Final report

Improving bovine respiratory disease control through the characterisation of pathogen-host interactions

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Abstract

Bovine respiratory disease (BRD) is the major cause of illness and death in feedlot cattle, costing the Australian feedlot sector $60-100M annually in treatment costs and lost productivity. This project has investigated three areas of BRD research. Firstly, if molecules circulating in blood could be used to identify cattle infected with viruses linked to BRD. Molecules were identified, one associated with bovine herpesvirus 1 infections and one with bovine viral diarrhea virus 1 infections, both viruses commonly linked to BRD. These molecules (biomarkers) have the potential to improve the identification of cattle with BRD. Secondly, tissues collected from fatal cases of BRD were analysed to determine the range of viruses present. While bovine herpesvirus 1 and bovine viral diarrhea virus 1 were frequently detected, other viruses were also detected which have not been reported in fatal cases of BRD in Australia before. One of these viruses, bovine influenza D virus, has emerged globally as having associations to BRD. Other outputs of this investigation are a suite of molecular diagnostics that enable the detection of the bacterial and viral pathogens associated with BRD. Additional assays were developed for the detection of antimicrobial resistance (AMR) genes. An estimate for the heritability of BRD resistance has been determined and it is of sufficient accuracy to suggest that breeding for BRD resistance is plausible. Application of the molecular assays confirmed the presence of multiple pathogens in the upper respiratory tract of cattle being treated for BRD. Similarly, AMR genes were also detected in these samples, though the repertoire detected varied between the participating feedlots. Methods have been developed to enable the application of a next generation sequencing technology to detect the bacterial species and antimicrobial resistance genes in the nasal swabs of cattle being treated for BRD. These approaches have the capacity to support antimicrobial stewardship programs within the sector. The outputs of this project will provide the feedlot sector with new knowledge and tools to improve BRD management and reduce the losses associated with this disease.

Executive summary

Background

The research described in this report was conducted to improve the management and prevention of bovine respiratory disease (BRD) in Australian feedlot cattle. The research is targeted to the Australian feedlot sector where BRD is the most important cause of cattle morbidity and mortality. BRD costs the sector between $60-100M annually in lost productivity and as a result, research is required to improve the prevention and management of this disease. In addition, BRD is a major contributor to the therapeutic use of antimicrobials in feedlot cattle. Consequently, reducing the incidence of this disease will reduce the use of antimicrobials in livestock, thus contributing to industry antimicrobial stewardship in response to global concerns of increasing rates of resistance to these drugs.

Objectives

The objectives of this project were to provide tools for the improved management of BRD in feedlot cattle, delivered via a multifaceted approach based on an understanding of host-pathogen interactions, including:

1. Development of new diagnostic assays for pathogens in the bovine respiratory disease complex.
2. Characterisation of the DNA and RNA viromes present in the tissues of cattle that have died of BRD to inform the development of novel vaccines to protect animals from BRD.
3. Identification of putative biomarkers for clinically healthy animals with respect to BRD, to contribute to improving the diagnosis of the disease.
4. Identification of potential biomarkers in the blood of cattle infected with bovine herpesvirus 1 and bovine viral diarrhoea virus 1, two the viruses frequently associated with BRD in Australia. These biomarkers will improve the diagnosis of BRD cases and underpin treatment decisions.

Methodology

A semi-structured literature review was conducted to identify potential microRNA biomarkers for BRD clinical. Followed by experimental testing of candidate biomarkers using a quantitative PCR array with extracts from cattle of known infection status.

A semi-structure literature review was conducted to identify putative DNA and RNA viruses associated with BRD in Australia. This identified several studies from overseas and one study of Australian feedlot cattle. Assays were developed to test for these viruses in samples from fatal BRD cases.

Sample collections from previous BRD were evaluated for their suitability for used in identifying genetic markers for BRD susceptibility/resistance.

Genotyping data was obtained from a third party organisation for cattle that used in another study. Correlating animal genotypes with

Nasal swab samples were collected from two feedlots for analysis using the molecular diagnostic assays developed in this study for BRD associated pathogens and antimicrobial resistant (AMR) genes.

A subset of samples from the feedlots were also analysed using Oxford Nanopore Technology (ONT) sequencing to evaluate agreement with isolation of selected bacterial pathogens, molecular detection of AMR genes and molecular detection of viruses with DNA genomes.

Results/key findings

This study has identified two potential biomarkers of infection for two of the viruses which are strongly associated with BRD development in Australian feedlot cattle.

The DNA/RNA virome present in tissues collected from cases of fatal BRD in Australian feedlot cattle was characterised. Consistent with published studies, there is a wider variety of viruses present in these tissues than the four or five viruses historically associated with BRD.

The genome wide analyses determine an estimate for the heritability of BRD resistance of 0.20 ± 0.04, this supports further investigations to improve this estimate.

Within the sampled populations unexpected high viral loads of bovine herpesvirus 1 were detected in vaccinated cattle.

Oxford Nanopore Technologies (ONT) sequencing proved to be highly amenable to detecting a wide range of bacteria in nasal swabs collected from cattle being treated for BRD. The application of strategies to remove bovine DNA from samples prior to analyses is essential. In addition, ONT sequencing enabled the detection of AMR genes in nasal swabs of cattle being treated for BRD.

Benefits to industry

The results of this project have the potential to improve the identification and management of cattle affected by BRD, the most important cause of morbidity and mortality in Australian feedlot cattle. Putative biomarkers for BRD were identified and with further development these could be used to identify BRD affected cattle. The capacity to objectively identify cattle with BRD will underpin decisions to treat affected animals and enhance antimicrobial stewardship.

A robust estimated breeding value has been determined for BRD resistance. The inclusion of this estimate in breeding plans would enable the selection of cattle that are less likely to be affected by BRD during their time on feedlots.

New approaches have been developed to enable the detection of bacteria and AMR genes in cattle being treated for BRD. The molecular assays developed to detected AMR genes have the capacity to improve antimicrobial stewardship by enable the detection and monitoring of antimicrobial resistance in feedlot cattle.

Future research and recommendations

Further research is recommended to better understand the detection of bovine herpesvirus 1 in cattle being treated for BRD. Not only was the rate of detection higher than might be expect, the time of treatment for some of these animals was well outside the window of high BRD risk. Similarly, the viral loads of some animals were extraordinarily high and clearly warrants further investigation.

Further research is also required to increase the number of animals included in the BRD genetic resistance estimates. Identifying sources of already genotyped cattle that move through the feedlot system would be a cost effective way to achieve this.

The results of this study also support the further development of the ONT sequencing to support the feedlot sector in antimicrobial resistance monitoring. The experimental approaches utilised in this project suggest this is a highly feasible approach. However, it is likely that additional improvements in the technology, specifically the underpinning bioinformatics is required before it can be deployed throughout the sector without expert assistance.

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1. Background

Bovine respiratory disease is the leading cause of morbidity and mortality in Australian feedlot cattle resulting annual losses of approximately $60 to $100M annually. This project is divided into three components aimed improve the detection, control, and prevention of BRD.

1.1 Biomarkers for BRD

Currently, the diagnosis of BRD is principally conducted by feedlot pen-riders who observe and assess cattle in their pens for clinical signs of BRD according to predefined criteria. Animals which meet the threshold are moved from the home pen to a hospital area within the feedlot for further clinical assessment, with treatment administered if deemed necessary. The typical treatment will be long-lasting antimicrobial. As a consequence of the assessment procedures and the need for interpretations to be made by feedlot staff, it is likely that feedlot cattle assessed for BRD fall into three categories. The first group is disease animals which true BRD cases, where immediate treatment is essential. The second is healthy animals which do not have BRD, where treatment will have no benefit. The third group is likely made up of animals which are misclassified into either of the first two groups. Healthy animals which are misdiagnosed and treated for BRD likely represent an important source of economic loss to feedlot operators.

The purpose of this component of the study was to identify one or more components present in an animal bodily fluid which clearly identifies animals affected by BRD. These types of molecules are commonly referred to as “biomarkers”. Biomarkers can be proteins, metabolites, or nucleic acids. This project will focus on the identification of a class of biomarkers known as microRNAs (miRNA) which a small (19 to 22 bases) non-coding RNAs. These molecules are involved in the regulation of complex biological pathways and have been identified as useful biomarkers for many human diseases. They are also known to circulate and be stable in biological fluids such as blood, saliva and urine. In comparison to traditional pathogen based diagnostics, a key advantage of using biomarkers for disease diagnosis is they are ideally pathogen agnostic. This means that the ideal biomarker(s) would identify animals affected by BRD regardless of what viral or bacterial agents were involved in the development of disease. As discussed in the next section of this report there are numerous viruses and bacteria associated with BRD in feedlot cattle making the development of comprehensive pathogen based diagnostics problematic. Consequently, accurate biomarkers have the potential to reduce the number of tests required to identify true BRD cases. More importantly, an accurate biomarker could be used by feedlot staff in the decision making processes on whether or not to treat BRD cases. The capacity to do this, would have two benefits for industry. Firstly, it would reduce the number of animals which are treated, not affected by BRD. This would provide an economic return to feedlot operators through reduced treatment costs. Secondly, a biomarker would provide objective support for the decision to treat BRD affected animals. Collectively, these outputs would contribute to improving antimicrobial stewardship in the feedlot sector.

1.2 Virome of BRD

A well accepted model for BRD is changes in management, environment, and other factors can result in some cattle have reduced immune capacity due these stressors at feedlot arrival which persists during the first few weeks at the feedlot. At the same time cattle may be exposed to cattle from different sources and consequently exposed to and infected by viruses. The resulting infections can

be more severe due to the reduced immune capacity, rendering animals to severe secondary bacterial infections which are observed as BRD.

Historically, four viruses have been strongly associated with BRD, bovine herpesvirus 1 (BoHV-1), bovine viral diarrhea virus 1 (BVDV-1), bovine respiratory syncytial virus (BRSV) and bovine parainfluenza virus 3 (BPIV-3). These viruses have been detected in Australian feedlot cattle. More recently several studies have suggested that the range of viruses infecting feedlot cattle in other countries is much broader that these four viruses. The purpose of this component of the study was to determine the range of viruses present in the respiratory tissues of cattle that have died from BRD. This information is required to improve our understanding of the role and number of viruses involved in predisposing cattle to BRD. This new knowledge will be used by researchers and the veterinary pharmaceutical industry to develop new tools to enable feedlot operators to reduce losses associated with BRD. The results of this research will be used to inform the development of new diagnostic assays and prioritise which viruses should be the target for new vaccines.

1.3 The genetics of susceptibility/resistance to BRD

The availability of resistance/susceptibility genetic markers for BRD would enable these to be considered by cattle producers to improve overall feedlot performance. In the long term, these genetic markers could be used to produce of cattle with resistance to BRD to reduce the overall economic losses associated with this disease in Australian feedlots. In the short term, BRD genetic markers could be used identify those animals most at risk of developing BRD either before or on arrival at the feedlot. Once animals with increased risk are identified then the management could be modified to reduce their likelihood of developing disease.

Bovine Respiratory Disease (BRD) was rated as the major health problem by medium and large feedlots in Australia (72 feedlots were surveyed) with 64% of all illnesses and death attributed to the disease (www.mla.com.au). BRD is a major problem in feedlot cattle as it causes serious economic losses due to mortality, costs of metaphylactic and therapeutic use of antibodies, cost of labour, reduced growth performance of affected cattle and carcass merit (affects marbling scores negatively) (Duff and Galyean, 2007; Ribble et al., 1995). In an Australian study, 2,468 cattle were individually identified at feedlot entry and monitored until slaughter approximately 70 days later. It was noted that the growth rate reduced by 0.702 kg/animal/day in cattle treated for BRD and a reduction of 0.302 kg/animal/day was observed in cattle identified with anorexia (Irwin et al., 1979). CSIRO have noted on their website that the disease is estimated to cost the domestic industry $60 million a year (http://www.csiro.au/files/files/p72o.rtf). An American study calculated the economic loss associated with lower gains and treatment costs for BRD infection in a 1,000 cattle feedlot as US$13.90 per animal, which does not include labour and handling costs (Snowder et al., 2006).

1.4 Bacteria species associated with BRD

The three major risk factors for BRD are animal stress, viral infection, and bacterial infection. The mixing of cattle from different sources and wide environmental temperature fluctuations or low environmental temperatures (in Australia the peak incidence of BRD occurs in autumn and early winter (Irwin et al., 1979) have been identified as major factors in the initiation of disease outbreaks in feedlots (Cusack et al., 2007).

In the United States respiratory disease is the most common cause of mortalities in feedlot cattle with fibrinosuppurative bronchopneumonia (shipping fever pneumonia or pneumonic pasteurellosis) being

the most frequent disease. Gagea et al. (2006) summarised previous studies and pointed out that the most common isolates from animals infected with fibrinosuppurative bronchopneumonia were *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni* (*somnus*), however, concurrent infection with multiple bacterial and viral pathogens was frequently observed. Most recently authors have reported *Mycoplasma bovis* associated with chronic pneumonia and polyarthritis in feedlot cattle (Haines et al., 2004; Shahriar et al., 2002). Gagea et al. (2006) determined the pathogens associated with mortality or severe morbidity in 72 Ontorio beef feedlots and reported that bacteria isolated from the lungs were *M. bovis* (82%), *Mycoplasma arginini* (72%), *Ureaplasma diversum* (25%), *M. haemolytica* (27%), *P. multocida* (19%), *H*. *somni* (14%) and *Trueperella*. (formerly *Arcanobacterium*) *pyogenes* (19%). Some of the viral agents produce only mild clinical signs by themselves but combined with other viral or bacterial agents and stress severe signs and death may occur. Studies have shown that susceptibility to *M. haemolytica* is greatly increased during active viral respiratory infection, resulting in rapid onset of a severe and even lethal pleuropneumonia (Czuprynski et al., 2004). A review from America stated that viral agents often predispose animals to bacterial infections, and *M. haemolytica* is the most frequently isolated organism in cattle with BRD (Duff and Galyean, 2007). An Australian study covering the period of 2010 to 2012 recorded the bacterial species present in the nasal cavity of 1,484 healthy cattle for export and revealed a prevalence of *M. haemolytica*, *P. multocida*, *M. bovis* and *H. somni* of 13.4%, 26%, 4.8% and 42%, respectively (Moore et al., 2015).

1.5 Diagnostics and Vaccines

Vaccines are available commercially in Australia for some of the viruses, such as Rhinogard™ (BoHV-1) and Pestigard™ (BVDV-1). There are also vaccines available for *M. haemolytica* alone or in combination with BoHV-1. Rhinogard™ (Zoetis Australia) contains live strain of BoHV-1, that contains an inactive copy of the thymidine kinase gene. Rhinogard™ is typically delivered as a single intranasal dose to feedlot cattle at induction. Pestigard™ (Zoetis Australia) is an inactivated BVDV-1 vaccine that contains multiple strains of BVDV-1 genotype 1c. It is typically administered by the injection of two doses, three to four weeks apart. Bovilis MH+IBR™ (Coopers®) contains inactivated two inactivated strains of *M. haemolytica* and one strain of BoHV-1. It is administered via two subcutaneous injections that can be delivered between 14 and 180 days apart. Specific vaccines are also available for *M. haemolytica*, that require a single-dose or multiple doses. Bovilis MH SS RTU (Coopers®) contains two strains of *M. haemolytica* and is single-dose vaccine that stimulates immune responses within one week. The immune responses are reported to persist for at least 25 weeks. Bovilis MH™ (Coopers®) contains two inactivated strains of *M. haemolytica*, and is administered via two subcutaneous injections, 21 to 28 days apart.

Australian studies of the bacterial species involved in the BRD complex are very limited. So far the main emphasis had been on *M. haemolytica* and the development of a vaccine for this bacteria species. The ability to diagnose bacteria involved in BRD is less than optimal. It would certainly help to have cost-effective, quantitative methods to accurately detect animals afflicted with or likely to develop BRD.

