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Bovine Respiratory Disease Pathogen Antimicrobial Resistance Surveillance and Genetic Sequencing

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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, tetracycline, 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. In previous work (B.FLT.3004) the first cases of AMR in *P. multocida*, the most prevalent BRD pathogen on Australian feedlots, was identified, with variable feedlot-dependent frequency of resistance to macrolides, tetracyclines as well as dual resistance. For the first time in Australia, *Mycoplasma bovis* was shown to be a significant BRD pathogen, was often found co-associated with *P. multocida*, and emerging resistance to macrolides was observed. No antimicrobial resistance was observed in *H. somni* and a single macrolide-resistant *M. haemolytica* isolate was suggestive of a gene transfer event with *P. multocida.*

In this extension project which maintained the AMR surveillance programme begun in 2019 for a further two years, the co-association between *Pasteurella multocida* and *Mycoplasma bovis* as the most common BRD pathogens isolated from Australian feedlots was strengthened. Newly described ST394 and globally significant ST79 were the predominant *P. multocida* sequence types causing BRD on Australian feedlots, with ST394 isolates more likely to carry antimicrobial resistance genes. Prevalence of resistance to macrolides, tetracyclines and dual resistance among *P. multocida* had increased to 48.8%, 30.2% and 27.9%, respectively, compared to 2019, but these results may have been impacted by the larger number of samples received from feedlots N2 and S1 where resistant strains are endemic. Additionally, several more macrolide-resistant *M. haemolytica* isolates were detected at different feedlots that did not take part in the 2019 study (e.g. V1). Antimicrobial resistance was still negligible in *H. somni* and whole genome sequence analysis of the previous *H. somni* collection revealed that the isolates were genetically homogeneous, with four subclades identified, but no geographical, temporal or disease syndrome associated clustering evident.

A unique Integrative conjugative elements (ICE), with most similarity to an international *H. somni* mobile genetic element, was identified in the MDR 2019 *P. multocida* isolates that appear to be confined to feedlot Q1, though no isolates were obtained from Qld feedlots in 2021 to verify this. Tetracycline and beta-lactam resistance genes were mapped to this ICE, but the genetic basis for tilmicosin resistance in these isolates could not be determined from long range sequencing.

The majority of tetracycline (*tet(H)-tet (R)*) and macrolide (*msr(E)-mph(E)*) resistance genes identified in the remaining *P. multocida* isolates were located on distinct small (4.6 kb and 7-7.7kb plasmids, respectively) with dual-resistant strains possessing both plasmids. A plasmid also appears to be the genetic element encoding macrolide resistance (on the basis of long-range sequencing) in the single *M. haemolytica* isolate that possessed *msr(E)-mph(E)* genes and was isolated from the same BRD case as a macrolide-resistant *P. multocida.* However, it is possible this plasmid may be integrated into the chromosome as plasmids could not be isolated from this *M. haemolytica* strain.

Confirmation that *Mycoplasma bovis* is a bona fide BRD pathogen in Australia was obtained through combined PCR detection and histopathological investigations. BHV-1 and BVDV-1 were the most common viruses detected in lung samples from both BRD and non-BRD affected cattle at post-mortem but neither was strongly associated with *Mycoplasma* detection. Whole genome sequence analysis of *Mycoplasma bovis* isolates from the 2019 collection that showed reduce susceptibility to macrolides (tylosin, tilmicosin and tildipirosin only) confirmed that all isolates belonged to the dominant Australian sequence type (ST54) also causing bovine mastitis, were closely related genetically and the two of the tylosin-resistant strains possessed a known point mutation associated with macrolide resistance in the 23S rRNA gene domain V.

The results shed further light on both the unique pathogenesis of BRD in Australia and the evolution of antimicrobial resistance in the main causal pathogens. They suggest that varying the antimicrobial selection pressure, either by antimicrobial drug rotation, or through the introduction of non-antimicrobial control methods such as efficacious autogenous vaccines (considered a viable alternative given the limited genetic variation shown among the isolates) may be strategies for preventing AMR prevalence from increasing. The feedlot industry is now well placed to continue AMR surveillance of BRD pathogens in fulfilment of its antimicrobial stewardship requirements.

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1 Background

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. 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. Tetracycline (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. Extended-spectrum cephalosporin ceftiofur (Excenel or Excede, classified as a third line treatment by the AVA) is often used for non-responders or 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 to prevent severe outbreaks.

Previous MLA research (B.FLT.3004) has provided up to date antimicrobial resistance (AMR) data on BRD pathogens isolated from Australian feedlots. Whilst the majority were susceptible to antimicrobials, resistance had emerged in *P. multocida*, the most common pathogen isolated. A significant proportion (23.1%) of the 2019 *P. multocida* collection was resistant to macrolides with some isolates also resistant to tetracycline, a trait possibly associated with chlortetracycline use (1). A small number of multidrug-resistant *P. multocida* isolates were also obtained from Queensland and short read whole genome sequencing suggested that these isolates may contain integrative conjugative elements (ICEs). *Mycoplasma bovis* was a significant pathogen commonly associated with *P. multocida*. The feedlots participating in the 2019 study still possessed sample kits provided to them and in this extension project it was proposed to continue sampling for another 6 months to increase sample size and follow the trends established in the 2019 targeted AMR surveillance study. Furthermore, the antimicrobial susceptibility testing methodology would be transferred to the referring veterinary diagnostic microbiology laboratories so they can provide each feedlot with local AMR surveillance data and contribute their results to the national database, significantly saving on double handling of isolates. Lastly, the *H. somni* and reduced susceptibility *Mycoplasma bovis* collections would be subjected to whole genome sequencing and disease associations made with viral pathogens using histopathology and PCR.

Additionally, it was proposed to characterise the genetic mechanisms responsible for recently identified resistance in the most common BRD pathogen, *P. multocida* and conduct a molecular risk assessment on the likelihood of resistance rapidly transferring to other strains and species. The emergence of resistance to frontline drugs in *P. multocida* and its risk of transfer to other agents of

BRD such as *M. haemolytica* and *H. somni* may compromise successful treatment, increase costs and result in more frequent use of reserve agents such as ceftiofur for BRD treatment. Understanding the risks posed by resistance development and its mechanism of transfer as well as developing strategies to reduce its impact such as drug rotation and non-antimicrobial control alternatives were identifed as the main benefits arising from this project.

1.1 Previous studies

1.1.1 International studies

In the recent past North American feedlots have observed a rapid rise in resistant variants of the BRD pathogens, *P. multocida* and *M. haemolytica*, resulting in infections which could not be treated with first- and second-line antibiotics prescribed for BRD. It has now been established that the rapid dissemination of resistance in North American feedlots was facilitated by a specific type of mobile genetic element known as Integrative Conjugative Elements (ICEs). Although such elements are characteristically found integrated within bacterial chromosomes, they are distinguished from other groups of mobile genetic elements by the presence of genes that facilitate plasmid-like movement between non-progeny cells (i.e., lateral gene transfer). Consequently, they have the capacity to disperse gene clusters, often including resistance genes as cargos, within defined boundaries of the genetic element.

The most significant observations reported from the genomic data presented in project B.FLT.3004 were: (i) the presence of gene clusters that confer plasmid-like mobility and (ii) presence of tetracycline (*tet*(H)-*tet*(R))and macrolide (*mph*(E)-*msr*(E)) resistance genes (both reported from ICEs in North American *P. multocida* isolates) in the genomes of the cohort of isolates studied from Australia (1). This led to the hypothesis that variants of the ICEs reported from North America may be present in Australian *P. multocida* genomes, however, association of ICEs with the resistance genes could not be confirmed in the B.FLT.3004 study. This is primarily because of the inherent nature of sequence data assembled from Illumina short read technology which, although extremely accurate for genotype prediction, generally assembles fragmented genomes. In addition, the preliminary Illumina sequencing results were suggestive that some resistance plasmids also may be be circulating within the local resistant *P. multocida* cohort. Therefore, an extension project using a different state-of-the-art Oxford nanopore genome sequencing technique (long read sequencing) was planned in an effort to completely close the genomes for a handful of resistant isolates. This would enable characterisation of the mobile genetic elements and their association with resistance genes of interest.

Identification of resistance mechanisms for early detection of evolution of drug-resistant variants of pathogens will enable lot feeders and veterinarians to better manage their herds, prevent animal loss and assist Australia in maintaining the quality and quantity of produce required to feed the local population and meet export demands.

1.1.2 Australian studies

There have been no previous studies on the identification of mobile genetic elements responsible for resistance dissemination in BRD pathogens isolated from Australian feedlots. The recent Al-

hamami et al. (1) study, derived from B.FLT.3004, was the first to report the presence of resistance genes in *P. multocida* isolated from BRD cases from Australian feedlots.

2 Project objectives

2.1 Conduct 6 months of antimicrobial resistance surveillance data from a minimum of 8 Australian feedlots.

2.2 Determine if in-feed use of tetracycline is correlated with increased antimicrobial resistance of bovine respiratory disease pathogens.

2.3 Determine if meta-phylaxis at feedlot entry with macrolides is associated with an increased risk of antimicrobial resistance of pathogens.

2.4 Characterise the genetic mechanisms responsible for recently identified resistance in *Pasteurella multocida*

2.5 Complete whole of genome sequencing of *Histophilus somni* and *Mycoplasma bovis* collections for surveillance of antimicrobial resistance genes.

3 Methodology

3.1 Conduct 6 months of antimicrobial resistance surveillance data from a minimum of 8 Australian feedlots.

Given both the reduced density of cattle in most feedlots during 2020, combined with issues relating to the COVID-19 pandemic and transport and sampling logistics, the AMR surveillance component of the study was extended into 2021. In 2020, isolates were obtained from feedlots in Queensland (Q2 and Q3 only), NSW (N1, N2 and N4), and SA (S1). In 2021, no isolates were submitted from Queensland, however a large number of isolates were obtained from NSW (N1-N4) and isolates were also obtained from a feedlot in Victoria (V1) and SA (S1).

3.1.1 Sample collection, isolation and identification of feedlot BRD pathogens

Isolate numbers per BRD bacterial species for 2020 and 2021 are shown in Table 1. Details on the sampling methodology, isolation and identification of each BRD pathogen can be found in the B.FLT.3004 Final Report and are briefly described as follows. Individually numbered and labelled sample packs supplied to each feedlot in 2019 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).

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.

Each veterinary diagnostic laboratory followed their standard operating procedures for isolation and identification of BRD pathogens. The plan for the 2020/2021 sampling period was for VDLs to introduce MIC testing of BRD pathogens as part of their NATA accredited range of tests, but this was not achieved at EMAI, Qld BSL or CSU during the project. Hence isolates from these labs were pooled and transported to the ACARE Antimicrobial Resistance Laboratory for MIC testing in single shipments at the end of each BRD season in 2020 and 2021. In 2021, ACE laboratories was purchased by Apiam, allowing feedlots in southern NSW and Victoria better access to sample processing.

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 2020-2021 collection period.

Bacterial species	2020	2021	Total
Mannheimia haemolytica	3	10	13
Pasteurella multocida	10	43	53
Histophilus somni	13	19	33
Streptococcocus dysgalactiae	0	2	2
Trueperella pyogenes	0	13	13
Mycoplasma bovis	12	49	51
Total	38	136	165

3.1.2 Antimicrobial susceptibility testing

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) (Table 2). Seventeen antimicrobials were tested: ampicillin, ceftiofur, chlortetracycline, clindamycin, danofloxacin, enrofloxacin, florfenicol, gentamicin, neomycin, oxytetracycline, penicillin, sulphadimethoxine, spectinomycin, tiamulin, tilmicosin, trimethoprim/sulfamethoxazole and tulathromycin. 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
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

MIC breakpoints are taken from Clinical and Laboratory Standards Institute (2018).

[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 were 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.

3.2 Determine if in-feed use of tetracycline is correlated with increased antimicrobial resistance of bovine respiratory disease pathogens.

In B.FLT.3004, tetracycline/macrolide dual-resistant *P. multocida* isolates were obtained from two NSW feedlots (N1 and N4) that routinely incorporated metaphylactic use of chlortetracycline into their production cycles during the 2019 targeted AMR surveillance programme. However, the number of isolates obtained from these feedlots was too low in 2019 for accurate statistical comparison. To increase the number of isolates, more intensive sampling was undertaken in 2021 at N4. Metaphylactic treatment at N4 occurs around 14-20 days post-induction during peak susceptibility and is typically undertaken for a 10 day period. Results were compared with the proportion of resistant isolates obtained from other feedlots participating in the AMR surveillance.

3.3 Determine if metaphylaxis at feedlot entry with macrolides is associated with an increased risk of antimicrobial resistance of pathogens.

It was not possible to find a participating feedlot that routinely practiced the administration of tilmicosin at feedlot entry during the 2020/2021 study period willing to take part in the study. However, it was possible to include the 2020 and 2021 AMR surveillance cases in the pathogen association study to strengthen the case that *Mycoplasma bovis* is strongly associated with *P. multocida* infection compared to the other feedlots (as shown for the 2019 BRD AMR study).

A total 163 lungs that were sampled from BRD affected cattle at post-mortem between 2019 and 2021 for investigation of their BRD bacterial pathogen status met the study inclusion criteria. The lung samples and/or deep lung swabs underwent culture (+/-) PCR to determine their infection status for *Mycoplasma bovis*, *P. multocida, M. haemolytica, and H. somni. M. bovis* was detected in 97 samples, *P. multocida* in 63 samples, *M. haemolytica* in 15 samples and *H. somni* in 42 samples. No BRD pathogens were detected in 31 of the samples, however 7 of these had mixed infections comprised of other bacteria that were not included in the selection criteria including *Trueperella pyogenes*, *Helcococcus ovis* and *Streptococcus dysgalactiae* (data not shown).

