella multocida*, *Histophilus somni, Trueperella pyogenes* and *Mycoplasma bovis*. Antimicrobial agents used to treat BRD in Australia include tulathromycin, tetracyclines (oxytetracycline and chlortetracycline), tilmicosin and ceftiofur. Internationally, antimicrobial resistance (AMR) has rapidly developed in BRD pathogens in North America due to the widespread dissemination of mobile genetic elements called integrative conjugative elements (ICEs) containing multiple resistance genes. Only two previous studies (1993 and 2014), on AMR in BRD pathogens have been conducted in Australia and found negligible levels of resistance. The current study was undertaken to obtain baseline antimicrobial susceptibility data on existing and prospective collections of Australian BRD isolates, develop guidelines and training materials for the collection of diagnostic specimens from cases of BRD, undertake case sampling studies at eight Australian feedlots, and perform genetic testing of selected isolates using whole genome sequencing.

A guide to post-mortem procedure and aseptic sampling from suspected BRD cases was developed in conjunction with feedlot veterinarians and trialled at a feedlot (50 post-mortems in 2018) for further refinement. Sample and sundry packs containing additional equipment required by feedlots to obtain samples aseptically were then designed to facilitate collection during the 2019 Australia-wide targeted surveillance study. BRD isolates submitted to Veterinary Diagnostic Laboratories from 2014-2019 (passive surveillance) and the 2019 targeted surveillance were subjected to antimicrobial susceptibility testing with the most significant pathogens (*P. multocida* and *M. haemolytica*) also subjected to whole genome sequenced to identify resistance genes and enable international comparison. A total of 358 BRD pathogen isolates, not including *Mycoplasma*, were identified and susceptibility tested. In the 2019 collection, *P. multocida* was most frequently isolated (65 isolates, 38%), followed by *H. somni* (35 isolates, 20%), *T. pyogenes* and *M. haemolytica* (both 33 isolates; 19%), and *Bibersteinia trehalosi* (5 isolates; 3%). Mixed infections with one or more pathogens were common and *Mycoplasma* infections either as a sole agent or in mixed infections were significant (e.g. 27/47 Qld submissions; 22/32 northern NSW submissions).

From the total (2014 to 2019) collection*, M. haemolytica* isolates (n = 88) were susceptible to all tested antimicrobials except for a single 2019 isolate resistant to macrolides. *H. somni* isolates (n = 70) were susceptible to all tested antimicrobials, as were *Mycoplasma bovis* isolates (n = 50), apart from three isolates each resistant to tetracycline and tilmicosin. Resistance was most prevalent in *P. multocida* isolates (n = 140) and was first detected in 2016/2017. In 2019, 15 of 65 isolates (23.1%) were resistant to macrolides. A further three isolates from Qld possessed an aminopenicillin-tetracycline-tilmicosin resistance phenotype, first identified in 2016-2018 isolates. Eleven isolates (16.9%) were resistant to tetracycline, six of which were also resistant to macrolides. Whole genome sequencing of *P. multocida* and *M. haemolytica* isolates identified the linked genes *msr(E)* and *mph(E)* encoding macrolide resistance and *tet(R)-tet(H)* or *tet(Y)* encoding tetracycline resistance. Furthermore, isolates with an aminopenicillin-tetracycline-tilmicosin resistance phenotype possessed the bla_{ROB-1} ß-lactamase gene and *tet(R)-tet(H)*. The basis of tilmicosin resistance in these isolates could not be determined by whole genome sequencing. Further long range DNA sequencing is required to fully determine the mechanisms contributing to this resistance profile as well as identify the genetic context of resistance to tulathromycin and tetracycline.

Whilst resistance levels are low by international standards, the results confirm the first emergence of AMR to frontline antimicrobials used to treat BRD in Australia, which should be closely monitored. Feedlots are now equipped to conduct their own local AMR surveillance and integrate the findings into their antimicrobial stewardship programmes. Rotation of antimicrobials could be considered along with non-antimicrobial management and husbandry practices to limit AMR emergence and spread.

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1 Background

Antimicrobial resistance (AMR) has been recognised as a global threat to human and animal health, with bodies such as the World Health Organisation (WHO) and the World Organisation for Animal Health (Office International des Epizooties [OIE]) calling on all nations to take urgent action to address the growing threat. Whilst the list of human use only drug classes will continue to grow as new drugs are discovered and developed, many of the traditional drug classes that are the mainstay of antimicrobial therapy are currently registered as both human and animal treatments (the so called "shared drug classes"). For the shared drug classes, harmonization of prescribing practices between human and animal health, reducing total antibiotic use as well as limiting the use of critically important drug classes through adoption of prudent use guidelines, and regular monitoring of AMR through surveillance, are designed to maintain the lifespan of shared drug classes whilst new classes are developed. It is important that the Australian feedlot industry understands and adapts to this new environment and adopts antimicrobial stewardship principles that allow it to continue to treat and prevent bacterial infections in animals with confidence. It should actively encourage AMR surveillance to continue to assure the public that its products have the highest standards of animal welfare and food safety with minimal impact on the environment.

Bovine respiratory disease (BRD) is the most important infectious disease affecting feedlot cattle and the main indicator for therapeutic use of antimicrobials in the feedlot industry. BRD has been estimated to cost the Australian feedlot industry in excess of $40 million annually. Initiated by a complex of viruses (bovine herpes virus, bovine respiratory syncytial virus, bovine parainfluenza virus and bovine viral diarrhoea virus), secondary bacterial infection by commensals of the upper airway quickly follows, resulting in pneumonia causing high mortality if untreated. Hence early detection and treatment with appropriate antimicrobials is essential for optimal production. The main bacterial causes of BRD are *Mannheimia haemolytica, Pasteurella multocida*, *Histophilus somni* and *Mycoplasma bovis*, with *M. haemolytica* being the most important. *Trueperella pyogenes* is also a relatively common opportunistic invader of the lung parenchyma. Many feedlot cattle in Australia are vaccinated against *M. haemolytica* and bovine herpes virus, but no vaccines exist for the other bacterial causes other than autogenous vaccines. The macrolide tulathromycin (Draxxin, classified as a second line agent by the Australian Veterinary Association (AVA)), is the mainstay of treatment of BRD in Australian feedlots often being used as a primary treatment of the disease. Oxytetracycline (Engemycin, classified as a first line treatment by the AVA) can also be used in the treatment of BRD, but is not considered to be as clinically effective as Draxxin, and is often used as a secondary line of treatment by feedlot consulting veterinarians. The extended-spectrum cephalosporin ceftiofur (Excenel or Excede, classified as a third line treatment by the AVA) is used for non-responders to second line treatment particularly when a shorter withholding period is required to meet strict export slaughter intervals. Some Australian feedlots use chlortetracycline in the feed as a metaphylactic treatment in severe outbreaks or for high risk cattle. Some also use tilmicosin (Micotil, classified as a first line treatment by the AVA) for individual animal treatment or as a metaphylactic treatment for high risk cattle.

To determine the antimicrobial susceptibility profile of BRD isolates, samples cannot be taken from the live animal, they must be collected aseptically from the lung lesions during post-mortem. Whilst some studies have used nasopharyngeal swabs from live animals these are not usually

representative of the organisms causing disease in the lower respiratory tract. Conducting post-mortems on all untreated or treated pen deaths and/or humane euthanasia cases is undertaken at Australian feedlots, but it is labour intensive and technically demanding to include regular collection and sampling of BRD specimens without adequate training.

1.1 Previous studies

1.1.1 International studies

Lubbers and Turnidge (2015) reviewed antimicrobial susceptibility testing and bovine respiratory disease and the challenges posed by this particular veterinary setting in terms of accurate diagnostic sampling to yield relevant isolates. They concluded, that rather than be guided by individual culture and susceptibility results, bovine feedlot practitioners should develop "cumulative antibiograms" of their herds (obtaining a number of post-mortem isolates during peak times when bovine respiratory disease is prevalent [in particular from treatment failures] and comparing susceptibility profiles across years to detect emerging resistance).

Garch et al. (2016) monitored antimicrobial susceptibility in bovine respiratory pathogens (*Mannheimia, Histophilus* and *Pasteurella*) obtained from European cattle between 2009-2012. They concluded that the majority of pathogens remained susceptible to registered drugs apart from a low to moderate level of resistance to tetracycline (3.0-12.0%) and emerging resistance to macrolides (0–4.0%).

In a risk factors study in Canadian feedlots, Noyes et al (2015) concluded that the identification of resistant isolates among bovine respiratory disease pathogens was relatively rare. Nevertheless, exposure to antimicrobial drugs in pen mates was associated with increased odds of recovering multidrug-resistant *M. haemolytica*.

Dedonder and Apley (2015) reviewed the literature documenting resistance in bovine respiratory pathogens in the US and identified 16 articles where resistance was reported. Studies between 1994 and 2008 confirmed the trend of low levels of cross-resistance among the macrolides, fluctuating levels of resistance to tetracycline, but uniform susceptibility to florfenicol, ceftiofur and fluoroquinolones. Lubbers and Hanzlicek (2013) examined the prevalence of resistance among BRD pathogens from submissions to the Kansas State University Veterinary Diagnostic Laboratory and identified an alarming trend of increasing MDR between 2009 (42% of isolates) and 2011 (63% of isolates). By 2011, a total of 25% of the isolates were resistant to four of six antimicrobials, with only ceftiofur and florfenicol showing uniform susceptibility. The genes associated with macrolide resistance in BRD isolates have been identified as *erm*, *msr* and *mph*.

Anholt et al. (2017) conducted an AMR surveillance study on bovine BRD isolates in Alberta, Canada and concluded that compared to previous studies, an increasing trend of resistance in BRD pathogens against the antimicrobials used to manage the disease in Alberta. Alarmingly, multidrug resistance was high in all target pathogens with 47.2% of the isolates resistant to four or five antimicrobial classes and 24.0% resistance to six to nine classes.

Increased resistance to multiple agents in international studies is attributed to the widespread movement of integrative conjugative elements (ICEs) among BRD isolates. In 2012, the first ICE

ICEPmu1 was identified in a *P. multocida* isolate from a case of bovine respiratory disease (Michael et al., 2012a,2012b). ICEs are mobile DNA segments that can accumulate multiple AMR genes and integrate into the bacterial chromosome at very specific sites. ICEPmu1 contains 12 AMR genes including genes imparting resistance to macrolides, florfenicol, aminoglycosides, sulphonamides, amoxycillin and tetracyclines and similar ICEs have subsequently been identified in *M. haemolytica* and *H. somni* indicating cross-species transfer. Isolates containing these ICEs are often resistant to all drugs registered for the treatment of bovine respiratory disease (in North America) except ceftiofur and fluoroquinolones. This presents a very concerning trend in beef feedlot medicine, as it is possible for an isolate to move from full susceptibility to resistant to nearly all possible treatment choices in a single genetic event. Klima et al (2014) investigated BRD mortalities in feedlots in Canada, Texas and Nebraska and concluded that over one third of the US isolates were resistant to more than seven antimicrobial classes, including aminoglycosides, penicillins, fluoroquinolones, lincosamides, macrolides, pleuromutilins, and tetracyclines. Nearly all these isolates possessed an ICE, however the isolates were not clonally related, indicating movement of similar ICEs among distinct isolates rather than dominance of one particular sub-type.

1.1.2 Australian studies

There have been few studies undertaken in Australia specifically on AMR of bovine respiratory disease isolates. Stephens (2003) reported that 25/25 *Mannheimia haemolytica* and 24/25 *Pasteurella multocida* were fully susceptible to tilmicosin based on available breakpoints at the time. To the best of our knowledge no further published studies have been undertaken on *M. haemolytica* or *P. multocida*. Recently Goldspink et al (2014) examined the antimicrobial susceptibility patterns of 53 *Histophilus somni* isolates originating from feedlot cattle, with 51 isolates originating from bovine respiratory disease and one isolate each from cases of thrombotic meningioencephalitis and vaginitis. The isolates were tested for susceptibility to ceftiofur, enrofloxacin, florfenicol, tetracycline, tilmicosin and tulathromycin, using Clinical Laboratory Standards Institute (CLSI) disc diffusion and minimum inhibitory concentration testing. However tulathromycin MIC testing was only performed for 43 isolates. All isolates were susceptible to all six antimicrobial agents, except for a single tetracycline-resistant isolate. No other Australian studies have been undertaken.

Whilst this does not provide evidence that all BRD bacterial isolates from Australian feedlot cattle are pan-susceptible as there are many reasons why an antimicrobial treatment could fail in addition to bacterial resistance, it does suggest that there has been relatively little incentive to further investigate isolate resistance profiles. However, the recent McDonald's paper on Antimicrobial Use Policy for Beef and Dairy Beef mandates that feedlots conduct their own AMR surveillance on BRD pathogens to guide their antimicrobial choices for the treatment of BRD.

Given the paucity of data on Australian BRD isolates, the present study was undertaken to:

1) Establish protocols for conducting aseptic sampling of BRD cases and submission of samples for culture of BRD pathogens
2) Form collaborative relationships with Veterinary Diagnostic Laboratories servicing the feedlot industries to supply BRD isolates to establish an Australia-wide collection
3) Conduct antimicrobial susceptibility testing on BRD isolates over three time periods (2014-2017; 2018; 2019) to identify resistance trends
4) Submit isolates for whole genome sequencing to identify the genetic basis of any resistance identified and the genetic relationships of Australian isolates to international isolates.

2 Project objectives

2.1 Obtain baseline antimicrobial susceptibility data on existing and prospective collections of Australian BRD isolates including *Mycoplasma bovis*.

2.2 Develop guidelines and training materials for the collection of diagnostic specimens from cases of BRD for culture and susceptibility testing.

2.3 Undertake case sampling studies at eight Australian feedlots that differ in their antimicrobial treatment regimes during 2018/2019 BRD seasons to link clinical, epidemiological and pathological data with microbiological culture and susceptibility testing.

2.4 Undertake genetic testing of selected isolates using whole genome sequencing to compare with international BRD isolates and conduct a molecular-based risk assessment on the likelihood of Australian BRD isolates acquiring resistance and in particular ICEs.

2.5 Hold an ACARE workshop in late 2019 (sponsored by the pharmaceutical industry and major research stakeholders) at project completion to disseminate project findings (with Dr Brian Lubbers Kansas State University as the Keynote Speaker) and develop Tips and Tools brochures for distribution.

3 Methodology

3.1 Design of training materials and sample submission packs

A guide to post-mortem procedure and aseptic sampling from suspected BRD cases was developed in conjunction with feedlot veterinarians and trialled at Feedlot S1 in 2018 for further refinement (Appendix 9.1). A total of 50 post-mortems were undertaken to refine the technique. Post-mortems were facilitated by the use of a reciprocating saw to remove the rib cage and expose the damaged lung. The ideal sample was obtained from the junction of healthy and damaged tissue. Following sampling of one lung side, it was important to remove the entire lungs to determine if sampling needed to be undertaken on the other side.

Sample packs (Fig. 1; shipped to each participating feedlot) and sundry packs (containing additional equipment required by the feedlot to obtain samples aseptically) were then designed to facilitate collection of samples during the 2019 Australia-wide study. Individually numbered and labelled sample packs contained gauze (for wiping the lung with ethanol prior to sampling), two sterile swabs and transport media (for obtaining swab samples of affected tissue from two different regions of the lung if required), a yellow topped sample container (for a formalin fixed sample of the affected lung for histopathology), and two whirl packs (for a fresh tissue sample of the affected lung). It was requested that the animal ID also be provided for each sample obtained to enable tracking of treatment histories as required.

Following exposure of the affected lung, the lung surface (junction of normal and diseased tissue) was swabbed with ethanol soaked gauze and cut with a sterile scalpel blade. A swab was then aseptically inserted deep into the tissue, placed immediately in transport media and sealed. The scalpel was then used to obtain a fresh tissue sample that was doubly sealed in two whirl packs and

placed in a hard receptacle. The fresh tissue sample facilitates the isolation of *Mycoplasma* and also provides the Diagnostic Microbiologist at the referring veterinary diagnostic laboratory with another chance to isolate BRD pathogens if the swab samples were contaminated. Finally, a second fresh tissue sample was placed in the yellow topped sample container and 10% buffered formalin added. The sample pack was then immediately stored at 4°C, whilst the sample in formalin was allowed to fix at room temperature for 24hr before being placed back in the sample bag.

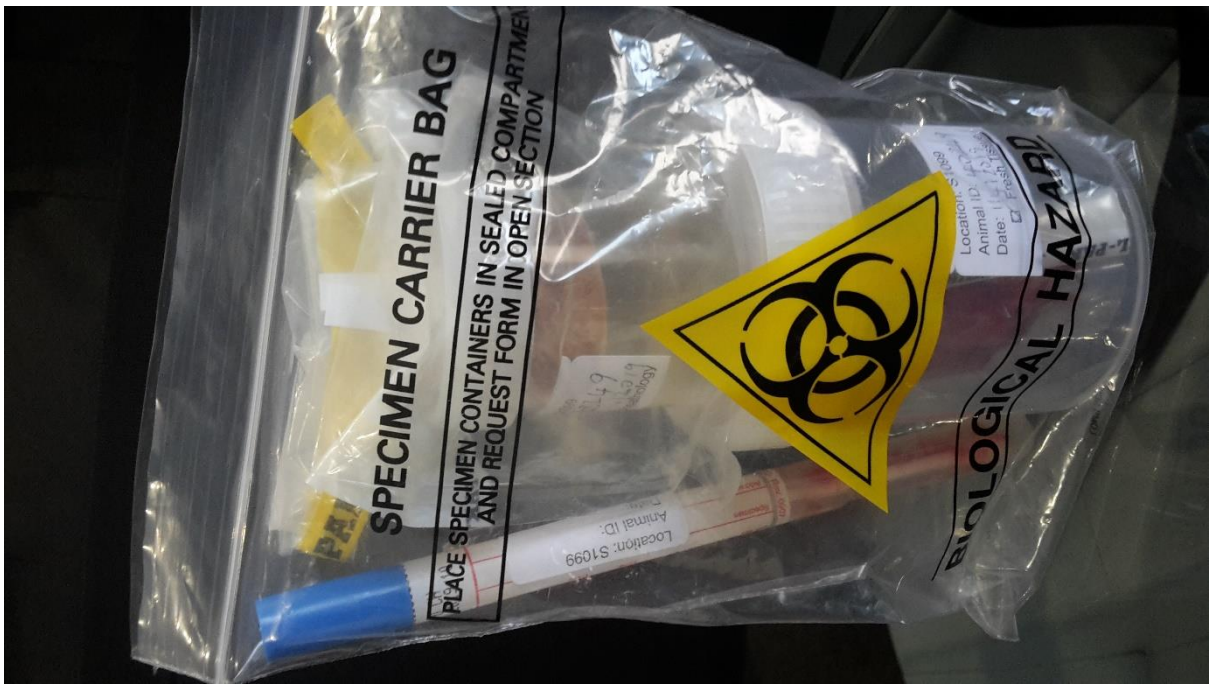

Fig. 1: A sample pack containing samples of fresh and fixed lung tissue.

3.2 Collaboration with feedlot veterinarians, recruitment of feedlots and veterinary diagnostic laboratories

Feedlots recruited for the targeted surveillance are listed in Appendix 9.2 (removed from final version as lists confidential information). Prof Darren Trott, Dr Manouchehr Khazandi and Dr Emilie Flattot visited nine feedlots and four veterinary diagnostic laboratories (VDL) in April 2019 to coordinate sample collection, transport and receipt by the VDLs. A total of 11 feedlots were provided with sample packs with samples consistently obtained from Q1-3; N1-2 and S1 throughout the 2019 BRD season. N4 provided samples at the commencement and conclusion of the study and N3 at the conclusion of the study, whilst V1 provided a single sample which grew a *Mycoplasma*. S2 and S3 also dropped out of the study without submitting samples. Sampling was undertaken collaboratively with feedlot veterinarians servicing each feedlot who were responsible for teaching sampling technique to each of the animal health crews.

3.3 Sampling procedure, pooling of samples and transport

During the pilot study at Feedlot S1 in 2018, the amount of time samples could be pooled and stored (to avoid excessive courier costs) was determined.

3.4 Isolation, identification and storage of bovine respiratory disease pathogens

3.4.1 Mannheimia, Pasteurella, Histophilus, Bibersteinia, Trueperella

Each veterinary diagnostic laboratory followed their standard operating procedures for isolation and identification of BRD pathogens. Isolates were pooled and transported to the ACARE Antimicrobial Resistance Laboratory in single shipments at the end of the BRD season in September-December 2019.

For *Mannheimia, Pasteurella* and *Bibersteinia* isolates, swab samples containing pure cultures of the organisms were inoculated directly onto Sheep Blood agar (SBA-Thermofisher Scientific) and incubated aerobically at 37˚C for 48 hours. For *Histophilus*, swabs were inoculated onto Chocolate Agar incubated in a 5% CO_2 atmosphere at 37˚C for 48 hours. *Trueperella* swabs were inoculated onto SBA and incubated in a 5% CO_2 atmosphere at 37˚C for 48 hours. Growth on each plate was assessed after 24 hours of incubation for the presence of contamination and typical colony appearance and smell of each pathogen.

Single colonies from suspicious microbial growth were subcultured to obtain pure growth for confirming microbial identification using Matrix Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectroscopy (MicroflexTM LT/SH Bruker Daltonics, Leipzig, Germany). Once confirmed isolates were inoculated into 1ml Trypone Soya Broth or Veterinary Fastidious Media plus 20% glycerol and stored at -80˚C for subsequent susceptibility testing. The breakdown of isolates by year is shown in Table 1.

Table 1. Major bovine respiratory disease isolates obtained in the 2014-2019 collection period.

Bacterial species	2014/2015	2016/2017	2018	2019	Total
Mannheimia haemolytica	11	23	21	33	88
Pasteurella multocida	12	40	23	65	140
Histophilus somni	13	11	11	35	70
Bibersteinia trehalosi	-	-	-	5	5
Trueperella pyogenes	-	-	22	33	55
Total	36	74	77	171	358

In 2014-2018, all BRD isolates obtained from veterinary diagnostic laboratories in NSW, Queensland, Victoria and South Australia were supplied to the ACARE laboratory for antimicrobial susceptibility

testing (passive surveillance isolates). In 2019, samples were also obtained from the feedlots taking part in the targeted surveillance component, significantly increasing the total number of isolates.