Recently a new infrared detection method has been established and it is said to work better than conventional clinical scoring systems to detect signs of bovine respiratory disease. However, orbital scans have to be taken with an infrared broadband camera, which has to be done by qualified infrared thermography technicians (Schaefer et al., 2006). The use of nitric oxide as a treatment has also been investigated and it was concluded that nitric oxide might be an effective adjuvant in treating BRD (Schaefer et al., 2006). However, this again relies on specialised equipment, such as gas tanks,

breathing activated delivery systems, and takes 20 minutes to administer for four days (Schaefer et al., 2006). Morbidity and mortality from BRD have been reported to increase with mixing of calves from different sources and assembly of calves from widely separated geographic locations (Cusack et al., 2003). The morbidity and mortality from BRD appears to be caused by infectious challenge encountered by naïve cattle mixed with cattle previously exposed to BRD infectious agents (Cusack et al., 2003). The stress of establishing of a new social hierarchy affects the immune system negatively and makes the naïve animals more prone to disease causing bacterial strains.

The discovery of a new species of *Mannheimia* in Australian feedlot cattle recently formalised as *Mannheimia pernigra* (Blackall et al., 2001; Kuhnert et al., 2021) and the recent isolation of *Bibersteinia* (*Pasteurella*) *trehalosi* from an 18-month-old Hereford bull with respiratory distress and subsequent mortality (Turni, unpublished data) points to the incomplete knowledge of bacterial species involved in the BRD complex. This lack of knowledge hinders the development of specific diagnostic tests and also preventative strategies to be implemented.

1.6 Where to from here?

The first step to tackling the bacterial component of BRD as part of this project was to obtain a complete picture of the bacteria that involved. The survey was performed, and species identified, allowing diagnostic methods can be developed to identify the key species involved in BRD. To be really helpful to the feedlot, these diagnostic tests need to be easy with a quick turnaround time to obtain results fast (preferably done in the feedlot itself) to support decision making processes.

An alternative assay to the standard, well recognised multiplex PCR assay is the Luminex microbead-based suspension array. These assays are a rapid, high-throughput method used for the detection of multiple analytes in solution and have been used for the identification and serotyping of bacterial species (Christopher-Hennings et al., 2013; Lin et al., 2013). The concept of the Luminex assay is a serovar specific PCR amplifying a product (typically up to 300 base pairs) with a reverse primer that is biotinylated on its 5’ end and a forward primer that has a TAG sequence attached that is separated from the sequence by an internal spacer element. This means an amplicon that is formed if reverse and forward primers bind specifically, and amplification takes place will contain a single stranded TAG sequence and is fluorescent. This TAG sequence will bind to an anti-TAG sequence of the microsphere in the Luminex hybridisation step. The fluorescent beads are then analysed on the Luminex platform.

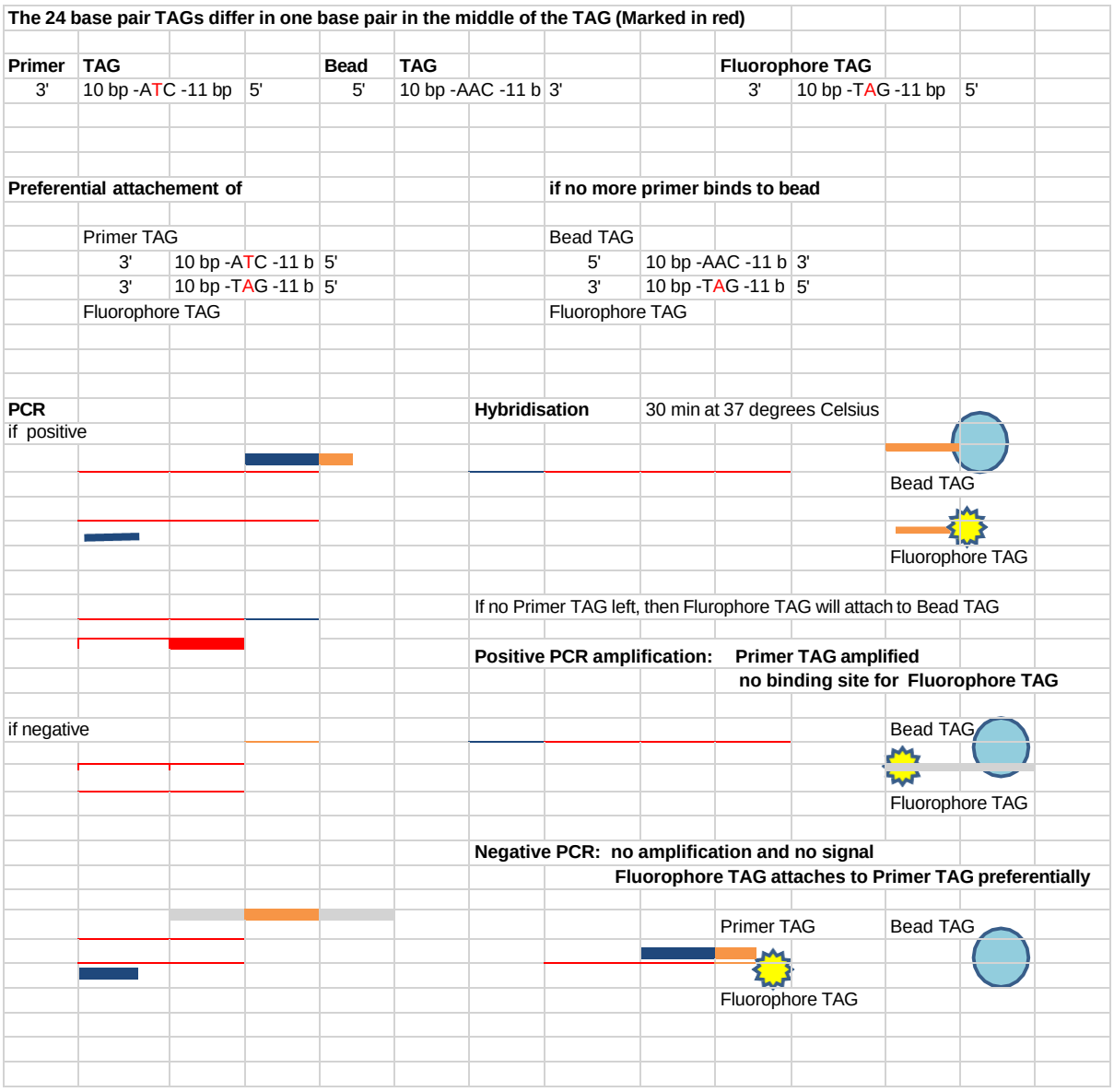

Figure 1.1. Principle of the Luminex based assays for the multiplex bacteria species-specific assay.

In the Queensland Alliance for Agriculture and Food Innovation (QAAFI) Microbiology laboratory the Luminex principle has been slightly altered to allow for the reaction components, such as beads and fluorophore, to be used for multiple assays. The major difference are the reverse primer is not biotinylated and the TAG attached to the forward primer has no spacer. This means that if there is amplification the TAG sequence will be amplified and there is no single stranded TAG sequence that would enable binding to the bead (**Figure 1**). In the hybridisation step, a fluorophore TAG is added which is complementary to the primer TAG. The fluorophore TAG is complementary to the bead TAG with the exception of one base pair, hence it preferentially binds to the primer TAG. This means that there will be a positive signal if the sequences were amplified, as the fluorophore binds to the beads. If there is no amplification then the fluorophore will preferentially bind to the primer TAG and there will be a negative reaction (**Figure 1**.1).

The aim was to develop a bead-based assay that identifies *B. trehalosi, H. somni, M. haemolytica, M. bovis, P. multocida* and *T. pyogenes*.

The second aim was to develop similar bead-based assays to detect antimicrobial resistance genes.

1.7 Antibiotic resistance

In Australia, the antimicrobials of choice for BRD are oxytetracycline, trimethoprim potentiated sulfonamides, tilmicosin and ceftiofur (Cusack et al., 2003). In recent years, florfenicol and tulathromycin have also been registered for use in cattle (APVMA, 2013). Resistance to neomycin, tetracycline and penicillin have been observed in BRD linked isolates of *P. multocida* from Queensland feedlots (Cusack et al., 2003). In the light of antimicrobial resistance issues, it is important to monitor resistance levels to maintain an effective control of BRD.

There are different classes and sub-classes of antimicrobials (**Error! Reference source not found.**). These antimicrobials target different structures in the bacterial cells.

Table 1.1. The groups and classes of antimicrobials and their modes of actions with an emphasis on those relevant for used in animals. Antimicrobials often used in Australian cattle are highlighted in bold.

Antimicrobial class	**Antimicrobial subclass**	**Examples of antimicrobials**	**Action/notes for cattle use**
Aminoglycosides		amikacin gentamicin Neomycin streptomycin tobramycin	Protein synthesis inhibitors Neomycin has residue issues
β-lactam/β-lactamase inhibitors combinations		amoxicillin-clavulinic acid	Blocks inactivating enzymes (β-lactamases)
Cephems	cephalosporin	Exist as 1st, 2nd and 3rd generation agents. **ceftiofur (**3rd generation)	Cell wall synthesis inhibitor Ceftiofur is specifically for respiratory disease caused by *Mannheimia haemolytica*, *Pasteurella multocida* and *Histophilus somni*
Folate pathway inhibitors		sulfonamides, **trimethoprim-sulfamethoxazole**	Inhibitor of essential metabolites (folate)
Glycopeptides		vancomycin	Inhibit cell wall formation
Lincosamides		lincomycin	Inhibition of protein synthesis
Macrolides 14-membered rings 15-membered rings 16-membered rings		erythromycin **tulathromycin** Tylosin & t**ilmicosin**	Protein synthesis inhibitors Tylosin mainly used for mycoplasma infections.

Penicillins	penicillin aminopenicillin	**penicillin** amoxicillin ampicillin	Inhibit cell wall synthesis
Polypeptides		bacitracin polymyxin	Cell wall synthesis inhibitor
Phenicols		Chloramphenicol **florfenicol**	Protein synthesis inhibitors
Quinolones	quinolone fluoroquinolones	nalidixic acid ciprofloxacin enrofloxacin marbofloxacin orbifloxacin	Inhibition of nucleic acid replication and transcription
Tetracyclines		tetracycline chlortetracycline **oxytetracycline**	Protein synthesis inhibitors
Miscellaneous	nitrofurans	nitrofurantoin	Inhibit a number of microbial enzyme systems

1.7.1 Antimicrobial Resistance Genes

There is increasing evidence that the results of phenotypic antimicrobial resistance assays can be confidently predicted by genotypic methods that detect the genes that confer antimicrobial resistance (Neuert et al., 2018). As well, high throughput methods, such as bead-linked molecular assays offer the capacity to screen for large numbers of resistance genes in a multiplex fashion. Hence, this review has focussed on identifying the resistance genes found in bacterial species associated with respiratory disease problems in cattle.

Antimicrobial resistance seems to mirror the usage of antimicrobials used in the field and hence the patterns can differ from different parts of the world. A Canadian report that feedlot cattle given a single injection of oxytetracycline or tulathromycin (as well as having feed with added chlortetracycline and monensin) resulted in a high level of resistance to tetracycline associated with the *tet*(H) gene (Holman et al., 2018). Studies also found a strong linkage of some genomic types of *M. haemolytica* being linked with particular resistance genes (Clawson et al., 2016). Resistance in bovine bacterial pathogens to a range of antimicrobials have been noted. In many countries, a common situation is resistance to tetracycline (Alhamami et al., 2021; Holman et al., 2018; Kehrenberg and Schwarz, 2001). In Japan, a study looked at characterization of quinolone-resistant *M. haemolytica* and reported that the prevalence of quinolone resistance was rising over time (Katsuda et al., 2009). Fluoroquinolone resistance was also reported from *M. bovis* in Israel (Lysnyansky et al., 2009).

It is important to note that many of these genes exist on transferrable elements, which means that this resistance can spread quite easily among bacteria (Michael et al., 2012b). Not surprisingly, there is wider genetic diversity of strains within a bacterial species on feedlots and the patterns of resistance can be variable as well (Klima et al., 2011).

There is some published data on the resistance patterns present in Australian bacterial isolates associated with BRD. A study from 2015 showed little evidence that there was resistance to six antimicrobial agents (ceftiofur, enrofloxacin, florfenicol, tetracycline, tilmicosin and tulathromycin) in *H. somni* isolates (Goldspink et al., 2015). A study in 2017 reported high level of resistance above 70% against tulathromycin and oxytetracycline for *M. haemolytica* and *P. multocida*, while the levels of resistance to oxytetracycline and penicillin were high for *H. somni* with 67% and 52% retrospectively (Timsit et al., 2017). In 2021 a study reported low resistance again to tetracycline (12.9%), tilmicosin (13.6%), tulathromycin/gamithromycin (12.1%) and ampicillin/penicillin (4.6%) against 140 *P. multocida*, while only one *M. haemolytica* isolate of 88 isolates showed resistance to macrolides (Alhamami et al., 2021).

Table 1.2. Antimicrobial resistance genes identified in the scientific literature.

Antimicrobial	Resistance gene	Enzymes/proteins	Country	Reference
aminoglycosides, such as kanamycin and neomycin	aphA	β-acid phosphatase/ phosphotransferase	USA Canada	Clawson et al. (2016) Cameron and McAllister (2016)
aminoglycosides gentamicin	aadB	2''-aminoglycoside nucleotidyl-transferase	USA	Clawson et al. (2016)
β-lactams	bla_{OXA-2}	β-lactamase inhibitor	USA	Clawson et al. (2016)
β-lactams	*cfx*A2 and *cfx*A3	class A β-lactamase	Multiple	Cameron and McAllister (2016)
β-lactam	bla_{SHV}, bla_{TEM1}, bla_{ctx-M}	β-lactamase inhibitors	Canada	Beukers et al. (2018)
	$blaROB_{-1}$ and cat2 (specific for M. haemolytica)	β -lactamase *gene and* catalase 2	USA	Owen et al. (2017)
ceftiofur	bla_{CMY-2} and bla_{CTX-M}	β-lactamase inhibitors	Multiple	Cameron and McAllister (2016)
chloramphenicol and florfenicol	floR	florfenicol/ chloramphenicol resistance protein	USA Germany Canada	Clawson et al. (2016) Michael et al. (2012a) Klima et al. (2014)
erythromycin	ermF and dfrA14 (specific to *H. somni*)	erythromycin ribosomal methylase and trimethoprim resistance *gene*	USA	Owen et al. (2017)
	ermX and $blaROB_{-1}$	erythromycin ribosomal methylase and beta-lactamase *gene*	Canada	Klima et al. (2011) Xu et al. (2018)
fluoroquinolones	qnrS and oqxB	quinolone resistance *genes and* efflux pump membrane transporter	Canada	Beukers et al. (2018)

gentamycin	aadB	2''-aminoglycoside nucleotidyl-transferase	Germany Canada	Michael et al. (2012a) Klima et al. (2014)
	aadA25	2''-aminoglycoside nucleotidyl-transferase	Canada	Klima et al. (2014)
kanamycin. neomycin	aphA1	class B acid phosphatase/phosphotransferase	Germany Canada	Michael et al. (2012a) Klima et al. (2014)
kanamycin. neomycin	aph3-Ia	class B acid phosphatase/phosphotransferase	US	Owen et al. (2017)
Macrolides	erm(A), erm(B), erm(F), and erm(X)	erythromycin ribosomal methylase	Canada	Clawson et al. (2016)
	erm(42), msrE and mphE	erythromycin ribosomal methylase, Macrolide efflux protein, macrolide phosphotransferase gene	USA	Clawson et al. (2016)
macrolide (tylosin)	*cfx*A2 and *cfx*A3	erythromycin ribosomal methylase	Multiple	Owen et al. (2017)
macrolide – (gamithromycin)	msr(E)-mph(E) operon	macrolide efflux protein, macrolide phosphotransferase gene	USA	DeDonder et al. (2016)
macrolide and lincosamide	erm(42)	rRNA methylase	Germany	(Kadlec et al., 2011)
macrolide-triamilide resistance	msr(E)-mph(E)	macrolide transporter gene and macrolide phosphotransferase gene	Germany	(Kadlec et al., 2011)
macrolide tilmicosin/ tulathromycin	msr(E)-mph(E)	macrolide efflux protein, macrolide phosphotransferase gene	Germany Canada	DeDonder et al. (2016) Klima et al. (2014)
macrolide	erm(A), erm(B), erm(C), erm(F) and mef(A)	rRNA methylase and macrolide efflux *gene*	Canada	Beukers et al. (2018)
penicillin	mecC, SCCmec type XI	penicillin binding protein	Multiple	Cameron and McAllister (2016)
penicillins, first- and second-generation cephalosporins	bla_{OXA-2}	β-lactamase inhibitors	Germany	Michael et al. (2012a)
phenicol resistance	floR	florfenicol/ chloramphenicol resistance protein	USA	Owen et al. (2017)
streptomycin/ sulphonamides/ tetracyclines	aphA1, strA, strB, sul2, tet(R) – tet(H)	class B acid phosphatase/phosphotransferase, aminoglycoside phosphotransferase, sulfonamide-resistant	Germany Canada	Alhamami et al. (2021) Klima et al. (2014)