3.4 Characterise the genetic mechanisms responsible for recently identified resistance in *Pasteurella multocida*

3.4.1 Strain selection for long read sequencing

Based on data generated in project B.FLT.3004, twelve genomes with varied combination of resistance determinants and mobile genetic elements (ICE or plasmids) capable of disseminating them were selected for long range sequencing. Genomes selected for long read sequencing and their characteristic features are presented in Table 3. Additionally, further analysis of *P. multocida* genomes obtained during B.FLT.3004 has identified the *P. multocida* capsular type, somatic antigen serotype and multilocus sequence type for each Australian BRD isolate (se Supplementary Figure 1). The majority of Australian isolates belong to either ST79, a well described international clone, or ST394, a recently discovered novel ST. ST394 isolates were significantly more likely to possess resistance genes compared to the ST79 and remaining STs of lower prevalence.

Table 3. List of genomes sequenced on Oxford NanoPore multiplexed sequencing run.

ACARE Ref:	BRD Ref:	Phenotype	State of isolation	BRD pathogen	Resistance Genotype (Illumina)
016*	17BRD-035		QLD	*P. multocida*	*parB, bla*$_{ROB1}$*, tetR/tetH*
047*	18BRD-001		QLD	*P. multocida*	*parB, bla* $_{ROB1}$*, tetR/tetH*
078	19BRD-003		SA	*P. multocida*	*gyrA*
082	19BRD-011	*Pm*3(M^RT^R)	NSW	*P. multocida*	*[msrE, mphE], [tetR/tetH]*
091*	19BRD-032		QLD	*P. multocida*	*parB, bla* $_{ROB1}$*, [tetR/tetH]*
100*	19BRD-057		QLD	*P. multocida*	*parB, bla* $_{ROB1}$*, [tetR/tetH]*
108$^{\$}$	19BRD-085	*Pm*2(M^R)	NSW	*P. multocida*	*[msrE, mphE]*
117	19BRD-106	*Pm*5(M^RT^R)	NSW	*P. multocida*	*[msrE, mphE], [tetR/tetH]*
120	19BRD-112		NSW	*P. multocida*	*[msrE, mphE], [tetR/tetH]*
141	17BRD-067		SA	*M. haemolytica*	*gyrA*
179	19BRD-019		SA	*M. haemolytica*	*gyrA*
188$^{\$}$	19BRD-084	*Mh*1(M^R)	NSW	*M. haemolytica*	*[msrE, mphE], gyrA*

Genomes marked with an asterisk (*) indicate isolates with ICE and with a $ sign indicates isolates collected from the same animal and identical macrolide resistance gene profile. [Genes in brackets] indicate clustering of resistance genes on the same genomic scaffold.

3.4.2 Oxford Nanopore sequencing

Genomic DNA for Nanopore sequencing was prepared using the XS-buffer protocol (2) with modifications. Briefly, genomic DNA from 2mL of bacterial cells growing at mid-log phase was collected by centrifugation (7000 rpm, 5 min) and resuspended in 1 mL of XS buffer. The cell suspension was incubated at 70°C till the solution was clear indicating complete lysis. The resuspended bacteria were then briskly vortexed for 5sec to maximise lysis and placed on ice for 30min to allow for the precipitation of cellular debris. The cell debris were removed by centrifugation (14000 rpm, 10min) and the supernatant was collected in a fresh Eppendorf tube. DNA was precipitated with an equal volume of isopropanol. The precipitated DNA was washed twice with 70% ethanol, before resuspending gently in 100µL of TE buffer and allowed to dissolve at 50°C for 1hr. On complete resuspension, the DNA solution was allowed to cool to room temperature and treated with 5µL of RNAse in a total volume of 200µL (PureLink™ RNase A 20 mg/mL from Invitrogen) for 1hr at 37°C. The DNA was purified using phenol/chloroform extraction and resuspended in 100µL of DNase free sdH_2O. Quality and quantity of DNA was initially monitored using a nanodrop spectrophotometer, followed by Qbit estimation for quantification of DNA prior to

preparation of sequencing libraries. Multiplexed sequencing libraries were prepared using Oxford Nanopore Technologies' (ONT) Rapid Barcoding Sequencing Kit (SQK-RBK004) with 500 ng of input genomic DNA per sample and sequenced using a R9.4.1 flowcell (FLO-MIN106) at the DNA sequencing facility at the ithree institute at UTS.

3.4.3 *Assembly of genome sequences*

Guppy base caller was used to extract long-read sequences from the multiplexed Nanopore run. Sequence pools representing each genome were demultiplexed using Deepbinner (3). FastQC, FastP (4) and pycoQC (5) was used for quality control purposes. MultiQC (6) was used to collate quality control datasets into a single file. The Nanopore long read sequences were co-assembled with Illumina sequences using Unicycler (7).

3.4.4 Sequence analysis and annotation

Preliminary genome annotations were generated using Prokka (8) and on the RAST server (9). Resistance gene annotations predicted by the automated annotation pipelines were confirmed using ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/). Regions of the genome representing Integrative Conjugative Elements (ICE) were initially identified by aligning genomes with sequence of published ICEs downloaded from GenBank. Following this, individual genes in the Integrative Conjugative Elements were manually curated by iterative BLASTn (10) searches. Multilocus sequence type of the genomes were analysed using the PubMLST portal and different schemes available in it (11, 12). Sequence of the 23S rRNA gene were aligned using ClustalW Multiple Sequence Alignment tool available online. Figures representing alignment of ICEs in the different genomes were generated using EasyFig v2.2.2. Other gene maps were generated using SnapGene and manipulated using PhotoShop.

3.4.5 Plasmid biology

A total of eight isolates were selected for further analysis based on existing phenotypic and genetic information; five resistant isolates and three sensitive isolates (Table 4).

Table 4. Summary table detailing the isolates used during this study, along-side their origin state and year, the observed resistances, and the *P. multocida* sequence type.

Isolate Designation	BRD Isolate	Bacterial Species	Origin	Resistance	Sequence Type
*$Mh1(M^R)$	19BRD-084	*M. haemolytica*	2019 NSW	Macrolide	NA
*$Pm2(M^R)$	19BRD-085	*P. multocida*	2019 NSW	Macrolide	394
$Pm3(M^RT^R)$	19BRD-011	*P. multocida*	2019 NSW	Macrolide and Tetracycline	394
$Pm4(T^R)$	19BRD-111	*P. multocida*	2019 NSW	Tetracycline	394
$Pm5(M^RT^R)$	19BRD-106	*P. multocida*	2019 NSW	Macrolide and Tetracycline	394
*Pm*6	19BRD-130	*P. multocida*	2019 NSW	Sensitive	13
*Pm*7	19BRD-007	*P. multocida*	2019 SA	Sensitive	79
*Pm*8	19BRD-093	*P. multocida*	2019 NSW	Sensitive	394

* Both isolates derived from BRD-affected lung tissue from the same animal

3.4.5.1 ***Spontaneous plasmid loss***

Each isolate was subcultured daily on sheep blood agar (SBA) plates for 12 days. After the 12 subcultures, disc diffusion antimicrobial susceptibility testing was conducted, as well as MIC testing.

3.4.5.2 ***Plasmid curing***

Plasmid curing was conducted using the agents Acridine Orange, Acriflavine, and Rifampicin, using a method adapted from Trevors (1986). 0.5 McFarland standards were made of each isolate, 20 μl of which was added into 1 ml LB broth. The curing agents were filter-sterilised, and then added at concentrations that ranged from 0.5 μg/ml to 100 μg/ml, depending on the isolate and curing agent selected. The bacterial suspensions were then incubated overnight at 37°C. All concentrations of plasmid curing agent that allowed for bacterial growth were plated, ranging from 10 μl to 50 μl per plate depending on the amount of growth, onto SBA. This was incubated overnight at 37°C. After confirming colony growth, individual colonies were examined for loss of resistance by 'patching' individual colonies onto a master SBA plate, and selective antibiotic plates depending on the resistance profile of the isolate. Patching involves taking a single colony with a pipette tip or toothpick, and replica-streaking it onto both non-selective and selective SBA plates. Any colonies that did not grow on the selective plates were examined for resistance by antimicrobial susceptibility testing using both MIC and disc diffusion.

3.4.5.3 ***Restriction digest***

Plasmids were extracted using a NuceloSpin Plasmid DNA extraction kit according the manufacturer's instructions (MACHERY-NAGEL, Germany). Plasmid DNA samples were then

subjected to restriction digestion for 1 h at 37 °C using enzymes *Bam*HI and *Hin*dIII. The digestion mix consisted of 2 μl buffer, 14 μl water, 2 μl of the enzyme *Bam*HI, and/or 2 μl of the enzyme *Hin*dIII (equivalent to 4 units of each restriction enzyme). After incubation, a gel loading dye was added to 1 X concentration and the mix was loaded onto a precast 50 ml 1% agarose gel dissolved in Tris-acetic acid ethylenediaminetetraacetic acid (TAE) buffer premixed with 5 μl of the RedSafe DNA intercalating dye. The DNA-agarose gel was then subjected to electrophoresis in TAE buffer for 45 minutes at 120 V on a Bio-Rad electrophoresis unit, including a 1 kb Plus DNA ladder (Thermo Fisher Scientific) or 1 kb Hyperladder (Bioline) as a size marker.

3.4.5.4 *Plasmid sequencing*

Plasmids were extracted as described in the restriction digest stage and sent externally to SA Pathology for long range sequencing. Sequences were then checked to conform to the maps generated by Dr Piklu Bhattacharya at UTS from the long range sequencing data.

3.4.5.5 *Electroporation*

Electroporation was carried out as conducted by Hoskins and Lax (13) with some adjustments. The *P. multocida* isolates *Pm*2(M^R), *Pm*3(M^RT^R), *Pm*5(M^RT^R), which had been plasmid cured previously, and had lost one or more resistances, and *Pm*8, a sensitive control isolate, were prepared for electroporation by allowing cells to grow in 15 ml LB and 37°C for 24 h. Following this, cells were centrifuged for 15 min at 4000 rpm. After removing the supernatant, cells were washed with 10% glycerol. Approximately 15 ml of 10% glycerol was added to the cells, which were then re-suspended. This mix was centrifuged for 15 minutes at 4000 rpm. The washing step was repeated two more times. Plasmid DNA, extracted from *Pm*3(M^RT^R) was added to the washed cells, in varying amounts depending on plasmid recovery.

3.5 Whole of genome sequencing of *Histophilus somni* and *Mycoplasma bovis* collections for surveillance of antimicrobial resistance genes.

3.5.1 DNA extraction and sequencing of *H. somni* isolates

All *H. somni* isolates from 2014-2019 were susceptible to all tested antimicrobials. Therefore, whole-genome sequencing (WGS) was conducted on the entire historical *H. somni* isolate collection as follows. DNA from the isolates was extracted using a MagMAX Multi-sample DNA extraction kit (Thermofisher Scientific, USA). DNA libraries were prepared using an IIlumina Nextera XT Library Preparation Kit, with slight variation from the manufacturer's instructions (increased tagmentation time of seven minutes). WGS was performed on a NextSeq 500 Illumina platform to obtain 2 x 150 bp paired-end sequences. The sequencing adapters were AGATCGGAAGAGCACACGTCTGAACTCCAGTCA and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT. Isolates with less than 30x sequencing coverage were removed. Reads were trimmed using the software Trimmomatic v0.38 (14) to remove sequencing adapters and low-quality bases. FASTQC v0.11.4 (15) was used to check the quality of raw and cleaned reads. De novo genome assembly of the isolates was performed on cleaned reads using SPAdes v3.12.0 (16). Assemblies were checked with Quast v4.5 (17) for the number of contigs and contig N50. A total of 65 isolates, QLD (n=33), NSW (n=22) and SA (n=10), were retained that passed the above described quality filters.

3.5.2 Phylogeny

The genetic relationships between isolates were examined using single nucleotide polymorphisms (SNPs) found from cleaned WGS reads for the 65 isolates mapped to a *H. somni* complete genome (NCBI Assembly Accession: NZ_CP018804.1). The software Snippy v4.6.0 (https://github.com/tseemann/snippy) was used to call core SNPs (SNPs that can be determined in all isolates). A maximum likelihood (ML) phylogenetic tree was constructed using the model GTRCAT and a rapid bootstrap analysis; with 100 bootstraps for the best-scoring ML tree conducted, using RAxML v8.2.10 (18) . This was followed by recombination removal using ClonalFrameML v1.12 (19). The final phylogenetic tree was manipulated with iTOL (https://itol.embl.de/) for display.

3.5.3 DNA extraction and sequencing of *M. bovis* isolates showing reduced susceptibility

Five *M. bovis* isolates from the 2018-2019 collection were selected for whole genome sequencing (Mb1 [SA], Mb2 [Qld], Mb3 [Qld], Mb4 [NSW], and Mb5 [NSW]). These isolates showed reduced susceptibility in MIC testing and non-wild type MIC values for erythromycin, lincomycin, tylosin, tilmicosin, and tildipirosin. Serial subcultures of the selected isolates were undertaken three times in modified Eaton broth/agar media to ensure purity. At each subculture, colonies were picked from the agar plates after incubation for 5-7 days and re-cultured in media containing high antibiotic concentrations to confirm growth continuity and purity. MIC values were obtained again after the final subculture to confirm stability of the phenotype.