3.4.2 Mycoplasma bovis

Samples of lung tissue (SA only) and swabs of pure cultures from Veterinary Diagnostic Laboratories in NSW and Qld were subjected to 16S rDNA PCR to confirm identification as *M. bovis* using the technique of McAuliffe et al. (2005). A total of 24 isolates collected in 2017-2018 and 27 isolates collected in 2019 were susceptibility tested. Tissue samples (approximately 1-2 cm^3) or swabs were immediately placed in *Mycoplasma* broth base medium (Eaton broth). All cultured samples were incubated at 37°C for 7-10 days in a CO_2 incubator. Aliquots were plated onto selective *Mycoplasma* (Eaton media) agar, incubated at 37°C for 7-10 days under CO_2 conditions and examined for typical *Mycoplasma* colonies using a stereomicroscope at 10× magnification power. For long-term storage, the pure cultures of each isolate were made in antibiotic-free Eaton medium supplemented with glycerol (20% v/v) in 2 ml aliquots in micro-tubes and stored at -80°C. Typical *Mycoplasma* colonies were subjected to two to five rounds of successive sub-culturing on Eaton agar to confirm purity and again the *Mycoplasma* cultured isolates were identified by using specific primers for the *M. bovis* 16S rDNA gene before MIC testing.

3.5 Antimicrobial susceptibility testing

3.5.1 *Mannheimia, Pasteurella* and *Histophilus* isolates

Minimum Inhibitory Concentration (MIC) antimicrobial susceptibility testing was performed by broth microdilution using Veterinary Reference Card panels (Sensititre®, Trek Diagnostics, ThermoFisher Scientific), specifically, the Bovine BOP07F panel (CLSI, 2018a). The ThermoScientific™Sensititre™ SWIN™Software System was used to interpret the MIC values manually using a Sensititre VizionTM viewing system. The data system uses CLSI breakpoint recommendations but they are not veterinary based. MIC values were manually interpreted on antimicrobials used in veterinary medicine using CLSI veterinary breakpoints (CLSI, 2018b). Nineteen antimicrobials were tested: ampicillin, ceftiofur, clindamycin, danofloxacin, enrofloxacin, florfenicol, gentamicin, neomycin, tetracycline, penicillin, sulphadimethoxine, spectinomycin, tiamulin, tilmicosin, trimethoprim/sulfamethoxazole, tulathromycin, tylosin tartrate, tildipirosin and gamithromycin. These antimicrobials are currently registered for use in food animals in the US and Canada to treat BRD as well as other infections in cattle (Portis et al., 2012). However, in Australia ceftiofur, oxytetracycline, tilmicosin and tulathromycin are the most commonly used antimicrobials to treat BRD infection in feedlot cattle. Control reference strains included *S. aureus* ATCC 29213, *S. pneumoniae* ATCC 49619, *M. haemolytica* ATCC 33396, *E. coli* ATCC 25922 and *E. coli* ATCC 35218. Breakpoints are listed in Table 2. Direct colony suspension was used to prepare the bacterial inoculum equivalent to a 0.5 McFarland Standard, using 5ml demineralized water. A 10µl aliquot of the suspension was transferred into a tube of 11mL Sensititre Mueller-Hinton broth to give an inoculum of 1×10^5 cfu/mL. After vortexing the Sensititre plate was inoculated, plates were sealed with seal strips and incubated at 35°C for 18 h (CLSI, 2018a). The minimum inhibitory concentrations (MIC) were interpreted and MIC_{50}, MIC_{90}, MIC range and the % resistant for each isolate determined.

Table 2. MIC breakpoints for the major bovine respiratory disease pathogens.

Antimicrobial Agent	MIC breakpoint (µg/ml)		
	Susceptible	**Intermediate**	**Resistant**
Ampicillin	≤0.03	0.06-0.12	≥0.25
Ceftiofur	≤2	4	≥8
Clindamycin[d]	-	-	-
Danofloxacin[a]	≤0.25	0.5	≥1
Enrofloxacin	≤0.25	0.5-1	≥2
Florfenicol	≤2	4	≥8
Gamithromycin	≤4	8	≥16
Gentamicin[d]	-	-	-
Neomycin[d]	-	-	-
Tetracycline	≤2	4	≥8
Penicillin	≤0.25	0.5	≥1
Sulphadimethoxine[d]	-	-	-
Spectinomycin	≤32	64	≥128
Tiamulin[d]	-	-	-
Tilmicosin[b]	≤8	16	≥32
Trimethoprim/sulfamethoxazole[d]	-	-	-
Tulathromycin	≤16	32	≥64
Tylosin tartrate[d]	-	-	-
Tildipirosin[c]	≤8	16	≥32

MIC breakpoints are taken from CLSI (2018b).

[a] For danofloxacin, we used CLSI breakpoints for cattle validated for the following bacteria: *M. haemolytica* and *P. multocida*.

[b] For tilmicosin, we used CLSI breakpoints for cattle, but only validated for *M. haemolytica.*

[c] For tildipirosin, in *M. haemolytica* the CLSI breakpoints was S=≤4, I=8 and R=≥16.

[d] For clindamycin, gentamicin, neomycin, tiamulin, trimethoprim/sulfamethoxazole and tylosin, no CLSI breakpoints for BRD isolates from cattle exist.

3.5.2 Trueperella pyogenes

Susceptibility testing of the *T. pyogenes* isolates was performed as described for the main BRD bacteria except that 10 µL of the 0.5 McFarland suspension was inoculated into 11 mL of Sensititre Mueller-Hinton broth infused with 5% lysed horse blood, and 50uL of the final suspension was then inoculated into the Sensititre vet bovine/swine BOPO6F plate.

Plates were read using a Vizion plate reader with the end points interpreted as the minimum concentration well in which there was no growth of the organism. Resistance was interpreted using the CLSI breakpoints for *T. pyogenes* where available (CLSI, 2018b), coupled with reference to human clinical breakpoints and those available for Gram-negative *Pasteurella multocida* and Gram-positive *Corynebacterium* spp (CLSI, 2018b). *Streptococcus pneumoniae* ATCC® 49619 was included as a control organism.

3.5.3 *Mycoplasma* isolates

The MIC testing was performed following the protocol of Hannan (2000). All the ingredients used for the preparation of Eaton medium were supplied by Thermo Fisher Scientific (Thebarton, South Australia 5031, Australia) or Sigma-Aldrich (12 Anella Ave, Castle Hill NSW 2154). Each *M. bovis* strain was subcultured from frozen stocks of a pure culture into 10 ml of supplemented Eaton's Medium and was incubated at 37°C until a typical colour change was observed in the culture medium (typically 48 to 72 h). To determine cell density in each *M. bovis* broth culture, one aliquot was thawed and serially diluted to a level of 10^{-5} in Eaton's Medium. Each selected dilution was applied to a 90 mm plate of solidified (agar) of Eaton's Medium. Inoculated plates were incubated at 37°C + 5% CO2 for 3-5 days, until readily visible colonies were produced. Colonies were enumerated and viable count in each undiluted culture was calculated (expressed as Colony- Forming Units per mL (CFU/mL).

Each diluted culture was used to inoculate a set of prepared microtitre wells containing the antimicrobial dilutions. A 100 µl aliquot of culture was added to each of these wells and to the growth control well. The well containing 200 µl of antibiotic-free broth remained uninoculated to produce the negative control. During inoculation, test item concentrations in the wells were halved due to addition of an equal volume of standardized inoculum. Thus, the final range concentrations in the MIC test were 0.004 µg/mL to 128 µg/mL. Immediately after inoculation, microtitre plates were placed in plastic boxes with loose-fitting lids and with damp paper towels in the base; to minimize evaporation of culture medium from the wells. Boxes containing plates were incubated at 37°C for a period of 24-48 hrs. Plates were inspected daily. If no growth was evident in the positive control wells, plates were re-incubated for an additional 1-2 days of incubation. For each culture, MIC results were read as soon as adequate growth was evident in the positive control wells.

3.6 Subspecific differentiation of *Mannheimia haemolytica* and *Pasteurella multocida* isolates

Enterobacterial repetitive intergenic consensus (ERIC)-PCR was undertaken on the *P. multocida* and *M. haemolytica* collections using the method described by Oliviera et al. (2013) with minor modification. Briefly, DNA was extracted using the boiled lysate method and the assay was performed in a 25 µL-reaction mixture containing 100 ng of template DNA, 1.2µM of primer (OPG13 CTCTCCGCCA), 2.5 µL of 10X buffer (500mM KCl and 100mM Tris-HCl), 3mM $MgCl_2$, 0.23mM of each deoxynucleoside triphosphate and 0.75 Units of Taq DNA polymerase. The ERIC-PCR assay was conducted for 30 cycles consisting of denaturation (94°C) for 30 seconds, annealing (50°C) for 1 minute, and extension (72°C) for 2 minutes in a thermal cycler. Twelve microliters of each reaction mixture was loaded onto an 18-cm-long 2% agarose gel; electrophoresis was performed at 70V in Tris-acetate-EDTA buffer for 3.5 hours. A 1-kb ladder was included as a size reference. Gels were stained with Gel red and photographed. The ERIC-PCR genomic fingerprints from 200 bp to 3 kb were compared and cluster analysis of similarity matrices was performed by the unweighted pair group method using arithmetic averages using Bionumerics. Dendrograms containing all 140 *P. multocida* isolates and 88 *M. haemolytica* isolates were constructed to assess overall genetic diversity.

3.7 Whole genome sequence analysis of *Mannheimia haemolytica* and *Pasteurella multocida* isolates

Whole genome sequence analysis was undertaken at the I3 Institute, University of Technology, Sydney. A total of 175 isolates collected from cattle with BRD were received from The ACARE Reference Laboratory in two batches in October 2019: (1) 119 BRD *Pasteurella multocida* (ACARE001-ACARE120); (2) 68 BRD *Mannheimia haemolytica* (ACARE121- ACARE188). These included all isolates from the 2014-2019 collection except 21 *P. multocida* isolates and 20 *M. haemolytica* isolates (mainly passive surveillance isolates) obtained in the final two months of 2019. These additional isolates were shipped to the I3 Institute in February 2020 and will be included in the analysis prepared for publication.

Isolates were resurrected on blood agar plates and DNA was extracted from monocultures growing overnight in brain-heart infusion broth. Four isolates (ACARE158, ACARE173, ACARE181 and ACARE186) appeared to be contaminated on blood agar plates. Colonies representing distinct morphologies from the suspected contaminated stocks were purified and sequenced separately, leading to a total of 192 genomes in the sequencing run. ACARE089 dropped out from the sequenced pool as no data was generated in the multiplexed HiSeq run.

Genomes were assembled using Shovill, and assembly statistics are presented in file S1. For specimens recovered from contaminated stocks received at I3, sequence of the 16s rRNA gene was extracted and used for identification of the sub-cultured colony variants representing the genome of interest. As highlighted in the notes column of file S1, colonies from all isolates except ACARE158, represented the expected BRD pathogen and were used in genomic analyses.

3.8 Histopathological analysis of *Mycoplasma*-infected tissues

Fresh lung samples obtained at post-mortem were placed in formalin and submitted with fresh tissue and swab samples from the same animal. Formalin fixed tissue was sent to The University of Adelaide Veterinary Diagnostic Laboratory where samples were trimmed, placed in paraffin and sectioned onto a histological slide. Slides were stained with hematoxylin and eosin and examined under light microscopy.

3.9 Statistical analysis

The QBSL and EMAI data on pathogen incidence was compiled into three contingency tables for comparison with *M. haemolytica, H. somni* and *P. multocida*, with *M. bovis* being the constant. A Fisher's exact test was carried out comparing each of the bacterial pathogens against *M. bovis* using an alpha value of 0.05. The alpha value was decreased to 0.017 following Bonferroni adjustment for repetitive measures. Using the contingency tables for each bacterial interaction with *M. bovis* an odds ratio was also manually calculated.

4 Results

4.1 Sampling procedure and transport

A total of 50 lung samples from BRD cases at S1 feedlot were submitted for culture and susceptibility testing in 2018 to further refine the guidelines for collection. A total of 38 samples yielded growth of bacteria, with 21 yielding a known BRD pathogen or additional pathogenic bacteria known to cause respiratory disease. All three BRD pathogens were isolated (*Mannheimia haemolytica* n = 2, *Pasteurella multocida* n = 3, *Histophilus somni* n = 6) and additional bacteria obtained included *Bibersteinia trehalosi* (n = 1), *Trueperella pyogenes* (n = 3), *Aerococcus viridans* (n = 1), *Streptococcus lutetiensis* (n = 2) and *Acinetobacter lwoffi* (n = 3).

Pooling of samples was considered due to the cost involved in submitting single samples. However, we determined that the longest time samples can stay refrigerated and still yield a viable BRD culture when submitted is approximately 3 days. Interestingly, in samples stored for longer than 1 week, a high prevalence of *Psychrobacter*, an organism that can proliferate at low temperatures in transport media, was identified. *Psychrobacter* has a similar colony appearance to *H. somni*. Therefore twice weekly submissions was identified as the most frequent interval that enabled some pooling of samples with no detrimental effects on sample quality.

4.2 Isolation, identification and storage of bovine respiratory disease pathogens (targeted and passive surveillance)

For the targeted surveillance study, a total of 171 isolates were received up until the end of December 2019 with high rates of successful culture of BRD pathogens without contamination from the feedlots, demonstrating that the sampling procedures being followed by the feedlots were adequate. For example, 47 submissions were received by the Queensland Biosecurity Sciences Laboratory (QBSL) and only eight (17.0%) did not yield the growth of a pathogen. Additionally 33 samples were received by EMAI and only three failed to yield a significant bacterial culture. Fifteen

submissions were received from the Charles Sturt University VDL (CSUVDL) and only three (20%) yielded no growth, mostly likely because they were chronic lesions. The laboratories remarked that there was little if any contamination of samples resulting from poor sampling technique. Swab samples obtained during post-mortem rarely yielded contaminants; these were more likely to be seen in the whole tissue samples submitted for culture

Of the main BRD bacterial pathogens in the 2019 collection, *P. multocida* showed the highest prevalence (65 isolates, 38%), followed by *H. somni* (35 isolates, 20%), *T. pyogenes* and *M. haemolytica* (both with 33 isolates; 19%), and *Bibersteinia trehalosi* (5 isolates; 3%). Additional bacteria isolated that could possibly cause pathology in BRD cases included *A.lwoffi* (3 isolates), *Streptococcus dysgalactiae* (3 isolates), *Pseudomonas spp.* (1 isolate) and *Helcococcus ovis* (1 isolate). Mixed infections with one or more pathogens were reasonably common and *Mycoplasma* infections either as a sole agent or in mixed infections were significant. For example, 27 of 47 submissions (57.4%) received by QBSL yielded *Mycoplasma bovis* isolates and in seven cases (14.9%), *Mycoplasma bovis* was the sole agent identified. In NSW, 22 of 32 submissions received by EMAI (68.8%) were positive by *Mycoplasma* spp. PCR, with the majority of samples identified as *M. bovis*, but only a single case was not a mixed infection with another BRD agent. In the 15 submissions received by CSUVDL, *Mycoplasma* spp. were isolated in three cases, one as a mixed infection. In mixed infections derived from submissions to EMAI and QBSL, *Mycoplasma bovis* isolation was strongly associated with *P. multocida* infection (p=0.011; Odds ratio 4.4), suggesting by analogy with respiratory disease in other host species that *Mycoplasma bovis* may be the primary bacterial pathogen and *P. multocida* the secondary pathogen in these cases. CSUVDL samples were not included in these calculations as they did not perform *Mycoplasma* PCR on the samples. QBSL's high success rate in growing and identifying *M. bovis* is attributed to direct plating of samples onto *Mycoplasma* selective agar followed by MALDI-TOF identification. EMAI also attempted *Mycoplasma* culture using this methodology half way through the sampling period (up until that time they were using *Mycoplasma* PCR), and were also successful in culturing *Mycoplasma*. EMAI and CSUVDL also identified a number of other species of *Mycoplasma* in samples, including *Mycoplasma alkalescens*.

4.3 Antimicrobial susceptibility testing

4.3.1 *Mannheimia, Pasteurella* and *Histophilus* isolates

MIC distribution tables for the *M. haemolytica*, *P. multocida* and *H. somni* isolates obtained in 2014/2015, 2016/2017, 2018 and 2019 are listed in Appendix 9.3. The *M. haemolytica* isolates were pan susceptible to all antimicrobials tested that had CLSI break points available except for a single isolate from the 2019 collection (from NSW from feedlot N1) that was resistant to the macrolides gamithromycin (MIC ≥ 8µg/ml), tulathromycin (MIC ≥ 32µg/ml) and tilmicosin (MIC ≥ 16µg/ml). A *P. multocida* isolate that was also resistant to macrolides together with *H. somni* (pan susceptible) and *T. pyogenes* isolates were obtained from the same lung lesions. This animal was treated with tulathromycin on the 8th March 2019 and was euthanised on the 3rd of May 2019.

Resistance among *P. multocida* from BRD cases was first identified in the 2016/2017 collection. Four isolates (three from Queensland and one from Victoria) were resistant to tetracycline (7.5%), one of which was also resistant to aminopenicillins and tilmicosin, and a single isolate (2.5%) supplied by a NSW operation was resistant to all three macrolides. In 2018, one isolate from Queensland had the

aminopenicillin-tetracycline-tilmicosin resistance phenotype and a second isolate from Queensland was resistant to aminopenicillins. One isolate from NSW was resistant to aminopenicillins only and a single isolate from SA was resistant to all three macrolides. This isolate was obtained from a steer with a history of metaphylactic use of tilmicosin on feedlot entry together with a single administration of Draxxin for BRD followed by euthanasia due to non-response. The *P. multocida* isolate was obtained as a light growth and was part of a mixed infection with *T. pyogenes* in one lung lobe, whereas a second lung lobe yielded a *H. somni* isolate susceptible to all antimicrobials.

In 2019, the availability of the targeted surveillance isolates significantly increased the number of *P. multocida* isolates for susceptibility testing (n=65) and the percentage of isolates resistant to macrolides, tetracycline and aminopenicillins. A total of 15 isolates (23.1%) were resistant to all three macrolides. A further three isolates from Qld possessed the aminopenicillin-tetracycline-tilmicosin resistance phenotype, previously identified in 2015-2018 isolates, increasing the percentage of isolates resistant to tilmicosin (27.7%). Eleven isolates (16.9%) were resistant to tetracycline, but an additional two isolates had tetracycline MICs of 4µg/mL (intermediate value) and carried tetracycline resistance genes (see section 4.5 below) confirming that they should be regarded as having a resistant phenotype.

The distribution of isolates according to feedlot (targeted surveillance isolates) or veterinary diagnostic laboratory (passive surveillance isolates) for 2019 are shown in Table 3. The percent resistant to each antimicrobial over time is shown in Fig. 2.

Table 3. Distribution of 2019 targeted and passive surveillance *Pasteurella multocida* isolates by feedlot and resistance profile.

Feedlot	Total Isolates	Pan-Susceptible	Tet-R	Mac-R	Tet/Mac-R	Pen-Tet-Mac-R
N1	8	3		5		
N2	7	1	3		3	
N4	4		1	1	2	
Q1	3	1				2*
Q2	7	6		1		
Q3	2	2				
S1	7	4		3		
NSW**	10	10				
Qld**	17	16				1*
Total	65	43	4	10	5	3*

* These isolates were resistant to tilmicosin only
** Passive surveillance isolates from each state. Note that one isolate from 2018 was also resistant to Pen-Tet-Mac.
Tet-R: resistant to tetracyclines
Mac-R: resistant to the macrolides tilmicosin, tulathromycin and gamithromycin
Pen-R: resistant to β-lactams (aminopenicillins)

Some interesting associations were found when the *P. multocida* isolates are split into their feedlot of origin (Table 3). The majority of isolates that were resistant to tetracyclines, including five isolates that were also resistant to macrolides, were restricted to two feedlots (N2 and N4). Both feedlots have a history of in feed use of chlortetracycline for BRD control shortly after induction. By comparison, the feedlots that yielded susceptible isolates or isolates resistant to macrolides only had

no history of the use chlortetracycline in the feed for BRD control or used it intermittently. The exception to this was Q1, where two isolates resistant to tetracycline, aminopenicillins and the macrolide tilmicosin (but not tulathromycin or gamithromycin) were obtained but there was no history of chlortetracycline use. However, these animals had limited access to medicated grazing supplement containing oxytetracycline.

Treatment histories were obtained for all animals yielding isolates showing resistance to antimicrobials except for two animals from Q1 where animal ID numbers were not supplied with the samples (Table 4). An additional isolate from Q1 (Q1057) was not MIC tested and is currently missing from the BRD isolate collection. Qld BSL has been contacted to see if this strain can be found.

Table 4. Treatment histories for animals yielding *Pasteurella multocida* isolates that were resistant to one or more antimicrobials.

Sample ID Number	Resistance	Date of entry	Treatment date	Treatment type	Date of death
N1034*	Mac-R	28/02/2019	31/03/2019	Draxxin	03/05/2019
N1041	Mac-R	02/04/2019	22/04/2019	Draxxin	20/05/2019
N1045	Mac-R	30/04/2019	15/05/2019	Draxxin	20/05/2019
N1061	Mac-R	17/04/2019	13/05/2019	Draxxin	22/05/2019
N1062	Mac-R	18/04/2019	20/04/2019 08/05/2019	Draxxin Engemycin	22/05/2019
N2052**	Tet-R	03/04/2019	05/05/2019	Draxxin	09/05/2019
N2027**	Tet-R	14/05/2019	31/05/2019 05/06/2019	Draxxin Engemycin	02/07/2019
N2092**	Tet-R	11/07/2019	NA	Nil	02/09/2019
N2056**	Mac-Tet-R	03/04/2019	25/04/2019	Micotil	08/05/2019
N2030**	Mac-Tet-R	08/04/2019	26/04/2019	Micotil	07/05/2019
N2050**	Mac-Tet-R	04/06/2019	21/06/2019 27/06/2019	Draxxin Engemycin	01/07/2019
N4036**	Tet-R	09/04/2019	07/05/2019 13/05/2019	Draxxin Engemycin	14/05/2019
N4037**	Mac-R	21/10/2019	18/11/2019	Draxxin	19/11/2019
N4034**	Mac-Tet-R	05/04/2019	26/04/2019 02/05/2019	Draxxin Engemycin	13/05/2019
N4016**	Mac-Tet-R	14/05/2019	26/04/2019 02/05/2019	Draxxin Engemycin	14/05/2019
Q1070***	Mac-Tet-Pen-R	Sample missing animal ID number			
Q1172***	Mac-Tet-Pen-R	Sample missing animal ID number			
Q1057***§	Isolate missing from database	06/03/2019	03/04/2019 12/04/2019 18/04/2019	Micotil Engemycin Moxylan	08/05/2019
Q2008a	Mac-R	16/05/2019	04/06/2019 10/06/2019	Draxxin Engemycin	20/06/2019
S1026	Mac-R		26/06/2019	Draxxin	11/07/2019

			03/07/2019	Engemycin	
S1040	Mac-R		24/06/2019	Draxxin	26/06/2019
S1042	Mac-R		13/06/2019	Draxxin	17/06/2019

* This sample also yielded a *Mannheimia haemolytica* isolate resistant to tulathromycin
** These animals received in- feed chlortetracycline early in their feeding period.
*** These animals had access to medicated prolix containing oxytetraxcycline early in their feed period.
§ currently seeking resubmission of isolate from Qld BSL.