		dihydropteroate synthase, tetracycline resistance protein		
	sulII, strA and catAIII	sulfonamide-resistant dihydropteroate synthase,e aminoglycoside phosphotransferase, catalase 2	Germany	Kehrenberg et al. (2001)
streptomycin	strA and strB	aminoglycoside phosphotransferase	Germany	Michael et al. (2012a)
streptomycin/ spectinomycin	aadA25	2''-aminoglycoside nucleotidyl-transferase	Germany	Michael et al. (2012a)
streptomycin	strB, strA	aminoglycoside phosphotransferase	USA	Clawson et al. (2016)
streptomycin and spectinomycin	aadA15	2''-aminoglycoside nucleotidyl-transferase	USA	Clawson et al. (2016)
streptomycin	strA, strB	aminoglycoside phosphotransferase	USA	Owen et al. (2017)
sulfonamides	sul2	sulfonamide-resistant dihydropteroate synthase	Multiple	Michael et al. (2012a)
sulphonamide sulfamethoxazole	sul2	sulfonamide-resistant dihydropteroate synthase	USA	Clawson et al. (2016)
sulfonamide resistance	sul1 and sul2	sulfonamide-resistant dihydropteroate synthase	Canada North America	Holman et al. (2018) Beukers et al. (2018)
sulfonamide resistance	sul2	sulfonamide-resistant dihydropteroate synthase	USA	Owen et al. (2017)
oxytetracycline	tet(H)	tetracycline resistance protein	Canada	Klima et al. (2011) Klima et al. (2014)
tetracycline efflux genes	tet(B), tet(C), tet(H), tet(L)	tetracycline resistance protein	Canada	Holman et al. (2018)
tetracycline ribosomal protection genes	tet(M) and tet(W)	tetracycline resistance protein	Canada	Clawson et al. (2016)
tetracycline	tet(R)	tetracycline resistance protein	USA Canada	Clawson et al. (2016) Klima et al. (2014)
tetracycline resistance	tet(H)	tetracycline resistance protein	US	Owen et al. (2017)
tetracylcine	tet(B), tet(G) and tet(H)	tetracycline resistance protein	Germany	Kehrenberg et al. (2001)
tetracycline	tet(R)-tet(H)	tetracycline resistance protein	Germany	Michael et al. (2012a)
tetracycline	tet(A), tet(B), tet(M), tet(O), tet(Q), tet(W)	Tetracycline resistance protein	Canada	Beukers et al. (2018)
macrolide-triamilide resistance	msr(E)-mph(E)	macrolide transporter gene and macrolide phosphotransferase gene	Australia	Alhamami et al. (2021)

tetracycline	tet(Y)	tetracycline resistance protein	Australia	Alhamami et al. (2021)

To cover the key possible resistance genes of importance in the treatment of BRD in Australian, assays for tetracyclines, beta-lactams, cephems, penicillins, macrolides, sulfonamides and phenicol resistance genes have been developed (Table **1.3**).

Table 1.3. Luminex was developed to detect the resistant genes listed. The example of antimicrobial given for each class is the antimicrobial of choice and are registered for use for BRD affected cattle in Australia.

Class	**Resistance genes**
Beta-lactams	*bla*$_{\text{OXA-2}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTXM-1}}$, *bla*$_{\text{KPC}}$, *bla*$_{\text{CMY-2}}$, ***bla*$_{\text{ROB-1}}$** and *cfx*A
Cephems Example: **ceftiofur**	*bla*$_{\text{CMY-2}}$ *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{TEM}}$
Penicillins Example: **penicillin**	*bla*$_{\text{OXA-2}}$
Macrolides Example: **tulathromycin**, **tilmicosin**	*erm*(A), *erm*(B), *erm*(C), *erm*(F), *erm*(42), *erm*(X), ***msr*(E)**, ***mph*(E)** and *mef*(A)
Phenicols Example: **florfenicol**	***floR***
Sulfonamides Example: combination of folate pathway inhibitor and suoponamide: trimethoprim-sulfamethoxazole	*sul1* and ***sul2***
Tetracyclines Example: **oxytetracycline**	*tet*(A), ***tet*(B)**, *tet*(C), *tet*(D), *tet*(E), ***tet*(H)**, *tet*(G), *tet*(H), ***tet*(L)**, ***tet*(M)**, ***tet*(O)**, *tet*(Q), ***tet*(R)**, ***tet*(W)** and ***tet*(Y)**

2. Objectives

Provide industry with a range of novel approaches to assist effective management of BRD in feedlot cattle, delivered via a multifaceted approach based on an understanding of host-pathogen interactions, including the potential development of:

1. **Novel diagnostic tests for bovine pathogens**

   This objective has been successfully met with the development of a new diagnostic assay for bovine adenovirus. The project also developed new diagnostic multiplex assays for the detection of antimicrobial resistance genes.

   Molecular assays developed in this project:

   - Quantitative Real-time PCR - Bovine parvovirus
   - Quantitative Real-time PCR – Bovine adenovirus
   - Multiplex Luminex assays for bacterial pathogens: *M. bovis, P. multocida*, *T. pyogenes*, *H. somni*, *M. haemolytica* and *B. trehalosi*.
   - Multiplex Luminex assays for the detection of 36 antimicrobial resistance genes.

2. **Novel vaccines against bovine pathogens**

   While the development of new vaccines was outside the scope of this project the characterisation of the DNA and RNA viromes present in the tissues of cattle that have died of BRD could be used to inform new targets for vaccine development. These studies reported the detection of several viruses in diseased tissue for the first time in Australian feedlot cattle. Further studies may be required to determine which of these agents should be priorities for vaccine development.

3. **Biomarkers for the identification of clinically normal animals**

   The expression of the majority the miRNA molecules evaluated in this study to date did not change in when infected and uninfected animals were tested in this study. As the expression of these molecules did not change regardless of the infection status of the animals, they could potentially be used as biomarkers for clinically normal animals. It is likely given the dynamic nature of miRNA expression that a suite of miRNAs will be required to identify unaffected animals.

4. **Biomarkers for the identification of clinically disease animals**

   Potential biomarkers have been identified for cattle infected with bovine herpesvirus 1 and bovine viral diarrhoea virus 1. The miRNA linked to bovine herpesvirus 1 infection was down-regulated. While the miRNA linked to bovine viral diarrhoea virus 1 was up regulated. Similar to defining health animals a suite of miRNAs are likely to be required to identify BRD affected cattle. Multiplexing technologies are available that could facilitate the development of an assay for this purpose, with overlapping miRNAs from the previous objective.

5. **Laboratory assays for the detection of biomarkers in serum, components of serum or other bodily fluids**

   Potential microRNA biomarkers for the BRD associated virus BoHV-1 and BVDV-1 were confirmed in cattle infected with these viruses.

6. Novel genetic markers for bovine respiratory disease resistance/susceptibility

Using a BayesR based genomic model a heritability estimate of BRD treatment was 0.20 ±0.04. This estimate is consistent with other estimates in the scientific literature and could be included into relevant breeding plans to select for cattle that are more resistant to BRD.

7. Oxford Nanopore Technology sequencing for detection of antimicrobial resistance genes.

The application of Oxford Nanopore Technology (ONT) sequencing was investigated for its potential to enable the detection of antimicrobial resistance genes. The results of the study suggest that ONT sequencing could be utilised to detect the AMR genes present in cattle that are undergoing treatment for BRD. Importantly, the ONT workflows can be successfully implemented by feedlot staff to successfully generate high quality sequence data. However, further development of the post-sequencing analyses is required to enable field application.

3. Methodology

3.1 Review of BRD biomarkers

To identify potential biomarkers for BRD, the PubMed database (November 2017) was interrogated with combinations of selected keywords. Published references were imported into EndNote. The references were subsequently ranked by scanning of the title, keywords and abstract for relevance to BRD and biomarkers.

Publications were ranked according to the following scale:

5 – Directly relevant to current study, definitely review all publications with this score.

4 – Relevant to current study, review most recent publications (2015 to 2017).

3 – Some relevance to the current study, assess most recent publications for inclusion (2016 to 2017).

≤2 – No relevance to the current study, not reviewed.

After the ranking was complete, all references with scores of 5 or 4 were reviewed. Those references considered to be relevant to or potentially relevant to BRD were tabulated and summarised in this report.

3.2 Circulating microRNA profiles in BRD cases and controls

3.2.1 Phase I Target identification

During this initial phase of screening the circulating miRNA it was proposed to use quantitative real-time PCR (qPCR) arrays. Several companies market these arrays which are based on the known range of bovine miRNAs and sold in panels associated with specific cellular pathways. While these arrays are designed to detect miRNA, there several different formats used in each. For the miRNA(s) to be detectable by qPCR it is first converted to a complementary DNA (cDNA) copy. Once the cDNA made it is then used as a template in the qPCR reaction.

TaqMan™ MicroRNA Assay (ABI/Thermofisher Scientific): This assay uses a specific hairpin primer to facilitate the synthesis of the miRNA cDNA copy. The principal disadvantage of this process is that a single cDNA reaction is required for each miRNA of interest. It can therefore be very expensive to screen for multiple miRNA. In addition, it is logistically a more time-consuming experiment due to the requirement for multiple cDNA reactions (one per target miRNA). There are also potential issues in comparing results for different miRNAs, as each cDNA synthesis is an independent reaction.

miSCRIPT™ (Qiagen): The principal advantage of this approach is it adds a generic nucleic tag onto all miRNAs in the extract of interest. This tag can then be used to convert all the miRNAs in the sample to cDNA which can then be detected and/or quantified by qPCR. As all the miRNA in the sample have been converted to cDNA, the same cDNA reaction can be used to detect multiple miRNAs. This reduces the overall costs and improves the efficiencies of screening for many different miRNAs.

miRCURY™ (Exiqon/Qiagen): This assay uses the same approach to generate cDNA as the miSCRIPT system and is therefore amenable to efficient screening of many miRNAs. The key difference in this system is it uses primers in the qPCR process which have modified bases known as, locked nucleic

acids (LNA). Primers with LNA bases have much higher melting temperatures in qPCR assays. As a consequence, LNA primers can be used develop highly specific qPCR assays which target short sequences, making them potentially ideal for the detection of miRNAs. This platform is widely considered the market leader in the qPCR detection of miRNAs.

3.2.2 Phase II miRNA qPCR Array

Sample selection: For these initial proof of concept studies samples were drawn from two cattle infection studies. The first of these studies, was conducted as part of B.FLT.0232 which compared the *in vivo* properties of five BVDV-1 strains. While the second study, involved the induction of an experimental form of BRD using a primary BoHV-1 challenge followed by a secondary *Mannheimia haemolytica* challenge, where cattle typically exhibit mild respiratory signs of disease.

It was decided to use these samples for three reasons. The first reason was the infecting pathogen was known, as reported previously it is possible that each pathogen may cause changes in the expression/regulation of different miRNAs. Thus it was considered essentially to know which pathogen was involved in the infection. For the NBRDI samples, while serology is available for the case/control samples and associations between seroconversion and BRD risk were demonstrated, these associations are very difficult to attribute at the animal level.

The second reason was the time of infection was known and therefore samples could be tested for differential miRNA expression at the time when peak virus was detected in the trial samples. Whereas the use of field samples would mean that the time of primary infection would be unknown. Thus any changes in miRNA expression may have return to normal levels or be altered in some other way in response to unknown factors (e.g. coinfections, stress, dietary changes etc).

The third reason was for some of these multiple blood samples were collected across the experiments. Consequently, if a potential biomarker was identified at peak infection, it would be possible to evaluate its temporal expression of the biomarker. Understanding temporal expression is important when evaluating field samples as the time of infection is unknown.

Serum samples (200 μL) were extracted using miReasy extraction kit. The extracted miRNA samples were converted to complementary DNA (cDNA) for subsequent qPCR analyses.

A custom miCURY miRNA qPCR array was designed based on the literature review detailed in the previous milestone report. The qPCR array It is laid out in a 96 well plate with each cDNA sample being tested in 32 wells, thus three samples tested per array. The array qPCR array was designed based on miRCURY qPCR system. The 32 wells per cDNA sample, test for miRNA which have been identified as being up regulated (n=26), down regulated (n=2) and no change (n=2) in cattle infection studies.

3.3 Genotyping of selected feedlot cattle

4.3.1 Genotyping with DNA from nasal swabs

Nasal swabs (n=10) collected during the national BRD initiative (NBRDI, B.FLT.0225) were selected to determine the feasibility of extracting sufficient quantities of bovine genomic DNA for genotyping for susceptibility/resistance to BRD.

Genomic DNA extraction and amplification: Material resuspended from nasal swabs were extracted using the DNeasy Blood & Tissue Kit (QIAGEN). All extracts were subsequently analysed using a Nanodrop UV spectrophotometer to assess the quantity and purity of genomic DNA. Where the

preliminary analyses suggest low quantities of nucleic acids, the amount of DNA was increased using a REPLI-g UltraFast Mini Kit (QIAGEN).

Quantitative Real-time PCR (qPCR): The amount of bovine DNA in sample extracts and amplification reactions were assessed using qPCR with Eukaryotic 18S rRNA assay (Thermo Fisher Scientific)

Genotyping: Samples were genotyped using the Illumina BovineHD Genotyping BeadChip by Neogen Australia (Gatton). The BovineHD Genotyping BeadChip which can detect more than 777,000 single nucleotide polymorphisms (SNPs) across the genome.

4.3.2 Genotyping with DNA from stored blood samples

Genomic DNA extraction: Genomic DNA was extracted from stored blood clots from blood samples (n =204) collected as part of Project No B.FLT.0164. The samples have been stored at -70°C since they were processed for serum harvesting in September 2017. After thawing samples were examined and the material located near the surface of the clot removed as this was anticipated to correspond to the white blood cells (commonly referred to as buffy coat). Once relocated to a fresh tube the material was extracted using a Puregene Blood Kit (Qiagen) according to the manufacturer's instructions. The quantity and purity of the extract DNA samples were evaluated using UV spectroscopy. Selected samples were further analysed by pulse field gel electrophoresis to evaluate the .

Genotyping: Selected samples (total animals n = 186; BRD cases n = 93; controls n = 93) were submitted to Neogen Australia for genotyping analysis on the BovineHD Genotyping BeadChip which can detect more than 777,000 single nucleotide polymorphisms (SNPs) across the genome.

3.4 Characterisation of the DNA virome in respiratory tissues of BRD deaths

3.4.1 The BRD DNA virome

Samples: The samples used in this study were sourced from the tissues collected from animals included in the National Bovine Respiratory Disease Initiative (NBRDI) as part of project B.FLT.0225. During the course of the project tissue samples were collected from 127 cattle. The tissues were collected by feedlot staff, who were asked to sample either trachea or lungs with pathology considered to be associated with disease. For most animals both lung and tracheal samples were submitted. To reduce the number of samples to be analysed, were both samples were submitted a portion of each tissue was co-extract, consequently each animal only had one extract in the study. Approximately 50mg of tissue(s) was extracted using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's instructions, except the RNase was not added to the extraction buffer.

Quantitative Real-time PCR - Bovine parvovirus:

This assay was designed to amplify products from the VP2 gene of bovine parvoviruses. The assay should react with all known bovine parvoviruses, though there is limited sequence data currently available for this virus.

Amplification primers used:

BPV_VP2_F 5' - ACT GGA TGT CAG GAC CGG G - 3'

BPV_VP2_R5' – CAT GGT CAC CAA TGC TCC C - 3'

BPV_VP2_Probe BPV_VP2_R 5' FAM– AAC ACA CAA CGC GAC TCT GCA AAC ACA-BHQ1 - 3'

Amplified using Quantitect™ (Qiagen) Master Mix using the manufacturer's instructions, with 2 μL of each extract as template.

Conventional bovine parvovirus PCR assay:

This assay is designed to amplify products from the VP2 gene of bovine parvoviruses. The assay should react with all known bovine parvoviruses, though there is limited sequence data currently available for this virus.