3.5.4 Nucleic Acid Extraction and WGS analysis

For the isolates Mb1-Mb5, 500 µL of broth culture was used for DNA extraction using DNeasy® Blood and Tissue (QIAGEN kit) following the manufacturer's instructions. Samples were eluted in a final volume of 100 µL using the elution buffer provided with the kit and assessed for purity and concentration using Tape Station, then held at -20^{o}C until sent for WGS at UTS. DNA was sequenced as described above for *H. somni*.

3.5.5 Phylogeny

Multiple genome alignment was performed using BioEdit software (https://bioedit.software.informer.com) with *M. bovis* PG45, 1982-M6152, and 2019-043682 on NCBI's Gene database as the reference genomes. SNPs and non-synonymous mutations relative to M. bovis PG45 were identified using Snippy v4.6.0 as described above. Gene features were analyzed and tabulated and further annotated by searching NCBI's Gene database to identify their functional roles.

3.6 Histopathological analysis of *Mycoplasma*-infected tissues and association with specific BRD-associated viruses

3.6.1 Histopathology

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. It was not possible to develop immunohistochemistry in the B.FLT.3011 extension project due to COVID-related issues with importing antibodies from international laboratories and the fact that many VDLs in North America had shut down their immunohistochemistry services due to the pandemic.

Fixed histological sections were combined with those analysed in B.FLT.3004 to enable large enough numbers for statistical comparison on the likelihood of detection of *Mycoplasma bovis* being associated with small necrotic foci in BRD-affected lung tissue.

3.6.2 Application of PCR to BRD and non-BRD affected fresh lung tissue for virus and *Mycoplasma* detection

To investigate the significance of BRD viruses to co-infection with *Mycoplasma bovis* in BRD cases, a subset of 60-70 fresh lung samples (together with formalin fixed lung tissue for histological sections) were obtained from feedlot S1 (approximately half from post-mortem cases of BRD and half from post-mortem cases from other diseases). All lung samples were diced while still frozen, and 200 mg of lung sample was homogenised in 1 mL of sterile PBS using a three-way stopcock and syringes. The homogenate was then centrifuged at 2000 rpm at 4° C for 15 minutes and the supernatant was used for DNA and RNA extraction. The DNA was extracted with DNeasy Blood and tissue Kit (QIAGEN Inc., Hilden, Germany) and RNA was extracted with QIAamp Viral RNA Mini Kit (QIAGEN Inc., Hilden, Germany). Viral RNA was kept on ice and immediately converted to cDNA with QuantiTect Reverse Transcription Kit (QIAGEN Inc., Hilden, Germany).

Total DNA, for BHV-1 detection, and cDNA, for other RNA viruses, were used directly for PCR amplification of the targeted genes with AllTaq™ Master Mix Kit (QIAGEN Inc., Hilden, Germany). PCR setup was run in a final volume of 20 µL that included 5 µL of 4x AllTaq Master Mix, 0.25 µM of forward primer and reverse primer, RNase free water and up to 1 µg DNA/cDNA per reaction. All PCR thermal conditions, except first nested PCR for BHV-1, were as follows: 1 cycle of initial activation for 2 min at 95 °C; 40 cycles of denaturing at 94 °C for 10 s, annealing temperature for 10 s, elongation at 72 °C for 30 s; and final elongation at 72 °C for 5 min. BHV-1 first nested PCR cycle was as follows: 1 cycle of initial activation for 2 min at 95 °C; 10 cycles of denaturing at 94 °C for 20 s, annealing at 65 °C for 45 s, elongation at 72 °C for 45 s; 15 cycle of denaturing at 94 °C for 20 s, annealing at 54 °C for 45 s, elongation at 72 °C for 45 s; 10 cycle of denaturing at 94 °C for 20 s, annealing at 60 °C for 45 s, elongation at 72 °C for 45 s, and final elongation at 72 °C for 5 min.

For *Mycoplasma* detection, a PCR assay was undertaken on the extracted DNA, using the Alltaq:Master Mix Kit protocol (Qiagen, Hilden, Germany) (Appendix C). The PCR reaction mix for one reaction included the template DNA extract (4 µl), All taq master mix (5 µl), RNase-free water (9 µl), and the primers (1 µl of each primer). The primers used were specific to *Mycoplasma bovis.* The forward primer was FH- M. bov FusBroa545 F: GATGATTGACGCCGTTGTTGAT and the reverse primer was FH- M.bov Fusbroa545 R: CCAGCATRATTGTTTGACCTGT. Once combined, the PCR reaction was incubated in a thermocycler (T100TM Thermal Cycler, Bio-Rad). PCR reaction conditions consisted of cycles of 94°C for 5 minutes, followed by 94°C for 1 minute, followed by 55°C for 30 seconds, followed by 72°C for 30 seconds and 72°C for 10 minutes.

3.7 Statistical analysis

The feedlot data on AMR incidence data for tetracycline-, macrolide- and both tetracycline and macrolide-resistant variants among the *P. multocida* isolates was compiled into 2 x 2 contingency tables for comparison of Feedlot N4 (which routinely practiced metaphylactic use of chlortetracycline) compared to the other feedlots for the 2021 AMR surveillance data using a Fisher's exact test. For co-association of *Mycoplasma bovis* infection with other BRD pathogens, Pearson Chi-Square Independence tests were used, however, the alpha significance value of 0.05 underwent Bonferroni correction to a significance value of 0.017, to compensate for increased risk of type I errors when three statistical tests were conducted on the same data. The Odds ratio (OR) test was calculated through SPSS which measured the strength of the association between *M. bovis* co-infection with *P. multocida, H. somni* and *M. haemolytica*. Lastly, Chi-Square and Fisher's exact tests were used to determine co-association of *Mycoplasma bovis* and pathognomonic, small necrotic foci observed in lung tissue histological sections.

4 Results

4.1 Antimicrobial resistance surveillance 2020/2021.

Apart from a single *P. multocida* isolate from feedlot S1 that was resistant to macrolides, all BRD isolates (i.e. *M. haemolytica*, *P. multocida* and *H. somni*) from the 2020 sampling period were pan-susceptible to the tested antimicrobials. For 2021, an interesting resistance was observed on one NSW feedlot (N3) and the Victorian feedlot (V1) with the identification of an additional three *M. haemolytica* isolates that were resistant to macrolides, matching with the single macrolide-resistant isolate identified in NSW in 2019 (all isolates exhibiting tulathromycin MICs of ≥ 64 µg/ml and tilmicosin MICs of ≥ 32 µg/ml).

Among *P. multocida* isolates, the following trends were observed (Table 5). Firstly, in 2019, feedlot N2 recorded a number of tetracycline-resistant and tetracycline/macrolide dual-resistant isolates. In 2021, all five *P. multocida* isolates obtained from this feedlot were pan-susceptible. Secondly, tetracycline/macrolide dual-resistant isolates still remained dominant on feedlot N4 in 2021, with just over half (57%) the total number *P. multocida* isolates (n=21) having this phenotype. A single tetracycline-resistant isolate was also obtained from N4 in 2021, but the remaining isolates were all pan-susceptible. Thirdly, macrolide-resistant *P. multocida* remain the dominant phenotype isolated from S1 (89%; 9 isolates in total) and lastly, a single macrolide-resistant *P. multocida* isolate was obtained from V1 (20% of 5 isolates).

Table 5. Distribution of 2021 targeted AMR surveillance of *Pasteurella multocida* (*Mannheimia haemolytica*) isolates by feedlot and resistance profile.

Feedlot	Total Isolates	Pan-Susceptible[1]	Tet-R[2]	Mac-R[3]	Tet/Mac-R[4]
N1	0				
N2	5	5			
N3	3 (2)	3		(2)	
N4	21 (6)	8 (6)	1		12
S1	9 (1)	1 (1)		8	
V1	5 (1)	4		1 (1)	
Total	43 (10)	21 (7)	1	9 (3)	12

[1] Isolates in this category exhibited MICs that were below the susceptible breakpoint shown in Table 2.

[2] Isolates in this category exhibited tetracycline MICs that were above the resistance breakpoint of ≥ 8 µg/ml

[3] Isolates in this category exhibited macrolide (tulathromycin and tilmicosin) MICs that were above the resistance breakpoints of ≥ 64 µg/ml and ≥ 32 µg/ml, respectively.

[4] Isolates in this category exhibited dual resistance to both macrolides and tetracycline

Table 6 MIC distribution of *P. multocida* BRD isolates 2020 - 2021

		Pasteurella multocida **(n = 53)**																	
		Distribution of MICs (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	0-8.42
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-8.42
Chlortetracycline	Cattle					60.4	7.5	3.8	3.8	22.6	1.9						0.5	0.5	14.19-38.58
Clindamycin	Cattle				3.8	0	0	0	9.4	13.2	60.4	13.2					16	16	-
Danofloxacin	Cattle			100	0	0	0										0.12	0.12	0-8.42
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-8.42
Florfenicol	Cattle				79.2	20.8	0	0	0	0							0.25	0.5	0-8.42
Gentamicin	Cattle						30.2	62.2	3.8	3.8	0						2	2	-
Neomycin	Cattle								81.1	13.2	3.8	1.9					4	8	-
Oxytetracycline	Cattle					66.0	1.9	3.8	0	3.8	24.5						0.5	0.5	17.20-42.55
Penicillin	Cattle			98.1	1.9	0	0	0	0	0							0.12	0.12	2.7-46.3
Sulphadimethoxine	Cattle														94.3	5.7	256	256	-
Spectinomycin	Cattle									18.9	73.6	7.5	0				16	16	0-8.42
Tiamulin	Cattle					0	5.7	0	0	1.9	64.1	28.3					16	32	-
Tilmicosin	Cattle								49.1	9.4	0	3.8	15.1	22.6			8	8	25.12-52.13
Trimethoprim/sulfamethoxazole	Cattle							100									2	2	-
Tulathromycin	Cattle						39.6	15.1	1.9	1.9	0	0	41.5				2	8	28.42-55.82

The antimicrobial concentration ranges tested are contained in white area. Susceptible and resistance breakpoints are indicated in vertical green and red lines.

Table 7 MIC distribution of *M. haemolytica* BRD isolates 2020-2021

		***Mannheimia haemolytica* (n = 13)**																	
		Distribution of MICs (µg/mL) (%)															**MIC50**	**MIC90**	**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**	**(µg/ml)**	**(µg/ml)**	
Ampicillin	Cattle				100	0	0	0	0	0							0.25	0.25	0-28.54
Ceftiofur	Cattle				100	0	0	0	0	0							0.25	0.25	0-28.54
Chlortetracycline	Cattle					69.2	23.1	7.7	0	0							0.5	0.5	0-28.54
Clindamycin	Cattle				0	0	0	0	15.4	76.9	7.7						8	8	-
Danofloxacin	Cattle			100	0	0	0										0.12	0.12	0-28.54
Enrofloxacin	Cattle			100	0	0	0	0									0.12	0.12	0-28.54
Florfenicol	Cattle				7.7	84.6	7.7	0	0	0							0.5	0.5	0-28.54
Gentamicin	Cattle						23.1	76.9	0	0	0						2	2	-
Neomycin	Cattle								53.8	46.2	0	0					4	8	-
Oxytetracycline	Cattle					76.9	23.1	0	0	0							0.5	0.5	0-28.54
Penicillin	Cattle			61.5	38.5	0	0	0	0	0							0.12	0.25	0-28.54
Sulphadimethoxine	Cattle														92.3	7.7	256	256	0-32.1
Spectinomycin	Cattle									7.7	84.6	7.7	0				16	32	0-28.54
Tiamulin	Cattle					0	0	0	0	0	69.2	30.8					16	32	-
Tilmicosin	Cattle								7.7	69.2	0	0	7.7	15.4			4	4	6.16-54.02
Trimethoprim/sulfamethoxazole	Cattle							100									2	2	-
Tulathromycin	Cattle						0	0	15.4	53.8	7.7	0	0	23.1			8	8	6.16-54.02

The antimicrobial concentration ranges tested are contained in white area. Susceptible and resistance breakpoints are indicated in vertical green and red lines.

Table 8 MIC distribution of *H. somni* BRD isolates 2020 - 2021

		Histophilus somni **(n = 33)**																	
		Distribution of MICs (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			97	0	1	0	0	0	0	0						0.25	0.25	-
Ceftiofur	Cattle				97	3	0	0	0	0							0.25	0.25	0-12.98
Chlortetracycline	Cattle					100											0.5	0.5	0-12.98
Clindamycin	Cattle				6.1	45.4	42.4	6.1	0	0	0						0.5	1	-
Danofloxacin	Cattle			100	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				94.0	3.0	3.0	0	0	0							0.25	0.25	0-12.98
Gentamicin	Cattle						0	3.0	27.3	60.6	9.1						8	8	-
Neomycin	Cattle								3.0	3.0	36.4	54.6	3.0				16	32	-
Oxytetracycline	Cattle					100	0	0	0	0	0						0.5	0.5	0-12.98
Penicillin	Cattle			100	0	0	0	0	0	0							0.12	0.12	0-12.98
Sulphadimethoxine	Cattle														54.6	45.4	256	512	-
Spectinomycin	Cattle									24.2	33.3	42.5	0				32	32	0-12.98
Tiamulin	Cattle					6.1	30.3	48.5	9.1	3.0	3.0	0	0				2	2	-
Tilmicosin	Cattle								51.5	48.5	0	0	0				4	8	0-12.98
Trimethoprim/sulfamethoxazole	Cattle							100									2	2	-
Tulathromycin	Cattle						6.1	3.0	33.3	30.3	27.3	0	0				8	16	0-12.98

The antimicrobial concentration ranges tested are contained in white area. Susceptible and resistance breakpoints are indicated in vertical green and red lines.