All resistant isolates were obtained from animals that had been treated with antimicrobials except for a single untreated pen death. Draxxin (tulathromycin) was used as the sole treatment in 8/18 cases, Draxxin, followed by Engemycin (oxytetracycline) in another 8/18 cases and Micotil (tilmicosin) in 2/18 cases. If the *P. multocida* isolate from Q1057 can be found it will be interesting to determine its resistance profile as it was treated with the three antimicrobials that two additional isolates (Q1070 and Q1172) were resistant to (tilmicosin, tetracycline and amoxicillin). Unfortunately, animal ID numbers were not provided with these samples.

The 70 *H. somni* isolates were susceptible to all tested antimicrobials.

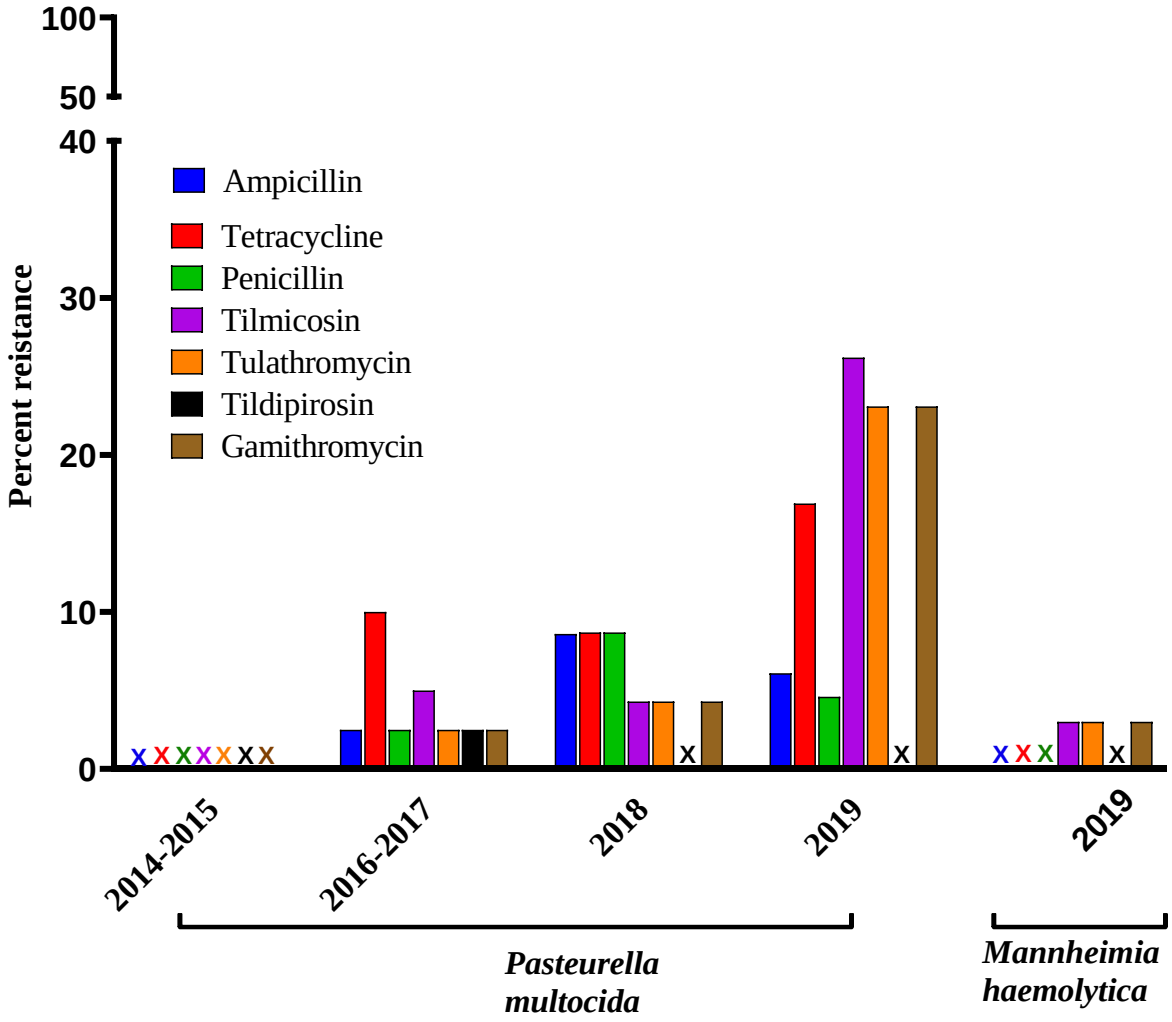

Fig 2. Percent of Bovine Respiratory Disease isolates resistant to ampicillin, tetracycline and individual macrolides over time.

4.3.2 Trueperella pyogenes

MIC testing has been performed on all *T. pyogenes* isolates with the results of the first 46 isolates analysed. All isolates were susceptible to β-lactams (penicillin, ampicillin and ceftiofur). Penicillin is the recommended agent for the treatment of *T. pyogenes* infections in feedlot cattle. One third of the isolates were resistant to oxytetracycline and just over 1/3 of the isolates were resistant to the macrolides tilmicosin and tulathromycin, with 26% of isolates resistant to both antimicrobials.

Table 4: MIC_{50}, MIC_{90} and percent resistant among 46 *T. pyogenes* isolates.

Class	Category	Antimicrobial	MIC_{50}	MIC_{90}	%Resistance
Aminoglycoside	prohibited	Gentamicin	≤1	≤1	0
	first line	Neomycin	8	8	0
		Spectinomycin	≤8	≤8	2
Fluoroquinolone	prohibited	Enrofloxacin	0.5	>2	22
	prohibited	Danofloxacin	1	>1	89
Macrolide	first line	Tylosin	≤0.5	>32	39
	second line	Tulathromycin	≤1	>64	35
	first line	Tilmicosin	≤4	>64	35
β**- lactam**	first line	Ampicillin	≤0.25	≤0.25	0
	first line	Penicillin	≤0.12	≤0.12	0
	third line	Ceftiofur	0.5	1	0
Lincosamide		Clindamycin	≤0.25	>16	39
Phenicol	first line	Florfenicol	0.5	0.5	0
Tetracycline	first line	Oxytetracycline	0.5	1	33
		Chlortetracycline	≤0.5	8	33
Pleuromutilin	second line	Tiamulin	≤0.5	≤0.5	0
Trimethoprim-sulphonamides	second line	Trimethoprim sulpha	≤2/38	≤2/38	0
Sulphonamides	first line	Sulphahdimethoxine	≤256	>256	11

4.3.3 *Mycoplasma* isolates

MIC data for the *Mycoplasma* isolates collected in 2017-2018 vs 2019 are shown in Tables 5 and 6. Minor differences in MIC range were observed between the two sampling periods. Extrapolating from the breakpoints established for other BRD pathogens, the *Mycoplasma* isolates were uniformly susceptible to tetracyclines and macrolides, apart from three 2019 isolates that showed resistance to tilmicosin only and three isolates from 2017/2018 that had tetracycline MICs of 8µg/ml.

Table 5. Summary of the MIC_{50} and MIC_{90} values of the 24 *M. bovis* strains isolated from cattle in Australia in 2017-2018.

Antibiotic group	Antibiotic	MIC Range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Fluoroquinolones	Enrofloxacin	1-4	2	4
Tetracyclines	Tetracycline	0.25-8	2	8
Macrolides	Tulathromycin	0.063-0.25	0.125	0.25
	Tylosin	0.008-1	0.008	0.125
	Tilmicosin	0.063-0.5	0.125	0.125
	Erythromycin	2-128	32	128
	Tildipirosin	0.125-32	0.25	8
Lincosamide	Lincomycin	0.5-128	16	128
Phenicol	Florfenicol	0.5-64	32	64
Pleuromutilins	Tiamulin	0.031-1	0.125	0.5
Aminocyclitol	Spectinomycin	0.063-0.25	0.125	0.25

Table 6: Summary of the MIC_{50} and MIC_{90} values of the 26 *M. bovis* strains isolated from cattle in Australia in 2019.

Antibiotic group	Antibiotic	MIC Range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Fluoroquinolones	Enrofloxacin	0.25-4	1	4
Tetracyclines	Tetracycline	0.25-4	2	4
Macrolides	Tulathromycin	0.063-0.5	0.25	0.5
	Tylosin	0125-32	0.5	32
	Tilmicosin	0.063-128	0.5	128
	Erythromycin	8-128	32	128
	Tildipirosin	0.125-16	2	1
	Gamithromycin	0.063-2	0.5	2
Lincosamide	Lincomycin	0.5-16	4	16
Phenicol	Florfenicol	0.25-32	8	32
Pleuromutilins	Tiamulin	0.063-4	0.5	4
Aminocyclitol	Spectinomycin	0.125-4	1	4

4.4 Subspecific differentiation of *Mannheimia haemolytica* and *Pasteurella multocida* isolates

Genetic fingerprinting using ERIC-PCR applied to the 2014-2018 collection of *P. multocida* isolates identified four main genetic clusters of isolates. Interestingly, the majority of resistant isolates were located within group 4 of the dendrogram suggesting that there may be something inherent in the genome of these isolates driving acquisition of resistance (such as an integrative conjugative element or ICE). However, the inclusion of the 2019 *P. multocida* isolates paints a much more complex picture with further genetic groups identified and resistance isolates distributed into a further two genetic clusters in addition to the previously described group 4 (See Appendix 9.4). By contrast, genetic fingerprinting of the *M. haemolytica* isolates showed that they were genetically homogeneous. It must be remembered that ERIC-PCR is an imperfect technique that is often used as a screening tool prior to whole genome sequencing and single nucleotide polymorphism (SNP)

analysis (see Section 4.5), which is now recognised as the gold standard technique for molecular epidemiology applications.

4.5 Whole genome sequence analysis of *Mannheimia haemolytica* and *Pasteurella multocida* isolates

4.5.1 Resistance genotyping and correlation with phenotype of the isolates included in the cohort

An increase in the frequency of isolation of tetracycline- and macrolide-resistant *P. multocida* was observed over the 3 sampling years and a single *M. haemolytica* isolate from 2019 was resistant to macrolides. The genomes were therefore sequenced to identify the resistance genes and gather preliminary information on their context. We used sequence data of all genes identified to have contributed towards the *M. haemolytica* resistance profile, including tetracycline and macrolide resistance, in a recent study (Snyder et al, 2019) to probe the sequenced genomes. Presented in Table 1 (Appendix 9.5) are all resistance genes that exhibit 98% identity over 100% of the query length. Except for 4 isolates, the *tet(H)-tet (R)* genes typical of Tn5706 transposon are present in all isolates that exhibit tetracycline resistance, while isolates with macrolide resistance harbour the *msr(E)-mph(E)* genes. Five isolates were confirmed to contain both *tet(H)-tet(R)* and *msr(E)-mph(E)*. The *tet(Y)* tetracycline resistance gene is also present in *P. multocida* genomes from ACARE19, ACARE22, ACARE23 and ACARE24. The bla_{ROB-1} ß-lactamase gene from *Actinobacillus pleuropneumoniae* is present in *P. multocida* isolates ACARE16, ACARE47, ACARE48, ACARE91, ACARE97 and ACARE100. Most of the resistance genes were located on the same genomic scaffold (highlighted as genes in bold fonts within square brackets), which may indicate clustering of the resistance genes on a single replicon in the respective genomes. However, verification of the presence of these linked resistance genes on mobile plasmids or other genetic elements is beyond the capacity of Illumina sequencing approaches. Deep sequencing will now be undertaken on a subset of resistant isolates to confirm if these resistance genes are plasmid- or chromosomally-encoded and whether they are associated with an ICE.

We downloaded 24 completely assembled *Pasteurella* and *Mannheimia* plasmid sequences (Details in File S2) available in GenBank RefSeq on the 18th of February and looked for their presence within our genomes. All *Pasteurella* isolates, except ACARE 078, most likely have two unnamed plasmids, 1 (28,093bp, accession number NZ_CP020349.1) and 2 (34,596bp, accession number NZ_CP020348.1) from *P. multicoda* subspecies *septica* strain CIRMBP-0873 collected from a wild rabbit in France. In addition, the isolates may also harbour a variant of an unnamed 325,255bp plasmid (accession no: NZ_CP020346.1) isolated from *P. multocida* subsp. *multocida* strain CIRMBP-088 also isolated from a wild rabbit in France. None of the four *Mannheimia* plasmids (or close variants) included in our genome wide search appear to have been present in the *Mannheimia* genomes sequenced in this cohort. It remains to be determined using deep sequencing if the single macrolide-resistant *M. haemolytica* isolate possesses *msr(E)-mph(E)* on either a plasmid or the chromosome (i.e. associated with an ICE) and if plasmid-mediated, whether it acquired the plasmid from *P. multocida*.

It is noteworthy that all resistant genotypes matched with their corresponding resistant phenotypes except in two cases. These isolates are currently being resequenced. While differences in expression of the genes is certainly an explanation for these mismatches, they may also arise from labelling

errors during sub-culturing and transportation of isolates at different stages of this project, thus resequencing should identify any mismatches.

4.5.2 Phylogeny of the *Mannheimia haemolytica* cohort

Whole genome SNP based phylogeny analysis of *M. haemolytica* using parSNP and M42548 as the reference genome, revealed a large group of 60 isolates clustering closely with the reference strain (Fig. 9.6a). The recombination filter flag in parSNP was switched on to improve resolution of ancestral relationships. Isolate ACARE141 was the most distantly related strain in the cohort, while isolates ACARE131, ACARE174, ACARE175 and ACARE127 shared a relatively more recent common ancestor with the large subclade. (N.B. Isolates ACARE158, ACARE173 and ACARE178 were identified as *Mannheimia varigena* and hence were dropped out of SNP based phylogeny analyses).

A total of 180 assembled *M. haemolytica* genomes were downloaded from GenBank RefSeq database (6th February) and included in an extended phylogeny analysis to identify distribution of resistance genotypes within phylogenetically related overseas isolates using the *M. haemolytica* strain 42548 genome as the reference. As seen in Fig 9.6b, overall clustering of Australian genomes did not change when overseas genomes were included in the phylogeny analysis and there was no specific co-relation of resistance profiles between Australian and international isolates.

ACARE141 retained its distant evolutionary relationship with the remaining isolates in the cohort. 16S rDNA BLAST analysis of ACARE141 revealed 98.89% identity over 100% length of the query sequence with *M. haemolytica* genome NCTC10643 (Accession no LR134495.1) collected from nasopharyngeal mucus of a sheep in Scotland.

4.5.3 Phylogeny of the *Pasteurella multicoda* cohort

Unlike the *M. haemolytica* cohort a SNP based phylogeny analysis could not be adopted for the *P. multocida* isolates sampled in this project as the genomes were genetically diverse (Fig. 9.6c). To capture this diversity, we embarked upon a marker gene-based phylogeny analysis (Phylosift) for *P. multocida* genomes. In contrast to the standard 7-11 housekeeping genes commonly used in marker gene phylogeny analysis, resolution of PhyloSift phylogenetic trees are more accurate as the software uses 37 prokaryotic marker genes to draw ancestral inferences. These 37 marker genes form 1% of an *E. coli* genome (approximately 4.5MB -5MB). As the genomes of *Pasteurella* are much smaller in size (nearly half that of *E. coli*), we believe the software would best capture and demonstrate phylogenetic relationships within a genetically diverse species cohort.

Genomes representing different subspecies of *P. multicoda* were included in the PhyloSift analysis to test whether clustering occurs based on subspecies classifications. As presented in Fig 9.6c, major subclades in the *Pasteurella* phylogenetic tree appeared to have clustered with representative genomes belonging to different subspecies. Isolate ACARE071 clustered with a *P. multicoda* subspecies *septica* strain collected from a human wound infection and ACARE078 appeared to be the most distantly related genome in this subset, not related to any reference genome included in this analysis.

Two hundred assembled *P. multocida* genomes representing different subspecies were available for downloading in GenBank RefSeq database on the 6th of February 2020 and used for an extended

phylogeny analysis to identify distribution of resistance genotypes within phylogenetically related overseas isolates with the Australian genomes sequenced in this cohort. As seen in Fig 9.6d, the overall clustering of the genomes did not change when overseas genomes were included and ACARE078 retained its distant evolutionary relation with the remaining of isolates in the cohort.

Most of the Australian isolates which exhibited similar resistance genotypes clustered together, likely indicating a higher percentage of genome wide sequence identity for the each of the ancestral subclades identified in this marker gene-based phylogeny analysis. However, there were no obvious co-relationships between the resistant Australian and international isolates, revealing that the Australian isolates cluster represents a distinct genetic subclade.

4.5.4 Identification of Integrative conjugative elements in the genomes

As tetracycline and macrolide resistant phenotypes have recently been attributed to the presence of specific ICEs in overseas genomes, one of the questions that was addressed in the course of this analysis was whether ICEs were present in the Australian cohort. We downloaded sequences of the ICE present in *P. multocida* strain 3358 (accession number CP029712.1, with macrolide resistance gene *mphE* and tetracycline resistance gene *tetH*) and *M. haemolytica 42548* (accession number CP005383.1, with tetracycline resistance genes tetH-tetR) and queried our genomes, with a focus on isolates which exhibited respective resistance genotypes and phenotypes. Our extensive analyses based on illumina short read sequence outputs indicate that five *P. multocida* genomes (ACARE016/17BRD035, ACARE047/18BRD-001, ACARE091/19BRD032, ACARE097/19BRD042 and ACARE100/19BRD057) likely have a variant of the ICE in *P. multicoda* 3358. These strains are worth investigating in more detail using long read sequencing which will resolve the different replicons that make up the genome of the strains and facilitate detailed characterisation of the ICE. A more detailed analysis of these five strains is supported by: (1) identical genotypic (bla_{ROB1} and *tetH-tetR*) and phenotypic (penicillin and tetracycline resistance) profile of these isolates as seen in Table 9.5, (2) presence of a different class of β-lactamase resistance gene (bla_{ROB1}) and other genes in the backbone of the *P. multicoda* 3358-ICE (data not presented) and, most importantly, (3) the distribution of these ICE in isolates obtained from different feedlots and different years that cluster into different phylogenetic sub-clades in Fig. 9.6c, including highly dissimilar isolates from the same feedlot (Q1) in Queensland isolated in 2019.

The five *P. multocida* isolates that potentially contain ICEs were also phenotypically resistant to tilmicosin but susceptible to gamithromycin and tulathromycin. No resistance gene was identified in these isolates that could explain this resistance phenotype.

4.6 Histopathological analysis of *Mycoplasma*-infected tissues

A total of 116 samples were included in the histopathology study that were subjected to *Mycoplasma bovis* by PCR and/or cultured for *Mycoplasma* and other BRD pathogens followed by identification using MALDI-TOF. Of the 116 samples, 10.3% (12/116) were positive solely for *Mycoplasma bovis*, 22.4% (26/116) yielded other BRD pathogens but not *Mycoplasma bovis*, 32.8% (38/116) yielded *Mycoplasma bovis* together with other BRD pathogens and 34.5% (40/116) were negative for all BRD pathogens. In a number of cases where *Mycoplasma bovis* was identified, a specific pathognomonic morphology was observed in histopathology sections. These well

demarcated regions of necrosis were observed in lung samples from 4/12 animals that were positive for *Mycoplasma bovis* but negative for other BRD pathogens, in 10/38 of animals that were co-infected with *Mycoplasma bovis* and other BRD pathogens, and in 2/40 of animals where no pathogens were isolated. The well demarcated area of necrosis, either coagulative or caseous, was surrounded by a well demarcated rim composed of inflammatory and matrix laying cells (Fig. 3). Some of the cells that had major involvement in this rim included neutrophils, fibroblasts and macrophages. In mixed infections, it was difficult to identify these characteristic lesions due to the more extensive inflammation typically associated with the more common BRD pathogens (*Mannheimia, Pasteurella* and *Histophilus*) (Fig. 4).

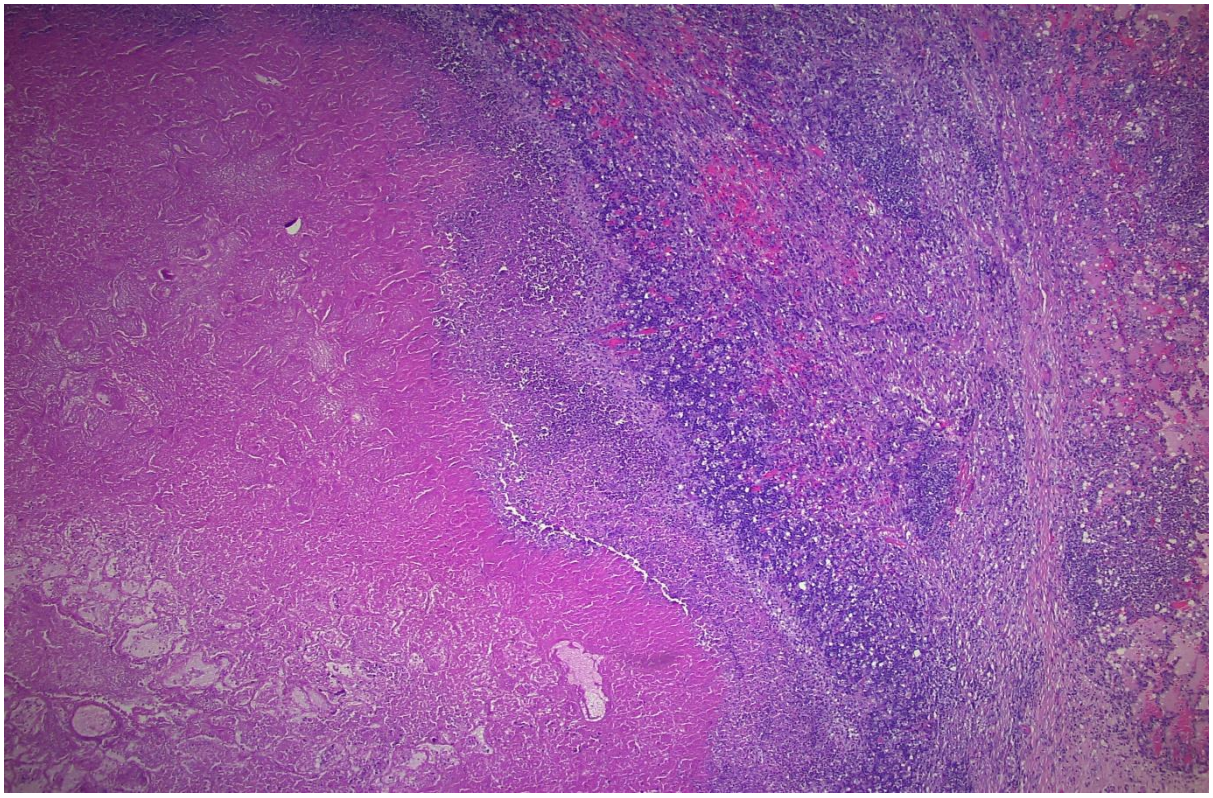

Fig. 3. Microscopic image of coagulative and caseous necrosis in a pneumonic lung. *Mycoplasma bovis* was the sole pathogen detected in this tissue.