Amplification primers used:

BPVfwd2 5' - CTG ACC ACG AAG TGC TAC GC - 3'

BPVrev2 5' – GCG TAG CAC TTC GTG GTC AG - 3'

Amplified using Platinum Taq™ (Thermofisher Scientific) using the manufacturer's instructions, with 2 μL of each extract as template.

Cycling conditions: 94 °C 3min; [94 °C 30sec, 55 °C 30 sec, 72 °C 60 sec] 40 cycles; 72 °C 10 min.

Consensus bovine adenovirus PCR assay:

This assay is based on highly conserved amino acid sequence motifs within the DNA polymerase enzymes from adenoviruses. They have been used to successfully amplify PCR products from viruses from a diverse range of hosts, including mammals and reptiles.

Amplification primers used:

Adeno pol F 5' – TNH GNG GNM GNT GYT AYC C - 3'

Adeno pol R 5'- GTD GCR AAN SHN CCR TAB ARN GMR TT - 3'

The amplification primers use were the same as those described by Wellehan et al. (2004), with one modification made to the second nucleotide of the forward primer, with the "M (A or C)" being replaced by "H (A or C or T)".

Amplified using Platinum Taq™ (Thermofisher Scientific) using the manufacturer's instructions, with 2 μL of each extract as template.

Cycling conditions: 94 °C 3min; [94 °C 30sec, 45 °C 60 sec, 72 °C 60 sec] 40 cycles; 72 °C 10 min.

Consensus herpesvirus conventional PCR:

This assay is based on highly conserved amino acid sequence motifs within the DNA polymerase enzymes from herpesviruses (VanDevanter et al., 1996). They have been used to successfully amplify PCR products from herpesviruses from a diverse range of hosts, including mammals, reptiles, and molluscs .

HerpCon_DFA_F 5' – GAY TTY GCN AGY YTN TAY CC - 3'

HerpCon_KGI_R 5'- GTC TTG CTC ACC AGN TCN CAN CCY TT - 3'

Amplified using Platinum Taq™ (Thermofisher Scientific) using the manufacturer's instructions, with 2 μL of each extract as template. The reactions also included 10% dimethyl sulfoxide and 20% glycerol.

PCR Cycling conditions: 94 °C 3min; [94 °C 30sec, 45 °C 60 sec, 72 °C 60 sec] 40 cycles; 72 °C 10 min.

3.4.2 The BRD RNA virome

Literature Review: One of the issues with characterising viromes from tissue samples is the potential for host nucleic acids to overwhelm the detection of pathogen nucleic acids. To address this issue, a review was undertaken of several recent studies which have attempted to describe the RNA viromes associated with BRD. The viruses common to these studies and associated with BRD were selected for further study.

Reverse-transcriptase quantitative real-time PCR assays (RT-qPCR): To determine the presence or absence of the RNA viruses identified as being associated with BRD, RT-qPCR assays were used to analyse total nucleic acid extractions from the lung tissue samples collected during B.FLT.0225. The data generated were then compiled with the DNA virus detection data, reported previously.

3.5 Development of new viral diagnostics

3.5.1 A novel diagnostic assay – DNA virus

Quantitative real-time PCR (qPCR) assay development: To inform the development of a qPCR specific for bovine adenovirus 3 (BAdV-3) sequences for two key adenovirus genes were downloaded from GenBank and aligned to identify well conserved sequence regions. The two genes selected were the DNA polymerase gene (Dpol) and the hexon gene. The Dpol gene was selected as a partial sequence was obtained for these gene during the characterisation of the DNA virome studies reported in Milestone 4. The hexon gene was also evaluated as sequences were available from a wider range of isolates compared to Dpol.

Evaluation of qPCR Assay: To determine the performance of the BAdV qPCR assay the tissue (combined lung and trachea) extracts from the cattle that died from BRD collected and prepared as part of B.FLT.0225 were tested. This testing was performed using standard qPCR conditions.

3.6 Evaluation of genetic markers for BRB susceptibility/resistance_2023

The genotype and phenotype data utilised in these analyses was provided by Angus Australia under a Material Transfer Agreement.

The phenotype data consisted of 1,213 Angus steers from two feedlots from the Angus BIN project (B.FLT.0159). The BRD trait was recorded as treated or not treated for BRD, with treated animals recorded from "Hospital" data for each feedlot. To be scored as treated steers had to be recorded with signs of BRD and treated with an appropriate antimicrobial either Draxxin (tulathromycin) or Miticol (tilmicosin).

These animals were genotyped with the ovine 50K Illumina SNP array Genotypes with a missing rate >0. 1, a minor allele frequency (MAF) of < 0.01 and those departing from the Hardy-Weinberg equilibrium at $P < 1 \times 10^{-8}$ were removed. After quality control, there were 46826 SNP remaining. Missing genotypes for individual animals were imputed using findhap4 (VanRaden et al., 2011).

3.6.1 Gene mapping

Two approaches were used to map genes affecting BRD.

In the GWAS approach, the linear mixed model fitted to the data using Equation 1:

Equation 1: y= intake cohort + feedlot + dentition + animal + SNP **+ error**

Where y was the phenotype for BRD (0= not treated, 1 = treated), intake cohort was the intake cohort into the feedlot, unique to property of origin and date, feedlot was the feedlot where the animal was recorded (1 or 2), dentition was the dentition of the animal (0,2), animal was the random animal effect, fitted to control for relationships between animal and population structure, SNP was the effect of each SNP (fitted in turn), with alleles coded as AA=0, AB=1 and BB =2), and error was a random error term. The animal effect was assumed ~ N(0,**G**sigmag^2), where **G** is the genomic relationship matrix constructed from all 46826 SNP following the approach described by Yang et al. (2011). This model was fitted for each SNP in turn (e.g. 46826) times and the P value and size of SNP effect recorded, using GCTA (Yang et al., 2011).

The BayesR approach was also implemented, with a similar model to above except all SNP were fitted simultaneously (Erbe et al., 2012).

In the BayesR, the variance associated with the ith SNP is assumed to come from one of four distributions as described by Equation 2.

Equation 2: $$\boldsymbol{\sigma^2_i} = \{\mathbf{0},\ \mathbf{10^{-4}}\boldsymbol{\sigma^2_g},\ \mathbf{10^{-3}}\boldsymbol{\sigma^2_g},\ \mathbf{10^{-2}}\boldsymbol{\sigma^2_g}\}$$

Where σ^2_g is the genetic variance of the trait.

This allows the BayesR model (Moser et al., 2015) to have a flexible SNP effect distribution which is a mixture of four possible normal distributions: $\boldsymbol{N(0,0)}, \boldsymbol{N(0,\ 10^{-4}\sigma^2_g)}, \boldsymbol{N(0,10^{-3}\sigma^2_g)}$ $\boldsymbol{N(0,10^{-2}\sigma^2_g)}$**,** all with a mean of 0 but with different variances. The posterior distributions were sampled using Markov Chain Monte Carlo (**MCMC**) with Gibbs Sampling in GCTB (Zeng et al., 2018)

with 25,000 iterations of which the first 5,000 are discarded as burn-in and thinned by 10 (2,000 MCMC samples).

To assess if any mutations of large effect were affecting BRD, the posterior inclusion probability was mapped against genome location.

3.6.2 Genomic predictions

Two strategies for genomic prediction using all markers were assessed, GBLUP and BayesR. BayesR was as described above. Genomic best linear unbiased prediction (GBLUP) fitted the model using Equation 3.

Equation 3: y = intake cohort + feedlot + dentition + animal + error

For example, as described for the GWAS but without the individual SNP effects. The animal effect from the model was the GEBV, and GCTA was used to fit the G-REML model (Yang et al., 2011).

To evaluate accuracies of GEBV from GBLUP and BayesR, five-fold cross-validation was used, with random grouping of animals such that all groups have approximately equal size. In each rotation of the cross validation the phenotypes of 1 group were masked and the remaining 4 groups were used to estimate the GEBV of the group without phenotypes. The accuracy of phenotype prediction for each group was calculated as the Pearson correlation between predictions and raw phenotypes of animals for which their phenotypes were masked, divided by the square root of the heritability for BRD. Accuracies were averaged across 5 groups and standard error (**SE**) was calculated as the standard deviation divided by the square root of the number of groups i.e., $SE = \frac{STD}{\sqrt{5}}$

3.7 Sample collection 2022

3.8 Sample extraction processes - Virology

On receipt at the laboratory, nasal swabs resuspended in 3 mL of PBS containing antimicrobial and antimycotics and stored at 4^{o}C until required.

3.8.1 Qiagen Puregene - Buccal Swab

Total nucleic acids were extracted from aliquots of the resuspended nasal swabs Once relocated to a fresh tube the material was extracted using the a the Puregene Buccal Swab protocol (Qiagen) according to the manufacturer's instructions. Samples were eluted in 50 µL DNA hydration solution and stored at 4^{o}C until required. The quantity and purity of the extract DNA samples were evaluated using UV spectroscopy. DEVIN Microbial Enrichment Kit

The Devin™ Microbial DNA Enrichment Kit is suitable for removing the white blood cells and isolating microbial DNA from whole blood, plasma or other body liquids. Extracted nucleic acids can be analysed by downstream application, such as real-time PCR and/or next-generation sequencing. This kit was tested for its capacity to remove bovine cells from the nasal swab samples. It was used according to the manufacturer's instructions. The time required for sample preparation using this method is approximately 120 min.

3.8.2 Molysis Basic 5 Bacterial Enrichment Kit

The Molysis Basic 5 Bacterial Enrichment Kit was also test for its capacity to enrich samples for microbial DNA. The kit was used to enrich a 1 mL aliquot of resuspended nasal swab using "Protocol 1: small size sample DNA isolation" as detailed in the manufacturer's instructions. At the end of this protocol, the samples were further enriched using the Puregene Buccal Swab protocol (Section 3.8.1). Samples were eluted in 50 µL DNA hydration solution and stored at 4^{o}C until required. The time required for sample preparation using this method is approximately 180 min.

3.8.3 PrepMan™ Ultra Sample Preparation Reagent

The PrepMan™ Ultra Sample Preparation Reagent (ThermoFisher Scientific) was evaluated as potential sample preparation method as it has minimal processing steps and required minimal technology requirements. Briefly, 500 µL of resuspended swab material was mixed with an equal volume of PrepMan™ Ultra Sample Preparation Reagent. The mixture was vortexed for 10-15 sec, heated at 100^{o}C for 10 min and allowed to cool at room temperature for 2 min. After centrifugation at 10,000 *g* for 2 min, the top 50% of the supernatant was carefully aspirated and stored at 4^{o}C until required. The time required for sample preparation using this method is approximately 15 min.

3.9 Oxford Nanopore Technologies Sequencing 2022

A schematic representation of the samples analysed by Oxford Nanopore Technologies (ONT) sequencing and their sources is shown in Section 4.6.

3.9.1 ONT Library Construction sequencing – Native barcoding

The extracts from selected nasal samples were prepared for ONT sequencing using the Ligation Sequencing gDNA - Native Barcoding (SQK-LSK109 with EXP-NBD104 and EXP-NBD114) kit according to the manufacturer's instructions. Briefly, this approach begins with the repair of the ends of all DNA extracts to be sequenced to ensure they are blunt ended. “Barcodes” are then ligated onto the repaired molecules, with a specific barcode allocated to each sample being analysed. Sequencing adapters are then ligated all DNA molecules in the samples of interested.

The minimal equipment requirements stated by the manufacturer are, gentle rotator mixer, magnetic separator (suitable for 1.5 mL microfuge tubes), microfuge, vortex mixer, thermal cycler, P1000 to P2 pipette and tips, ice bath, and timer.

Twelve barcodes are available for this kit, thus enabling the simultaneous sequencing of up to 12 samples on a single ONT flow cell. The estimated time for completion of libraries using this approach is 2 to 3 hrs.

Libraries were analysed on R9.4.1 (FLO-MIN106) flow cells according to the manufacturer's instructions.

3.9.2 Library Construction and sequencing – Rapid Barcoding

The extracts from selected nasal samples were prepared for ONT sequencing using Rapid Barcoding Sequencing Kit (SQK-RBK004) according to the manufacturer's instructions. Briefly, the prepared DNA (400 ng per sample) in extracts is simultaneously fragmented and barcoded using a transposon complex based approach. As the added barcodes include the adapters required for sequencing the samples are immediately ready for sequencing.

The minimal equipment requirements stated by the manufacturer are, thermal cycler (or heat block) for treatments at 30°C and 80°C, a microfuge, a P10 pipette and tips.

ONP rapid barcoding kit technology has the capacity to simultaneously sequence up to 96 samples. The estimated time for the completed of sequencing libraries is estimated to be 10 to 15 min.

Libraries were analysed on R9.4.1 (FLO-MIN106) flow cells according to the manufacturer's instructions.

3.9.2 ONT Data Analyses

Once sequencing was complete, the Fast_5 pass files were uploaded to The University of Queensland high performance computer cluster: Tinaroo. Guppy GPU v3.6.0 software was used for base-calling. The base-called sequences were then uploaded to Galaxy Australia to trim the adaptors and prepare the sequences for analyses. All sequences were initially screened against the bovine genome, with those sequences showing identity to this sequence were removed from the data and deemed to be “host genome”. The remaining sequence were compared to a custom database to identify those with identity to known taxa to the genus level.

4. Results

4.1 Review of BRD biomarkers

Initially 12 keyword combinations were used to identify publications with potential relevance to the current study. Collectively these searches returned 1,133 published studies. Of these 1,241 have been ranked using the criteria described above.

Due to the number of publications, only the results for the three keyword combinations of most relevance to this project were reviewed in detail in this report. A summary of these keyword searches to identify studies with relevance to BRD biomarkers is shown in Table 4.1. Each set of keywords identified a large number of studies (Table 4.1). While additional keyword(s) could have been added to refine the searching criteria this was not done to reduce the risk of missing important articles.

Table 4.1 Summary of the keyword searches and publication rankings. All searches were performed using the PubMed database.

				Ranking			
Keyword 1	Keyword 2	Keyword 3	Total publications	5	4	3	≤2
Biomarker	Bovine	Respiratory	72 (32 *)	30	13	20	9
Biomarker	Saliva	respiratory	95 (17 *	11	12	4	68
Bovine	Circulating	microRNA	64 (21 *)	11	5	13	35

*References reviewed

Summary of keywords – Biomarker, Bovine, Respiratory: The references examined clearly demonstrate that biomarkers can certainly be used to identify animals with BRD (Appendix A). However, what is also readily apparent is the complexity of this challenge. In most of the studies examined biomarkers were identified to differentiate infected and control animals, but for the most part there is minimal overlap between the biomarkers from each study. This suggests that the identified biomarker(s) are specific for the experimental model used to identify it. If a similar scenario applies to BRD in feedlots where many of the factors contributing to disease are unknown and/or variable a single BRD biomarker may not exist. A common theme are the acute phase proteins and the influence of stress as an overlay.

One feature of many of the references in this search was, despite some studies reported what appear to be very good results, are infrequently cited. It is difficult to explain this phenomenon, given the importance of BRD it is reasonable to expect that other researchers would have attempted to confirm these biomarkers in follow-up studies. One possible explanation is that the biomarkers were not confirmed in subsequent studies for reasons similar to those discussed above.

Key studies from this set of keywords:

Taxis et al. (2017) identified differential expression of circulating microRNAs in cattle following infection with Bovine viral diarrhoea virus 2. Differential expression was identified as early as Day 4 post-infection. The expression patterns identified have not been examined in any other systems.

Ferrari et al. (2010) utilised acoustic monitoring to identify the coughing of animals with BRD, from a practical point of view could be easily adopted. The main disadvantage would be that BRD could be well advanced before coughing develops.

Aich et al. (2009) identified several biomarkers for BRD. Using acute phase protein analyse and metabolomics. Clearly demonstrated that diseases and healthy animals can be differentiated. However, utilised complex analytics to do so. Utilised the dual bovine herpesvirus 1 / *Mannheimia haemolytic* challenge BRD model.

Summary of keywords – Biomarker, Saliva, respiratory:

Several interesting studies although main focus is on humans with a couple of studies on pigs (Appendix B). Review has focused on biomarkers of disease rather than detection of pathogens in saliva. Many of the human studies are focussed on tuberculosis and assessing responses to treatment. Overall suggests saliva is a very promising sample. Main issue is collection, as systems have been developed to optimise collection for humans, which are unlikely to work in cattle. Much of the work the testing of saliva has focused testing athletes for increase susceptibility to respiratory infections.