4.2 Determine if in-feed use of tetracycline is correlated with increased antimicrobial resistance of bovine respiratory disease pathogens.

In 2021, just over half (57%) of the *P. multocida* isolates obtained from feedlot N4 were found to be resistant to both tetracyclines and macrolides, a single isolate was tetracycline-resistant and the remainder were pan-susceptible to antibiotics. By comparison, no tetracycline-resistant or dual-resistant *P. multocida* were isolated from the remaining feedlots providing samples for the 2021 AMR surveillance period. Comparison of proportions in contingency tables (Table 6) confirmed that *P. multocida* isolates from N4 were significantly more likely to be tetracycline-resistant or dual-resistant compared to isolates from the other feedlots. However, it is important to note that some of the comparator feedlots still use metaphylaxis treatments with chlortetracycline at different times of the year during peak BRD incidence for high-risk cattle, including N2, which in 2021 only yielded pan-susceptible *P. multocida* isolates (compared to 2019 where 6 of 7 isolates were tetracycline-resistant, with 3 of these also resistant to macrolides), despite the fact that fewer isolates were obtained from N2 in 2021.

Table 6. Contingency tables showing the proportion of total tetracycline-resistant, total macrolide-resistant and total tetracycline/macrolide-resistant *Pasteurella multocida* isolated from feedlot N4 (which consistently practices metaphylactic use of chlortetracycline in the feed) compared to other feedlots sampled during the 2021 AMR surveillance study.

Feedlot	Tet-R Pm	Other Pm	Mac-R Pm	Other Pm	Tet-R/Mac-R Pm	Other Pm
N4	13[1]	8	12[2]	9	12[1]	9
Other (N1, N2, N3, S1, V1)	0	22	9	13	0	22
Total	13	30	21	22	12	31

[1] $P < 0.00001$

[2] $P = 0.5377$

4.3 Determine if metaphylaxis at feedlot entry with macrolides is associated with an increased risk of antimicrobial resistance of pathogens.

4.3.1 Tilmicosin administration at feedlot entry

No results possible.

4.3.2 *Co-association of Mycoplasma bovis with other BRD pathogens*

By combining the AMR surveillance data for 2019-2021 that satisfied the selection criteria, *Mycoplasma bovis* was detected in 97/163 (60%) of BRD-affected cattle lung samples, 28 (17.2%) of which yielded *Mycoplasma bovis* as the sole infectious agent. *P. multocida* was present in 39% of the lung samples, whilst *H. somni* and *M. haemolytica* were present in 26% and 9% of the lung samples, respectively. Application of the Chi Square test indicated that *Mycoplasma bovis* and *P. multocida*

had a strong association of co-infection (p=0.001), whilst *M. haemolytica* (p=0.553) and *H. somni* (p=0.467) did not have a significant association of co-infection with *Mycoplasma bovis*. The Odds ratio (OR) quantified the strength of the co-infection relationship and revealed that cattle infected with *Mycoplasma bovis* have 3.3 times the odds of being co-infected with *P. multocida* than cattle not infected with *Mycoplasma bovis* (95% CI:1.6-6.7).

4.4 Characterise the genetic mechanisms responsible for recently identified resistance in *Pasteurella multocida*

4.4.1 Long read sequencing

Seven genomes, (6 for *P. multocida* and 1 for *M. haemolytica*) were completely closed generating circular chromosomes and plasmids (Table 7). Except for 19BRD-003 (ACARE078) the average coverage of genome sequences ranged between 259x and 553x, including genomes which could not be circularised, providing an ideal opportunity for detailed context analysis of the genes of interest. Unlike other isolates in the long read sequencing cohort, 19BRD-003 (ACARE078) did not contain any antimicrobial resistance genes, but was phylogenetically distinct to other *P. multocida* isolates sampled in project B.FLT.3004 and was therefore included in this sequencing run. Although relatively fragmented, MLST and 16S rRNA gene analysis of the 19BRD-003 genome confirmed that it belonged to *M. haemolytica* ST1, the predominant ST associated with BRD in Australia. *M. haemolytica* isolates 19BRD-019 (ACARE179) and 19BRD-084 (ACARE188) also belonged to ST1; while 17BRD-067 (ACARE141), the only closed *M. haemolytica* genome in the cohort, belonged to a new ST which has not yet had a number designated in the pubMLST database for its allelic combination (*adk*=7, *aroE*=10, *deoD*=10, *gapDH*=10, *gnd*=9, *mdh*=10, *zwf*=12). All resistant *P. multocida* isolates included in this project belonged to ST394.

Table 7. Assembly statistics of genomes assembled using Unicycler hybrid assembly of Illumina short read and Oxford-Nanopore sequences. Rows in bold indicate completely closed genomes. The Contigs[#] column reports on number of contigs which were >= 5000 bp. Genome size indicates the size of genome calculated from the assembled contigs.

ACARE Ref.	BRD Ref.	Contigs[#]	Genome size	GC (%)	N50	N75	Avg. depth
Pasteurella multocida genomes							
016	**17BRD-035**	**1**	**2624884**	**40.23**	**2624884**	**2624884**	**344**
047	18BRD-001	3	2586086	40.27	1457552	1105582	273
078	19BRD-003	13	2656581	40.99	472454	280506	185
082	**19BRD-011**	**2**	**2611627**	**40.2**	**2599299**	**2599299**	**259**
091	19BRD-032	3	2553301	40.27	1447701	1080540	553
100	**19BRD-057**	**1**	**2586401**	**40.27**	**2586401**	**2586401**	**471**
108	**19BRD-085**	**2**	**2447144**	**40.3**	**2439433**	**2439433**	**456**
117	**19BRD-106**	**2**	**2478170**	**40.33**	**2466504**	**2466504**	**348**
120	**19BRD-112**	**2**	**2478131**	**40.33**	**2466465**	**2466465**	**385**
Mannheimia haemolytica genomes							
141	**17BRD-067**	**1**	**2354207**	**41.25**	**2352841**	**2352841**	**263**
179	19BRD-019	3	2699748	41.05	2667746	2667746	265
188	19BRD-084	2	2698129	41.07	2687589	2687589	272

Focussing on replicon sizes of the completely closed *P. multocida* genomes (Table 8), 17BRD-035 had the biggest genome (2624884 bp) consisting of a single chromosome only. Isolates 19BRD011 (ACARE082), 19BRD-085 (ACARE108), 19BRD-106 (ACARE117) and 19BRD-112 (ACARE120) each possessed a plasmid over 7kb in size. Alignment of p1_ACARE117 and p1_ACARE120 indicated 100% identity over the query length, indicating presence of identical plasmids in these two isolates.

Alignment of p1_ACARE082 and p1_ACARE108 revealed 99.41% identity over 98% query sequence. In addition, 19BRD011 (ACARE082), 19BRD-106 (ACARE117) and 19BRD-112 (ACARE120) had a second circular extra-chromosomal replicon, 4580bp in size and much higher genome coverage (11.5x-66.5x) raising the possibility of the presence of a small plasmid in these isolates. Alignment of replicon sequences indicated that the plasmids in isolates 19BRD-106 (p2_ACARE117) and 19BRD-112 (p2_ACARE120) were identical, while the plasmid in 19BRD011 (p2_ACARE082) had likely undergone internal recombination events when compared to p2_ACARE117 and p2_ACARE120 and had 99.1% identity over 98% of the query sequence. *P. multocida* isolate 19BRD-085 (ACARE108) and *M. haemolytica* isolate 19BRD-084 (ACARE188) were isolated from the same sample and both possessed a plasmid over 7.6kb. Alignment of p1_ACARE188 and p1_ACARE108 indicated that they had 99.1% identity over 97% query length.

Table 8. Replicon sizes in completely closed genomes. * indicates isolates collected from biopsy samples of the same animal.

ACARE Ref	BRD Ref	No of Contigs (>=5kb)	Chromosome size (nt)	Plasmid size (nt)	Additional <5kb replicon (coverage)
Pasteurella multocida genomes					
16	17BRD-035	1	2624884	N/A	
82	19BRD-011	2	2599299	7748 (p1_ACARE082)	4580 (11.54x)
100	19BRD-057	1	2586401	N/A	
108*	19BRD-085	2	2439433	7711 (p1_ACARE108)	
117	19BRD-106	2	2466504	7086(p1_ACARE117)	4580 (66.01x)
120	19BRD-112	2	2466465	7086(p1_ACARE120)	4580(48.85x)
Mannheimia haemolytica genomes					
141	17BRD-067	1	2352841	N/A	1366(546.32x)
188*	19BRD-084	2		7603 (p1_ACARE188)	

Given most smaller replicons in the genomes were under 8kb, it was imperative to check for the presence of genes which could possibly encode for a homologue of replication protein for stable maintenance in the genome. Preliminary annotations using RAST confirmed that both the 4580bp replicon and the comparatively larger replicons did have CDSs that could encode functional proteins which would support DNA replication (File S1). The 4580bp replicons also harboured the tetracycline resistance determinants, and the larger (>7kb) plasmids housed the macrolide resistance genes. Additionally, the larger replicons could also encode protein homologues which would support

plasmid mobilisation. A diagrammatic representation of the relative position of the different functional proteins of interest on the plasmid backbones are presented in Figures 1 and 2.

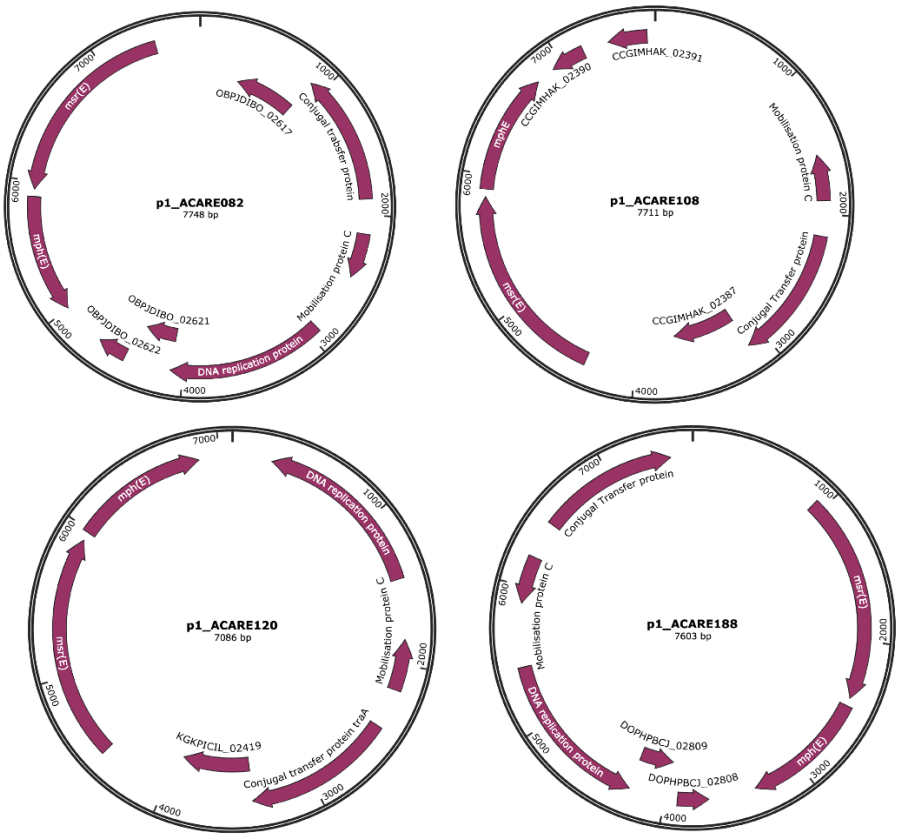

Figure 1: Diagrammatic representation of the functional proteins identified on the large plasmids >7kb and the *msrE-mphE* genes which confer macrolide resistance.

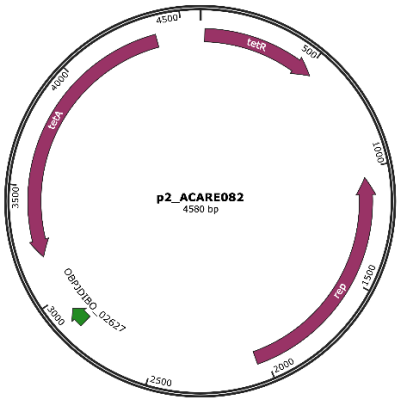

Figure 2. Diagrammatic representation of the functional proteins identified on the smaller 4.5kb plasmid and the *tetA-tetR* genes which confer macrolide resistance.