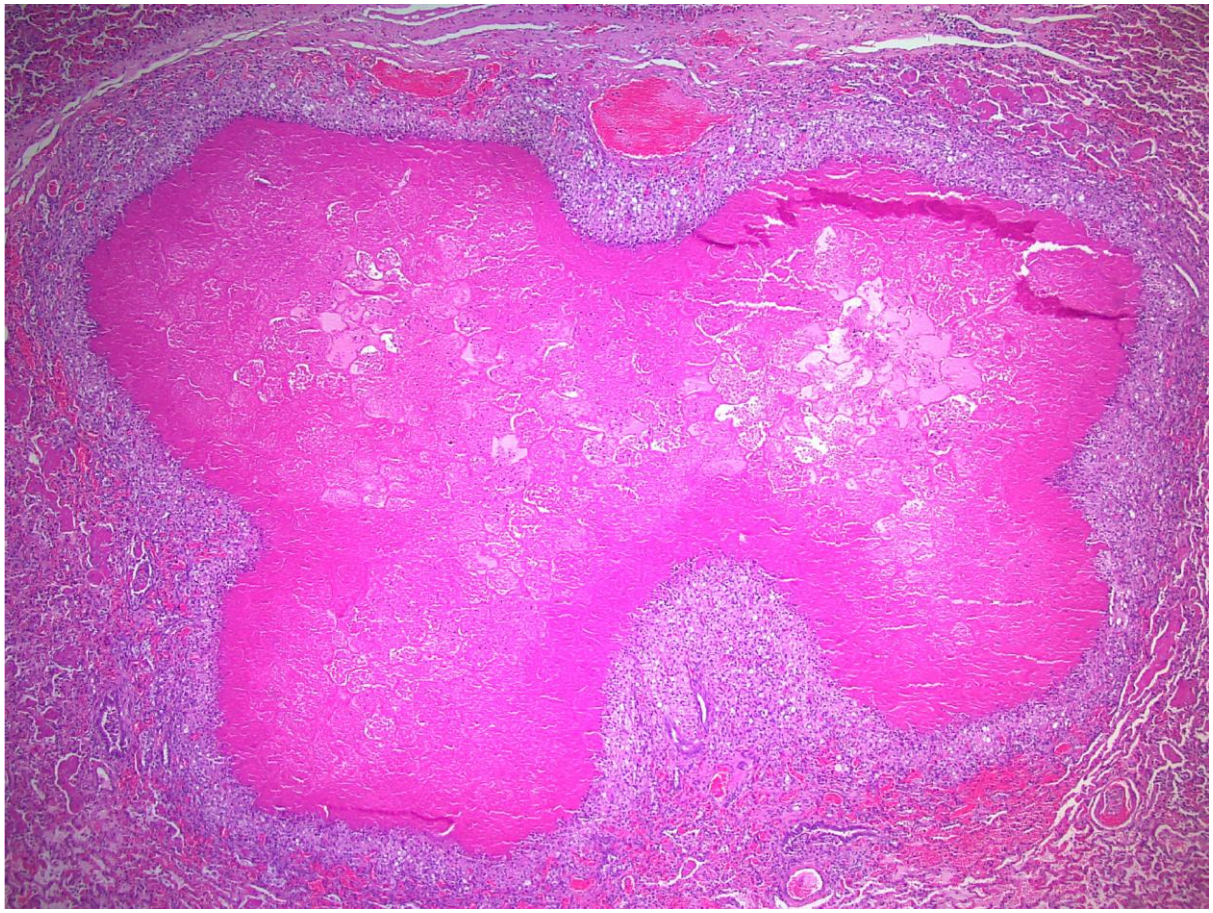

Figure 4. Histology of a section of pneumonic lung. Area of caseous and coagulative necrosis surrounded by well demarcated aggregation of leukocytes. Surrounding tissue appears hyperaemic with some areas of haemorrhage indicating a difference to sole *Mycoplasma bovis* infections. *Mycoplasma bovis, Histophilus somni* and *Trueperella pyogenes* were detected in this lung sample.

5 Discussion

5.1 Main study findings

As the first comprehensive survey of AMR in BRD pathogens in Australia, this study had six major findings.

1) *Pasteurella multocida* is the most common BRD pathogen isolated from Australian feedlots, and is often present in mixed infections, particularly with *Mycoplasma bovis*. Aseptic sampling technique and rapid transport to laboratories yielded a high proportion of significant BRD pathogens in comparison to no growth or contaminated specimens.
2) AMR has emerged in *P. multocida* with a significant proportion (23.1%) of 2019 isolates resistant to all three macrolides and a subset of these also resistant to tetracycline (9.2%).
3) AMR was negligible in three of the five main BRD pathogens (*Mannheimia haemolytica, Histophilus somni* and *Mycoplasma bovis*), which contrasts with many international studies documenting resistance in these pathogens.

4) The majority of *P. multocida* and *M. haemolytica* isolates subjected to whole genome sequencing do not appear to contain ICEs.
5) Whole genome sequencing also identified a range of plasmids in *P. multocida* isolates, though once again long range sequencing will be required to fully annotate and describe each plasmid. It is possible that one or more of these plasmids contains tetracycline resistance genes (*tet(H)-tet (R)*) within a well-recognised transposon. Macrolide resistance genes (*msr(E)-mph(E)*) are usually found in the chromosome. The single macrolide-resistant *M. haemolytica* isolate that possessed *msr(E)-mph(E)* genes was isolated from the same BRD case as a macrolide-resistant *P. multocida* isolate which may indicate horizontal movement of a mobile genetic element into *M. haemolytica*.
6) A small number (n = 5) of Queensland-source *P. multocida* isolates have an unusual resistance phenotype (aminopenicillin, tetracycline, tilmicosin resistance) and may possess an ICE, though long range (deep) sequencing will now be required to confirm this as well as identify the genetic basis of the tilmicosin resistance.

5.2 BRD pathogens isolated from post-mortem samples

The high rate of isolation of BRD pathogens from post-mortem specimens obtained during the 2019 BRD season confirms that the sampling technique, refined and technology transferred to feedlots distributed throughout Australia, was simple yet robust enough to yield significant results. The inclusion of two swabs per sample pack together with a fresh tissue sample aseptically collected into the two Whirlpaks provided often yielded multiple isolates from the same animal, significantly increasing isolation rates. This allowed the referring diagnostic microbiology laboratories a second chance to isolate pathogens from the fresh tissue sample if the deep lung swabs were contaminated or did not yield any significant growth. Furthermore, the fresh tissue samples made it possible to investigate *Mycoplasma* as a possible aetiological agent of BRD in Australia, and in some cases it was the sole agent identified. The pathognomonic areas of necrosis identified within the lung tissue typical of *Mycoplasma* infection add further weight to the hypothesis that it is a significant BRD pathogen in Australia. This supports previous non-culture based studies implicating it as having significant involvement in BRD in Australian feedlots (Horwood et al., 2014; Schibrowski et al., 2018).

The project led to a major change in processing of BRD samples by each laboratory. Samples for *Mycoplasma* isolation were directly inoculated onto selective agar as well as into enrichment media, rather than just enrichment media by itself. This enabled rapid identification of *Mycoplasma bovis* and other *Mycoplasma* species of significance using a combination of MALDI-TOF and PCR. If only enrichment media is used, it is difficult to determine to what extent *Mycoplasma* is involved in the lung pathology. Heavy pure growth of *Mycoplasma* spp. on selective agar provides a strong indication that it was a primary aetiological agent in the lung lesions. This was supported by histopathological analysis of BRD lung samples, which identified the typical small necrotic foci typically associated with *Mycoplasma* in a significant number of pure culture and mixed infections. *Mycoplasma* is resistant to β-lactam antibiotics such as ceftiofur as it lacks a peptidoglycan cell wall, however, it is unlikely that other antibiotics to which *Mycoplasma* is usually sensitive to (tulathromycin and tetracycline) would significantly penetrate the widespread necrotic foci identified in histological lesions, leading to possible treatment failure.

All six species of bacteria previously associated with BRD were isolated in the study (*M. haemolytica, P. multocida, H. somni, Mycoplasma bovis, T. pyogenes and B. trehalosi*), however, the prevalence of each pathogen differed considerably from some overseas studies. In Australia, widespread vaccination of cattle against *M. haemolytica* has likely resulted in reduced prevalence of what is

regarded internationally as the most significant bacterial pathogen causing BRD (Griffin et al., 2010). *P. multocida* was the most common pathogen identified at most feedlots in Australia that were sampled and the most likely pathogen to have resistance to antimicrobials, which may have influenced its increased detection. By comparison, in the North American study of Klima et al. (2014) *M. haemolytica* had a 91% prevalence and *P. multocida* only 8% prevalence, indicating marked differences in disease epidemiology between these two studies. However in the study of Timset et al. (2017), which focused on isolates obtained from transtracheal sampling of live animals rather than at post-mortem, *P. multocida* had the highest prevalence in cattle with BRD (54.8%), followed by *M. haemolytica* (30.5%) and *H. somni* (22.9%). It must be taken into consideration that Australian cattle inducted into feedlots are comparatively older than their North American counterparts and that Australian feedlot husbandry practices differ considerably compared to many feedlots in other countries in having much reduced stocking densities and significantly shorter production cycles (90-120 days).

In the present study, an association between *P. multocida* and *Mycoplasma* coinfection was also identified. *Mycoplasma* has been shown to be common in mixed BRD infections. For example in one North American study, *Mycoplasma* was detected in 25/34 BRD cases and was a major component of mixed infections (24/25) (Mehinagic et al., 2019). To the best of our knowledge, however, no previous studies have reported an association with *P. multocida*. It will be interesting to identify what viruses are associated with mixed *Mycoplasma/Pasteurella* infections in Australian feedlot cattle, as a recent study using immunohistochemistry found a high proportion of lung samples from BRD cases in Switzerland had persistent BPI3 and *Mycoplasma* infection (Mehinagic et al., 2019). Immunohistochemistry staining of the histological sections obtained for 2019 source samples could be used to retrospectively analyse the presence of the four main viral pathogens (IBR, BRSV, PI3 and BVD).

5.3 Antimicrobial resistance in *P. multocida*

A notable outcome of the study was the identification of a significant number (15/65; 23.1%) of 2019 *P. multocida* isolates that were resistant to all three macrolides, with a proportion of these also resistant to tetracycline (6/65; 9.2%). The macrolide-tetracycline-resistant isolates came from two feedlots that practice metaphylactic use of chlortetracycline in pens for approximately 1 week in duration soon after arrival. Use of chlortetracycline in the feed has been a common prophylaxis for cattle at high risk of developing BRD (Duff et al., 2000). For example, approximately 20% of feedlot cattle in the United States have been estimated to receive chlortetracycline as a prophylaxis (Miller et al., 2018), where it is often used to prevent histophilosis. Whilst a number of studies have focused on the effect of chlortetracycline on *E. coli* resistance genes in the gut (e.g. Miller et al., 2018), few have focused on respiratory pathogens. Timset et al. (2017) identified high rates (>70%) of resistance to tulathromycin and tetracycline in BRD isolates from cattle prophylactically treated with either tulathromycin on feedlot entry or two chlortetracycline pulses in the feed shortly after arrival.

In the present study, the isolation of *P. multocida* resistant to both macrolides and tetracycline could indicate that use of chlortetracycline in the feed may be driving this dual resistance though no statistical association was possible with the low number of samples obtained. More samples should definitely be obtained from feedlots using tetracycline in the feed (e.g. N2 and N4) compared to those that don't (e.g. Q2) considering that the *P. multocida* isolates from these two feedlots have become resistant to the two front line drugs used for the treatment of BRD in Australia and are only susceptible to ceftiofur. Consideration should be given to rotating additional first line antimicrobial agents at these establishments that are registered for the treatment of bovine respiratory disease

such as florfenicol, to which the isolates remain susceptible, before returning to tulathromycin. If further sampling confirms an association, use of chlortetracycline pulses in the feed in preference to other control measures should be discouraged unless there is no alternative, for example in severe outbreaks of BRD with large numbers of animals affected.

The increased rate of resistance to tulathromycin in the 2019 *P. multocida* study isolates (compared to previous years) and its widespread identification at five of the participating feedlots suggests that macrolide resistance in Australian feedlots is just emerging. However, the introduction of targeted surveillance and investigation of feedlot deaths may have artificially increased the prevalence or uncovered a previously unknown number of actual treatment failures due to development of resistance in the main bacterial pathogen*, P. multocida*. Analysis of the treatment records of BRD cases yielding resistant *P. multocida* isolates identified that the majority were treated with tulathromycin followed by oxytetracycline. The time interval from the last treatment to euthanasia varied greatly, but in five cases (four cases yielding tulathromycin-resistant and once case yielding tulathromycin/tetracycline resistant bacteria) euthanasia occurred within five days of the final treatment. These cases could therefore be regarded as treatment failures based on the isolation of heavy pure growths of *P. multocida* that was resistant to the treatment/s administered. It also must be remembered that obtaining isolates from post-mortem examinations for AMR surveillance purposes represents an extremely biased sample population as it does not take into account the many animals that respond to treatment (but cannot be sampled when they have an active infection). AMR surveillance should ideally be cross-sectional in design, taking single samples from as many feedlots as possible, but this was neither practical nor achievable in the current project, which firstly sought to establish reliable methods for sample collection from participating feedlots.

The 2019 AMR surveillance data were obtained following release of the McDonald's statement on Antibiotic Use Policy for Beef and Dairy Beef. Two statements listed in the McDonald's document are worth discussing in the context of the results obtained in the present study.

1) When antibiotics are prescribed by a veterinarian, McDonald's global position is one of responsible use, informed by resistance monitoring and susceptibility testing
2) McDonald's will encourage producers to adopt a tiered approach to antibiotic use; the lowest importance human drugs ranked as the first choice, and Highest Priority Critically Important Antibiotic's restricted to last choice.

To adhere to the first statement, industry is now well primed to conduct local surveillance of AMR in BRD pathogens using the sample kits provided. Sampling should occur at peak times during a typical BRD season (e.g. autumn and spring) when there is likely to be an increased number of cases. A suggested plan for sampling entails the following: 1) Aseptically cutting into the exposed infected lungs where there is a clear demarcation between normal and damaged tissue; 2) obtaining a deep swab sample of the junction of infected tissue as well as submitting a fresh tissue sample; 3) pooling samples for no longer than three days; and 4) expediting transport to ensure the samples remain chilled and do not languish at the courier company over the weekend. Four of the five laboratories supporting the project are equipped with MALDI-TOF and Sensititre AST systems and will be able to conduct AMR surveillance and provide each feedlot with their local susceptibility data. Data can then be pooled from each laboratory to obtain estimates of industry-wide prevalence of resistance.

The second statement suggests that a tiered approach to antimicrobial use be adopted to use High Importance antimicrobials first (by World Health Organisation Classification), and reserving the Highest Priority Critically Important Antimicrobials as a later choice. Australian Veterinary Association recommendations would indicate use of a first line antimicrobials (such as oxytetracycline or potentially florfenicol), followed by a second line antimicrobial (such as the macrolide, tulathromycin). Third line antimicrobials (ceftiofur) are only recommended for non-responses to tier 1 or tier 2 antibiotics, or in cases where there is a risk of exceeding the export slaughter interval in using a tier 1 or tier 2 antimicrobial.

This may not be the most ideal regime, however, for adequate treatment and control of BRD (given that tulathromycin is regarded as the most clinically effective antimicrobial agent for BRD by the industry). A system of drug rotation of first and second line drugs (for example incorporating florfenicol) could be considered where resistance has emerged to prevent its further spread. A recent systematic review and network meta-analysis of the use of injectable antimicrobials for metaphylaxis (i.e. disease control) at feedlot entry concluded that macrolides (and ceftiofur) are the most effective antibiotics for the reduction of BRD incidence in the first 45 days on feed in North America (O'Connor et al. 2019). Injectable oxytetracycline (and florfenicol) effectively controlled BRD compared with no antibiotics; however, they were less effective than macrolide (or ceftiofur) treatments. Ceftiofur is also not going to have any effect on *Mycoplasma* infections (as they lack a cell wall). The systematic review did conclude that greater use of oxytetracycline (and by analogy florfenicol) to treat cases of BRD during metaphylaxis may have advantages from an antimicrobial stewardship perspective. Florfenicol belongs to the same tier 1 (AVA recommendations) as oxytetracycline. It is important to realise, however, that plasmid- and ICE-mediated resistance to florfenicol occurs by a number of mechanisms, including both *cat* and *floR* genes and is widespread in North American but not European BRD isolates (Michael et al., 2018), thus it should only be considered as a possible rotation drug rather than a replacement drug.

The macrolide tilmicosin, which is also listed as a tier 1 antimicrobial (AVA recommendations), has been used occasionally for metaphylaxis in at risk cattle on feedlot entry in Australia. The results of the present study, however, suggests a more cautious approach with metaphylactic use needs to be taken given that tulathromycin resistance has now emerged and it is possible that tilmicosin could provide additional selection pressure. Additionally, the present study has uncovered evidence of an unknown mechanism of tilmicosin resistance in isolates that were also tetracycline and aminopenicillin-resistant and appear to be carrying an ICE.

The present study findings should be interpreted with respect to the recent study of Coetzee et al. (2019) who undertook a Bayesian analysis of treatment histories and susceptibility data for a large number *M. haemoytica* isolates obtained from BRD submissions to a North American diagnostic microbiology laboratory. The Coetzee et al. study concluded that use of a bacteriostatic drug (most significantly tulathromycin) followed by a bactericidal drug (most significantly ceftiofur) had the highest probability of returning isolates resistant to multiple antimicrobial agents, presumably due to antimicrobial interactions between the two drugs. They also concluded that use of multiple drug classes was more likely to be associated with resistance development rather than retreatment with the same drug class. This is compounded by the fact that as a β-lactam, ceftiofur works most efficiently against rapidly dividing rather than dormant bacterial cells and tulathromycin has a very long half-life and may still be present in the lungs at sub-MIC levels when animals present for

retreatments, resulting in a negative drug interaction. However, this should not be the case for treatment with fluoroquinolones as these are equally bactericidal against dividing or dormant bacteria. The Coetzee et al. study is confounded by the fact that resistance mechanisms and their association with ICEs were not investigated in the isolates and the results may only be applicable to feedlots in North America. Nevertheless, treatment outcomes research should definitely be undertaken in Australian feedlots, especially now that resistance to tier 1 and tier 2 antimicrobials has been detected.

Tulathromycin is still the first drug of choice for the treatment of BRD in Australian feedlots and the current project has uncovered the first evidence of widespread (in terms of number of feedlots) resistance to tulathromycin in *P. multocida* isolated from infected lungs at post-mortem and the first evidence of resistance emergence in *M. haemolytica.* It will be important for feedlot veterinarians to carefully monitor their animal health teams to ensure correct weights are obtained prior to administration of the correct dose of antimicrobial agent. Chronic underdosing is by far the biggest issue in the development of AMR in animal pathogens and correct dosing is a cornerstone of antimicrobial stewardship (Lloyd and Page, 2018).

5.4 Antimicrobial resistance in other BRD pathogens

Apart from a single isolate that was resistant to macrolides, the Australian *M. haemolytica* collection was pan-susceptible to the tested antimicrobial agents. This was also the case for the *H. somni* isolate collection. This differs substantially from a number of international studies. Anholt et al. (2017) undertook a large cross sectional study of AMR in BRD isolates in Canada. Among 745 isolates, all *Mannheimia* and *Pasteurella* were resistant to at least one antimicrobial. Multidrug resistance was high in all target pathogens with 47.2% of the isolates resistant to four or five antimicrobial classes and 24.0% resistance to six to nine classes. El Garch et al. (2016) monitored antimicrobial susceptibility in BRD pathogens (*Mannheimia, Histophilus* and *Pasteurella*) obtained from European cattle between 2009 and 2012. This followed an earlier study conducted on isolates obtained between the years 2002 and 2006 (de Jong et al., 2014). They concluded that the majority of European BRD pathogens remained susceptible to registered drugs apart from a low to moderate level of resistance to tetracycline (3.0-12.0%). However, emerging resistance to macrolides was noted (0–4.0%). Four *M. haemolytica* isolates were resistant to both tulathromycin and gamithromycin with high MICs exhibited (128 and 256 µg/ml, respectively). Three *P. multocida* were resistant to tulathromycin and two isolates to gamithromycin, once again with high MICs.

DeDonder and Apley (2015) reviewed the literature documenting resistance in BRD pathogens in the US and identified 16 articles where resistance was reported. Studies between 1994 and 2008 confirmed the trend of low levels of cross-resistance among the macrolides, fluctuating levels of resistance to tetracycline, but uniform susceptibility to florfenicol, ceftiofur and fluoroquinolones. Lubbers and Hanzlicek (2013) examined the prevalence of resistance among BRD pathogens from submissions to the Kansas State University Veterinary Diagnostic Laboratory and identified an alarming trend of increasing MDR between 2009 (42% of isolates) and 2011 (63% of isolates) including resistance to the newer generation macrolides tulathromycin and gamithromycin. By 2011, a total of 25% of the isolates were resistant to four of six antimicrobials, with only ceftiofur and florfenicol showing uniform susceptibility.