Gutierrez et al. (2017) identified changes in total adenosine deaminase activity were associated with inflammation and respiratory disorders. Was correlated to acute phase proteins. While not discussed in the paper if a substrate for adenosine deaminase could be identified which changes colour could be useful for crush-side testing (with appropriate controls). Plus, a test based on enzyme activity would amplify the detection signal therefore potential increasing likelihood of detect. Appears to warrant further investigation in cattle.

Dixon et al. (2016) comprehensive review of care diagnostics under evaluation mid 2014 to end of 2015. Eighty technologies were included – 25 were considered promising for measuring biomarkers in saliva and included wearable technology. A more recent review by Barbosa and Reis (2017) looked at the microfluidic devices developed in the period 2005-2016 capable of measuring protein biomarkers from the pM to fM range. The importance of these reviews are they suggest significant progress towards point of care diagnostics for humans. Some of these may have direct utility in veterinary applications, particularly once costs start to decrease, if appropriate markers are available.

Barbosa and Reis (2017) identified a biomarker for stress implicated in inflammatory processes that was positively associated with cortisol. No studies to date have been completed on cattle.

Summary of keywords – Bovine, disease, microRNA:

Several studies looking at the roles of bovine miRNA in infection and physiological pathways were summarised (Appendix C). While overall the profiles reported are generally quite complex, i.e. multiple miRNA either up regulated or down-regulated depending on the system being analysed, which supports any initially investigation into a new application looking as broad a range of miRNAs as possible. Another common theme is investigating the role of miRNAs in complex diseases such as mastitis, tuberculosis and Johne's disease.

Key studies from this set of keywords:

Stenfeldt et al. (2017) investigated changes in miRNA expression profiles in the sera of cattle infected with food and mouth disease virus (FMDV). The study was able to differentiate animals acutely infected, persistently infected and recovered animals. While this achievable it was several miRNA

biomarkers, some of which change their expression pattern overtime. Emphasises the need to explore a wide range of miRNAs.

Taxis et al. (2017) summarised previously.

Malvisi et al. (2016) one of several studies looking at the role of miRNAs in Johne's disease. Demonstrates the capacity to using miRNAs detect the different stages of disease. Several publications also report similar findings for tuberculosis.

Discussion:

Not unexpectedly it has proven difficult to identify a broadly applicable biomarker for BRD. The range of viral and bacterial pathogens and other factors that may contribute to the risk of BRD suggest that there are multiple cellular pathways which converge into disease. This is reflected in the number and range of potential biomarkers which have been identified for BRD. Acute phase proteins have arguable been the most well studied BRD biomarkers, despite this they have not been developed into generally available test for BRD.

As this project progresses there are three important elements that will be considered in the identification of BRD biomarkers. The first is the biomarker itself, which maybe protein, nucleic acid or metabolite. Biomarkers within all these categories have been identified in studies on BRD or BRD related pathogens. Though to date none appear to have moved from experimental trials to field application. The review of the associated literature suggests that the identification of biomarkers which segregate infected and control animals in a pen trial setting is readily achievable. However, to date the biomarkers appear to be specific for the conditions of the trial reported. When one component of the trial is change, such using a different pathogen, then the biomarker changes as well. Thus, translating an identified biomarker(s) into field test where a multitude of factors can potentially influence the development has not been reported yet. Consequently, it is envisaged that a suite of biomarkers will likely be required to improve the detected of BRD. In this context miRNAs will be a strong focus for the current study as they are involved in numerous biological pathways.

The second crucial element to be considered is the sample. The most commonly tested sample for the presence of biomarkers is blood/serum. Currently, collection of blood/serum is typically done via venepuncture and in large quantities, typically millilitres. Generally, it is necessary to restrain the animal during the collection process. The potentially less invasive sample of saliva has received some attention of late. Saliva is of particularly interest in human diagnostics where specialised collection tubes have been developed and the patient can be instructed to maximise the likelihood of successful collection. For cattle, it would seem that saliva could be problematic as the collection of consistent samples for testing could be challenging. Also of potential interest in the diagnosis of BRD is breath analysis and several studies have investigated this approach with cattle. It is difficult to see how breath analyses could be robustly adapted into a feedlot setting, within the constraints of current industry practices. Future improvements in the associated technologies may facilitate this. Overall blood/serum is probably the best sample for biomarker detection as if it can be efficiently collected it is consistent and scalable sample. Investigating the application of technologies used by human diabetics to efficiently collect small quantities of blood may be worthwhile once suitable biomarkers are identified.

The third key component is the testing platform. As would be expect ELISA and PCR have been widely used in the detection of biomarkers. While these are relatively straight forward in a laboratory setting they are more problematic in a field setting, albeit there have been significant advances in the field application of PCR in recent years. Despite these advances, it is difficult to see how they might be applied in a feedlot setting for a rapid return of a test result. As an example, if a test could be complete in 10 minutes, then minimum time to induct a pen of 200 animals would be over 30 hours, assuming the animal is held for the test result before a decision is made regarding what happens next to the animal. This clearly well outside the constraints of current industry standards. Testing at the hospital pen is more feasible as it is less time critical and also with fewer animals involved.

Once identified, the next stage in the application of the biomarker(s) is how it will be used to intervene in BRD management. There are two potential intervention points where a biomarker of BRD could be utilised. The first point is induction, if animals could be identified that were at increased risk of BRD it may be possible to intervene or change the intended management of the animal to reduce the severity of disease. A test of this type would most likely need to assess the immunological competence of the animal and how this was related to its BRD risk. A key challenge in an intervention at induction is time, while it may be possible to sample animals without affecting the induction process, the time required for subsequent testing of the sample creates a problem within the constraints of current industry practices, as discussed previously it could significantly affect induction time. Potentially if animals could be sampled as part of the induction process and tested, then the results could be added to an overall BRD risk assessment for the animal and or its pen mates. This approach would enable testing to occur post-induction and intervention may only occur if the test result suggested the presence of disease or a substantial risk of disease developing.

The second logical point of intervention would be at the hospital pen. At this point a biomarker(s) which confirmed BRD would be useful in supporting the decision of if and how to treat an animal. Ideally it would also support a reduction in BRD associated treatment costs by reducing the number of false BRD cases. The other advantages of hospital pen based biomarker assessments are fewer animals need to be sampled and tested. It would be less time critical compared to sampling and testing at induction. A positive biomarker test prior to treatment would also underpin the decision treat an animal. Ideally, the biomarker would indicate “disease”, therefore addressing one of the limitations of pathogen based diagnostics which can generally only detect known pathogens.

Based on this review of BRD biomarkers and some detection technology, this project will continue to the proposed plan to investigate circulating miRNA. These molecules are regulators of key cellular pathways involved in BRD such as, pathogen sensing, inflammation, and immune responses. It is anticipated that a suite of miRNAs will be required to accurately detect BRD in cattle, as they may be the common factors in the multiple pathways to BRD.

4.2 Analysis of circulating microRNA profiles in BRD cases and controls

4.2.1 Phase I Identification RT-qPCR platform

As the aim of this activity was to screen bovine sera for multiple miRNA, the first step was to identify the most suitable qPCR platform out of those described above for screening for circulating bovine miRNAs. We have used TaqMan™ MicroRNA qPCR assays for the past 10 years in our studies of the miRNAs encoded by bovine herpesvirus as the number of miRNAs being analysed have been limited. We have also reduced the cost of using this platform by designing our own hairpin primers for use in these qPCR assays. While this system is not ideal for screening miRNA expression experiments, we have a lot of experience with it and historical data to use as a reference point to evaluate the other systems.

miRCURY LNA miRNA PCR System: We have previously characterised the miRNA expression profile of cultured bovine cells using next generation sequencing technology (Glazov et al., 2009). These sequencing data were used to selected seven bovine miRNAs which express at different levels to evaluate the dynamic range of the miSCRIPT assay. The miSCRIPT assay has pre-designed assay components for the detection of human miRNA, consequently only bovine miRNAs with identical sequences to the respective human homologues were considered. Based on the sequencing and conservation data, selected miRNAs were bta-mir-145 with 0 sequence reads per million miRNAs (srpm), bta-mir-15a with 510 srpm, bta-mir-130a with 1,644 srpm, bta-mir-320 with 8,008 srpm, bta-mir-25 with 16,560 srpm, bta-mir-29a with 43,642 srpm and bta-let-7f with 930,889 srpm. The results of this analysis are shown in Fig. 4.1. Overall there was a poor correlation between the qPCR cycle threshold value (Ct) and the srpm for the miRNAs. The Ct values for bta-mir-15a, bta-mir-130a and bta-mir-320 did appear to show some correlation, however these results suggested a dynamic range of only two orders of magnitude. Some level of discrepancy was expected between the qPCR results and the sequencing data, biases in sequencing data have been reported previously.

Of most concern in this comparison were the results for bta-miR-25. We have developed an in-house hairpin assay for bta-miR-25 to reduce the costs associated with qPCR detection of this miRNA as it is routinely used as the control reaction in our viral miRNA studies. When this assay was used to detected bta-miR-25 in the same extracts used in the assessment of the miSCRIPT assay it typically yields a Ct value of approximately 20.5, which is much lower than the Ct = 29.9 from the miSCRIPT assay (Fig. 1). The reasons for this discrepancy are unclear as it was expected there would generally agreement between the two qPCR assays when used to analyse the same sample. This result suggests the miSCRIPT assay is far less sensitive compared the in-house hairpin assay and is therefore not suitable for screening bovine sera for circulating miRNAs where the miRNAs would be expected to be at low concentrations.

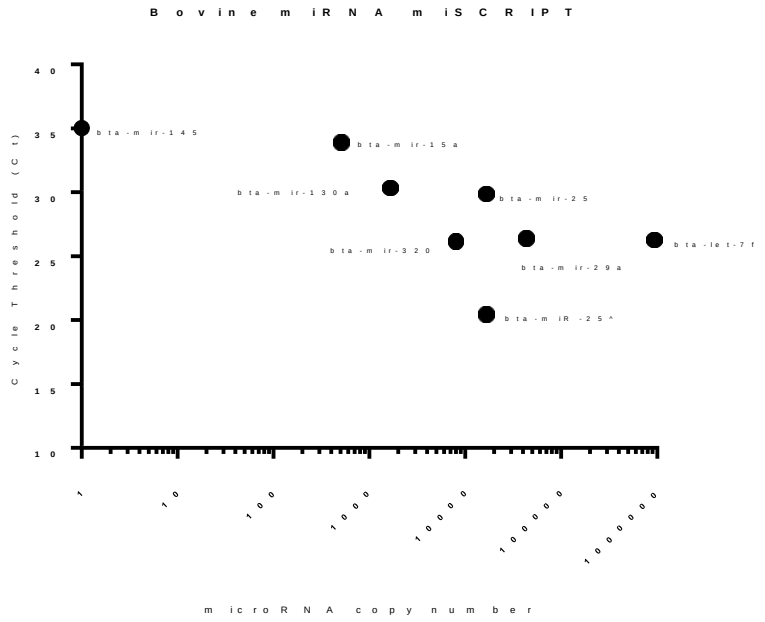

Fig. 4.1. Quantitative real-time PCR detection of selected bovine microRNAs expressed by MDBK cells using the miSCRIPT system. The cycle thresholds for each miRNA from the qPCR assay are plotted against the reads per million as determined using next generation sequencing. For bta-mir-145 the reads per million +1 to enable plotting on a logarithmic scale. The bta-miR-25^ represents a typically result using the in-house hairpin assay.

Previously, we have evaluated six TaqMan™ microRNA assays for the detection of bovine miRNAs. As with the miSCRIPT selection process was primarily based on the sequencing results of bovine miRNA expressed by the MDBK cells, albeit for different reasons. The selection of these miRNAs were based on their expression levels in response of infection of MDBK cells with bovine herpesvirus 1. The selected miRNAs were bta-mir-145 (0 srpm), bta-miR-205 (0 srpm), bta-miR-199a-3p (121 srpm), bta-mir-98 (592 srpm), bta-let-7c (1605 srpm) and bta-miR-25 (16,560 srpm). As result of applying different selection criteria only bta-miR-25 overlapped between the two qPCR platforms. Figure 2 illustrates the results of these TaqMan™ microRNA assays and the in-house qPCR assay for bta-miR-25. Similar to the miSCRIPT system, there was no correlation between the srpm and the Ct values (Fig. 4.2). However, in general the overall trends of qPCR results were more similar to the sequence data results, for example bta-miR-25 with the highest srpm yielded the lowest Ct value, while bta-miR-205 with 0 srpm yielded the highest Ct value (Fig. 4.2).

The in-house assay for bta-miR-25 yielded a higher Ct value compared to the commercial assay, suggesting it was less sensitive. Although these data are from cell extracts prepared at different times by different people, so direct comparison of these results is problematic and should be made with caution. We are in the process of comparing the in-house and TaqMan™ microRNA assays for bta-miR-25 bta-miR-25, to determine if the variation in Ct values is due to assay performance or differences in experimental set-up.

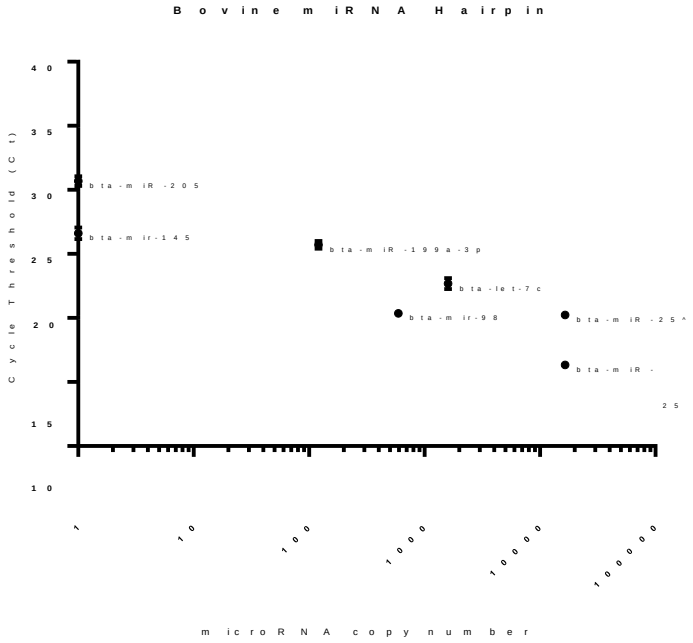

Fig. 4.2. Quantitative real-time PCR detection of selected bovine microRNAs expressed by MDBK cells using the Assay by Demand system. The cycle thresholds for each miRNA from the qPCR assay are plotted against the reads per million as determined using next generation sequencing. For bta-mir-145 and bta-miE-205 the reads per million +1 to enable plotting on a logarithmic scale. The bta-miR-25^ represents a typically result using the in-house hairpin assay.

4.2.2 Analysis of circulating miRNA in cattle of known infection status

Detection of circulating microRNA in cattle infected with BVDV-1

Of the 30 bovine miRNA evaluated the majority were detected in at least one animal at either Day 0 or Day 7 post-infection. The exceptions to this were bta-miR-154a, hsa-miR-34a-5p, mmu-miR-497a-5p and bta-miR-369-5p (data not shown). Five miRNA were detected in all cattle at Day 0 (n=10, Table 4.2). While Day 7 post-infection four miRNA were detected in all cattle tested (n=9, Table 4.1).

Three miRNAs were detected in all samples analysed (Table 4.1). Comparison of the average Ct values between the Day 0 and Day 7 samples, identified that the Ct value for miRNA hsa-miR-486-5p/bta-miR-486 was significantly higher in the Day 7 samples compared to Day 0 (Table 4.2). This suggests expression of this miRNA was reduced as a consequence of BVDV-1 infection.

Table 4.2 Circulating microRNA detected in cattle either prior to infection (Day 0, n=10) or after infection (Day 7, n=9) with BVDV-1 strain PI506. The average cycle threshold (Ct) is shown with the standard deviation in parenthesis.