The *bla*$_{ROB-1}$ gene was not identified on any of these plasmids. Of the four isolates (17BRD-035, 18BRD-001, 19BRD-032 and 19BRD-057) in which *bla*$_{ROB-1}$ was present, 17BRD-035 and 19BRD-057 (both ST394 isolates) were completely closed into a single circular chromosome. Preliminary annotation indicated the presence of two tandem copies of *bla*$_{ROB-1}$ gene and the *tetA-tetR* genes located approximately 35kb downstream on the chromosome. These four isolates were selected for long read sequence analysis as they had >95% sequence identity with regions of the IC-Element reported from *P. multocida* 36950 in North America. Alignment of *P. multocida* 36950 ICE with 17BRD-035 and 19BRD-057 led to the identification of an integrative conjugative element, where the major differences were observed in the resistance modules only. The backbone region of *P. multocida* 36950 ICE harboured genes essential for integration and conjugation of the ICE within defined boundaries (Figure 3). The IC-Element present in *P. multocida* 17BRD-035 was 68,898bp in size, which is much smaller than the ICE in *P. multocida* 36950, however, it had boundaries identical to that seen in *P. multocida* 36950, and all major gene homologues required for the movement of the integrative conjugative element. Differences were also seen in the number of hypothetical genes in addition to the resistance modules R1-Aus and R2-Aus. The R1-Aus penicillin resistance module consisted of two copies of the *bla*$_{ROB1}$ genes in tandem, bounded by two copies of insertion element IS*Apa1* both in the same orientation. The *bla*$_{ROB1}$ penicillinase module appears to be a local acquisition and the genetic signatures around it indicate that this acquisition has happened in the recent past. The R2-Aus module consisted of a tetracycline resistance gene and a metal efflux system. BLAST analysis of the ICE identified in 17BRD-035 indicated 99.87% identity over the entire length (Figure 3) with ICEHs1 64.9kb in length (Genbank Accession number MF136609) described recently from *Histophilus somni* strain AVI 1, isolated from clinical samples of BRD and characterised because of its ability to disseminate metal and drug resistance phenotypes.

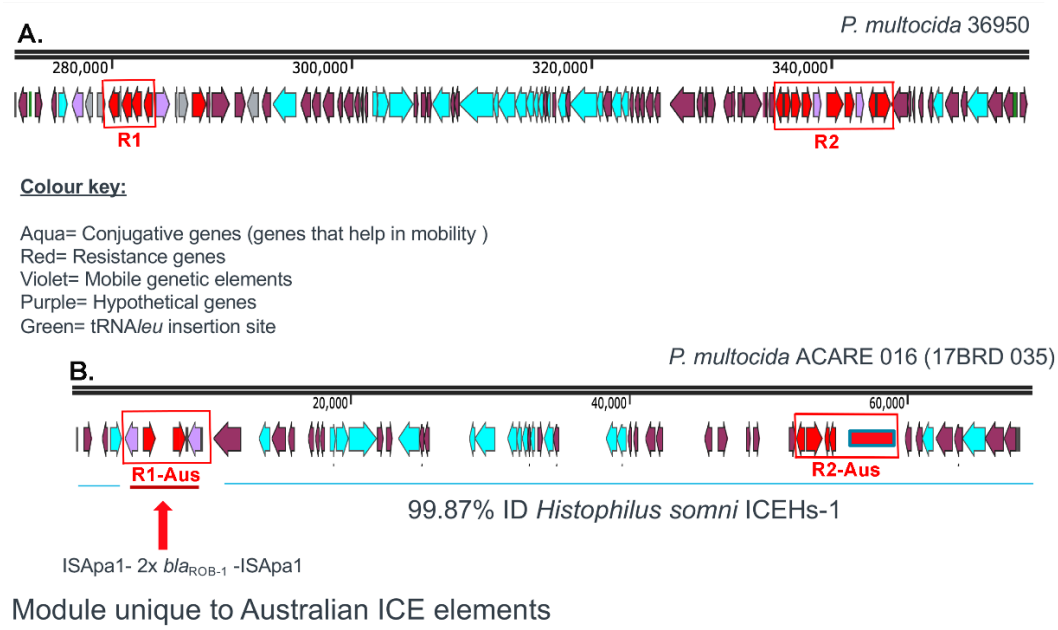

Figure 3: Diagrammatic comparison of ICE elements in *P. multocida* 36950 reported from North America (A) and 17BRD-035 (B) genome. The R1 module in *P. multocida* 36950 ICE consists of kanamycin, streptomycin and sulphonamide resistance genes. R2 module in *P. multocida* 36950 ICE consists of tetracycline resistance gene, aminoglycoside resistance gene, beta-lactamase resistance gene bla_{OXA-2} and the macrolide resistance module. The R1-Aus module consists of two copies of the bla_{ROB-1} gene and the R2-Aus module consists of Tetracycline resistance genes and a metal efflux region.

Alignment of the ICE characterised in 17BRD-035, with the remaining genomes (18BRD-001, 19BRD-032 and 19BRD-057) selected for this purpose indicated the presence of a near identical ICE element in all (Figure 4). In comparison to *P. multocida* 36950 ICE, they all have identical boundaries and conjugative modules. The R1-Aus bla_{ROB1} penicillinase module is also identical.

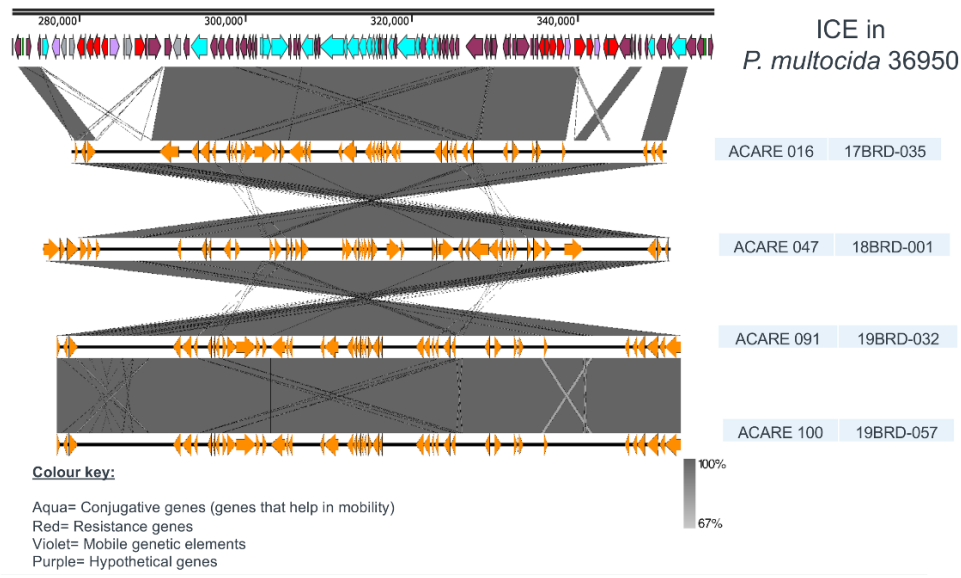

Figure 4. Alignment of 18-BRD-001, 19BRD-032 and 19BRD-057 chromosomes with the ICE characterised in 17BRD-035 and the reference *P. multocida* 36950 ICE.

4.4.2 Plasmid biology

4.4.2.1 *Plasmid curing experiments*

Rather than use BRD identification codes, in this section isolates are referred to by their resistance phenotype for ease of reading. Plasmid curing was successful for isolates *Pm*2(M^R), *Pm*3(M^RT^R), and *Pm*5(M^RT^R). *Pm*2(M^R) initially grew at >128 μg/ml in the presence of both macrolide antibiotics, and following plasmid curing, this decreased to 16 μg/ml. The MIC for tetracycline also decreased, from 16 μg/ml to 0.5 μg/ml. *Pm*3(M^RT^R) and *Pm*5(M^RT^R) had initial MICs of 64 μg/ml for tetracycline, and >128 μg/ml for both macrolide antibiotics. Both dual-resistant isolates, *Pm*3(M^RT^R) and *Pm*5(M^RT^R), became susceptible to macrolide antibiotics in preference to tetracycline resistance. In a single patch for isolate *Pm*3(M^RT^R), tetracycline resistance was lost first. Tetracycline resistance was unable to be cured in isolates *Pm*4(T^R) and *Pm*5(M^RT^R), and the macrolide resistance observed in *Mh*1(M^R) could not be cured.

4.4.2.2 *Electroporation experiments*

Back-transformation via electroporation was successful. Isolates *Pm*2 and *Pm*3, which had previously been "cured" of their plasmid containing the antimicrobial resistance genes and had become susceptible to both macrolide and tetracycline antibiotics, as well as the control sensitive isolate, *Pm*8, all successfully acquired the tetracycline resistance plasmid via electroporation. However, no isolate successfully acquired the macrolide resistance plasmid via electroporation.

4.4.2.3 *Plasmid extraction, profiling and restriction digestion*

Plasmids were successfully extracted from *Pm*2, *Pm*3, *Pm*4, and *Pm*5. Plasmid extraction from isolate *Mh*1 was unsuccessful. When visualised via gel electrophoresis, two bands were seen. A larger band was seen at approximately 7.7 kb, and a smaller band was seen at approximately 4.5 kb. However, in the plasmids isolated from *Pm*5, the larger band was at approximately 7.0 kb (Figure 2, Figure 3). This was confirmed to be a macrolide resistance-encoding plasmid, however it was smaller than the other macrolide resistance encoding plasmids visualised.

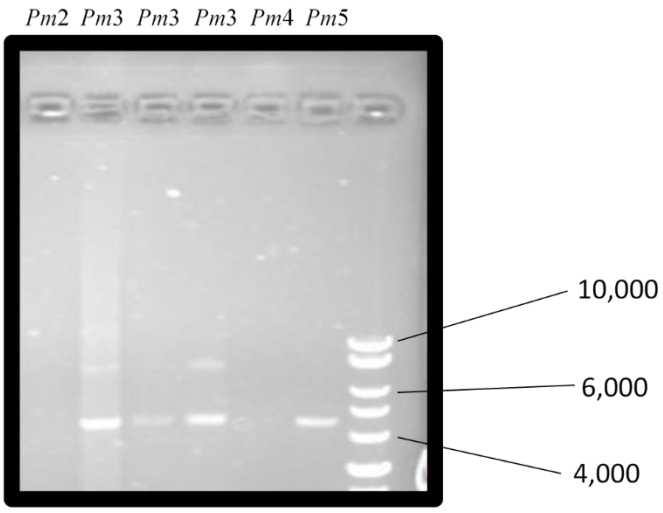

Figure 5. Gel electrophoresis of restriction digest of samples *Pm*2, *Pm*3, *Pm*4, and *Pm*5. A band is observed at approximately 7.7 kb for samples *Pm*2 and *Pm*3, a band is seen at approximately 7 kb.

The plasmids that were visualised via get electrophoresis matched the plasmid maps obtained from the long range (deep) sequencing of each isolate (Figure 1 above), confirming the presence of macrolide resistance genes (*msr*(E) and *mph*(E)) on similar 7603-7748 bp plasmids in isolates *Pm*2, *Pm*3, and *Pm*4, and a slightly smaller 7086 bp plasmid in *Pm*5. In tetracycline-resistant isolates, tetracycline resistance genes (tet(H) and tet(R)) were located on a much smaller 4580 bp plasmid. Thus it has been shown that dual resistant (macrolide and tetracycline-resistant) isolates possess two individual plasmids rather than both sets of resistance genes being located on a single plasmid.

4.5 Whole of genome sequencing of *Histophilus somni* and *Mycoplasma bovis* collections for surveillance of antimicrobial resistance genes.

4.5.1 Phylogenetic analysis of *Histophilus somni* isolates

For the 65 Australian *H. somni* feedlot cattle isolates that passed whole genome sequence data quality testing, a high proportion were collected during 2019 (30/65; 46.2%). Only 11,569 core SNPs were found among the 65 *H. somni* isolates, confirming their genetic homogeneity. Nevertheless, four distinct clusters were identified from the maximum likelihood tree constructed using the core SNPs (See supplementary Figure 2). A total of 31 of 65 isolates (47.7%), and the reference genome, belonged to Cluster 1, which comprised isolates obtained from QLD (16/65; 24.6%), NSW (10/65 (15.4%), and SA (5/65; 7.7%), obtained during the full range of years (2004-2019) and from the different feedlots within each state (Q2, Q3; N1, N3, N4). Cluster 2 comprised a smaller number of isolates originating from QLD (8/65; 12.3%), NSW (9/65; 13.8%) and SA (4/65; 6.2%), obtained over a similar time period (2015-2019) and once again from multiple feedlots (Q1, Q2; N1, N2). Cluster 3 consisted entirely of QLD (8/65; 12.3%) isolates obtained from 2015-2019, whereas Cluster 4 (the smallest but most genetically diverse cluster), also comprised isolates originating from QLD (1/65; 1.5%), NSW (3/65; 4.6% including feedlots N1 and N2), and SA (1/65; 1.5%) again over a similar time period (2015-2019). The isolates obtained from heart tissue were located in Clusters 1 (most closely related to a lung isolate from the same feedlot in NSW) and 4 (most closely related to a lung isolate

from a different feedlot in NSW), respectively. Thus no temporal, geographical or tissue associated clustering of the 65 Australian *H. somni* isolates was evident.

4.5.2 Phylogenetic analysis of *M. bovis* isolates showing reduced susceptibility

The five M. *bovis* isolates subjected to whole genome sequencing (Table 9) were genetically homogeneous, all belonging to ST54, the sequence type to which the majority of sequenced *M. bovis* isolates from Australia belong to (mainly isolates for cases of bovine mastitis) (see Supplementary Figures 3 and 4). Interestingly, *M. bovis* ST54 is also prevalent in China (which is suggestive of a possible Australian origin via export cattle). Based on a comparison of ST52 isolate genomes sourced from Australia and China, the five *M. bovis* isolates obtained from BRD-affected lung tissue formed a cluster, most closely related to isolates from cases of bovine mastitis in Australia.

In terms of matching MIC phenotype to known resistance mechanisms, isolates Mb4 and Mb5, which possessed high tylosin MICs both had defined point mutations in domain V of the 23S rRNA gene (A2058G). However, this mutation was not associated with resistance to any of the other veterinary macrolides. In particular, Mb2 and Mb3 had similar high MIC values for both tilmicosin and tildipirosin, however, no specific SNPs in other genes, including in the L22 ribosomal protein which has been associated with macrolide resistance in other veterinary mycoplasmas were identified.