The finding that Australian isolates of *Mycoplasma bovis* remain largely susceptible to antimicrobial agents, with only a comparatively small number of isolates found to be resistant to tilmicosin and tetracycline, is in contrast to several other studies. Gautier-Bouchardon et al. (2014) examined the AMR profiles of 27 and 46 *M. bovis* isolates, respectively obtained in 1978-1979 and in 2010-2012 from respiratory disease outbreaks in young cattle throughout France. The increase of the MIC_{50} of the isolates was substantial for tylosin, tilmicosin, tulathromycin and spectinomycin, and moderate for enrofloxacin, danofloxacin, marbofloxacin and oxytetracycline. No differences in MIC_{50} values were observed for gamithromycin and tildipirosin between the two sampling periods, with all *Mycoplasma bovis* isolates found to be resistant to both macrolides at relatively high levels (128 µg/mL). If referring to CLSI breakpoint MIC values published for the major BRD pathogens (S ≤ 4 µg/mL), most contemporary isolates would be classed as intermediately resistant to fluoroquinolones and resistant to macrolides, oxytetracycline, spectinomycin and florfenicol.

Similar proportions of resistance among contemporary *M. bovis* isolates from the Netherlands (2008-2014) were identified by Heuvelink et al. (2016) using both in house and commercially available broth microdilution methods. Fluoroquinolones appeared to be the most efficacious in inhibiting *M. bovis* growth, followed by tulathromycin and oxytetracycline with the highest MIC values obtained for erythromycin, tilmicosin, and tylosin. Tildipirosin and gamithromycin were not tested in this study. For tulathromycin, the MIC distribution was bimodal with approximately 52-64% of isolates (depending on the method) having MICs ≤ 1.0 µg/mL and 26-29% of isolates with MICs of 128 µg/mL or higher, which clearly indicates the presence of macrolide resistance determinants in some isolates. In the present study, reduced susceptibility to fluoroquinolones without any history of their use in feedlot cattle suggests that it may be an intrinsic resistance in *Mycoplasma* or an issue with susceptibility testing and not associated with chromosomal target gene point mutations, active efflux or other potential mechanisms. Whole genome sequencing of the *Mycoplasma* collection will be required to confirm this hypothesis.

5.5 Genetic analysis of *Pasteurella* and *Mannheimia* isolates

The results of ERIC-PCR and whole genome sequencing were in agreement that the *M. haemolytica* collection of isolates was homogeneous whereas the *P. multocida* collection was extremely diverse. A vaccine approach for control and prevention of *M. haemolytica* BRD infections has been very successfully applied in Australia (hence the lower incidence of disease), however, the same approach for the more prevalent *P. multocida* infections is unlikely to be successful due to their potentially high antigenic diversity. Whole genome sequencing and comparison with international genomes in available databases also confirmed that Australian *P. multocida* isolates from cases of BRD are unique. This is unsurprising given the ban on live animal importation, Australia's geography and border security, as well as differences in animal health and management within Australian feedlots. Australian BRD isolates have had the opportunity to evolve in isolation from the rest of the world and may have only recently developed resistance to macrolides and tetracyclines. Additionally, the resistant strains shown to contain known AMR genes appear to be genetically related. This could mean they are potentially derived from a common ancestor, or they have genetic restrictions on the uptake of mobile genetic elements including plasmids, transposons and ICEs.

5.6 Characterisation of possible mobile genetic elements in *P. multocida*

One of the limitations of Illumina sequencing is that the short sequence reads preclude the identification of gene clusters, plasmids and other mobile genetic elements. Nevertheless, it is the most rapid and cost effective method for identifying genetic traits in large collections of isolates. In the present study, whole genome sequencing identified known resistance determinants in *P. multocida* and a single *M. haemolytica* strain. This included *msr(E)* and *mph(E),* which encode macrolide efflux and phospotranserase proteins, respectively (Michael et al., 2018). These two genes, found in tandem and expressed from the same promoter, are predicted to be chromosomally encoded and impart higher MICs for gamithromycin and tulathromycin, and lower MICs for tildipirosin and tilmicosin (Kadlec et al., 2011).

Additionally, the current study identified *tet(H)-tet(R)* genes typically associated with the Tn*5706* transposon in all isolates that exhibited tetracycline resistance, with an unusual finding of the tetracycline efflux pump gene *tet(Y)* in four isolates. The Tn*5706* can be found on both chromosomes and plasmids in *P. multocida*, however, to the best of our knowledge, *tet(Y)* has not been described previously in the Pasteurellaceae family (Michael et al., 2018) and was associated with streptomycin (*strA, strB*), neomycin/kanamycin (*apha1*) and sulphonamide (*sul2*) resistance genes. *tet(Y)* was originally found in tandem with *tet(R)* on a multidrug resistance plasmid from a strain of freshwater *Aeromonas* spp (Gordon et al., 2011). Interestingly, the original plasmid also contained the florfenicol resistance gene *floR* in addition to the streptomycin and sulphonamide resistance genes. The unusual finding of a macrolide-resistant *M. haemolytica* strain (the only such strain in the collection) being isolated from the same lung sample as a macrolide-resistant *P. multocida* strain warrants further investigation and suggests potential recent transfer of some type of mobile genetic element between the two pathogens.

The unusual resistance phenotype (aminopenicillin-tetracycline-tilmicosin-resistance) of five *P. multocida* isolates from Qld warrants further investigation. These isolates appear to carry an ICE that may contain the core resistance genes *tet(R)-tet(H)* and the β-lactamase resistance gene $bla_{\text{ROB-1}}$. Furthermore, they are likely to be the only strains in the entire Australian BRD isolate collection that contain an ICE. Interestingly, the resistance to tilmicosin in these isolates currently remains unexplained, but could be related to point mutations in the 23 rRNA gene, which have been identified in other members of the Pasteurellaceae family (Michael et al., 2018). The $bla_{\text{ROB-1}}$ is an unusual gene to find in a *Pasteurella* isolate, but has recently been discovered in ICEs present in BRD isolates from North America (Stanford et al., 2020).

In conclusion, deep sequencing is now required in order to further delineate and identify the mobile genetic elements in the resistant strain collection responsible for the AMR phenotype observed. A selection of 10 strains that cover the main phenotypes for long range sequencing will facilitate the discovery of plasmids, transposons, insertion sequences and ICEs and allow a detailed molecular risk assessment to be undertaken on the transferability of the identified resistance genes and the likelihood of multidrug-resistant phenotypes developing in the future. The fact that the only Australian strains that may contain ICE are still susceptible to tulathromycin suggests that they are unlikely to rapidly increase and spread. Similarly, isolates showing dual resistance to tetracyclines and all macrolides except tildipirosin may not carry both resistance elements on the same mobile genetic element, which means it is unlikely that this resistance phenotype will spread horizontally.

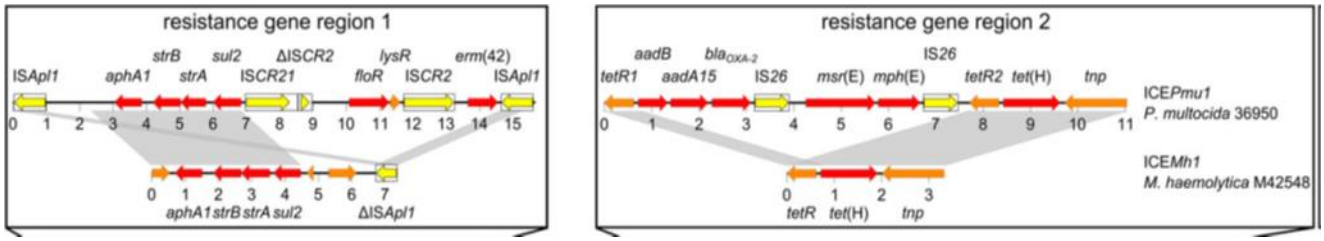

Fig. 5: Similar resistance encoding regions identified in ICEs from bovine respiratory disease pathogens *P. multocida* and *M. haemolytica* isolated in North America (Eidam et al. 2015).

5.7 Meeting Project Objectives

5.7.1 Obtain baseline antimicrobial susceptibility data on existing and prospective collections of Australian BRD isolates including *Mycoplasma bovis*.

This objective has been achieved. Passive surveillance isolates from 2014-2019 were obtained from each collaborating veterinary diagnostic laboratory and antimicrobial susceptibility tested. Resistance to tetracyclines and macrolides was first observed in these isolates.

5.7.2 Develop guidelines and training materials for the collection of diagnostic specimens from cases of BRD for culture and susceptibility testing.

This objective has been achieved. Technology was successful transferred to the collaborating feedlots who were provided with sundry kits to enable sampling.

5.7.3 Undertake case sampling studies at eight Australian feedlots that differ in their antimicrobial treatment regimes during 2018/2019 BRD seasons to link clinical, epidemiological and pathological data with microbiological culture and susceptibility testing.

This objective has been achieved. Initially 11 feedlots were invited to take part in the study with eight consistently submitting samples to their local veterinary diagnostic laboratory. Isolates from each VDL were sent to a centralised laboratory for re-identification and antimicrobial susceptibility testing. Pathological data has been obtained and linked to culture results, confirming *Mycoplasma* as a significant feedlot pathogen.

5.7.4 Undertake genetic testing of selected isolates using whole genome sequencing to compare with international BRD isolates and conduct a molecular-based risk

assessment on the likelihood of Australian BRD isolates acquiring resistance and in particular ICEs.

This objective has been achieved. Large collections of *P. multocida* and *M. haemolytica* isolates have been sent to University of Technology Sydney for whole genome sequencing using Illumina technology. This provides large numbers of low read sequences that are ideal for identifying genetic relationships between strains and AMR genes. Long range sequencing is now required to physically identify the location of resistance genes within isolates.

5.7.5 Hold an ACARE workshop in late 2019 (sponsored by the pharmaceutical industry and major research stakeholders) at project completion to disseminate project findings (with Dr Brian Lubbers Kansas State University as the Keynote Speaker) and develop Tips and Tools brochures for distribution.

ACARE workshop has been postponed until September 2020 due to COVID-19.

5.7.6 Study strengths and limitations

The project has achieved a significant milestone in establishing sampling techniques for BRD pathogens at post-mortem at eight feedlots distributed throughout Australia. All deaths on feedlots are subjected to a cursory post-mortem exam to confirm the cause of death/euthanasia and the industry can now conduct local AMR surveillance on samples submitted to participating VDLs to fulfil its obligations according to the McDonald's antimicrobial stewardship policy. Protocols at VDLs have been changed (particularly with the adoption of MALDI-TOF) to have a better chance of isolating *Mycoplasma* and confirming it as a significant pathogen in BRD cases in Australia. Emerging resistance to macrolides and tetracyclines was identified in *P. multocida* isolates and a single *M. haemolytica* isolate was found to be resistant to macrolides. For the remaining pathogens resistance was negligible (*H. somni*) or far less than what has been reported internationally (*Mycoplasma bovis* and *T. pyogenes*). Whole genome sequencing of isolates identified resistance genes responsible for the AMR phenotype and provided some indication of possible mobile genetic elements present in feedlot isolates.

The main study limitation was that approximately half the expected number of samples were received during the 2019 targeted surveillance study. Nevertheless, a high rate of pathogen isolation was obtained and resistance trends could be established, indicating that sampling technique was successfully transferred. Good sampling technique and transport to the laboratories relied upon instructions delivered by feedlot veterinarians and co-ordination with courier companies and it is recognised that feedlots have gone to some effort to factor sample collection at post-mortem into their busy schedule. Enormous effort and cost has been expended to ensure samples obtained from post mortems conducted on site, transported to the nearest laboratory and submitted for culture and susceptibility testing grow BRD pathogens and not contaminating bacteria.

Another limitation was the coordination of sample number with animal identification ID between feedlots,VDLs, The University of Adelaide and The University of Technology, Sydney. Some feedlots did not provide animal ID codes with every sample. The use of multiple spreadsheets by feedlots, VDLs and Research Organisations prevented the loss of data, but some mistakes in data entry were identified and rectified.

A final limitation was that due to cost and space limitations a fresh tissue sample from each feedlot was not stored at -80^{o}C by each VDL for later virus detection and/or isolation. Whilst histological sections can be retrospectively analysed for the presence of viruses and *Mycoplasma* by immunohistochemistry staining now that disease associations have been identified, particularly the association of *P. multocida* with *Mycoplasma bovis* infections. A targeted prospective study could now proceed to determine the significance of BPI3 in particular which has been previously associated with *Mycoplasma bovis* and chronic BRD lesions.

6 Conclusions/recommendations

6.1 Continue targeted AMR surveillance in 2020

Feedlots that took part in the targeted surveillance study still have a large number of sample containers left over from the 2019 survey. Sampling could continue for another six to nine months, commencing in April 2020, to increase the number of samples from lots that have returned resistant isolates to statistically determine associations with antimicrobial use. Feedlots that did not significantly take part in the 2019 survey (V1, S2 and S3) could be invited to join the 2020 survey to fulfil their obligations to conduct local surveillance as part of the McDonald's blueprint and increase/improve the diversity of isolates obtained. Each VDL apart from CSUVDL would be using MALDI-TOF for bacterial identification, adopting Sensititre antimicrobial susceptibility testing and thus would able to generate local AMR data for each feedlot as well as compile and submit data (rather than isolates) to be used in national AMR surveillance for the industry. This would significantly save on double handling of isolates and provide a mechanism for continued, sustainable surveillance following the conclusion of the MLA grant. With the savings made on susceptibility testing, frozen tissue samples could be archived for investigation of BRD viruses to identify co-associations.

6.2 Conduct long range (deep) whole genome sequencing on 10 selected isolates from the 2019 targeted AMR survey

The study has shown that the majority of bacteria isolated, including *Mycoplasma,* remain susceptible to the most common antimicrobials used to treat BRD cases in the industry. This is a point of difference with many international studies, in particular those from North America where multidrug-resistant ICEs have been found in all three of the main pathogenic bacteria isolated from BRD cases (*M. haemolytica, P. multocida* and *H. somni*). Resistance to macrolides has emerged in a small number of *P. multocida* isolates obtained from the majority of participating feedlots, with two feedlots also yielding isolates that were resistant to both macrolides and tetracycline. Skewed sampling that focuses on identifying BRD pathogens at post-mortem is likely to have selected for resistant bacteria in animals that do not respond to first or second line therapy. Nevertheless, it will be important to ensure that these bacteria do not spread further within the feedlot environment.

The Illumina data have given an overview of resistance mechanisms present in *Pasteurella* and a single *Mannheimia* isolate. Importantly, the majority of resistant strains do not appear to have ICEs in their genome. ICEs are possibly limited to a small number of Qld isolates with an unusual resistance phenotype that were nevertheless susceptible to tulathromycin. The basis of tilmicosin resistance in these isolates still remains to be determined. To conduct a proper molecular risk

assessment and determine the likelihood of resistance increasing, it is recommended that long range sequencing be conducted on a range of resistant and susceptible isolates to identify the genetic elements encoding resistance which possibly could include a combinations of plasmids, transposons and ICEs. UTS has the capacity to conduct this on a total of 10 strains at the same time. Recommended strains to include would be:

1) Two *Pasteurella* isolates that have the β-lactam/tetracycline/tilmicosin resistance phenotype, and appear to contain an ICE.

2) The *Pasteurella* isolate and the *Mannheimia* isolate from the same animal that were resistant to macrolides and a further two *Pasteurella* isolates from different feedlots that are macrolide-resistant.

3) Two *Pasteurella* isolates with macrolide and tetracycline resistance (one from each feedlot)

4) A fully susceptible *Pasteurella* isolate and a fully susceptible *Mannheimia* isolate.

6.3 Discuss with feedlot veterinarians about future opportunities to use florfenicol on a rotational basis to treat resistant infections before switching back to tulathromycin

Use of ceftiofur in the industry appears to be appropriate as a third line therapy for retreatments of cases where there is no response to tulathromycin or late BRD cases due to export slaughter intervals based on responses to a MLA sponsored questionnaire (Badger et al., 2020). Whilst being registered in Australia for the treatment of respiratory infections in cattle and pigs and classed as a first line antimicrobial by the AVA, florfenicol (Nuflor) is not favoured by feedlot veterinarians. Two injections by the intramuscular route must be given two days apart, which is not practical in a feedlot environment, though successful treatment of BRD is achieved with a single subcutaneous injection at double the recommended dose. Florfenicol is often used off label on a rotational basis by swine industry veterinarians to treat *E. coli* infections that have become resistant to aminoglycosides and other antimicrobial classes (P. McKenzie, personal communication). Use is limited as resistance to florfenicol can appear rapidly within a population and has been identified in BRD isolates internationally (Michael et al., 2018) including within ICEs (Fig. 6). Following review of this project a meeting of feedlot veterinarians (potentially at the conference planned in September 2020) could devise treatment plans for dealing with resistant bacteria identified in local AMR studies that may or may not include florfenicol as an option. Dr Brian Lubbers would be a Keynote Speaker to cover lessons learned from the development of resistance to multiple agents in North American feedlots.

6.4 Debate on the metaphylactic use of chlortetracycline in the feed and the prophylactic administration of tilmicosin at feedlot entry

The project has uncovered the first evidence that metaphylactic use of chlortetracycline pulses in the feed (as a first line treatment, which is entirely appropriate based on the AVA's prudent use guidelines), followed by the macrolide tulathromycin for individual case treatments (also entirely appropriate as second line therapy according to the AVA) may be selecting for *P. multocida* subpopulations resistant to both agents. Further sampling from the feedlots that generated dual resistant isolates should be undertaken to build up the number of cases to enable statistical analysis

to be undertaken to confirm or refute this hypothesis. Tilmicosin administration at feedlot entry for cattle deemed to be at high risk of developing BRD (a first line macrolide according to the AVA guidelines) could also provide selection pressure for macrolide-resistant sub-populations, particularly if it is followed up with individual tulathromycin treatments and could similarly be reviewed.

6.5 Significance of *Mycoplasma* as a BRD pathogen and retrospective immunohistochemistry analysis of histological sections

The project has confirmed that *Mycoplasma* are significant causes of BRD in Australia as both monoculture and mixed infections. A large heterogeneous collection of *Mycoplasma bovis* has been established in this project and it was shown to be susceptible to the major antimicrobial agents used to treat BRD (except for intrinsic resistance to ceftiofur), a clear point of difference with international studies. *Mycoplasma* was shown to be associated with *Pasteurella* infections and retrospective analysis of histological sections could now be undertaken using immunohistochemistry to visualise viral and *Mycoplasma* pathogens within lesions to indentify the key co-morbidities (in particular BPIV3). Whole genome sequencing of the *Mycoplasma bovis* collection could be another possible future project and would provide necessary data for a reverse vaccinology approach to prevent infections, given the high prevalence in mixed infections.

7 Key messages

7.1 Tools and tips for industry

Finalisation of the whole genome sequencing component of this project in February 2020 has confirmed the identification of genes associated with three main resistance mechanisms first identified in passive surveillance *P. multocida* isolates from submissions to VDLs in 2016-2018 and demonstrated in further *P. multocida* and a single *M. haemolytica* isolate obtained during the 2019 targeted surveillance study.

Resistance to macrolides through possession of *msr(E)* and *mph(E)* was the most common and widespread mechanism identified. It was found in association with tetracycline resistance mediated by *tet(R)-tet(H)* at a restricted number of feedlots in NSW that practice pulsing of chlortetracycline in the feed. Finally, a small number of isolates localised to Qld were resistant to aminopenicillins (bla_{ROB-1}), tetracycline (*tet(R)-tet(H)*) and tilmicosin (unknown) and appear to have an ICE. These isolates are still susceptible to tulathromycin (and hence should be eliminated at the first treatment) and do not appear to have spread beyond a single feedlot. Tips and tools will be developed with feedlot veterinarians and Dr Mandi Carr to outline that resistance has just emerged within the Australian industry, that it is low by international standards, and that further spread can be prevented by good antimicrobial stewardship practice. These will include:

1) The importance of conducting local AMR surveillance by investigating BRD mortalities during peak disease periods (autumn and spring), aseptically collecting samples using the appropriate tools and submitting to the nearest VDL.
2) Management and husbandry practices that reduce the occurrence of BRD.

3) The early detection and treatment of BRD cases including calculating and administrating the appropriate dose.
4) The judicious use of ceftiofur as a reserve agent
5) Possible rotation of first line drugs (tulathromycin to florfenicol and back to tulathromycin) to eliminate resistant subpopulations that may be present in the feedlot.
6) The importance of management practices (e.g. backgrounding, vaccination) to reduce BRD risk.

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9 Appendix

9.1 Post-mortem guide for obtaining BRD specimens – for feedlot and veterinary use only – obtain by written request to MLA

9.2 Feedlots and supporting diagnostic laboratories (removed due to Confidentiality)

9.3 MIC Distribution Tables for *Mannheimia, Pasteurella* and *Histophilus* isolates

Table 9.3.1. MIC distribution frequencies of *M. haemolytica* cattle isolates from Australia 2014-2015.

Mannheimia haemolytica (n = 11)																			
		Percentage of isolates with MICs distribution (µg/mL) b															MIC50 (µg/ml)	MIC90 (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-32.14
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-32.14
Clindamycin	Cattle				0	0	0	0	0	100	0						8	8	0-32.14
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-32.14
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-32.14
Florfenicol	Cattle				0	100	0	0	0	0							0.5	0.5	0-32.14
Gentamicin	Cattle						9.1	81.8	9.1	0	0						2	4	0-32.14
Neomycin	Cattle								54.5	45.5	0	0					4	8	0-32.14
Tetracycline	Cattle					90.9	9.1	0	0	0							0.5	1	0-32.14
Penicillin	Cattle			63.6	9.1	27.3	0	0	0	0							0.12	0.5	0-32.14
Sulphadimethoxine	Cattle														100		256	256	0-32.14
Spectinomycin	Cattle									0	9.1	90.9	0				16	32	0-32.14
Tiamulin	Cattle					0	0	0	0	36.4	63.6	0					8	16	0-32.14
Tilmicosin	Cattle							0	81.8	18.2	0						4	8	0-32.14
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-32.14
Tulathromycin	Cattle						0	0	63.6	18.2	18.2	0	0				4	8	0-32.14
Tylosin tartrate	Cattle					0	0	0	0	0	9.1	27.3	63.6				16	>32	0-32.14
Tildipirosin	Cattle						0	100	0	0	0						1	1	0-32.14
Gamithromycin	Cattle						0	100	0	0							1	1	0-32.14

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.2. MIC distribution frequencies of *M. haemolytica* cattle isolates from Australia 2016-2017.