	Ct Value			
miRNA[#]	**Day 0 (n=10)**	**Day 7 (n=9)**	**Regulation**	**P value[$]**
bta-miR-29a	33.78 (1.11)	N/A*		
ssc-miR-339-5p	N/A*	33.69 (1.03)		
bta-miR-29a	33.78 (1.11)	N/A*		
hsa-miR-29b-3p	36.60 (1.65)	N/A*		
hsa-miR-486-5p	31.56 (1.77)	33.84 (1.25)	Decrease	0.0052
hsa-miR-92a-3p	32.08 (2.15)	32.04 (1.25)	No change	0.9616
hsa-miR-24-3p	33.50 (1.81)	33.84 (1.89)	No change	0.6938

[#]*Bos taurus* (bta); *Sus scrofa* (ssc); *Homo sapiens* (hsa). Where the non-bovine homologue is listed it is to match the commercially available assay and the miRNA targets are 100% identical.

*Not applicable, miRNA not detected in all animals tested at this time point.

[$]Two-sided T test.

Detection of circulating microRNA in cattle infected with BoHV-1

Of the 30 bovine miRNA evaluated the 15 were detected in the sera of all animals pre-infection (Day 0) and post-infection (Day 7) (Table 2). Due to time constraints fewer BoHV-1 challenged animals have been tested, compared to the BVDV-1 animals, consequently no determination has been made regarding differential expression of any miRNAs detected when comparing the Day 0 and Day 7 results. Qualitatively, the results have identified some promising candidates for consideration as biomarkers of BoHV-1 infection (Table 3.3).

Table 4.3 Circulating microRNA detected in cattle either prior to infection (Day 0, n=4) or after infection (Day 7, n=4) with BoHV-1 strain Q3932. The mean cycle threshold (Ct) is shown with the standard deviation in parenthesis.

	Ct Value			
miRNA[#]	**Day 0**	**Day 7**	**P value**	**Regulation**
hsa-let-7b-5p	32.07 (2.66)	31.36 (1.72)	0.6697	No Change
hsa-let-7d-5p	33.19 (1.37)	31.11 (1.56)	0.0920	No Change
cfa-miR-144	35.48 (3.56)	32.96 (3.33)	0.3410	No Change
hsa-miR-185-5p	28.81 (1.56)	28.08 (1.17)	0.4823	No Change
hsa-miR-205-5p	34.58 (1.08)	31.51 (0.39)	0.0017	Increase
bta-miR-29a	28.36 (2.64)	29.78 (0.59)	0.3342	No Change
hsa-miR-29b-3p	32.36 (1.77)	33.33 (1.23)	0.4028	No Change
bta-miR-30e-5p	28.44 (1.21)	29.07 (0.63)	0.3913	No Change
ssc-miR-339-5p	27.22 (1.84)	28.2 (1.47)	0.4371	No Change
hsa-miR-486-5p	27.14 (1.65)	25.01 (1.26)	0.0860	No Change
hsa-miR-532-5p	34.10 (0.72)	33.05 (0.59)	0.0649	No Change
hsa-miR-92a-3p	26.42 (1.53)	25.46 (0.98)	0.3313	No Change
bta-miR-345-3p	36.27 (1.52)	35.80 (1.21)	0.6457	No Change
hsa-miR92b-3p	35.19 (0.78)	33.93 (1.11)	0.1126	No Change
hsa-miR-17-5p	28.22 (0.50)	27.45 (0.91)	0.1886	No Change

[#]*Bos taurus* (bta); *Sus scrofa* (ssc); *Homo sapiens* (hsa). Where the non-bovine homologue is listed it is to match the commercially available assay and the miRNA targets are 100% identical.

Discussion:

This preliminary study has used samples from infection trials with BoHV-1 and BVDV-1, the two viruses commonly associated with BRD in Australia to evaluate the expression of miRNA identified as potential biomarkers of viral infection. To date most promising results have been obtained from the samples from cattle infected with BoHV-1. The reasons for this are unknown, as many of the miRNAs were chosen based on a *in vivo* study of BVDV-2 in cattle. While BVDV-1 and BVDV-2 are separate viral species, it was considered reasonable to expect that there may be some overlap in miRNA expression patterns.

One issue which requires further investigation is why the miRNAs were more consistently detected in the Day 0 samples of the BoHV-1 challenged animals compared to the BVDV-1 challenged animals. One of the basic assumptions of this analysis is that all cattle prior to infection will have a similar miRNA expression profile which represents the "healthy" state. Thus we might expect that the miRNAs detected in the Day 0 samples would be similar, then in response to infection they change. This is a crucial element as without this, paired sera would be required from any animal being assessed. While this is achievable in a trial setting, it would be very difficult to implement in a field setting, if not impossible. Thus having a robust panel of miRNA to define "healthy" is essential for the application of miRNA as biomarkers in the field.

To date this study has focused on miRNA which have been reported to be up regulated in cattle studies. These same studies have also identified miRNA which have been down regulated. While down regulation of a biomarker is equally valid compared to up regulation, it can be more problematic to implement. As an example, if a negative result is obtained then the assay performance needs to be carefully evaluated to ensure the result is valid. While controls are important for any type of assay, they become critical when the result of interest is a negative. However if an up-regulated marker cannot be identified, robust down-regulated marker could still be developed into a test if appropriate controls can be identified.

4.3 Genotyping of cattle for BRD susceptibility/resistance

4.3.1 Genotyping using genomic DNA from bovine nasal swabs

Analysis of primary extracts from the nasal swabs suggested the amounts of nucleic acid were below the level of detection using UV spectroscopy. Subsequent analyses using qPCR suggest the samples did contain some bovine DNA, albeit at low levels

Due the low amounts of DNA in the extracts it was decided to use a genomic amplification protocol to increase the overall DNA content. These protocols are used to increase the amount of nucleic acid in rare samples. The amplification was performed using the REPLI-G kit. Subsequent analysis using UV spectroscopy confirmed substantial increases in the amounts of DNA in the samples. The amplification of DNA in the samples was successful, with the UV spectroscopic analysis demonstrated an increase in all samples. However, subsequent qPCR analysis suggested the amount of bovine DNA had decreased as the Ct value increased in the samples analysed (Table 4.4).

Table 4.4 Analyses of DNA extracts from nasal swabs for SNP analysis.

	Original DNA extractions		Amplified DNA extractions	
Sample	DNA concentration (ng/μL)	qPCR Ct	DNA concentration (ng/μL)	qPCR Ct
Nasal Swab 1	0	31.3	Not done	36.3
Nasal Swab 2	0	26.0	1012	Not done
Nasal Swab 3	0	23.8	1215	27.4
Nasal Swab 4	0	24.3	907	Not done
Nasal Swab 5	0	26.8	918	Not done
Nasal Swab 6	0	24.9	991	Not done
Nasal Swab 7	0	24.6	935	Not done
Nasal Swab 8	0	23.7	964	Not done
Nasal Swab 9	0	29.6	944	Not done
Nasal Swab 10	0	29.1	1017	Not done

Neogen was not able to obtain any useful information from the interrogation of the amplified samples using the bovine 770K SNP chip.

Discussion:

Very low amounts of bovine genomic DNA were obtained from the nasal swabs for bovine SNP chip analyses. Amplification of the amount of DNA in these extracts was successful, however the proportion of bovine DNA appeared to decrease. While the reasons for this are unclear, it maybe due to the preferential amplification of other sources of DNA in the samples, such as bacteria. The much smaller sizes of bacteria genomes compared to the bovine genome, probably mean they are present in much higher numbers in nasal swabs, thus during the molecular interactions the amplification reaction the bacterial genomes dominant the reaction.

Interrogation of these amplified DNA extracts using the Illumina high density SNP array did not yield any results. The primary reason for this is considered to be the low levels of bovine DNA in the

amplified extracts. It is feasible that sufficient bovine epithelial cells could be collected using a nasal swab to facilitate genotyping, it would need to be collected specifically for this purpose. In contrast the nasal swabs collected for the NBRDI were to determine the presence of pathogens, not to sample the epithelial cells.

Many tissue samples (lung and trachea) were collected during the NBRDI and these would likely provide sufficient quantities of DNA for analysis using the bovine SNP chip. However, the tissue collection only includes samples from cattle known to have died from BRD. Thus, any SNP analysis would be difficult to interpret without data from samples collected healthy cattle collected under similar conditions. It is not possible to substitute in samples from healthy cattle, as it is not known how they would have performed with respect to BRD under feedlot conditions, that is the phenotype (BRD) cannot be defined. As a consequence, such an approached would not be able to classify cattle with differing susceptibility or resistance to BRD and therefore reduce the likelihood of identifying SNPs associated with BRD.

4.3.2 Genotyping using genomic DNA from stored blood samples

The analysis of the preliminary DNA extracts demonstrated that seven of eight blood clots yielded genomic DNA (Fig. 4.3). However there was a wide variation in the yields and purity of the DNA (Table 4.5). Five samples yielded the amount of DNA required for genotyping recommended by Neogen (1 to 2 µg).

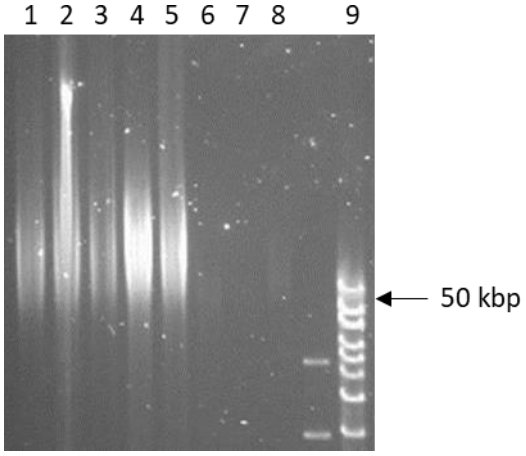

Fig. 4.3.2.1 Pulse field gel electrophoresis analysis of bovine DNA samples extracted from stored blood clots. Lane 1 Animal number 1383; Lane 2 Animal number 0883; Lane 3 Animal number 0840; Lane 4 Animal number 0814; Lane 5 Animal number 0993; Lane 6 Animal number 103; Lane 7 Animal number 240; Lane 8 Animal number 162; Lane 9 High molecular weight marker.

Table 4.5 Details of the selected samples for genotyping using the GGP Taurine 50K SNP Chip

Animal Number	Concentration (ng/mL)	Ratio Absorbance 260/280	Suitable for genotyping	Genotyping successful
1383	350	1.88	Yes	No
0883	223.5	1.87	Yes	Yes
0840	438.2	1.86	Yes	Yes
0814	144	1.89	Yes	Yes
0993	235.7	1.84	Yes	Yes
0103	4.3	2.29	No	Not done
0240	5.2	1.51	No	Not done
0162	3.9	2.18	No	Not done

Following the successful genotyping of four of the five animals in the preliminary analysis, the remaining samples from Project No B.FLT.0164 were extracted using the same procedure. This block of samples consisted of 302 blood clots from BRD cases and controls. Of these, genomic DNA was successfully isolated from 192 of these samples, which are comprised of 101 and 91 from BRD cases and controls, respectively. Overall 186 samples have yielded genomic DNA that appears to be suitable for genotyping.

One hundred and eighty six animals of known BRD status (50% controls, 50% BRD affected) from B.FLT.0164 were genotyped by Neogen using the Trop-Beef V2 assay, which includes 50045 genome wide SNP (after quality control, with genotypes with QC score <0.6 set to missing, monomorphic SNP excluded and SNP with all heterozygous calls excluded). The genotypes of all animals were imputed to 709768 SNP on the Bovine HD array (following further QC) using a multi-breed reference panel of 4065 cattle, and the Eagle program for phasing and Minimach3 for imputation (Das et al., 2016; Loh et al., 2016). The GCTA program was then used to build the genomic relationship matrix among all the animals using the 709768 SNP, then estimate the proportion of variance associated with the SNP (genomic heritability), and finally estimate genomic estimated breeding values (GEBV) using the best estimates of the variance components (Yang et al., 2011). A genome wide association study was also performed, fitting one SNP at a time to investigate evidence for any mutations of large effect.

Using this dataset, a genomic heritability for BRD resistance was estimated to be 0.52 (given a prevalence of approximately 1% of cases), albeit with a large standard error of ±0.2. This is encouraging, suggesting that progress could be made by selecting for BRD resistance if GEBV were available. GEBV were produced for all animals in the analysis, demonstrating a pipeline for GEBV for BRD resistance has been developed.

A preliminary genome wide association study identified SNP with moderate effect on BRD resistance on chromosomes eight and eleven (Fig. 4.3.2.1). These SNP should be validated in a large cohort of animals with defined BRD status.

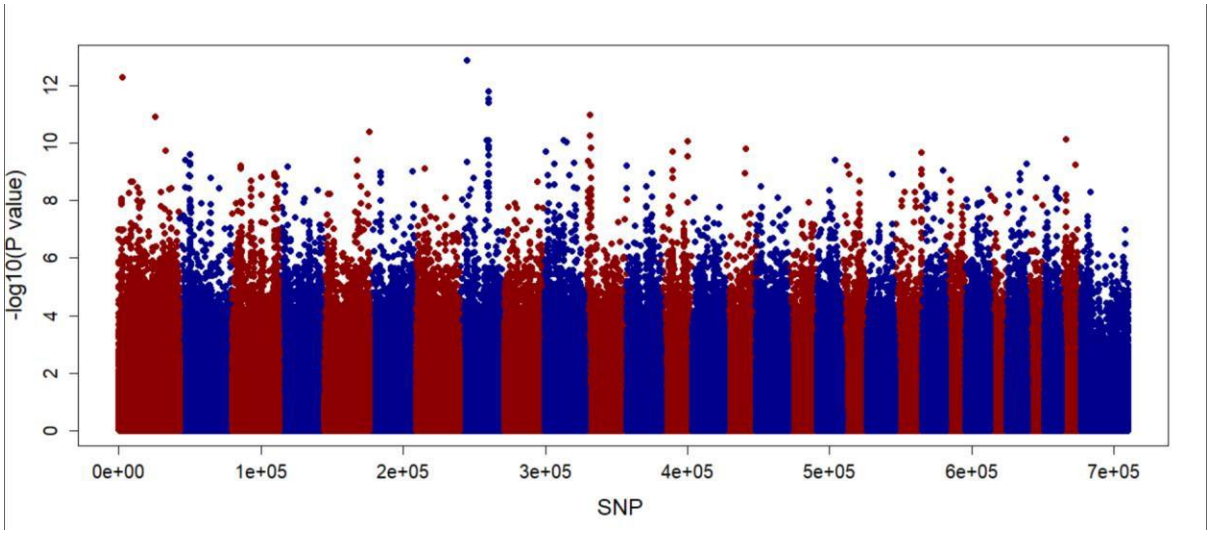

Figure 4.3.2.1. Genome wide association study for BRD resistance. SNPs are ordered by genome position on the x axis, the y axis is significance expressed as -log10(P value). Odd numbered chromosomes are coloured in red, even numbered chromosomes in blue.

4.3.3 Genotyping of third party feedlot population

As a consequence of closure of the Queensland/New South Wales border, plans to collect a series of induction samples from feedlot cattle (n=10,000) was not able to proceed. An alternative population of cattle was identified that had been involved in another feedlot study.

The population consistent of 1,666 steers that were distributed across three commercial feedlots. The BRD status of each animal was designated as BRD or no BRD based on the treatment records provided feedlots. They were all long feed steers with an average days on feed of 222 days. Samples for genotyping were taken at induction or soon thereafter for Feedlot 1 and 2, while at Feedlot 3 samples were collected in the chiller.

The provided genotyping data was used to build genetic models for the heritability of BRD treatment based on the assigned phenotype of each animal. Heritability of BRD treatment was higher from the BayesR approach than from the GREML approach, Table 4.3.3.1, but was broadly consistent with estimates from other studies that have reported values from 0.07 to 0.29 as recently reviewed by Neibergs (2020).

Table 4.3.3.1. Estimates of BRD treatment from GREML and BayesR methods.

Method	Heritability	Standard error
GREML	0.07	0.05
BayesR	0.20	0.04

The GWAS approach for mapping mutations affecting BRD demonstrated some evidence for a peak in the middle of Chromosome 5, and at the start of Chromosome 7, although no associations exceeded the genome wide threshold (Fig. 4.3.3.1A).

The BayesR analysis also indicated little evidence for mutations of very large effect on any chromosome, with most SNP having a similar probability of inclusion in the model. One SNP on Chromosome 5, and another on Chromosome 23, had the largest probabilities of inclusion, though these were not particularly elevated (Fig. 4.3.3.1B).