Table 9. Macrolide and florfenicol MICs and mutations identified in the 23S rRNA gene associated with macrolide resistance for the five selected *Mycoplasma bovis* isolates subjected to whole genome sequencing. No other SNPs were identified.

Isolate	Source	Erythromycin MIC (µg/ml)	Tylosin MIC (µg/ml)	Tilmicosin MIC (µg/ml)	Tildipirosin MIC (µg/ml)	Florfenicol MIC (µg/ml)	Known Mutations in 23S rRNA gene
Mb1 (ST54)	SA	>128	0.016	0.063	2	64	
Mb2 (ST54)	Qld	128	64	64	32	2	
Mb3 (ST54)	Qld	128	2	64	32	64	
Mb4 (ST54)	NSW	32	128	0.5	0.5	2	A2058G
Mb5 (ST54)	NSW	8	128	0.063	0.125	0.5	A2058G

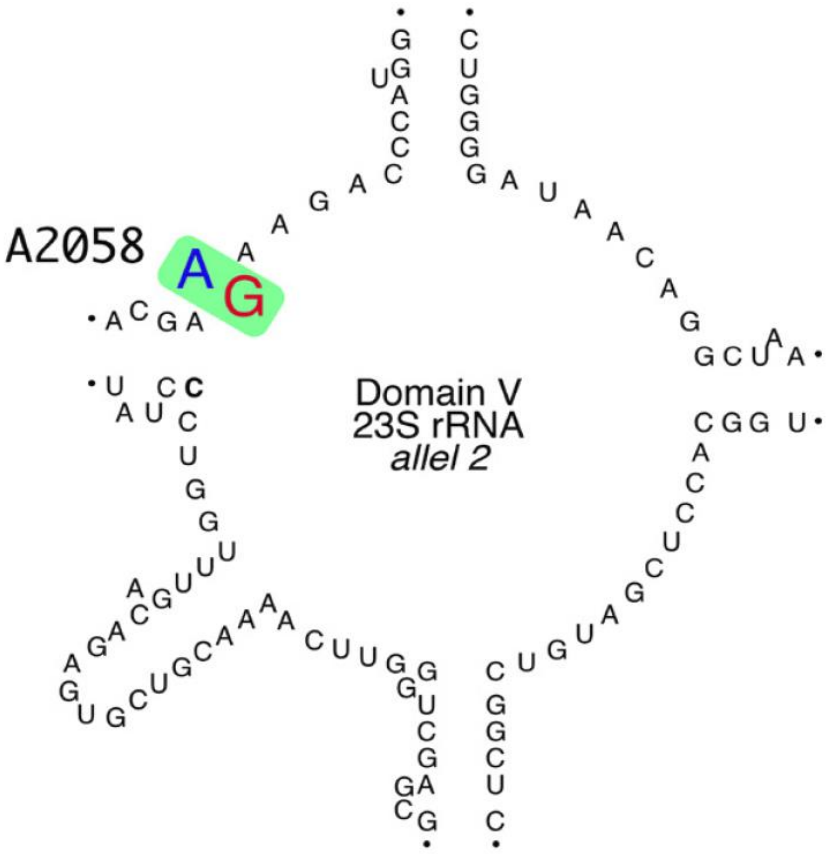

Figure 6. Defined point mutation at position A2058 in domain V of the *M. bovis* 23S rRNA gene associated with tylosin resistance.

4.6 Histopathological analysis of *Mycoplasma*-infected tissues

4.6.1 Pathognomonic lesions indicating *Mycoplasma bovis* infection

By combining available histopathological and microbiological diagnostic data obtained from the 2019-2021 AMR surveillance studies, a total of 155 samples that satisfied the selection criteria were included in the final analysis (detection of *Mycoplasma bovis* by PCR and/or culture applied together with accompanying formalin fixed lung tissue sample of significant quality to enable histopathological diagnosis) (Table 10).

Table 10. Correlation of *Mycoplasma bovis* detection with pathognomonic necrotic lung lesions in BRD-affected formalin fixed lung tissue sections.

Table of M. bovis correlation to Necrotic lesions			
M. bovis	Necrotic Lesions		
	Negative	Positive	Total
Negative	90 81.316 0.9274	2 10.684 7.0583	92
Positive	47 55.684 1.3542	16 7.3161 10.307	63
Total	137	18	155

* P<0.0001

Diagnostic sensitivity for necrotic foci indicating the presence of *Mycoplasma bovis* infection in BRD-affected lung tissue was 25%, whereas specificity was 98%. Similarly, in a study of >60 lung tissue sections from BRD-affected and non-BRD affected cattle, *Mycoplasma bovis* was more likely to be associated with BRD-affected tissue (Table 11).

Table 11. Association of *Mycoplasma bovis* detection in BRD-affected lung tissue samples compared to lung tissue samples obtained from other causes of death/euthanasia (e.g. intractable lameness).

Cause of Death	Total (n= 61) Lung samples		*Mycoplasma bovis* Positive**		*Mycoplasma bovis* negative	
		%		%		%
BRD	29	47.6	12	41.4	17	58.6
Other	32	52.4	4	12.5	28	87.5
Total	61	100	16	26.2	45	73.8

Overall, high rates of BHV-1 detection were observed in both BRD-affected and non-BRD lung tissue (Table 12). Lower frequencies of BVDV-1 were detected overall, and evidence of BVDV as the sole viral infection was only found in BRD affected tissue. There was no association with specific virus detection and whether the tissue was also positive for *Mycoplasma bovis* infection (data not shown).

Table 12. Detection of specific viruses in BRD-affected and non-BRD lung tissue at post mortem.

Pathogen	Positive outcomes (PCR)		
	Total (%)	Non-BRD (%)	BRD (%)
Only BHV-1	20/67 (29.85%)	14/32 (43.75%)	6/35 (17.14%)
Only BVDV-1	5/67 (7.46%)	0/32 (0%)	5/35 (14.29%)
BHV-1 & BVDV-1	3/67 (4.48%)	2/32 (6.25%)	1/35 (2.86%)
Only BRSV	0/67 (0%)	0/32 (0%)	0/35 (0%)
Only BPI3	0/67 (0%)	0/32 (0%)	0/35 (0%)
Only BCV	0/67 (0%)	0/32 (0%)	0/35 (0%)
Total	28/67 (41.79%)	16/32 (50%)	12/35 (34.28%)

5 Discussion

5.1 Main study findings

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

1) *Pasteurella multocida* and *Mycoplasma bovis* are the most common BRD pathogens isolated from Australian feedlots, and are significantly co-associated as a mixed infection. Newly described ST394 and globally significant ST79 are the predominant *P. multocida* sequence types present in Australian feedlots causing BRD, with ST394 isolates more likely to carry antimicrobial resistance genes.
2) Prevalence of resistance to macrolides, tetracyclines and dual resistance among *P. multocida* has increased to 48.8%, 30.2% and 27.9%, respectively, compared to 2019, but these results may have been impacted by the larger number of samples received from feedlots N2 and S1 where resistant strains are endemic. Additionally, several more macrolide-resistant *M. haemolytica* isolates were detected at different feedlots that did not take part in the 2019 study (e.g. V1).
3) Antimicrobial resistance was still negligible in *H. somni* and whole genome sequence analysis of the previous *H. somni* collection revealed that the isolates were genetically homogeneous, with four subclades identified, but no geographical, temporal or disease syndrome associated clustering.

4) A unique Integrative Conjugative Element (ICE), with most similarity to an international *H. somni* ICE, was identified in the MDR 2019 *P. multocida* isolates that appear to be confined to feedlot Q1, though no isolates were obtained from Qld feedlots in 2021 to verify this. Tetracycline and beta-lactam resistance genes were mapped to this ICE, but the genetic basis for tilmicosin resistance in these isolates could not be determined from long range sequencing.
5) The majority of tetracycline (*tet(H)-tet (R)*) and macrolide (*msr(E)-mph(E)*) resistance genes identified in the remaining *P. multocida* isolates are located on distinct small (4.6 kb and 7-7.7kb plasmids, respectively) with dual-resistant strains possessing both plasmids. A plasmid also appears to be the genetic element encoding macrolide resistance (on the basis of long-range sequencing) in the single *M. haemolytica* isolate that possessed *msr(E)-mph(E)* genes and was isolated from the same BRD case as a macrolide-resistant *P. multocida.* However, it is possible this small plasmid may have integrated into the chromosome as plasmids could not be isolated from this *M. haemolytica* strain.
6) Confirmation that *Mycoplasma bovis* is a bona fide BRD pathogen in Australia was obtained through combined PCR detection and histopathological investigations. BHV-1 and BVDV-1 were the most common viruses detected in lung samples from both BRD and non-BRD affected cattle at post-mortem but neither was strongly associated with *Mycoplasma* detection. Whole genome sequence analysis of *Mycoplasma bovis* isolates from the 2019 collection that showed reduce susceptibility to macrolides (tylosin, tilmicosin and tildipirosin only) confirmed that all the isolates belonged to the dominant Australian sequence type (ST54) also causing bovine mastitis, were closely related genetically and the two tylosin-resistant strains possessed a known point mutation associated with macrolide resistance in domain V of the 23S rRNA gene.

5.2 BRD pathogens isolated from post-mortem samples

In extending the surveillance data by an additional two years, the results confirm that both *P. multocida* and *Mycoplasma bovis* have remained the dominant BRD pathogens on Australian feedlots with certain feedlots also yielding a higher prevalence of sole infections with *H. somni*. The previous association between *P. multocida* and *Mycoplasma* coinfection first identified in the 2019 AMR surveillance programme, was maintained and in fact strengthened with the addition of the new data. To the best of our knowledge, however, there are still no previous studies internationally that have reported such a strong association between these two BRD pathogens as recorded in Australia. In a recent comprehensive North American study based on nasopharyngeal swabbing of calves at different key time points, *P. multocida* was the most common pathogen, followed by *M. bovis*, but mixed infections were not commented upon (20).

5.3 Antimicrobial resistance in *P. multocida* and *M. haemolytica*

A major finding of the extension project was the increased prevalence of resistance to macrolides, tetracyclines and dual resistance observed among *P. multocida* isolates compared to 2019. Whilst this may have been impacted by a skewed proportion of isolates coming from two main feedlots (N2 and S1), the results do confirm that AMR in BRD pathogens is very much a local feedlot issue, rather than a widespread phenomenon with little evidence of high rates of transmission between sites. This would tend to suggest that resistant genotypes that are found at post-mortem from diseased lungs

may actually be endemic to each particular feedlot and acquired following induction into the feedlot rather than existing in carrier animals prior to feedlot entry. This is further strengthened by the finding that most isolates of *P. multocida* from Australian feedlots belong to one of two clonal groups, the recently identified ST394 clonal lineage and the more internationally recognised ST79 clonal lineage. Interestingly, it appears that ST394 is considerably more likely to carry one or both unique resistance plasmids identified that encode either tetracycline resistance or macrolide resistance. Further studies should explore the range of sequence type recipients that these plasmids could possibly naturally transfer into by conjugation, or if this is not successful, by electroporation and transformation.

It is clear from the AMR surveillance that has been done to date, that the prevalence of resistance in BRD pathogens isolated from animals at post-mortem appears to be a relatively recent phenomenon (noted from historic analysis of isolates submitted to VDLs) and appears to be increasing, though not at a level where widespread treatment failures are a common issue. Metaphylactic use of chlortetracycline in the feed is still practiced as a cost-effective, streamlined and thus far effective strategy to manage high risk cattle and preventing widespread cases of BRD requiring large increases in the number of individual animal treatments. However, the perceived increased prevalence of drug-resistant strains suggest that introducing changes in selection pressure through alternative treatments such as florfenicol or autogenous vaccines may be particularly useful at this time where resistance may be increasing, but widespread treatment failures are uncommon.

One of the more interesting findings of this study was the detection of additional *M. haemolytica* isolates that were resistant to macrolides. Our initial hypothesis was that when this phenomenon was first observed in a 2019 isolate, it may be evidence of plasmid transfer from *P. multocida*, given that both isolates were obtained from the same animal. However, long range sequence analysis of these strains could not confirm or refute this hypothesis at this point in time, given the difficulty in identifying the macrolide resistance determinant and whether or not it has integrated into the *M. haemolytica* chromosome (see 5.5 below). The recent availability of additional isolates from different feedlots from the 2021 study may aid in discovering the exact mechanism of acquisition of this resistance.

5.4 Whole genome sequence analysis of *Histophilus somni* and *Mycoplasma bovis* isolates

Australian *H. somni* isolates were pan-susceptible to all 19 antimicrobials tested (including those registered for the treatment of *H. somni* infections) and genetically homogeneous. Nevertheless, four main clusters were identified based on whole-genome sequence analysis for SNPs. Only a limited number of studies have compared the phylogeny of *H. somni* isolates obtained from cases of BRD in cattle, and there is currently no multilocus sequence typing scheme that defines genetic variability in this genus and species (21). The 65 Australian *H. somni* isolates investigated in the present study were genetically homogeneous, with only 11,569 core SNPs identified within the collection, compared with another study documenting more genetic diversity in *H. somni* isolates obtained from United States of America (USA) (22). Nevertheless, the Australian collection could be divided into four phylogenetic clusters, each comprised of isolates obtained from the three main beef feedlot states in Australia (QLD, NSW, and SA) apart from Cluster 3, which consisted mostly of NSW isolates. This finding supported previous research in Australia, Brazil and Canada, which have

suggested that *H. somni* isolates have low genetic diversity overall and that the primary distribution model is clonal expansion (23, 24). The lack of temporal, geographical or tissue site specific clustering of isolates was further evidence of the persistence of multiple clonal lineages in widespread localities over time. It is likely that Australia's geographic isolation, strict quarantine restrictions, large distances between feedlots, and extensive animal management systems have provided limited opportunity for genetic recombination (particularly with other BRD pathogens) and further strain evolution, which may have influenced the antimicrobial susceptibility of the isolates.