Mannheimia Haemolytica (n = 23)																			
		Percentage of isolates with MICs distribution (µg/mL) b															**MIC_{50} (µg/ml)**	**MIC_{90} (µg/ml)**	**CI (95%)**
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-17.81
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-17.81
Clindamycin	Cattle				0	0	0	0	0	82.6	17.4						8	16	0-17.81
Danofloxacin	Cattle			100	0	0	0										0.12	0.12	0-17.81
Enrofloxacin	Cattle			95.7	0	0	4.3	0									0.12	1	0-17.81
Florfenicol	Cattle				4.3	91.3	4.3	0	0	0							0.5	1	0-17.81
Gentamicin	Cattle						4.3	95.7	0	0	0						2	2	0-17.81
Neomycin	Cattle								34.8	65.2	0	0					4	8	0-17.81
Tetracycline	Cattle					100	0	0	0	0							0.5	0.5	0-17.81
Penicillin	Cattle			39.1	52.2	8.7	0	0	0	0							0.25	0.5	0-17.81
Sulphadimethoxine	Cattle														100		256	256	0-17.81
Spectinomycin	Cattle									0	26.1	73.9	0				16	32	0-17.81
Tiamulin	Cattle					0	0	0	0	4.3	95.7	0					8	16	0-17.81
Tilmicosin	Cattle							0	34.8	56.5	8.7	0	0				4	8	0-17.81
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-17.81
Tulathromycin	Cattle						0	0	30.4	65.2	4.3	0	0				4	8	0-17.81
Tylosin tartrate	Cattle					0	0	0	0	0	0	100					32	32	0-17.81
Tildipirosin	Cattle						95.7	4.3	0	0	0						1	2	0-17.81
Gamithromycin	Cattle						0	100	0	0							1	1	0-17.81

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. A Cut-off values were used according to CLSI document VET08.

Table 9.3.3. MIC distribution frequencies of *M. haemolytica* cattle isolates from Australia 2018.

		Mannheimia haemolytica (n = 21)																	
		Percentage of isolates with MICs distribution (µg/mL)															MIC50 (µg/ml)	MIC90 (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-19.24
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-19.24
Clindamycin	Cattle				0	0	4.8	4.8	0	66.6	19.0	4.8					8	16	0-19.24
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-19.24
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-19.24
Florfenicol	Cattle				14.3	80.9	4.8	0	0	0							0.25	0.5	0-19.24
Gentamicin	Cattle						0	100	0	0	0						2	2	0-19.24
Neomycin	Cattle								14.3	85.7	0	0					4	8	0-19.24
Tetracycline						100	0	0	0	0							0.5	0.5	0-19.24
Penicillin	Cattle			47.6	52.4	0	0	0	0	0							0.12	0.25	0-19.24
Sulphadimethoxine	Cattle														100		256	256	0-19.24
Spectinomycin	Cattle									0	19.0	76.2	4.8				16	32	0-19.24
Tiamulin	Cattle					0	4.8	4.8	0	9.5	71.4	9.5					8	16	0-19.24
Tilmicosin	Cattle							0	38.1	61.9	0	0	0				4	8	0-19.24
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-19.24
Tulathromycin	Cattle						4.8	9.5	28.6	47.6	9.5	0	0				8	16	0-19.24
Tylosin tartrate	Cattle					0	0	0	0	4.8	4.8	9.5	80.9				32	32	0-19.24
Tildipirosin	Cattle						95.2	4.8	0	0	0						1	2	0-19.24
Gamithromycin	Cattle						95.2	4.8	0	0							1	2	0-19.24

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines., MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.4. MIC distribution frequencies of *M. haemolytica* cattle isolates from Australia 2019.

***Mannheimia haemolytica* (n = 33)**																			
		Percentage of isolates with MICs distribution (µg/mL)															**MIC_{50} (µg/ml)**	**MIC_{90} (µg/ml)**	**CI (95%)**
Antimicrobial Agent	**Source**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-12.98
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-12.98
Clindamycin	Cattle				0	0	0	0	6.1	63.6	30.3						8	16	0-12.98
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-12.98
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-12.98
Florfenicol	Cattle				0	97.0	3.0	0	0	0							0.5	0.5	0-12.98
Gentamicin	Cattle						0	97.0	3.0	0	0						2	2	0-12.98
Neomycin	Cattle								15.2	84.8	0	0					8	8	0-12.98
Tetracycline	Cattle					93.9	6.1	0	0	0							0.5	0.5	0-12.98
Penicillin	Cattle			75.8	24.2	0	0	0	0	0							0.12	0.25	0-12.98
Sulphadimethoxine	Cattle														87.9	12.1	256	512	0-12.98
Spectinomycin	Cattle									0	54.5	45.5	0				16	32	0-12.98
Tiamulin	Cattle					0	0	0	0	12.1	60.6	27.3					16	32	0-12.98
Tilmicosin	Cattle							6.1	27.3	60.6	3.0	3.0					4	8	0.16-17.51
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-12.98
Tulathromycin	Cattle						0	0	0	97.0	0	0	0	3.0			8	8	0.16-17.51
Tylosin tartrate	Cattle					0	0	0	0	0	0	15.2	84.8				32	≥64	0-12.98
Tildipirosin	Cattle						84.8	12.1	3.0	0	0						1	1	0-12.98
Gamithromycin	Cattle						90.9	6.1	0	0	3.0						1	2	0.16-17.51

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.5. MIC distribution frequencies of *P. multocida* cattle isolates from Australia 2014-2015.

***Pasteurella multocida* (n = 12)**

		Percentage of isolates with MICs distribution (µg/mL)															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-30.13
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-30.13
Clindamycin	Cattle				0	0	0	0	0	25.0	50.0	25.0					8	>16	0-30.13
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-30.13
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-30.13
Florfenicol	Cattle				75.0	25.0	0	0	0	0							0.25	0.5	0-30.13
Gentamicin	Cattle						25.0	50.0	25.0	0	0						2	4	0-30.13
Neomycin	Cattle								75.0	25.0	0	0					4	8	0-30.13
Tetracycline	Cattle					91.7	8.3	0	0	0							0.5	1	0-30.13
Penicillin	Cattle			100	0	0	0	0	0	0							0.12	0.12	0-30.13
Sulphadimethoxine	Cattle														100		256	256	0-30.13
Spectinomycin	Cattle									8.3	33.3	58.3	0				16	32	0-30.13
Tiamulin	Cattle					0	0	0	0	16.7	58.3	25.0					16	32	0-30.13
Tilmicosin	Cattle								83.3	16.7	0						4	8	0-30.13
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-30.13
Tulathromycin	Cattle						75.0	25.0	0	0	0	0	0				1	2	0-30.13
Tylosin tartrate	Cattle					0	0	0	0	8.3	33.3	58.3					16	32	0-30.13
Tildipirosin	Cattle						100	0	0	0	0						1	1	0-30.13
Gamithromycin	Cattle						100	0	0	0							1	1	0-30.13

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.6. MIC distribution frequencies of *P. multocida* isolates from Australia 2016-2017.

***Pasteurella multocida* (n = 40)**

		Percentage of isolates with MICs distribution (µg/mL)															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				97.5	0	2.5	0	0	0	0						0.25	1	0.13-14.73
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-10.91
Clindamycin	Cattle				0	0	0	2.5	5.0	15.0	42.5	35.0					8	≥16	0-10.91
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-10.91
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-10.91
Florfenicol	Cattle				62.5	37.5	0	0	0	0							0.25	0.5	0-10.91
Gentamicin	Cattle					10.0	12.5	52.5	22.5	0	2.5						2	4	0-10.91
Neomycin	Cattle								50	35	7.5	0	7.5				8	16	0-10.91
Tetracycline	Cattle					87.5	2.5	0	0	10.0							0.5	8	3.25-24.6
Penicillin	Cattle			92.5	5.0	0	0	2.5	0	0							0.12	0.25	0-10.91
Sulphadimethoxine	Cattle														100		256	256	0-10.91
Spectinomycin	Cattle									10.0	47.0	42.5	0				16	32	0-10.91
Tiamulin	Cattle					5.0	0	2.5	2.5	12.5	60.0	17.5					16	32	0-10.91
Tilmicosin	Cattle								67.5	27.5	0	2.5	0	2.5			4	8	0-10.91
Trimethoprim/ sulfamethoxazole	Cattle							100									0.12	2	0-10.91
Tulathromycin	Cattle						55.0	32.5	5.0	5.0	0	0	0	2.5			1	2	0.13-14.73
Tylosin tartrate	Cattle					0	2.5	0	2.5	10.0	55.0	22.5	7.5				16	32	0-10.91
Tildipirosin	Cattle						92.5	0	0	5.0	0	2.5					1	8	0.13-14.73
Gamithromycin	Cattle						90.0	2.5	0	5.0	2.5						1	8	0.13-14.73

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08

Table 9.3.7. MIC distribution frequencies of *P. multocida* cattle isolates from Australia 2018.

Pasteurella multocida (n = 23)

		Percentage of isolates with MICs distribution (µg/mL)															MIC50 (µg/ml)	MIC90 (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				91.3	0	0	0	0	0	4.3	4.3					0.25	0.25	15.2-29.51
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-17.81
Clindamycin	Cattle				0	0	0	4.3	0	13.0	13.0	69.6					8	>16	0-17.81
Danofloxacin	Cattle			95.7	4.3	0	0	0									0.12	0.12	0-17.81
Enrofloxacin	Cattle			95.7	4.3	0	0	0									0.12	0.12	0-17.81
Florfenicol	Cattle				56.5	43.5	0	0	0	0							0.25	0.5	0-17.81
Gentamicin	Cattle						21.7	56.5	21.7	0	0						2	4	0-17.81
Neomycin	Cattle								52.2	34.8	13.0	0					4	8	0-17.81
Tetracycline	Cattle					86.9	4.3	0	0	0	8.7						0.5	1	15.2-29.51
Penicillin	Cattle			91.3	0	0	0	0	0	8.7							0.12	0.12	1.52-29.51
Sulphadimethoxine	Cattle														100		256	256	0-17.81
Spectinomycin	Cattle									13.0	69.6	17.4	0				16	32	0-17.81
Tiamulin	Cattle					0	0	4.3	0	17.4	56.5	21.7					16	32	0-17.81
Tilmicosin	Cattle								65.2	26.1	0		4.3				4	8	0.23-23.97
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-17.81
Tulathromycin	Cattle						48.0	30.4	4.3	13.0	0	0	4.3				1	8	0.23-23.97
Tylosin tartrate	Cattle					0	0	0	4.3	30.4	34.8	30.4					16	32	0-17.81
Tildipirosin	Cattle						86.9	4.3	0	8.7	0						1	8	0-17.81
Gamithromycin	Cattle						95.7	0	0	0	4.3						1	>8	0.23-23.97

Table 9.3.8. MIC distribution frequencies of *P. multocida* cattle isolates from Australia 2019.

***Pasteurella multocida* (n = 65)**

		Percentage of isolates with MICs distribution (µg/mL)															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	Source	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512			
Ampicillin	Cattle				93.8	1.5	0	0	0	1.5	3.1						0.25	0.5	1.99-15.78
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-6.95
Clindamycin	Cattle				0	0	1.5	0	3.1	6.2	49.2	40.0					16	32	0-6.95
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-6.95
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-6.95
Florfenicol	Cattle				81.5	18.5	0	0	0	0							0.25	0.5	0-6.95
Gentamicin	Cattle						18.5	67.7	12.3	1.5	0						2	4	0-6.95
Neomycin	Cattle								69.2	12.3	18.5	0					4	8	0-6.95
Tetracycline	Cattle					75.4	4.6	0	3.1	15.4	1.5						0.5	8	9.14-28.68
Penicillin	Cattle			92.3	3.1	0	0	0	0	0	4.6						0.12	0.25	1.2-13.76
Sulphadimethoxine	Cattle														100		256	256	0-6.95
Spectinomycin	Cattle									10.7	63.1	26.2	0				16	32	0-6.95
Tiamulin	Cattle					1.5	0	1.5	3.1	18.5	60	15.4					16	32	0-6.95
Tilmicosin	Cattle							27.7	35.4	9.2	1.5	26.2					8	32	16.38-38.76
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-6.95
Tulathromycin	Cattle									75.4	1.5	0	23.1				8	64	13.9-35.5
Tylosin tartrate	Cattle					0	1.5	0	3.1	9.2	64.6	21.5					16	32	0-6.95
Tildipirosin	Cattle						72.3	9.2	7.7	6.2	4.6						1	2	0-6.95
Gamithromycin	Cattle						76.9	0	0	0	23.1						1	16	13.9-35.48

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.9. MIC distribution frequencies of *H. somni* cattle isolates from Australia 2014-2015.

Histophilus somni (n = 13)																			
		Percentage of isolates with MICs distribution (µg/mL)[b]															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	**Source**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-28.34
Ceftiofur	Cattle				76.9	7.7	7.7	7.7	0	0							0.25	0.5	0-28.34
Clindamycin	Cattle				15.4	61.5	23.1	0	0	0	0						0.5	1	0-28.34
Danofloxacin	Cattle			92.3	7.7	0	0	0									0.12	0.12	0-28.34
Enrofloxacin	Cattle			92.3	7.7	0	0	0									0.12	0.12	0-28.34
Florfenicol	Cattle				100	0	0	0	0	0							0.2.5	0.25	0-28.34
Gentamicin	Cattle						23.0	7.7	30.8	23.1	15.4						8	16	0-28.34
Neomycin	Cattle								7.7	46.1	23.1	23.1					16	32	0-28.34
Tetracycline	Cattle					0	92.3	7.7	0	0							1	1	0-28.34
Penicillin	Cattle			84.6	15.4	0	0	0	0	0							0.12	0.25	0-28.34
Sulphadimethoxine	Cattle														100		256	256	0-28.34
Spectinomycin	Cattle									69.2	30.8	0	0				8	16	0-28.34
Tiamulin	Cattle					30.8	69.2	0	0	0	0	0					0.5	1	0-28.34
Tilmicosin	Cattle							0	61.5	38.5	0						4	8	0-28.34
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-28.34
Tulathromycin	Cattle						30.8	0	23.0	30.8	15.4	0	0				8	16	0-28.34
Tylosin tartrate	Cattle					30.8	32.1	23.1	23.0	0	0	0					2	4	0-28.34
Tildipirosin	Cattle						0	53.8	46.2	0	0	0					2	4	0-28.34
Gamithromycin	Cattle						100	0	0	0							0.5	0.5	0-28.34

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. A Cut-off values were used according to CLSI document VET08. This collection was tested for susceptibility to oxytetracycline.

Table 9.3.9. MIC distribution frequencies of *H. somni* cattle isolates from Australia 2016-2017.

Histophilus somni (n = 11)																			
		Percentage of isolates with MICs distribution (µg/mL)[b]															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	**Source**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-32.14
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-32.14
Clindamycin	Cattle				9.1	72.7	18.2	0	0	0	0						0.5	1	0-32.14
Danofloxacin	Cattle			100	0	0	0										0.12	0.12	0-32.14
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-32.14
Florfenicol	Cattle				100	0	0	0	0	0							0.2.5	0.25	0-32.14
Gentamicin	Cattle						9.1	9.1	18.2	18.2	45.4						8	16	0-32.14
Neomycin	Cattle								72.7	18.2	9.1	45.4					4	8	0-32.14
Tetracycline	Cattle					0	100	0	0	0							1	1	0-32.14
Penicillin	Cattle			100	0	0	0	0	0	0							0.12	0.25	0-32.14
Sulphadimethoxine	Cattle														100		256	256	0-32.14
Spectinomycin	Cattle									9.1	81.8	9.1	0				8	16	0-32.14
Tiamulin	Cattle					27.3	45.4	27.3	0	0	0	0					0.5	1	0-32.14
Tilmicosin	Cattle							0	54.5	45.5	0						4	8	0-32.14
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-32.14
Tulathromycin	Cattle						9.1	18.2	18.2	36.3	18.2	0	0				8	16	0-32.14
Tylosin tartrate	Cattle					9.1	18.2	18.2	54.5	0	0	0					2	4	0-32.14
Tildipirosin	Cattle						18.2	63.6	18.2	0	0						2	4	0-32.14
Gamithromycin	Cattle						90.0	9.1	0	0							0.5	0.5	0-32.14

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines. A Cut-off values were used according to CLSI document VET08.

Table 9.3.10. MIC distribution frequencies of *H. somni* cattle isolates from Australia 2018.

Histophilus somni (n = 11)																			
		Percentage of isolates with MICs distribution (µg/mL)[b]															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	**Source**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-32.14
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-32.14
Clindamycin	Cattle				27.3	72.7	0	0	0	0	0						0.25	0.5	0-32.14
Danofloxacin	Cattle			100	0	0	0										0.12	0.12	0-32.14
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-32.14
Florfenicol	Cattle				100	0	0	0	0	0							0.2.5	0.25	0-32.14
Gentamicin	Cattle						36.3	45.5	9.1	9.1	0						1	2	0-32.14
Neomycin	Cattle								72.7	18.2	9.1	0					4	8	0-32.14
Tetracycline	Cattle					0	100	0	0	0							0.5	0.5	0-32.14
Penicillin	Cattle			90.9	9.1	0	0	0	0	0							0.12	0.25	0-32.14
Sulphadimethoxine	Cattle														100		256	256	0-32.14
Spectinomycin	Cattle									63.6	36.4	0	0				8	16	0-32.14
Tiamulin	Cattle					9.1	81.8	9.1	0	0	0	0					0.5	1	0-32.14
Tilmicosin	Cattle							0	90.9	9.1	0	0	0				4	8	0-32.14
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-32.14
Tulathromycin	Cattle						9.1	18,2	54,5	9.1	9.1	0	0				2	4	0-32.14
Tylosin tartrate	Cattle					9.1	18.2	72.7	0	0	0	0					2	1	0-32.14
Tildipirosin	Cattle						9.1	81.8	0	9.1	0						2	8	0-32.14
Gamithromycin	Cattle						100	0	0	0							1	1	0-32.14

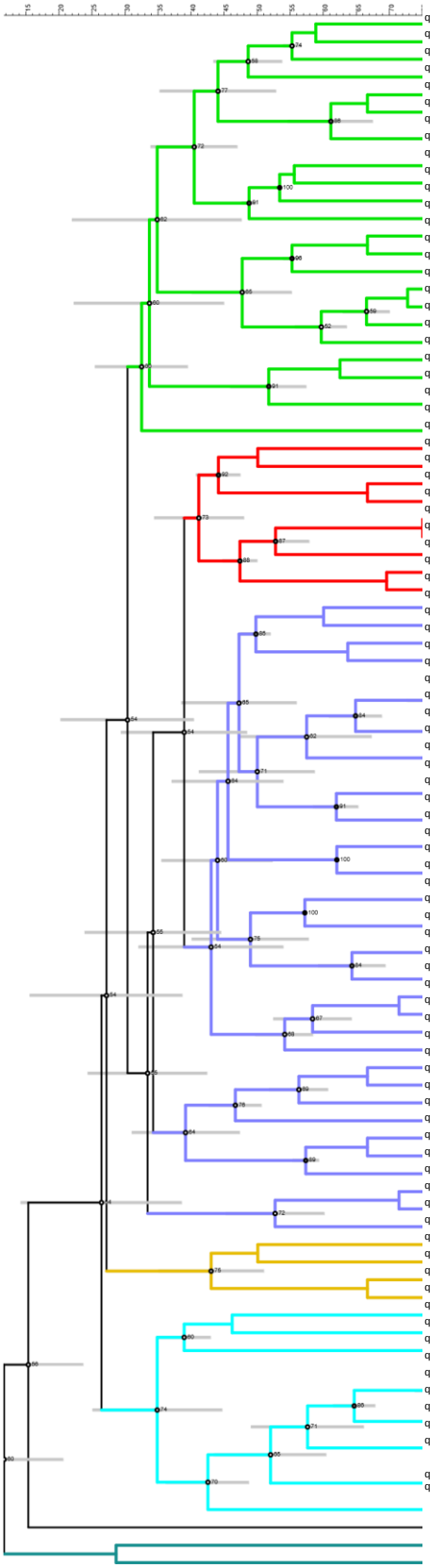
The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines., MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

Table 9.3.11. MIC distribution frequencies of *H. somni* cattle isolates from Australia 2019.