Conclusion

This was limited evidence for mutations of large effect affecting BRD treatment. However genomic predictions, using all genome wide markers simultaneously, were moderately accurate when a BayesR approach was used. Expansion of this approach to include more animals and a diversity of breeds is recommended to successfully develop a GEBV for BRD resistance for the Australian beef industry.

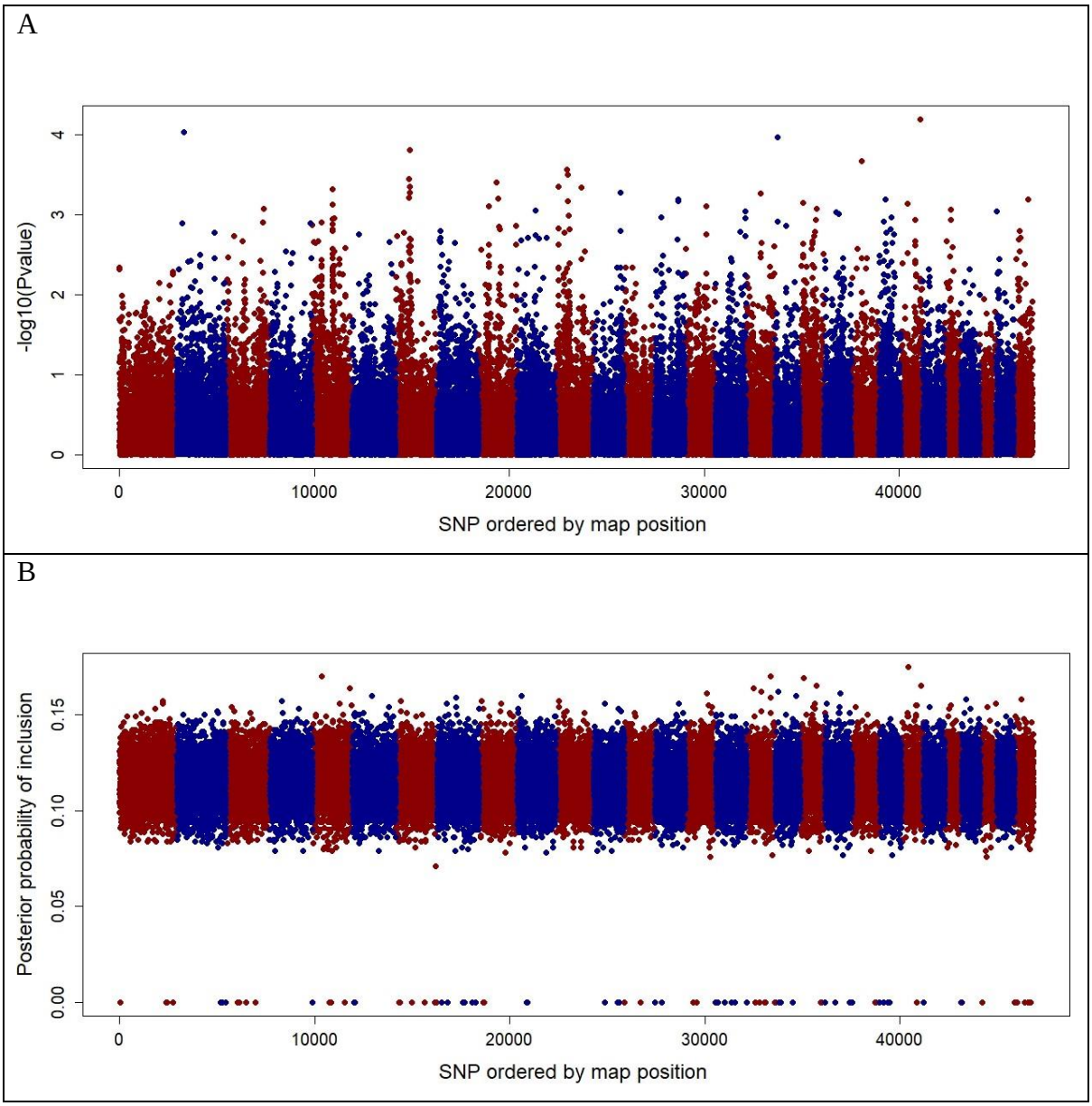

Figure 4.3.3.1. Genotyping to identify associations with risk of being treated for BRD. A. Genome wide association study fitting one SNP at a time, and B. Posterior probability of inclusion in the BayesR prediction model for 46826 SNP. Odd chromosomes are coloured in red, even chromosomes are coloured in blue.

Genomic predictions from BayesR analysis were more considerably accurate from GBLUP, Table 4.3.3.2.

Table 4.3.3.2. Accuracies of genomic prediction for BRD treatment from GBLUP and BayesR methods.

Method	Accuracy	Standard error
GREML	0.11	0.02
BayesR	0.23	0.01

The accuracies we observed for GBLUP were very similar to accuracies of genomic prediction observed in a comparable number of Holstein-Friesian cattle by (Hoff et al., 2019).

Accuracies of genomic prediction from BayesR were considerably greater than from GBLUP, suggesting this is the method of choice for deriving GEBV for BRD treatment. Accuracies of 0.23 are quite useful for selection, for example in sheep, selection on GEBV with similar accuracies for eating quality has resulted in considerable gains.

The accuracies were also very much in line with theoretical predictions we had made earlier, given the size of the reference set and incidence of BRD, Figure 4.3.3.3. This suggests that even more accurate GEBV could be derived with larger reference sets.

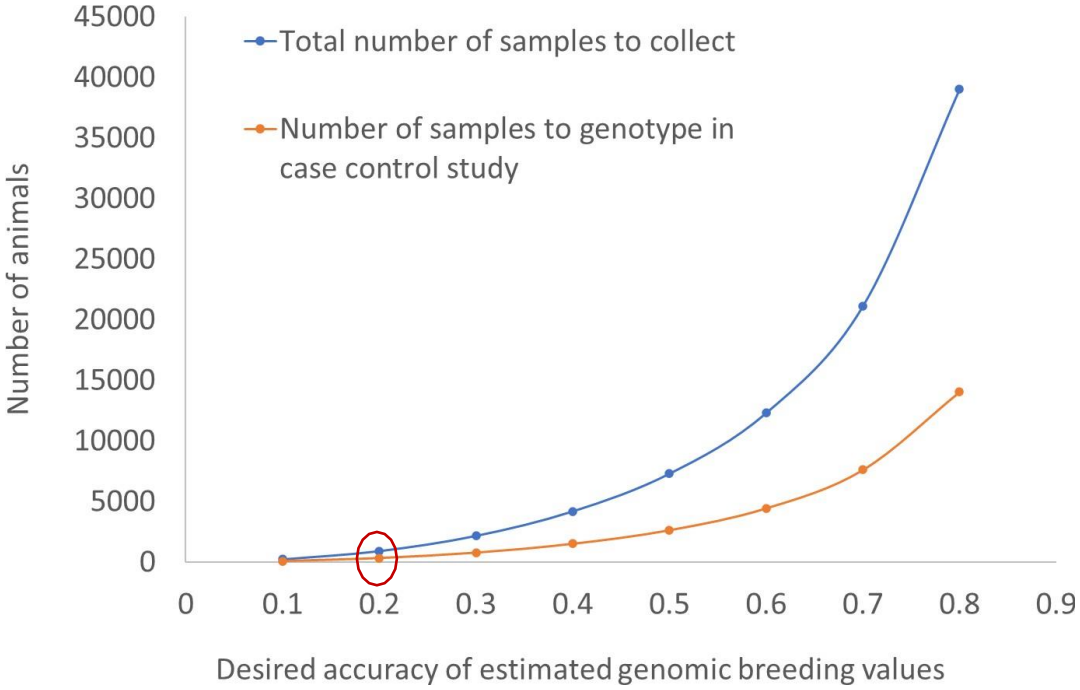

Figure 4.3.3.3. Estimates of number of samples required to achieve a desired accuracy of GEBV for BRD resistance, based on deterministic predictions of Lee and Wray (2013). The red circle indicates the current size of the reference/accuracy achieved.

4.4 Characterisation of the virome in respiratory tissues of BRD deaths

4.4.1 The DNA virome

Bovine parvovirus PCR analyses: All the samples tested (n=110) with the bovine parvovirus qPCR assay were non-reactive, except for one. The one sample which reacted with the bovine parvovirus qPCR assay, yielding a Ct value of 7.33. The Ct value of the adenovirus control was 23.4. The amplification curve of the reactive sample was not logarithmic and had an atypically amplification curve. To determine if the observed reactivity was spurious or not, the reactive sample was re-tested using the conventional parvovirus PCR. The sample did not react in this assay, with no evidence of any amplicon (data not shown). As a result, it was concluded that none of the samples tested contained any evidence of bovine parvovirus.

Bovine adenovirus PCR analyses: Many of the samples tested (n=110) tested with the bovine adenovirus PCR assay yielded multiple amplicons. Fig. 4.4 shows the range of amplicons from a typical assay from 56 samples. Despite the range of amplicons generated four samples (Number 75, 84, 87 and 88) contain amplicons in the expected size range of approximately 500 bp. These amplicons were excised from the gel and directly sequenced.

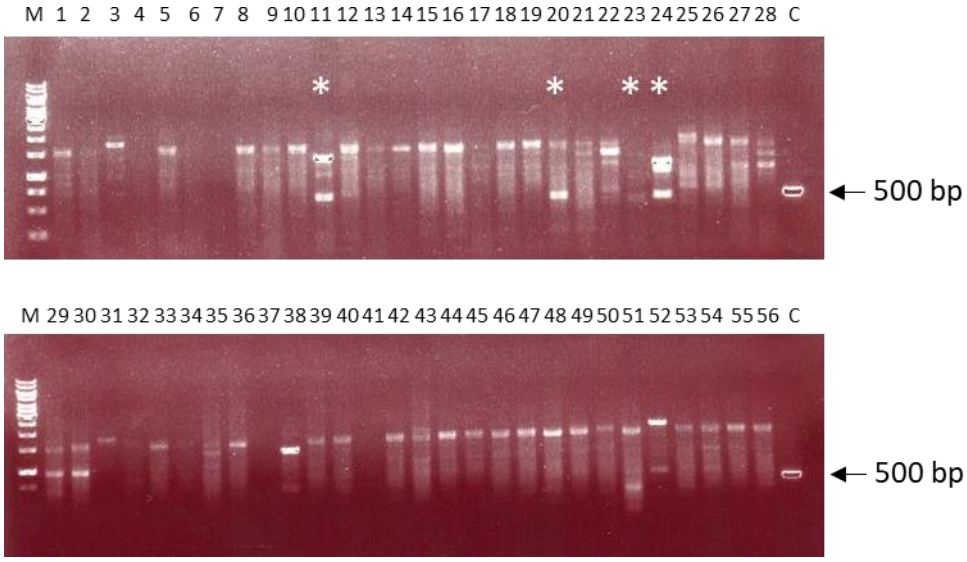

Figure 4.4 Conventional PCR analyses using consensus primers adenovirus of tissue sample extracts from cattle that died from bovine respiratory disease. A control amplicon (C) from a known adenovirus was included to illustrate the expected size (500 bp). Lanes 1 to 28: Sample Extracts 55 to 92; Lanes 29 36: Sample extracts 93 to 100; Lane 37: No template control; Lanes 38 to 56: Sample extracts 101 to 119. Asterix denote samples with amplicons of the expect size 500 bp in samples 75, 84, 87 and 88. Lane M: Molecular weight marker.

Sample 75 amplicon: Sequence analysis of the putative adenovirus amplicon from Sample 75 yield excellent sequence data (data not shown). Database sequence comparisons with this sequence did not identify any sequence matches with adenoviruses or any other viral species. At the nucleotide sequence level there no significant matches to the database using the blastN algorithm.

When compared to the polypeptide sequence database using the blastX algorithm, a 100% sequence identity was observed with a hypothetical protein from *Porphyromonas levii*. The second ranked database match was with a polypeptide from *Chlamydia trachomatis* known as "trigger factor" with 91% amino acid sequence similarity. The bacterial polypeptides referred to as "trigger factor" are molecular chaperones involved in translation. *Porphyromonas levii* has been isolated from the bovine rumen. Thus, it seems likely that the amplicon is a non-specific PCR amplicon generated from the extracted nucleic acid, due to either post-mortem contaminated or contamination during sample collection.

Sample 84 amplicon: The direct sequencing data from amplicon had a 100% at the nucleotide level with bovine adenovirus type 3 DNA polymerase gene.

Sample 87 amplicon: This amplicon could not be directly sequenced. It was subsequently cloned and sequenced. The resulting nucleotide sequence had a significant match to *Bibersteinia trehalose*, 98% at the nucleotide level. *Bibersteinia trehalose* is known to be associated with BRD in feedlot cattle.

Sample 88 amplicon: This amplicon could not be directly sequenced. It was subsequently cloned and sequenced. While excellent sequencing data resulted from the selected clone, no significant matches were identified through blastN searching.

Sequence analyses of other prominent amplicons in Figure 1 did not yield any virus specific sequences (data not shown).

Bovine herpesvirus PCR analyses: Samples previously testing positive for bovine herpesvirus 1 (n=58) were excluded from these analyses. While the presence of other herpesviruses cannot be excluded from these samples, the amount of bovine herpesvirus 1 in these samples (based on qPCR analyses) would have made it extremely difficult to identify other herpesvirus species. The consensus herpesvirus PCR assay was shown to amplify the expected amplicons from isolates of bovine herpesvirus 1 and bovine herpesvirus 5. However, the assay did not amplify any amplicons from the samples included in part of the study (n=52).

Discussion:

This study has analysed extracts from tissue samples collected from cattle that died of BRD during the National Bovine Respiratory Initiative (NBRDI) for the presence of viruses with DNA genomes to evaluate associations with BRD. Previous analyses of these samples identified that 58 of these tissue extracts were positive for bovine herpesvirus 1. The current analyses identified one sample that was positive for a virus with a DNA genome, bovine adenovirus 3. No evidence was found indicating any of the sample extracts contain bovine parvovirus genetic material.

Previous analyses of these samples identified that all samples, except for one, contained genetic material from at least one of the eight pathogens (four viruses and four bacteria) they were tested for. While not specifically analysed the samples most likely also contain genetic from the bovine host. Consequently, the detection of unknown viral pathogens with DNA genomes was considered to be problematic using next generation sequencing (NGS) methodologies. Particularly as many of the samples contain high quantities of bacterial DNA as demonstrated by the analyses conducted during the NBRDI. Given there are relatively few DNA viruses associated with respiratory disease in cattle it was decided to use a more focused technology to identify other viruses. As a result, several PCR assays were used to interrogate the samples. Some of these assays utilised PCR primers with sequences based on very highly conserved amino acid motifs in enzymes which are essential to virus replication. For adenovirus and herpesvirus this enzyme is DNA polymerase which is essential to generate new

copies of the viral genome. While the amino acid motifs within the DNA polymerases of these viruses are highly conserved, the encoding DNA is less constrained and can mutate if the functionality of the motif is not altered. As a result, to use these amino acid motifs in PCR assays they are reverse translated into all the possible codons for each amino acid, yielding highly degenerate PCR primers (see primer sets described for the adenovirus and herpesvirus consensus assays). The potential difficulty in using these types of primer sets was clearly evident in the amplicon profiles generated using the adenovirus primers (Fig. 4.4). While varying conditions were utilised to improve the specificity of this assay, they did not increase the frequency of generating amplicons of the expected size (data not shown).

The "new" virus detected in this study was bovine adenovirus 3. When the data generated in this study is combined with the existing dataset for these samples, it suggests the DNA virome of BRD samples in Australia is limited to bovine herpesvirus and bovine adenovirus. This conclusion is consistent with recent studies which aimed to describe the diversity of viruses associated with BRD affected animals in North America.

Ng et al (2015) examined the respiratory virome of young dairy cattle using nasopharyngeal and pharyngeal recess swabs from healthy and disease animals using next generation sequencing (NGS) methodologies. Specific qPCR assays were designed to detect those viruses (two DNA and six RNA) for which reasonable amounts of sequencing data was identified in the NGS datasets. The qPCR assays were then used to test for these viruses in samples from healthy and diseased calves to test for associations with disease. In respect to DNA viruses, the study evaluated bovine adenovirus 3 and parvovirus 2, of these only the first virus was associated with BRD.