Similarly, the small numbers of *Mycoplasma bovis* isolates submitted for whole genome sequencing were also genetically homogeneous, even though they showed phenotypic variability in their susceptibility to macrolide antibiotics. All belonged to ST54, a sequence type also identified from cases of bovine mastitis in Australia and China; suggesting the relative homogeneity of this pathogen in Australia beef and dairy cattle. Much greater genetic diversity among *Mycoplasma bovis* isolates has been observed in international studies. Further, whole genome sequencing of antibiotic-susceptible isolates from Australia is likely to confirm this homogeneity. Whilst a single point mutation was observed in two of the sequenced strains that has been previously linked to macrolide resistance, no further mutations within the genomes could explain the unusual macrolide resistance phenotype. However, this unusual resistance phenotype to tilmicosin, tildipirosin and tylosin has been identified in *M. bovis* isolated from Nth American feedlot cattle in a recent study (20) and its genetic basis remains to be determined.

5.5 Long range genetic analysis of *Pasteurella* and *Mannheimia* isolates and characterisation of possible mobile genetic elements in *P. multocida*

In the course of this project, we have successfully linked resistance genes of interest to unique mobile genetic elements that have not previously been described in *P. multocida* or any of the other BRD pathogens, though the identified ICE is most closely related to an element identified in *H. somni* and was similar in structure to other ICEs identified in *P. multocida* from Nth American feedlots. The tetracycline resistance genes were present both on small plasmids (4850bp) and ICEs equipped with the entire cargo of genes that can support lateral movement and rapid dispersal. The macrolide resistance appeared to be localised on plasmids which range in size between 7kb and 7.7kb but have gene homologues that can support mobilisation and replication. To the best of our knowledge, this group of small resistance plasmids have never been identified in *Pasteurella* or other closely related BRD-associated bacteria and may possibly be unique to Australia.

The resistance plasmids and ICE characterised in this project need regular surveillance within BRD-affected cattle in Australia, more so because they may evolve rapidly by acquisition of new resistance genes and the small macrolide resistance plasmid also appears to have the ability to disperse rapidly, thereby amplifying the resistance problem under antibiotic selection pressure. The prevalence of isolates carrying the plasmid/s obtained from BRD post-mortems appears to have increased in the 2021 AMR surveillance, though it was an interesting observation that no dual resistant isolates were obtained from N2, which had previously yielded such isolates in 2019.

5.6 Meeting Project Objectives

5.6.1 Conduct 6 months of antimicrobial resistance surveillance data from a minimum of 8 Australian feedlots.

This objective has been achieved by extending the survey into 2021. Despite many attempts, the absence of isolates from Queensland feedlots in 2021 is a significant drawback as it was not possible to monitor the frequency of the unique ICE identified in MDR *P. multocida* isolates. Additionally, not all VDLs were able to upskill and offer MIC testing for BRD pathogens as a stand alone NATA accredited test apart from ACE Laboratories.

5.6.2 Determine if in-feed use of tetracycline is correlated with increased antimicrobial resistance of bovine respiratory disease pathogens.

This objective has been achieved. However, whilst the highest proportion of dual-resistant *P. multocida* from feedlot N4 may indicate an associated risk of metaphylactic use of chlortetracycline, no dual-resistant *P. multocida* were identified at the second feedlot (N2) that also had a history of use. In a much larger longitudinal study undertaken in Canada, metaphylactic use of chlortetracycline at feedlot induction was not associated with tetracycline resistance development, however, parenteral metaphylactic use of macrolides was associated with macrolide resistance (20). When compared to the resistance issue in Australia, encouragingly, tetracycline and macrolide resistance was confirmed to be encoded on separate small plasmids, therefore changing the selection pressure may cause the strains to lose their plasmids, as confirmed in the laboratory following successive subcultures in the absence of macrolide or tetracycline in the bacteriological media.

5.6.3 Determine if metaphylaxis at feedlot entry with macrolides is associated with an increased risk of antimicrobial resistance of pathogens.

It was not possible to achieve this objective. However, analysis of the combined data from 2019-2021 AMR surveillance revealed the strong co-association of *P. multocida* and *Mycoplasma bovis* infection in Australian BRD cases.

5.6.4 Characterise the genetic mechanisms responsible for recently identified resistance in *Pasteurella multocida*

This objective has been achieved. Both the ICE and the two distinct plasmid groups have been sequenced and mapped. However it was not possible to confirm if the same plasmid was also present in the macrolide-resistant *M. haemolytica* isolate at this point in time.

5.6.5 Complete whole of genome sequencing of *Histophilus somni* and *Mycoplasma bovis* collections for surveillance of antimicrobial resistance genes

This objective was achieved.

5.6.6 Study strengths and limitations

The project has continued the significant milestones established in B.FLT.3004 by further isolation of BRD pathogens and characterisation of the genetic basis of resistance; affording greater understanding of pathogenesis, ecology and epidemiology within Australia.

The main study limitations were that no samples were obtained from Qld feedlots during 2021 to complement the numbers of samples received from NSW, Victorian and South Australian feedlots and the state based VDLs in Qld and NSW were still not able to offer NATA accredited MIC testing of BRD pathogens at the conclusion of the project.

Another limitation was that antimicrobial susceptibility testing of more recent (2020-2021) *M. bovis* isolates as well as antibiotic-susceptible isolates from 2018-2019 could not be undertaken due to the cost limitations (Mycoplasma are slow growing and require expensive bacteriological media, we have now established a pipeline extending from isolation, identification, susceptibility testing, DNA extraction, sequencing and bioinformatics analysis.

A third limitation was that despite our best efforts in the laboratory, we still have not been able to determine/confirm the genetic basis of macrolide resistance in the *M. haemolytica* isolate, however, as more strains were identified in the 2021 AMR surveillance programme, further investigation may confirm that the plasmid may have integrated into the chromosome, as we suspect.

6 Conclusions/recommendations

6.1 Autogenous vaccine programme to challenge antimicrobial selection pressure

Non-antimicrobial control methods should be further explored that can provide an immediate solution for industry. A recently approved Meat Donor Company project will now aim to develop and test the efficacy of autogenous vaccines for each of the main BRD pathogens (*P. multocida, H. somni, Mycoplasma bovis*), using whole genome sequencing to characterise the isolates present at a feedlot and thus provide a more nuanced selection process of potential vaccine strains.

6.2 Further experiments on plasmid biology and rate of transfer

Natural conjugation experiments should be undertaken using the ICE-containing *P. multocida* strains, which are resistant to beta-lactams, as the recipient strains, thus making it possible to determine how successfully or efficiently the macrolide resistance plasmid transfers into isolates of the same or different sequence type (i.e. genetic background), given that two of the ICE-containing isolates are ST394 and one belongs to ST125. It may even be possible to examine natural transfer of the smaller tetracycline resistance plasmid, given that one of the Qld strains from 2018 is only resistant to beta-lactams and likely contains an ICE that is missing tetracycline resistance genes.

6.3 Further sequencing and plasmid biology experiments on the macrolide-resistant *Mannheimia haemolytica* isolates

We are close to understanding the genetic basis of resistance in the macrolide-resistant *M. haemolytica* isolate, and are now much closer given that the results of the 2021 AMR survey confirm that it is not a unique isolate, with multiple isolates now identified from more than one feedlot. It is important to determine if the putative *M. haemolytica* plasmid is inserted into the chromosome, as suggested by long range sequencing, which would confirm that both the *P. multocida* and *M. haemolytica* strains contain similar 7.7 kb plasmids, with alignments indicating that they share 99.1% identity over 97% query length. This would suggest that the macrolide resistance plasmid can transfer between bacterial species.

6.4 Metaphylactic use of chlortetracycline in the feed and antimicrobial resistance

Extensive sampling was undertaken at feedlot N4 where a high proportion of tetracycline and tetracycline/macrolide resistant *P. multocida* had previously been reported, but sample size was too small for any statistical comparison, with the hypothesis developed that chlortetracycline metaphylaxis may be a risk factor for dual resistance. Whilst a high proportion of dual-resistant *P. multocida* was identified, and there was a significant association, it was important to note that on feedlot N2, no dual-resistant *P. multocida* were obtained, despite the continued use of chlortetracycline metaphylaxis, albeit at a reduced frequency compared to N4. Deciphering the mobile genetic elements responsible for resistance has identified a small tetracycline resistance plasmid that is much more difficult to remove from the parent strain once antimicrobial selection pressure is removed. So far, this plasmid has only been identified in ST394 isolates from N2 and N4. Further research is required to understand the ecology of this resistance and associated risk factors, but it is interesting to note that in a recent Canadian study, macrolide metaphylaxis at feedlot entry was identified as a risk factor for the isolation of resistant strains, but chlortetracycline metaphylaxis was not (20).

6.5 Significance of *Mycoplasma* and viruses as initiators of BRD infection

The extension project further confirmed the key role for *Mycoplasma bovis* infection in BRD in Australia and its co-association with *P. multocida*. In an effort to decipher the role of primary viral infection in BRD pathogenesis, post mortem-collected fresh lung samples were investigated for virus detection. While BHV-1 and BVDV1 were most prevalent, no association with *Mycoplasma* infection was identified and high rates of virus infection were identified in both BRD and non-BRD affected lung tissue. Further investigation using immunohistochemistry should now be undertaken, in addition to detecting virus infection at feedlot entry and live animals at first BRD pull. Combining this with live sampling for bacterial investigation, either through nasopharyngeal swabbing (which has its drawbacks) or bronchioalveolar lavage would provide greater insight into what appears to be a unique pathogenesis for BRD in Australia.

7 Key messages

7.1 Tools and tips for industry

Key findings from the extension project should be transferred to industry and most feedlots now have the capability of conducting their own on farm AMR surveillance. The results confirm that resistance in *P. multocida* is mostly small plasmid-mediated with each plasmid identified as encoding resistance to a single antibiotic class and dual resistance associated with carriage of both plasmids. ICEs appear to be rare and confined (thus far) to a single feedlot. This is an important finding as it suggests that in the absence of antibiotic selection pressure through drug rotation or the introduction of alternative non-antimicrobial control measures, the plasmids may be readily lost, though further laboratory work is required to confirm this. Secondly, it has not been determined how frequent natural genetic transfer of the plasmid has driven the development of resistance in Australian feedlots (as opposed to clonal expansion of the strain carrying the plasmid). Lastly, it is highly probable that if natural transfer is frequent among *P. multocida* strains, it is also like to occur between species, as possibly may have happened in the case of the macrolide-resistant *M. haemolytica* isolates.

It is also clear that *P. multocida* isolates from feedlot cattle in Australia are mainly confined to two genetic lineages, ST394 and ST79. Combined with the identification of genetic homogeneity in both *H. somni* and (very likely) *Mycoplasma bovis* isolated from Australian feedlot cattle, the introduction of autogeneous vaccines as a control measure is definitely worth investment given that antigenic variation is likely to be low among all three pathogens.