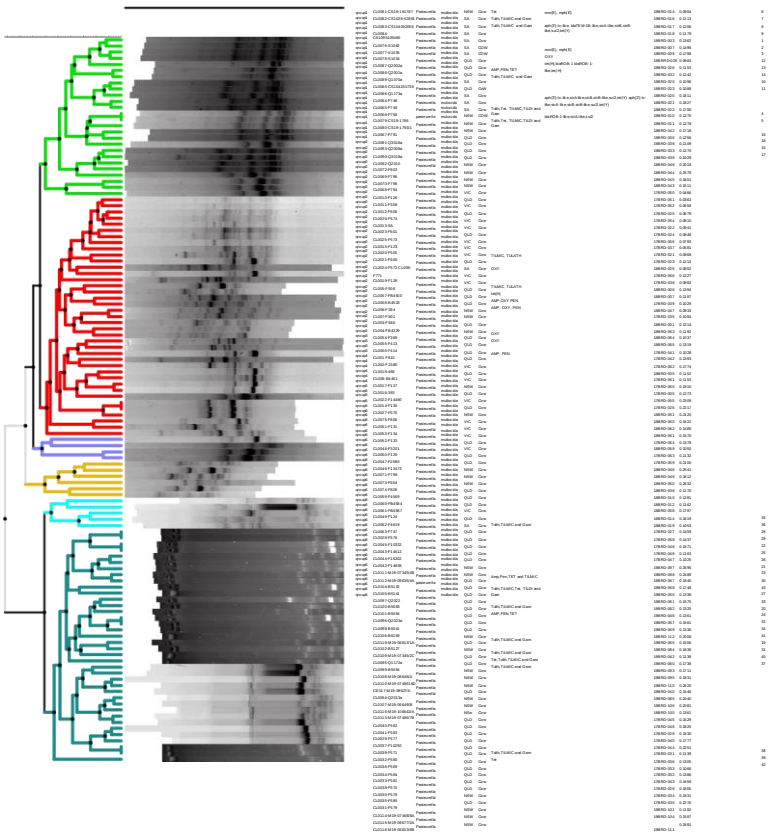
Histophilus somni (n = 35)																			
		Percentage of isolates with MICs distribution (µg/mL)[b]															MIC_{50} (µg/ml)	MIC_{90} (µg/ml)	CI (95%)
Antimicrobial Agent	**Source**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**			
Ampicillin	Cattle				100	0	0	0	0	0	0						0.25	0.25	0-12.32
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.5	0-12.32
Clindamycin	Cattle				0	94.3	2.9	2.9	0	0	0						0.5	1	0-12.32
Danofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-12.32
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-12.32
Florfenicol	Cattle				100	0	0	0	0	0							0.2.5	0.25	0-12.32
Gentamicin	Cattle						2.9	5.7	77.1	14.3	0						4	8	0-12.32
Neomycin	Cattle								0	8.6	74.3	17.1					16	32	0-12.32
Tetracycline	Cattle					2.9	65.7	31.4	0	0							1	2	0-12.32
Penicillin	Cattle			100	0	0	0	0	0	0							0.12	0.12	0-12.32
Sulphadimethoxine	Cattle														74.3	25.7	256	>256	0-12.32
Spectinomycin	Cattle									2.9	60.0	37.1	0				16	32	0-12.32
Tiamulin	Cattle					5.7	60.0	31.4	2.9	0	0	0					1	2	0-12.32
Tilmicosin	Cattle							14.3	60.0	25.7	0						4	8	0-12.32
Trimethoprim/ sulfamethoxazole	Cattle							100									2	2	0-12.32
Tulathromycin	Cattle						0	0	0	100	0	0	0				8	8	0-12.32
Tylosin tartrate	Cattle					0	0	5.7	65.7	25.7	2.9	0					4	8	0-12.32
Tildipirosin	Cattle						8.6	51.4	40.0	0	0						2	4	0-12.32
Gamithromycin	Cattle						97.1	2.9	0	0							1	1	0-12.32

The dilution ranges tested are those contained in the white area. Values above this range indicate MIC values higher than the highest concentration within the range. Values corresponding to the lowest concentration tested indicated MIC values lower or equal to the lowest concentration within the range. When available, susceptible and resistance breakpoints are indicated in vertical green and red lines., MIC's > higher concentration available are indicated in the shaded region. A Cut-off values were used according to CLSI document VET08.

9.4 *Pasteurella* and *Mannheimia* phylogenetic trees



group1	17BRD-013TG	Mannheimia haemolytica	QLD	Cow	Tulth, TILMIC andGAM
group1	17BRD-016TG	Mannheimia haemolytica	NSW	Cow	
group1	18BRD-003TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-069TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-074TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-078TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-075TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-080TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-011TG	Mannheimia haemolytica	QLD	Cow	
group1	18BRD-038TG	Mannheimia haemolytica	NSW	Cow	
group1	17BRD-012TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-079TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-073TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-077TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-072TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-015TG	Mannheimia haemolytica	QLD	Cow	
group1	19BRD-027TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-068TG	Mannheimia haemolytica	QLD	Cow	
group1	17BRD-070TG	Mannheimia haemolytica	QLD	Cow	
group1	19BRD-044TG	Mannheimia haemolytica	QLD	Cow	
group1	19BRD-084TG	Mannheimia haemolytica	NSW	Cow	
group1	18BRD-040TG	Mannheimia haemolytica	NSW	Cow	
group1	18BRD-041TG	Mannheimia haemolytica	NSW	Cow	
group1	19BRD-051TG	Mannheimia haemolytica	QLD	Cow	
group2	17BRD-007TG	Mannheimia haemolytica	QLD	Cow	
group2	17BRD-009TG	Mannheimia haemolytica	QLD	Cow	
group2	17BRD-003TG	Mannheimia haemolytica	QLD	Cow	
group2	17BRD-004TG	Mannheimia haemolytica	QLD	Cow	
group2	19BRD-076TG	Mannheimia haemolytica	QLD	Cow	
group2	19BRD-078TG	Mannheimia haemolytica	QLD	Cow	
group2	17BRD-001TG	Mannheimia haemolytica	QLD	Cow	
group2	18BRD-016TG	Mannheimia haemolytica	QLD	Cow	
group2	18BRD-017TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-011TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-160TG	Mannheimia haemolytica	NSW	Cow	
group3	17BRD-071TG	Mannheimia haemolytica	NSW	Cow	
group3	19BRD-161TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-170TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-181TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-189TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-172TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-163TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-186TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-004TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-185TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-024TG	Mannheimia haemolytica	SA	Cow	
group3	18BRD-013TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-015TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-034TG	Mannheimia haemolytica	NSW	Cow	
group3	19BRD-002TG	Mannheimia . haemolytica	SA	Cow	
group3	19BRD-006TG	Mannheimia . haemolytica	SA	Cow	
group3	18BRD-035TG	Mannheimia haemolytica	NSW	Cow	
group3	18BRD-023TG	Mannheimia haemolytica	SA	Cow	
group3	18BRD-032TG	Mannheimia haemolytica	NSW	Cow	
group3	17BRD-006TG	Mannheimia haemolytica	QLD	Cow	
group3	18BRD-033TG	Mannheimia haemolytica	NSW	Cow	
group3	18BRD-036TG	Mannheimia haemolytica	NSW	Cow	
group3	18BRD-037TG	Mannheimia haemolytica	NSW	Cow	
group3	19BRD-019TG	Mannheimia . haemolytica	SA	Cow	
group3	19BRD-187TG	Mannheimia haemolytica	QLD	Cow	
group3	19BRD-188TG	Mannheimia haemolytica	QLD	Cow	
group3	17BRD-017TG	Mannheimia haemolytica	NSW	Cow	
group3	17BRD-014TG	Mannheimia haemolytica	NSW	Cow	
group3	17BRD-008TG	Mannheimia haemolytica	QLD	Cow	
group3	17BRD-019TG	Mannheimia haemolytica	QLD	Cow	
group3	17BRD-076TG	Mannheimia haemolytica	QLD	Cow	
group3	17BRD-018TG	Mannheimia haemolytica	QLD	Cow	
group3	17BRD-020TG	Mannheimia haemolytica	QLD	Cow	
group4	17BRD-065TG	Mannheimia haemolytica	VIC	Cow	
group4	17BRD-002TG	Mannheimia haemolytica	QLD	Cow	
group4	19BRD-045TG	Mannheimia . haemolytica	QLD	Cow	
group4	18BRD-002TG	Mannheimia haemolytica	QLD	Cow	
group5	19BRD-114TG	Mannheimia haemolytica	SA	Cow	
group5	19BRD-052TG	Mannheimia . haemolytica	QLD	Cow	
group5	19BRD-071TG	Mannheimia haemolytica	QLD	Cow	
group5	18BRD-009TG	Mannheimia haemolytica	QLD	Cow	
group5	19BRD-117TG	Mannheimia haemolytica	NSW	Cow	
group5	19BRD-118TG	Mannheimia haemolytica	NSW	Cow	
group5	19BRD-115TG	Mannheimia haemolytica	SA	Cow	
group5	17BRD-010TG	Mannheimia haemolytica	QLD	Cow	
group5	19BRD-121TG	Mannheimia haemolytica	NSW	Cow	
group5	19BRD-122TG	Mannheimia haemolytica	NSW	Cow	
group5	19BRD-119TG	Mannheimia haemolytica	NSW	Cow	
group5	19BRD-120TG	Mannheimia haemolytica	NSW	Cow	
	19BRD-123TG	Mannheimia haemolytica	NSW	Cow	
group6	17BRD-067TG	Mannheimia haemolytica	VIC	Cow	
group6	18BRD-039TG	Mannheimia haemolytica	NSW	Cow	
	19BRD-012TG	Mannheimia . haemolytica	NSW	Cow	



9.5 Whole genome sequencing Resistant phenotype vs genotype

Co-relation between resistance phenotype and genotype of isolates in the cohort

Biochemical ID	*Genome Reference*	*BRD Reference*	*TET*		*TULATH*		*Resistance genenotype*
P.multocida	*ACARE 001*	*17BRD-005*	*<=*	*0.5*	*=*	*4*	
P.multocida	*ACARE 002*	*17BRD-021*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 003*	*17BRD-022*	*=*	*1*	*<=*	*1*	
P.multocida	*ACARE 004*	*17BRD-023*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 005*	*17BRD-024*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 006*	*17BRD-025*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 007*	*17BRD-026*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 008*	*17BRD-027*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 009*	*17BRD-028*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 010*	*17BRD-029*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 011*	*17BRD-030*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 012*	*17BRD-031*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 013*	*17BRD-032*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 014*	*17BRD-033*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 015*	*17BRD-034*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 016*	*17BRD-035*	*>*	***8***	*=*	*2*	*parB, blaROB1, tetR, tetH*
P.multocida	*ACARE 017*	*17BRD-036*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 018*	*17BRD-037*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 019*	*17BRD-038*	*>*	***8***	*=*	*2*	***[strA, strB, aphA1, sul2, tet(Y)]***
P.multocida	*ACARE 020*	*17BRD-039*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 021*	*17BRD-040*	*<=*	*0.5*	*>*	*64*	***[msrE, mphE]***
P.multocida	*ACARE 022*	*17BRD-041*	*>*	***8***	*=*	*2*	***[strA, strB, aphA1, sul2, tet(Y)]***

P.multocida	*ACARE 023*	*17BRD-042*	>	***8***	=	*2*	***[strA, strB, aphA1, sul2, tet(Y)]***
P.multocida	*ACARE 024*	*17BRD-043*	<=	*0.5*	=	*4*	
P.multocida	*ACARE 025*	*17BRD-044*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 026*	*17BRD-045*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 027*	*17BRD-046*	<=	*0.5*	=	*8*	
P.multocida	*ACARE 028*	*17BRD-047*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 029*	*17BRD-048*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 030*	*17BRD-049*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 031*	*17BRD-050*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 032*	*17BRD-051*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 033*	*17BRD-052*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 034*	*17BRD-053*	>	***8***	=	*2*	***[strA, strB, aphA1, sul2, tet(Y)]***
P.multocida	*ACARE 035*	*17BRD-054*	=	*1*	<=	*1*	
P.multocida	*ACARE 036*	*17BRD-055*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 037*	*17BRD-056*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 038*	*17BRD-057*	<=	*0.5*	>	*64*	***[msrE, mphE]***
P.multocida	*ACARE 039*	*17BRD-058*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 040*	*17BRD-059*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 041*	*17BRD-060*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 042*	*17BRD-061*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 043*	*17BRD-062*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 044*	*17BRD-063*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 046*	*17BRD-066*	<=	*0.5*	<=	*1*	
P.multocida	*ACARE 047*	*18BRD-001*	>	***8***	=	*2*	*parB, blaROB1, tetR, tetH*
P.multocida	*ACARE 048*	*18BRD-005*	<=	*0.5*	=	*2*	***[blaROB1, strA, sul2]***
P.multocida	*ACARE 049*	*18BRD-006*	<=	*0.5*	=	*8*	
P.multocida	*ACARE 050*	*18BRD-007*	<=	*0.5*	=	*2*	
P.multocida	*ACARE 051*	*18BRD-008*	<=	*0.5*	=	*8*	

P.multocida	*ACARE 052*	*18BRD-010*	*<=*	*0.5*	*=*	*8*	
P.multocida	*ACARE 053*	*18BRD-012*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 054*	*18BRD-014*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 055*	*18BRD-019*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 056*	*18BRD-020*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 057*	*18BRD-021*	*<=*	*0.5*	*=*	*4*	
P.multocida	*ACARE 058*	*18BRD-022*	*<=*	*0.5*	*=*	*2*	
P.multocida	*ACARE 059*	*18BRD-025*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 060*	*18BRD-042*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 061*	*18BRD-043*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 062*	*18BRD-044*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 063*	*18BRD-045*	*=*	*1*	*<=*	*1*	
P.multocida	*ACARE 064*	*18BRD-046*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 065*	*18BRD-047*	*>*	***8***	*<=*	*1*	***[tetR, tetH]***
P.multocida	*ACARE 066*	*18BRD-048*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 067*	*18BRD-049*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 068*	*18BRD-050*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 069*	*18BRD-051*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 070*	*18BRD-058*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 071*	*18BRD-059*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 072*	*18BRD-060*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 073*	*18BRD-061*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 074*	*18BRD-062*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 075*	*18BRD-063*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 076*	*18BRD-064*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 077*	*18BRD-065*	*<=*	*0.5*	*<=*	*1*	
P.multocida	*ACARE 078*	*19BRD-003*	*<=*	*0.5*	*<=*	*8*	*gyrA**
P.multocida	*ACARE 079*	*19BRD-007*	*<=*	*0.5*	*<=*	*8*	

P.multocida	*ACARE 080*	*19BRD-009*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 081*	*19BRD-010*	***=***	***8***	*<=*	*8*	***[tetR, tetH]***
P.multocida	*ACARE 082*	*19BRD-011*	***=***	***8***	*>*	***64***	***[msrE, mphE], [tetR, tetH]***
P.multocida	*ACARE 083*	*19BRD-014*	***=***	***8***	*<=*	*8*	***[tetR, tetH]***
P.multocida	*ACARE 084*	*19BRD-016*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 085*	*19BRD-017*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 086*	*19BRD-018*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 087*	*19BRD-020*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 088*	*19BRD-023*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 089*	*19BRD-025*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 090*	*19BRD-026*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 091*	*19BRD-032*	***=***	***8***	*<=*	*8*	*parB, blaROB1,* ***[tetR, tetH]***
P.multocida	*ACARE 092*	*19BRD-033*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 093*	*19BRD-036*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 094*	*19BRD-038*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 095*	*19BRD-039*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 096*	*19BRD-040*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 097*	*19BRD-042*	***=***	***8***	*<=*	*8*	*parB, blaROB1, [****tetR, tetH]***
P.multocida	*ACARE 098*	*19BRD-046*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 099*	*19BRD-050*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 100*	*19BRD-057*	***=***	***8***	*<=*	*8*	*parB, blaROB1, [****tetR, tetH]***
P.multocida	*ACARE 101*	*19BRD-060*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 102*	*19BRD-061*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 103*	*19BRD-062*	*=*	*1*	*<=*	*8*	
P.multocida	*ACARE 104*	*19BRD-065*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 105*	*19BRD-067*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 106*	*19BRD-068*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 107*	*19BRD-072*	*<=*	*0.5*	*<=*	*8*	

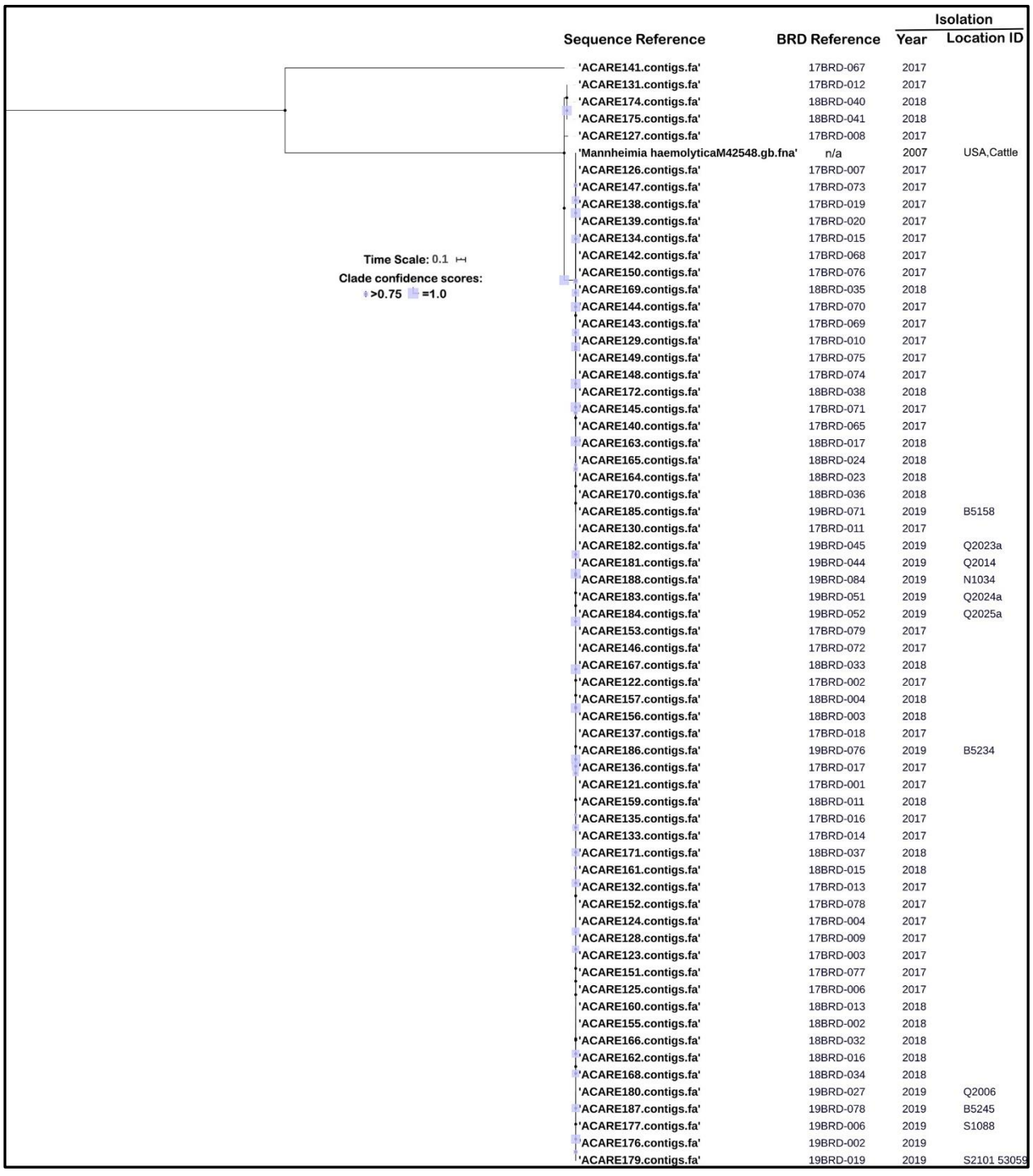
P.multocida	*ACARE 108*	*19BRD-085*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 109*	*19BRD-093*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 110*	*19BRD-094*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 111*	*19BRD-095*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 112*	*19BRD-097*	*<=*	*0.5*	*<=*	*8*	
P.multocida	*ACARE 113*	*19BRD-098*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 114*	*19BRD-100*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE]***
P.multocida	*ACARE 115**	*19BRD-101**	*<=*	*0.5*	*>*	***64***	*n/a*
P.multocida	*ACARE 116*	*19BRD-104*	***=***	***8***	*<=*	*8*	***[tetR, tetH]***
P.multocida	*ACARE 117*	*19BRD-106*	***=***	***8***	*>*	***64***	***[msrE, mphE], [tetR, tetH]***
P.multocida	*ACARE 118**	*19BRD-110**	*=*	*4*	*>*	***64***	***[tetR, tetH]***
P.multocida	*ACARE 119*	*19BRD-111*	*=*	*4*	*<=*	*8*	***[tetR, tetH]***
P.multocida	*ACARE 120*	*19BRD-112*	***=***	***8***	*>*	***64***	***[msrE, mphE], [tetR, tetH]***
M. haemolytica	*ACARE 121*	*17BRD-001*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 122*	*17BRD-002*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 123*	*17BRD-003*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 124*	*17BRD-004*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 125*	*17BRD-006*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 126*	*17BRD-007*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 127*	*17BRD-008*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 128*	*17BRD-009*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 129*	*17BRD-010*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 130*	*17BRD-011*	*<=*	*0.5*	*=*	*8*	***[strA, strB, sul2],*** *gyrA**
M. haemolytica	*ACARE 131*	*17BRD-012*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 132*	*17BRD-013*	*<=*	*0.5*	*=*	*16*	*gyrA**
M. haemolytica	*ACARE 133*	*17BRD-014*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 134*	*17BRD-015*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 135*	*17BRD-016*	*<=*	*0.5*	*=*	*16*	*gyrA**

M. haemolytica	*ACARE 136*	*17BRD-017*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 137*	*17BRD-018*	*<=*	*0.5*	*=*	*16*	*gyrA**
M. haemolytica	*ACARE 138*	*17BRD-019*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 139*	*17BRD-020*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 140*	*17BRD-065*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 141*	*17BRD-067*	*=*	*1*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 142*	*17BRD-068*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 143*	*17BRD-069*	*<=*	*0.5*	*=*	*8*	***[strA, strB, sul2]**, gyrA**
M. haemolytica	*ACARE 144*	*17BRD-070*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 145*	*17BRD-071*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 146*	*17BRD-072*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 147*	*17BRD-073*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 148*	*17BRD-074*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 149*	*17BRD-075*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 150*	*17BRD-076*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 151*	*17BRD-077*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 152*	*17BRD-078*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 153*	*17BRD-079*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 154*	*17BRD-080*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 155*	*18BRD-002*	*<=*	*0.5*	*=*	*16*	*gyrA**
M. haemolytica	*ACARE 156*	*18BRD-003*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 157*	*18BRD-004*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 158*	*18BRD-009*	*=*	*1*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 159*	*18BRD-011*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 160*	*18BRD-013*	*<=*	*0.5*	*=*	*16*	*gyrA**
M. haemolytica	*ACARE 161*	*18BRD-015*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 162*	*18BRD-016*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 163*	*18BRD-017*	*<=*	*0.5*	*=*	*4*	*gyrA**

M. haemolytica	*ACARE 164*	*18BRD-023*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 165*	*18BRD-024*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 166*	*18BRD-032*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 167*	*18BRD-033*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 168*	*18BRD-034*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 169*	*18BRD-035*	*<=*	*0.5*	*=*	*2*	*gyrA**
M. haemolytica	*ACARE 170*	*18BRD-036*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 171*	*18BRD-037*	*<=*	*0.5*	*=*	*8*	*gyrA**
M. haemolytica	*ACARE 172*	*18BRD-038*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 173*	*18BRD-039*	*<=*	*0.5*	*=*	*2*	*gyrA**
M. haemolytica	*ACARE 174*	*18BRD-040*	*<=*	*0.5*	*<=*	*1*	*gyrA**
M. haemolytica	*ACARE 175*	*18BRD-041*	*<=*	*0.5*	*=*	*4*	*gyrA**
M. haemolytica	*ACARE 176*	*19BRD-002*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 177*	*19BRD-006*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 178*	*19BRD-012*	*n/a*		*n/a*		*gyrA**
M. haemolytica	*ACARE 179*	*19BRD-019*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 180*	*19BRD-027*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 181*	*19BRD-044*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 182*	*19BRD-045*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 183*	*19BRD-051*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 184*	*19BRD-052*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 185*	*19BRD-071*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 186*	*19BRD-076*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 187*	*19BRD-078*	*<=*	*0.5*	*<=*	*8*	*gyrA**
M. haemolytica	*ACARE 188*	*19BRD-084*	*<=*	*0.5*	*>*	***64***	***[msrE, mphE],*** *gyrA**

Resistance genes on the same genomic scaffolds are highlighted in bold font and placed within [square brackets].
For isolates indicated with an * in the 'Genome Reference' and 'ACARE Reference' columns genotype does not match with phenotype data.
gyrA*= 99.96% identity with M. haemolytica 42584 gene

9.6 Whole genome sequencing phylogenetic trees



Sequence Reference	BRD Reference	Isolation Year	Isolation Location ID
'ACARE141.contigs.fa'	17BRD-067	2017	
'ACARE131.contigs.fa'	17BRD-012	2017	
'ACARE174.contigs.fa'	18BRD-040	2018	
'ACARE175.contigs.fa'	18BRD-041	2018	
'ACARE127.contigs.fa'	17BRD-008	2017	
'Mannheimia haemolyticaM42548.gb.fna'	n/a	2007	USA,Cattle
'ACARE126.contigs.fa'	17BRD-007	2017	
'ACARE147.contigs.fa'	17BRD-073	2017	
'ACARE138.contigs.fa'	17BRD-019	2017	
'ACARE139.contigs.fa'	17BRD-020	2017	
'ACARE134.contigs.fa'	17BRD-015	2017	
'ACARE142.contigs.fa'	17BRD-068	2017	
'ACARE150.contigs.fa'	17BRD-076	2017	
'ACARE169.contigs.fa'	18BRD-035	2018	
'ACARE144.contigs.fa'	17BRD-070	2017	
'ACARE143.contigs.fa'	17BRD-069	2017	
'ACARE129.contigs.fa'	17BRD-010	2017	
'ACARE149.contigs.fa'	17BRD-075	2017	
'ACARE148.contigs.fa'	17BRD-074	2017	
'ACARE172.contigs.fa'	18BRD-038	2018	
'ACARE145.contigs.fa'	17BRD-071	2017	
'ACARE140.contigs.fa'	17BRD-065	2017	
'ACARE163.contigs.fa'	18BRD-017	2018	
'ACARE165.contigs.fa'	18BRD-024	2018	
'ACARE164.contigs.fa'	18BRD-023	2018	
'ACARE170.contigs.fa'	18BRD-036	2018	
'ACARE185.contigs.fa'	19BRD-071	2019	B5158
'ACARE130.contigs.fa'	17BRD-011	2017	
'ACARE182.contigs.fa'	19BRD-045	2019	Q2023a
'ACARE181.contigs.fa'	19BRD-044	2019	Q2014
'ACARE188.contigs.fa'	19BRD-084	2019	N1034
'ACARE183.contigs.fa'	19BRD-051	2019	Q2024a
'ACARE184.contigs.fa'	19BRD-052	2019	Q2025a
'ACARE153.contigs.fa'	17BRD-079	2017	
'ACARE146.contigs.fa'	17BRD-072	2017	
'ACARE167.contigs.fa'	18BRD-033	2018	
'ACARE122.contigs.fa'	17BRD-002	2017	
'ACARE157.contigs.fa'	18BRD-004	2018	
'ACARE156.contigs.fa'	18BRD-003	2018	
'ACARE137.contigs.fa'	17BRD-018	2017	
'ACARE186.contigs.fa'	19BRD-076	2019	B5234
'ACARE136.contigs.fa'	17BRD-017	2017	
'ACARE121.contigs.fa'	17BRD-001	2017	
'ACARE159.contigs.fa'	18BRD-011	2018	
'ACARE135.contigs.fa'	17BRD-016	2017	
'ACARE133.contigs.fa'	17BRD-014	2017	
'ACARE171.contigs.fa'	18BRD-037	2018	
'ACARE161.contigs.fa'	18BRD-015	2018	
'ACARE132.contigs.fa'	17BRD-013	2017	
'ACARE152.contigs.fa'	17BRD-078	2017	
'ACARE124.contigs.fa'	17BRD-004	2017	
'ACARE128.contigs.fa'	17BRD-009	2017	
'ACARE123.contigs.fa'	17BRD-003	2017	
'ACARE151.contigs.fa'	17BRD-077	2017	
'ACARE125.contigs.fa'	17BRD-006	2017	
'ACARE160.contigs.fa'	18BRD-013	2018	
'ACARE155.contigs.fa'	18BRD-002	2018	
'ACARE166.contigs.fa'	18BRD-032	2018	
'ACARE162.contigs.fa'	18BRD-016	2018	
'ACARE168.contigs.fa'	18BRD-034	2018	
'ACARE180.contigs.fa'	19BRD-027	2019	Q2006
'ACARE187.contigs.fa'	19BRD-078	2019	B5245
'ACARE177.contigs.fa'	19BRD-006	2019	S1088
'ACARE176.contigs.fa'	19BRD-002	2019	
'ACARE179.contigs.fa'	19BRD-019	2019	S2101 53059

Fig. 9.6a: SNP phylogeny of genomes sequenced in the project. Tree scale indicates substitutions per site. Clades with confidence scores ≥0.75 and 1 are presented as violet circles.

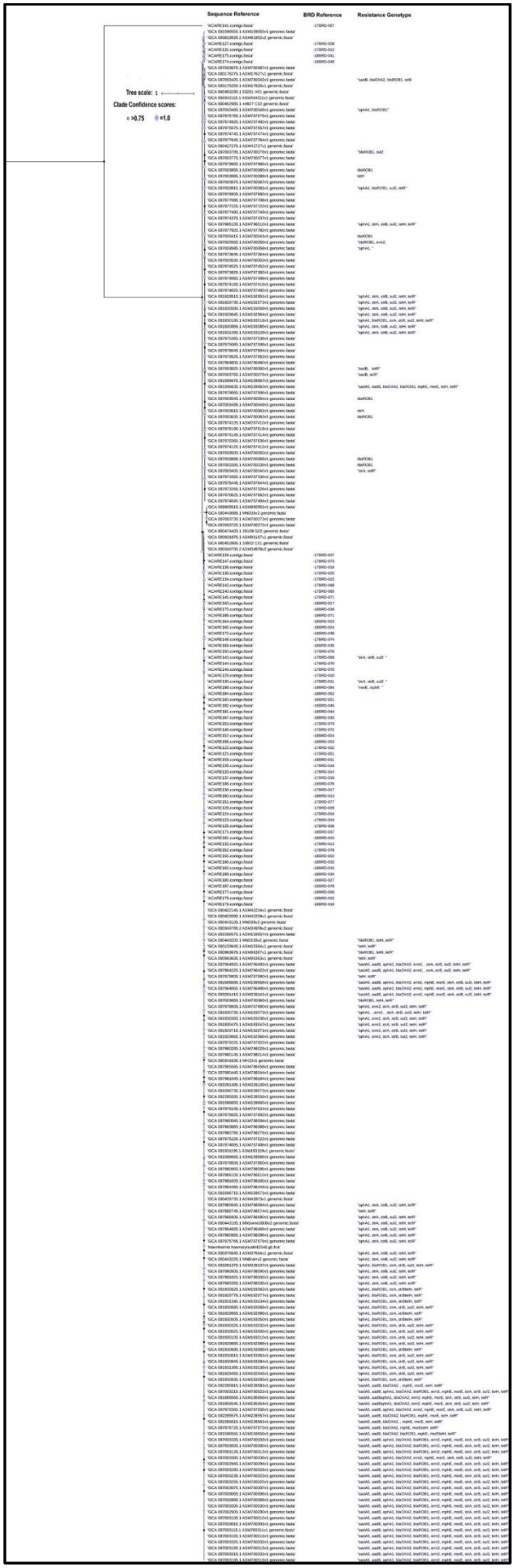
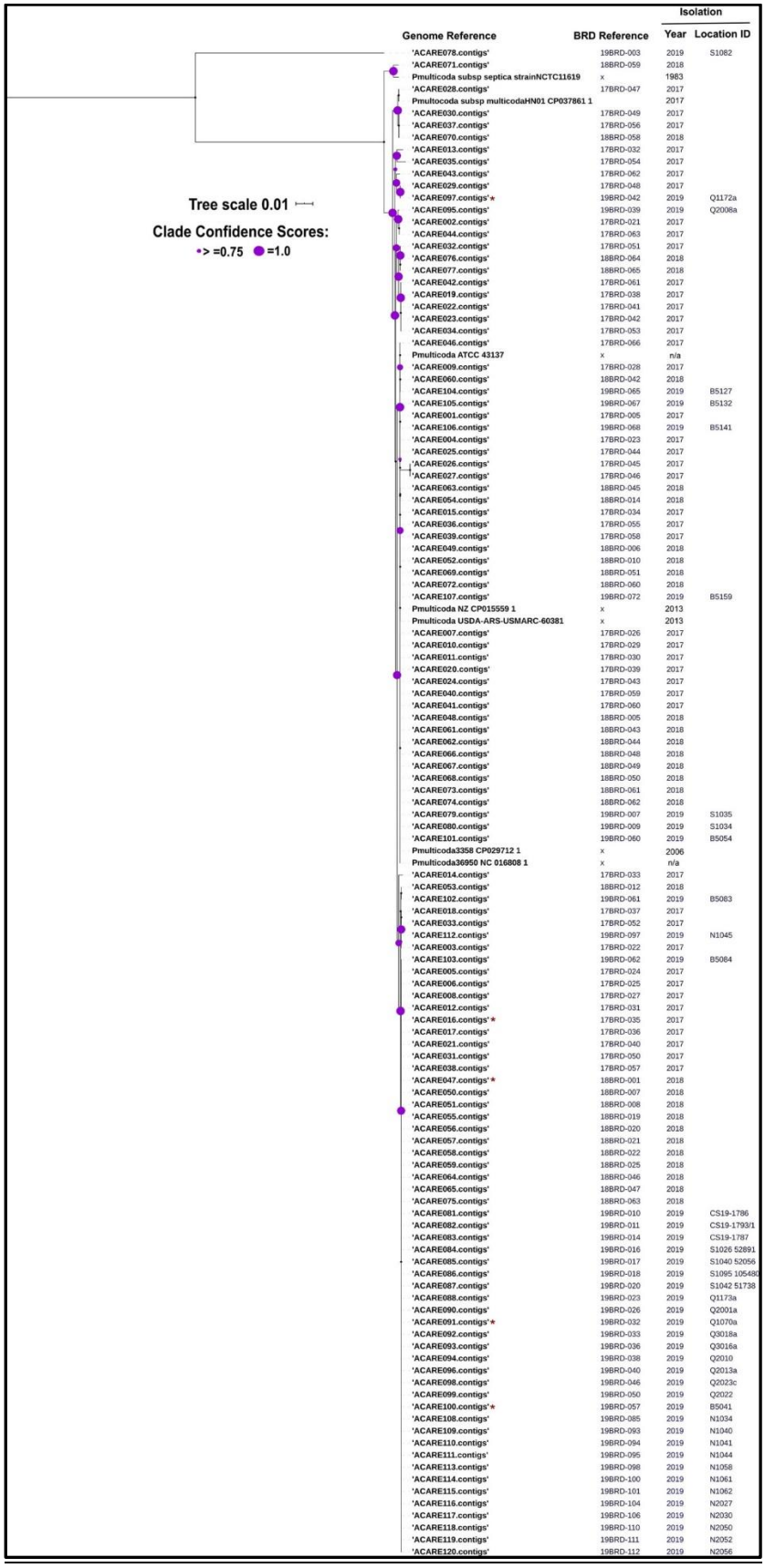

Sequence Reference
BRD Reference
Resistance Genotype
Tree scale: 1
Clade Confidence scores:
= >0.75
=1.0

Fig. 9.6b: SNP phylogeny of 256 *Mannheimia haemolytica* genomes. Tree scale indicates substitutions per site. Clades with confidence scores ≥0.75 and 1 are presented as violet circles.



Genome Reference	BRD Reference	Isolation Year	Isolation Location ID
'ACARE078.contigs'	19BRD-003	2019	S1082
'ACARE071.contigs'	18BRD-059	2018	
Pmulticoda subsp septica strainNCTC11619	x	1983	
'ACARE028.contigs'	17BRD-047	2017	
Pmultocoda subsp multicodaHN01 CP037861 1		2017	
'ACARE030.contigs'	17BRD-049	2017	
'ACARE037.contigs'	17BRD-056	2017	
'ACARE070.contigs'	18BRD-058	2018	
'ACARE013.contigs'	17BRD-032	2017	
'ACARE035.contigs'	17BRD-054	2017	
'ACARE043.contigs'	17BRD-062	2017	
'ACARE029.contigs'	17BRD-048	2017	
'ACARE097.contigs'*	19BRD-042	2019	Q1172a
'ACARE095.contigs'	19BRD-039	2019	Q2008a
'ACARE002.contigs'	17BRD-021	2017	
'ACARE044.contigs'	17BRD-063	2017	
'ACARE032.contigs'	17BRD-051	2017	
'ACARE076.contigs'	18BRD-064	2018	
'ACARE077.contigs'	18BRD-065	2018	
'ACARE042.contigs'	17BRD-061	2017	
'ACARE019.contigs'	17BRD-038	2017	
'ACARE022.contigs'	17BRD-041	2017	
'ACARE023.contigs'	17BRD-042	2017	
'ACARE034.contigs'	17BRD-053	2017	
'ACARE046.contigs'	17BRD-066	2017	
Pmulticoda ATCC 43137	x	n/a	
'ACARE009.contigs'	17BRD-028	2017	
'ACARE060.contigs'	18BRD-042	2018	
'ACARE104.contigs'	19BRD-065	2019	B5127
'ACARE105.contigs'	19BRD-067	2019	B5132
'ACARE001.contigs'	17BRD-005	2017	
'ACARE106.contigs'	19BRD-068	2019	B5141
'ACARE004.contigs'	17BRD-023	2017	
'ACARE025.contigs'	17BRD-044	2017	
'ACARE026.contigs'	17BRD-045	2017	
'ACARE027.contigs'	17BRD-046	2017	
'ACARE063.contigs'	18BRD-045	2018	
'ACARE054.contigs'	18BRD-014	2018	
'ACARE015.contigs'	17BRD-034	2017	
'ACARE036.contigs'	17BRD-055	2017	
'ACARE039.contigs'	17BRD-058	2017	
'ACARE049.contigs'	18BRD-006	2018	
'ACARE052.contigs'	18BRD-010	2018	
'ACARE069.contigs'	18BRD-051	2018	
'ACARE072.contigs'	18BRD-060	2018	
'ACARE107.contigs'	19BRD-072	2019	B5159
Pmulticoda NZ CP015559 1	x	2013	
Pmulticoda USDA-ARS-USMARC-60381	x	2013	
'ACARE007.contigs'	17BRD-026	2017	
'ACARE010.contigs'	17BRD-029	2017	
'ACARE011.contigs'	17BRD-030	2017	
'ACARE020.contigs'	17BRD-039	2017	
'ACARE024.contigs'	17BRD-043	2017	
'ACARE040.contigs'	17BRD-059	2017	
'ACARE041.contigs'	17BRD-060	2017	
'ACARE048.contigs'	18BRD-005	2018	
'ACARE061.contigs'	18BRD-043	2018	
'ACARE062.contigs'	18BRD-044	2018	
'ACARE066.contigs'	18BRD-048	2018	
'ACARE067.contigs'	18BRD-049	2018	
'ACARE068.contigs'	18BRD-050	2018	
'ACARE073.contigs'	18BRD-061	2018	
'ACARE074.contigs'	18BRD-062	2018	
'ACARE079.contigs'	19BRD-007	2019	S1035
'ACARE080.contigs'	19BRD-009	2019	S1034
'ACARE101.contigs'	19BRD-060	2019	B5054
Pmulticoda3358 CP029712 1	x	2006	
Pmulticoda36950 NC 016808 1	x	n/a	
'ACARE014.contigs'	17BRD-033	2017	
'ACARE053.contigs'	18BRD-012	2018	
'ACARE102.contigs'	19BRD-061	2019	B5083
'ACARE018.contigs'	17BRD-037	2017	
'ACARE033.contigs'	17BRD-052	2017	
'ACARE112.contigs'	19BRD-097	2019	N1045
'ACARE003.contigs'	17BRD-022	2017	
'ACARE103.contigs'	19BRD-062	2019	B5084
'ACARE005.contigs'	17BRD-024	2017	
'ACARE006.contigs'	17BRD-025	2017	
'ACARE008.contigs'	17BRD-027	2017	
'ACARE012.contigs'	17BRD-031	2017	
'ACARE016.contigs'*	17BRD-035	2017	
'ACARE017.contigs'	17BRD-036	2017	
'ACARE021.contigs'	17BRD-040	2017	
'ACARE031.contigs'	17BRD-050	2017	
'ACARE038.contigs'	17BRD-057	2017	
'ACARE047.contigs'*	18BRD-001	2018	
'ACARE050.contigs'	18BRD-007	2018	
'ACARE051.contigs'	18BRD-008	2018	
'ACARE055.contigs'	18BRD-019	2018	
'ACARE056.contigs'	18BRD-020	2018	
'ACARE057.contigs'	18BRD-021	2018	
'ACARE058.contigs'	18BRD-022	2018	
'ACARE059.contigs'	18BRD-025	2018	
'ACARE064.contigs'	18BRD-046	2018	
'ACARE065.contigs'	18BRD-047	2018	
'ACARE075.contigs'	18BRD-063	2018	
'ACARE081.contigs'	19BRD-010	2019	CS19-1786
'ACARE082.contigs'	19BRD-011	2019	CS19-1793/1
'ACARE083.contigs'	19BRD-014	2019	CS19-1787
'ACARE084.contigs'	19BRD-016	2019	S1026 52891
'ACARE085.contigs'	19BRD-017	2019	S1040 52056
'ACARE086.contigs'	19BRD-018	2019	S1095 10548(
'ACARE087.contigs'	19BRD-020	2019	S1042 51738
'ACARE088.contigs'	19BRD-023	2019	Q1173a
'ACARE090.contigs'	19BRD-026	2019	Q2001a
'ACARE091.contigs'*	19BRD-032	2019	Q1070a
'ACARE092.contigs'	19BRD-033	2019	Q3018a
'ACARE093.contigs'	19BRD-036	2019	Q3016a
'ACARE094.contigs'	19BRD-038	2019	Q2010
'ACARE096.contigs'	19BRD-040	2019	Q2013a
'ACARE098.contigs'	19BRD-046	2019	Q2023c
'ACARE099.contigs'	19BRD-050	2019	Q2022
'ACARE100.contigs'*	19BRD-057	2019	B5041
'ACARE108.contigs'	19BRD-085	2019	N1034
'ACARE109.contigs'	19BRD-093	2019	N1040
'ACARE110.contigs'	19BRD-094	2019	N1041
'ACARE111.contigs'	19BRD-095	2019	N1044
'ACARE113.contigs'	19BRD-098	2019	N1058
'ACARE114.contigs'	19BRD-100	2019	N1061
'ACARE115.contigs'	19BRD-101	2019	N1062
'ACARE116.contigs'	19BRD-104	2019	N2027
'ACARE117.contigs'	19BRD-106	2019	N2030
'ACARE118.contigs'	19BRD-110	2019	N2050
'ACARE119.contigs'	19BRD-111	2019	N2052
'ACARE120.contigs'	19BRD-112	2019	N2056

Fig. 9.6c: PhyloSift phylogeny of genomes sequenced in the project. Tree scale indicates substitutions per site. Clades with confidence scores ≥0.75 and 1 are presented as violet circles. Red

stars indicate isolates which may harbour a variant of the Integrative conjugative element in *P multicoda* 3358 genome.

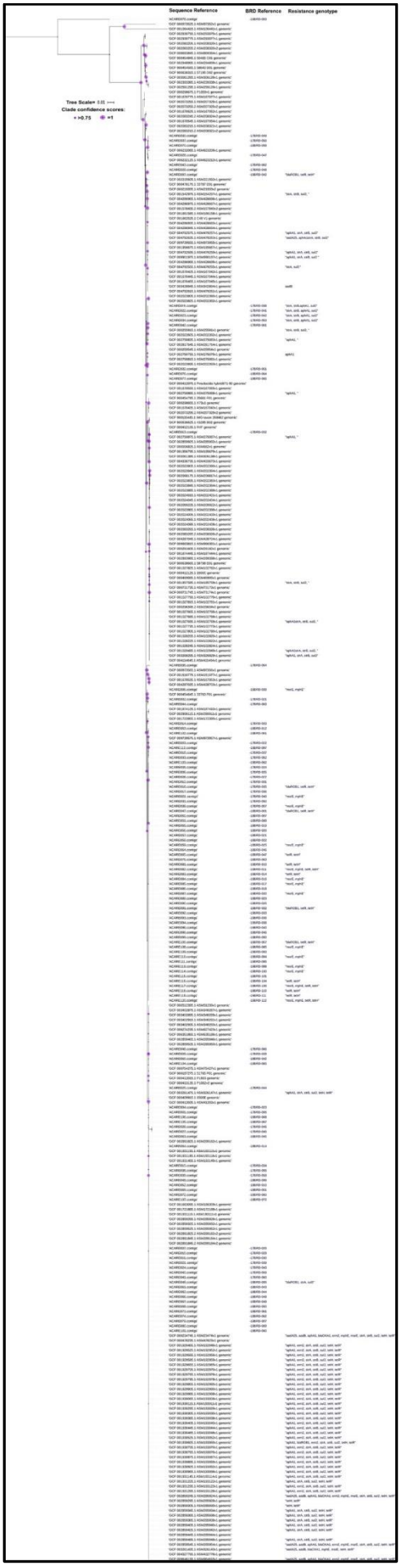

Sequence Reference
BRD Reference
Resistance genotype
Tree Scale= 0.01
Clade confidence scores:
≥ 0.75
= 1

Fig 9.6d: PhyloSift phylogeny of 318 *Pasteurella multicoda* genomes. Tree scale indicates substitutions per site. Clades with confidence scores ≥0.75 and 1 are presented as violet circles.