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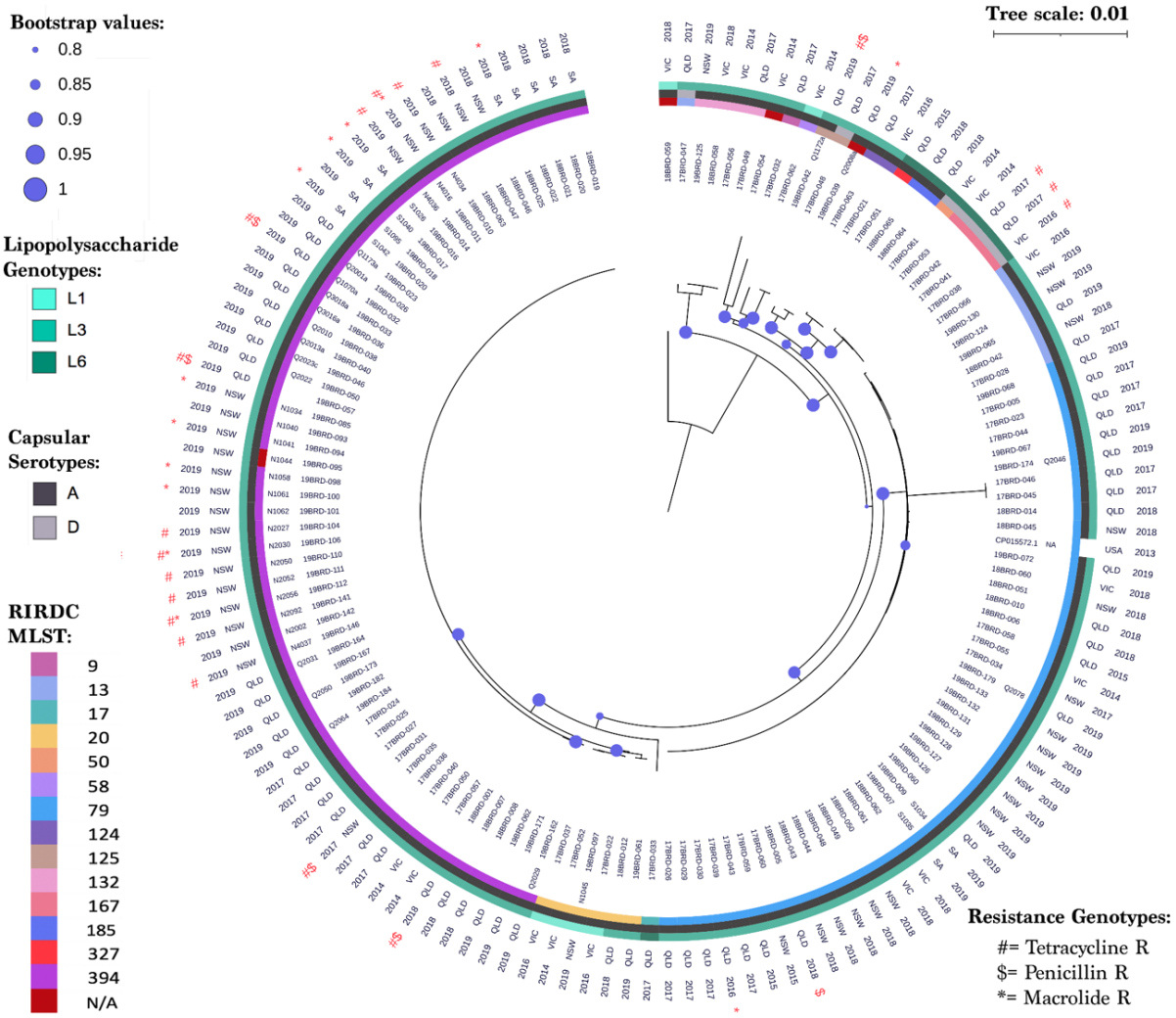
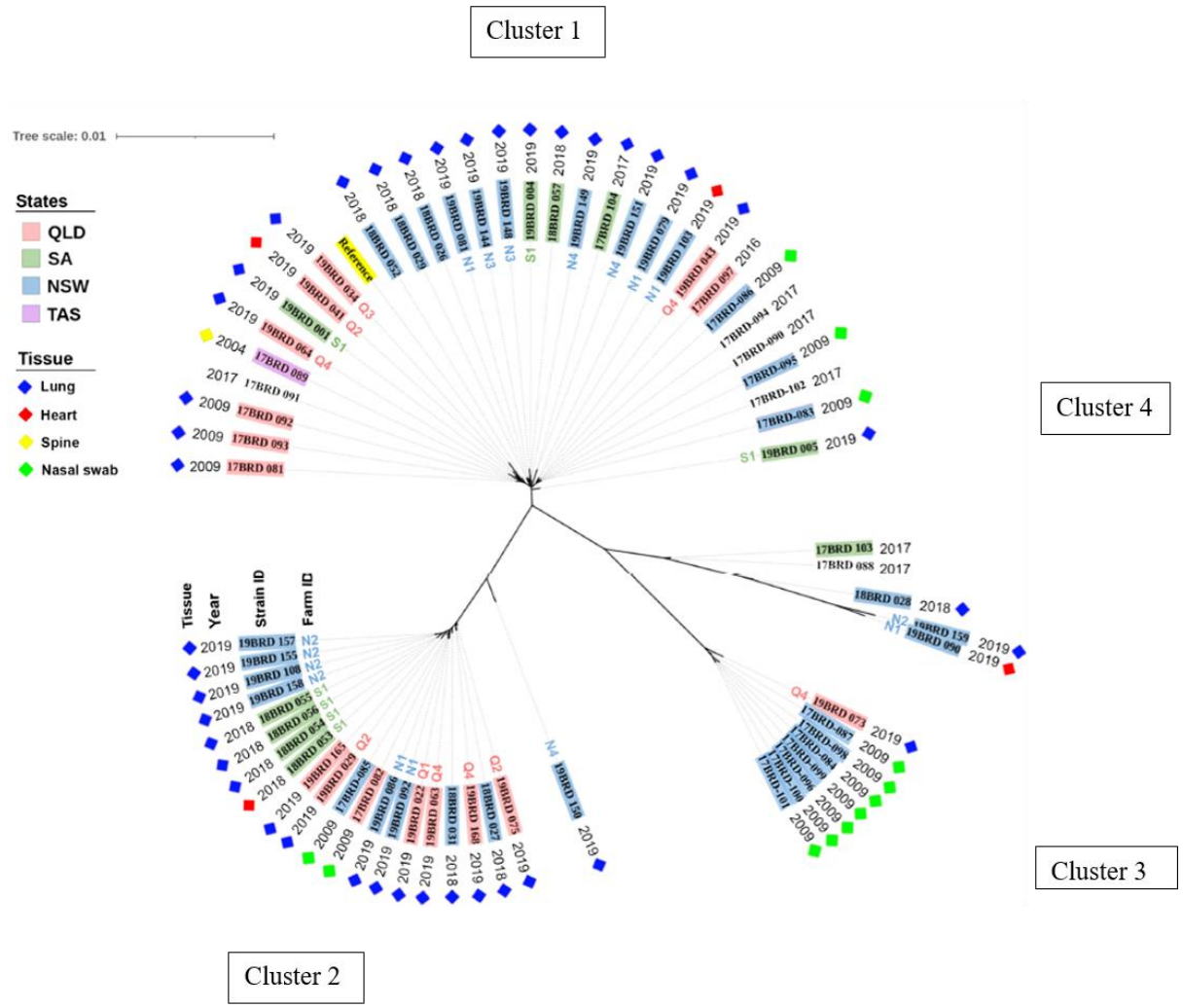
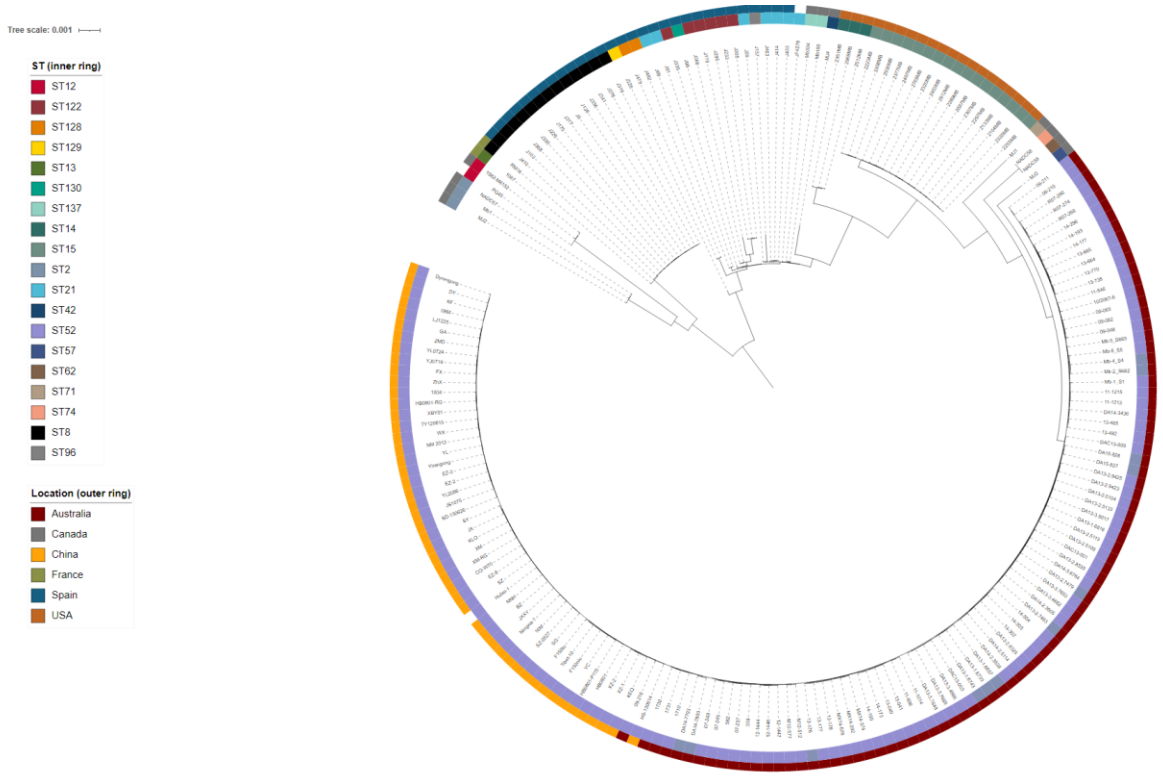


Supplementary Figure 1 – *Pasteurella multocida* Phylogenetic Tree. Phylogenetic tree constructed using PhyloSift. Alphabets in red along the tree branches indicate the separate clades. The isolates ID numbers appear in the innermost text ring, with the farm ID (where available) presented adjacent to it. The coloured circles from outside to inwards represent lipopolysaccharide genotypes, Capsular Serotypes and RIRDC Multi-locus Sequence Types. The outer text rings indicate the Australian state of isolation and the year of collection. The red signs (*, $, #) designate isolate genotypes corresponding to macrolide, penicillin and tetracycline resistance respectively.

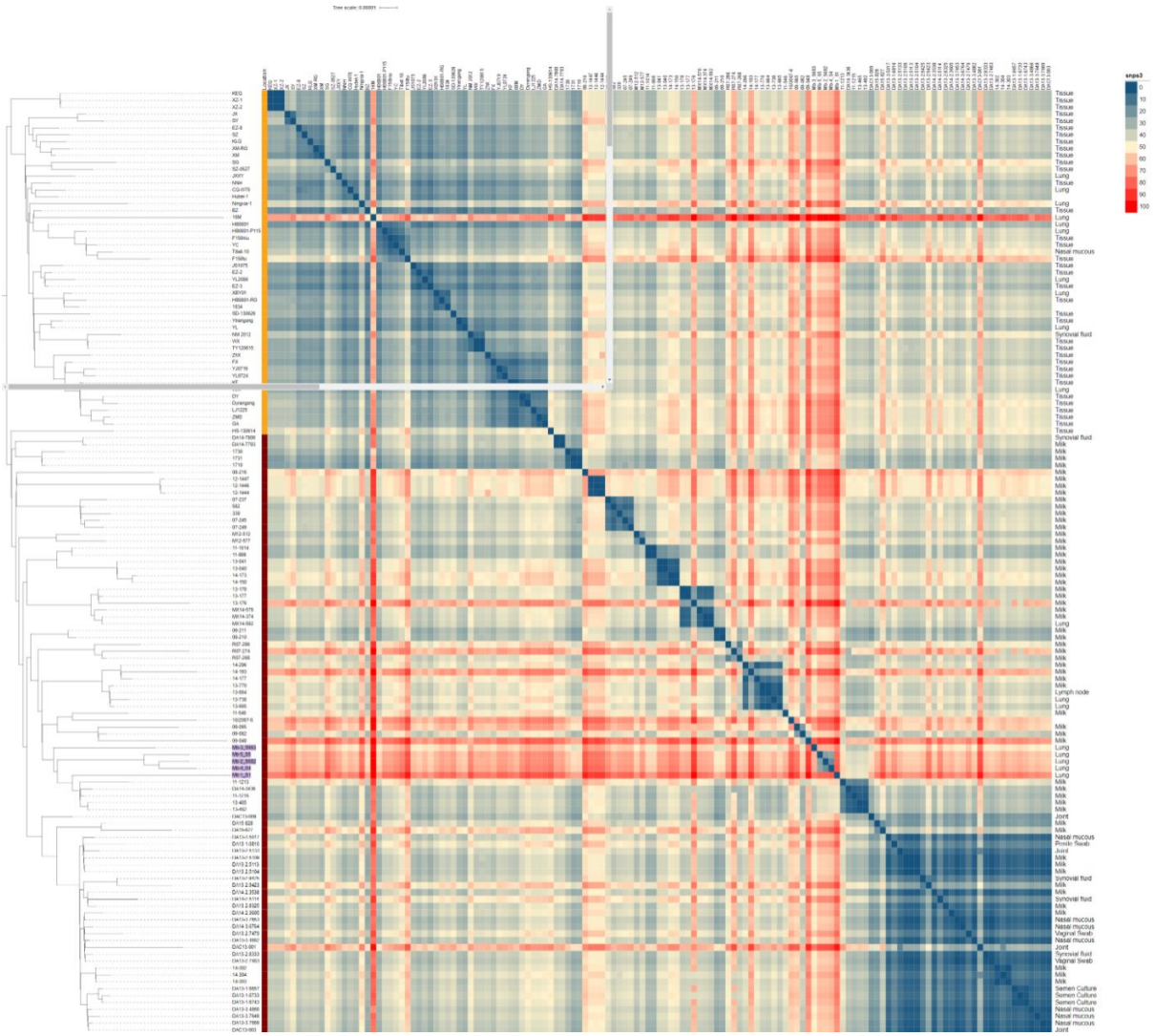


Supplementary Figure 2 – *Histophilus somni* phylogenetic tree. A maximum-likelihood phylogenetic tree generated based on core single nucleotide polymorphisms for 65 Histophilus somni isolates obtained from lung tissue or heart tissue, and nasal swabs from feedlot cattle in Australia during 2004-2019 together with an isolate from Tasmania (taken from the spine of an individual steer) and a reference isolate (NCBI accession: NZ_CP018804). The isolate identification number is coloured according to the Australian state where it originated (red, Queensland; blue, New South Weals; green, South Australia and purple; Tasmania). The red, blue, yellow or green diamonds next to the year of isolation represent the tissue sites where the isolate originated (lung, heart tissue, nasal swab, spine).

For isolates obtained in 2019, the feedlot ID (Q1-Q4; N1-N4 and S1) are also indicated. The reference genome (NZ_CP018804.1) is indicated in yellow.



Supplementary Figure 3 – *Mycoplasma bovis* phylogenetic tree



Supplementary Figure 4 – *Mycoplasma bovis ST52* phylogeny