The current study confirmed the presence of adenovirus 3 in one sample by amplicon sequencing and amplicons indicative of this virus were identified in a further three samples. The development of a specific qPCR assay for this virus was undertaken to re-evaluate the presence of this virus in the study tissue extracts. It is expected that the qPCR assay will have increased sensitivity that will facilitate the identification additional samples containing this virus. It would be of interest to use the adenovirus qPCR assay to evaluate the presence of bovine adenovirus 3 in the NBRDI hospital samples.

Another interesting finding of Ng et al (2015) was the failure to detect any of the viruses commonly associated with BRD in Australia, including bovine herpesvirus 1, bovine viral diarrhoea virus 1, bovine parainfluenza 3, bovine respiratory syncytial virus and the more recently associated bovine coronavirus. All of these viruses have been detected in the lung tissues used in the current study. It is not readily apparent why these viruses were not detected by Ng et al. (2015), there are however obvious differences in the study populations including, dairy cattle compared to beef cattle, young (27 to 60 days age) animals compared to older animals (18 to 24 months of age) and the type of sample upper respiratory tract swabs compared to tissue (lung and tracheal samples).

In a second study, Mitra et al. (2016) utilised NGS to compare the viromes of cattle affected and unaffected by BRD. The study animals were feedlot steers located either in the USA or Mexico. The study identified a wide range of viruses from both sick and health animals. Interestingly, some viruses were identified exclusively in health animals (e.g. bovine parainfluenza virus 3) and others exclusively in sick animals (e.g. bovine viral diarrhoea virus 2 and bovine torovirus). Mitra et al. (2016) did detect the viruses typically associated with BRD, however the frequency of detection was low. Despite the wide range of viruses identified the study did not identify any significant associations between disease and the viruses detected.

Consistent with published studies, the results for these analyses suggest the repertoire of viruses associated with BRD that have DNA genomes is limited. The current study suggests that bovine

adenovirus 3 is present in lung tissues of cattle which have died from BRD in Australian feedlots. The development of real-time qPCR assay for bovine adenovirus 3 was achieved.

4.4.2 The RNA virome

Examination of the literature identified five publications since 2015 which described metagenomic studies of samples collected from BRD cases and controls. Brief summaries of these studies review are given below:

Study 1: Ng et al. (Ng et al., 2015) this study utilised Californian dairy calves (27-60 days of age, specific breed is not mentioned but probably Holstein which dominant the Californian dairy industry). The study collected nasopharyngeal and pharyngeal recess swabs from BRD cases (n=50) and controls (n=50). Next generation sequencing (NGS) was performed on 10 pooled samples (five cases in each) and used the data to identify what viruses were present. The study subsequently used qPCR to determine the presence/absence of the viruses identified by NGS. Sequence reads corresponding to at least 12 viruses were identified in the NGS data (Table 4.6). The number of sequencing reads for these viruses were highly variable, with over 11,100 reads identified for bovine rhinitis A virus compared to 19 sequence reads for bovine herpesvirus 6. The qPCR data was then used to determine the association of these eight viruses with BRD in a case-control analyses. Three viruses were identified as being significantly associated with the BRD cases (Table 4.6). This study did not detect serval of the viruses typically associated with BRD including, bovine herpesvirus 1 (BoHV-1), bovine viral diarrhoea virus 1 (BVDV-1 or 2), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV-3) or bovine coronavirus (BoCV).

Table 4.6 The most commonly identified viruses identified in Californian dairy calves with BRD by next generation sequencing. The detection of selected virus in BRD cases and controls by qPCR is also shown, including associations with BRD, where tested. The type of genome for each virus is also shown. Significant associations are shaded. Modified from Ng et al. (Ng et al., 2015).

		Number positive (%)		
Virus	Genome	Case	Control	BRD Association (*p* value)
Bovine adenovirus 3	DNA	24 (48%)	5 (10%)	< 0.0001
Bovine adeno-associated virus	DNA	NT[#]	NT[#]	NT[#]
Bovine rhinitis A virus BSRI4	RNA	12 (24%)	4 (8%)	0.009
Bovine rhinitis B virus BSRI2	RNA	4 (8%)	1 (2%)	0.36
Bovine rhinitis B virus BSRI3	RNA	5 (10%)	1 (2%)	0.2
Bovine influenza D virus BSRI5	RNA	7 (14%)	0 (0%)	0.012
Bovine astrovirus BSRI1	RNA	4 (8%)	0 (0%)	0.117
Picobirnavirus BSRI1	RNA	1 (2%)	0 (0%)	1
Picobirnavirus BSRI2	RNA	NT[#]	NT[#]	NT[#]
Bovine parvovirus 2 BSRI	DNA	4 (8%)	1 (2%)	0.36
Bovine herpesvirus 6	DNA	NT[#]	NT[#]	NT[#]

[#] Not tested

Study 2: Mitra et al. (Mitra et al., 2016) collected a 103 nasal swabs from steers (227-363 kg) held in US (n=4) and Mexican (n=6) feedlots, with the sampled animals being classes as either a BRD case or control. The study reported the detection of over 20 types of viruses in the samples when analysed using NGS (Table 4.7). Following the identification of the viruses present in the samples the study subsequently used the sequencing data to determine associations with viruses and BRD cases (Table 3). At the widely utilised level of significance, $p < 0.05$, no significant associations between the presence of virus and BRD were detected (Table 3). However, using an adjusted level of significance, $p < 0.15$, two viruses Influenza D virus (p=0.134) and BRSV (p=0.126) were considered to be potentially associated with BRD. As the odds ratio for Influenza D virus was >1 (OR 2.94; CI95%0.71-12.1, p=0.134) compared to BRSV where the odds ratio was <1 (OR 0.18; CI95%0.02–1.64, p=0.126), a qPCR assay was design to test for it. The authors reported that the qPCR assay results were identical to the sequencing analysis for the Influenza D virus. Briefly, it was reported that eight and three samples tested positive from BRD cases and controls respectively by qPCR. Comparison of the qPCR results using the cycle threshold (Ct) values, the BRD cases Ct values ranged from 20.3 to 36.6 while the range for the healthy animals was 34.6 to 37. These ranges were reported to be significantly different (p=0.04).

Table 4.7: The viruses identified in Mexican and USA feedlot cattle by next generation sequencing of nasal swabs. The number of positive feedlots for each country are shown, along with the number of positive samples from BRD affected or control animals. Modified from (Mitra et al., 2016).

	Mexico			USA			
	Positive Feedlots (n=6)	Positive Cattle		Positive Feedlots (n=4)	Positive Cattle		BRD Assoc
Virus		BRD (n=27)	Control (n=27)		BRD (n=20)	Control (n=20)	*p*
Influenza D virus	4	8 (30%)	1 (4%)	1	–	2 (10%)	0.134
Bovine rhinitis A virus	4	5 (18%)	7 (27%)	4	18 (90%)	19 (95%)	0.466
Bovine rhinitis B virus	5	7 (26%)	3 (12%)	4	4 (20%)	8 (40%)	NT[#]
Enterovirus E	3	2 (7%)	2 (8%)	1	3 (15%)	–	0.265
Enterovirus F	2	1 (4%)	1 (4%)	1	1 (5%)	–	0.579
Ungulate bocaparvovirus 6	4	5 (18)	3 (12%)	2	1 (5%)	3 (15%)	0.968
Ungulate Tetraparvovirus 1	1	–	1 (4%)	2	5 (25%)	1 (5%)	0.721
BRSV	–	–	–	2	1 (5%)	4 (20%)	0.126
BVDV1	–	–	–	3	3 (15%)	1 (5%)	NT[#]
BVDV2	1	1 (4%)	–	–	–	–	NT[#]
BoCV	2	3 (11%)	–	4	11 (55%)	9 (45%)	0.212
Bovine nidovirus	2	1 (4%)	3 (12%)	–	–	–	0.324
BPV3	2	3 (11%)	–	1	4 (20%)	4 (20%)	0.529
BAdV3	1	3 (11%)	5 (19%)	1	–	1 (5%)	0.290
Bovine adeno-associated virus	1	3 (11%)	5 (19%)	–	–	–	0.448
BPIV3	–	–	1 (4%)	1	–	1 (5%)	NT[#]
Bovine torovirus	1	1 (4%)	–	–	–	–	NT[#]
BHV1	1	1 (4%)	–	–	–	–	NT[#]
Mouse salivary gland Hypertrophy virus	2	4 (15%)	–	–	–	–	0.984
Single-stranded circular DNA viruses	4	4 (15%)	4 (15%)	2	3 (15%)	–	NT[#]
Picobirnavirus	16	–	1 (4%)	–	–	–	NT[#]

[#] Not tested

Study 3: Zhang et al. (Zhang et al., 2019a) examined the viral metagenome of Canadian beef cattle using metagenomics and potential associations with BRD using swabs and tracheal washes from BRD cases (n=58) and controls (n=58). As with previous studies a wide range of viruses was detected in these samples with a total of 21 viruses being identified (Table 4.8). Zhang et al. (Zhang et al., 2019a) identified several significant associations between viruses and the risk of cattle being diagnosed with BRD. Two of the viruses, BRSV and BoCV were shown to increase the risk of BRD by 3.8 and 7.4 fold respectively. These are two of the viruses historically associated with BRD. Influenza D virus and bovine rhinitis B virus were both identified in the previously described studies (Table 2 and 3). It was also identified as being significantly associated with BRD by Ng et al. (Ng et al., 2015).

A surprising result of the Zhang et al. (Zhang et al., 2019a) study is that two viruses were negatively associated with the BRD cases (Table 4). The odds ratio for bovine nidovirus was 0.078 (95% CI 0.021–0.29, p=0.000), meaning that BRD cases were 92.2% less likely to test positive for this virus compared to the control animals. Similarly, BRD cases were 70.4% less likely to be positive for ungulate bocaparvovirus 6 (Odds ratio 0.296, 95%CI 0.108–0.814, p=0.019), compared to healthy animals.

Another interesting finding of this study was weak correlations between the viruses detected in swabs and tracheal washes from the same animals, suggesting that sampling location may influence the results of studies.

Table 4.8 Summary of the viruses detected by metagenomic sequencing in samples from Canadian feedlot cattle. The identity of the viruses are shown with the association with BRD as odds ratios and 95% confident intervals (CI) are shown, determined using case-control samples, where tested. Odds ratios with a p value <0.05 were considered statistically significant. Significant associations are shaded. Modified from Zhang et al. (Zhang et al., 2019a).

Virus	Odds Ratio	95% CI	p value
Influenza D virus	6.2	1.4–26.3	0.015
Influenza C virus	NT[#]	NT[#]	
Bovine rhinitis B virus	3.8	1.3–11.8	0.020
Bovine rhinitis A virus	5.7	0.98–32.6	0.052
Enterovirus E	4.26	0.70–25.74	0.11
BRSV	13.4	1.5–123.9	0.022
BPIV3	NT[#]	NT[#]	
BoCV	7.4	1.4–40.4	0.021
Bovine nidovirus	0.078	0.021–0.29	0.000
Bovine parvovirus 2	3.289	0.7–15.9	0.137
Bovine adeno-associated virus	NT[#]	NT[#]	
Ungulate tetraparvovirus 1	1.9	0.8–4.4	0.140
Ungulate bocaparvovirus 6	0.296	0.108–0.814	0.019
BVDV-1	NT[#]	NT[#]	
Bovine hepacivirus	NT[#]	NT[#]	
BAdV3	0.34	0.025–4.6	0.41
Bovine astrovirus	NT[#]	NT[#]	
Single stranded circDNA virus	NT[#]	NT[#]	
WU polyomavirus	0.42	0.08–2.19	0.30
Paramecium bursaria chlorella virus	NT[#]	NT[#]	
Human papillomavirus type 40	NT[#]	NT[#]	

[#] NT, Not tested

Study 4: Zhang et al. (Zhang et al., 2019b) explored the metagenomics of lung tissues (n=130) collected from diagnostic submissions from beef cattle (n=118) and dairy cattle (n=12) in central Canada. Lung samples were analysed using viral metagenomes, qPCR (BoHV-1, BRSV, BPIV3, BVDV, BoCV and *Mycoplasma bovis*), bacterial isolation (n=120) (*Mannheimia haemolytica*, *Histophilus somni*, *Pasteurella multocida* and *Bibersteinia trehalosi*) and also the histopathology of the lung tissues. As with the previously described studies, a wide range of viruses was detected in the metagenomic analyses, including several of the viruses commonly associated with BRD, such as BRSV, BPIV3, BAdV, BVDV-1 and BoCV (Table 4.9). *Mannheimia haemolytica* was clearly the most frequently identified pathogen, isolated from 48 of the samples (Table 5) and was significantly associated with fibrinous bronchopneumonia (Zhang et al., 2019b).

None of the viruses detected were significantly associated with any of the types of lung pathology identified in the study. In contrast, *M. haemolytica*, *H. somni* and *P. multocida* were positively associated with fibrinous bronchopneumonia, suppurative bronchopneumonia and bronchopneumonia/bronchointerstitial pneumonia respectively ($p<0.05$) (Zhang et al., 2019b).

The study also explored associations between multiple pathogens on a sample by sample basis for those samples (n=120) that were tested for all pathogens. *M. haemolytica* was more likely to be the only pathogen in a sample, while *H. somni, P. multocida and M. bovis* were more likely to be present with one or more of the other pathogens.

Table 4.9 Summary of the virological (n=130), bacteriological (culture, n=120) of lung samples from Canadian cattle. The number of positive samples for the pathogen of interest are shown with the percentage in parenthesis are shown as a function of the lung pathology identified in the lungs. Adapted from Zhang et al. (Zhang et al., 2019b).

	Lung Pathology[#]					
Pathogen	**FBP**	**SBP**	**IP**	**BIP**	**Bronchiolitis**	**BP & BIP**
Virology^						
BRSV	4 (3.1)	1 (0.8)	2 (1.5)	1 (0.8)	1 (0.8)	2 (1.5)
BPIV3	1 (0.8)	0 (0.0)	0 (0.0)	1 (0.8)	0 (0.0)	1 (0.8)
UTPV1	4 (3.1)	3 (2.3)	0 (0.0)	2 (1.5)	0 (0.0)	2 (1.5)
BPV2	9 (6.9)	3 (2.3)	1 (0.8)	1 (0.8)	1 (0.8)	1 (0.8)
BPV3	0 (0.0)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Bov. astrovirus	1 (0.8)	4 (3.1)	0 (0.0)	0 (0.0)	1 (0.8)	1 (0.8)
BVDV-1&2	5 (3.8)	1 (0.8)	0 (0.0)	0 (0.0)	2 (1.5)	0 (0.0)
Enterovirus E	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
BoCV	4 (3.1)	2 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Virus ND*	39 (30)	13 (10.0)	5 (3.8)	11 (8.5)	3 (2.3)	11 (8.5)
Bacteriology						
H. somni	5 (4.2)	10 (8.3)	0 (0.0)	1 (0.8)	1 (0.8)	2 (1.7)
P. multocida	7 (5.8)	6 (5.0)	1 (0.8)	0 (0.0)	3 (2.5)	7 (5.8)
M. bovis[$]	7 (5.8)	9 (7.5)	0 (0.0)	3 (2.5)	4 (3.3)	4 (3.3)
M. haemolytica	48 (40)	5 (4.2)	0 (0.0)	0 (0.0)	0 (0.0)	4 (3.3)
Bacteria ND*	4 (3.3)	4 (3.3)	4 (3.3)	7 (5.8)	2 (1.7)	7 (5.8)

[#]Type of lung pathology: FBP, fibrinous bronchopneumonia; SBP, suppurative bronchopneumonia; IP, interstitial pneumonia; BIP, bronchointerstitial pneumonia; BP & BIP bronchopneumonia + BIP. ^Only viruses detected in two or more samples are shown. *ND, not detected. [$]Determine by qPCR.

Study 5: Ambrose and Gravel (Ambrose and Gravel, 2017) analysed pools (n=6) of nasal swabs (n=36) collected from Australia feedlot cattle at the time of being treated for BRD, as part of project B.FLT.0225. Similar to other studies a broad range of viruses were detected (Table 6). Two of the viruses commonly associated with BRD, BVDV-1 and bovine coronavirus were detected in this study. As this study did not have access to matched samples from control cattle it was unable to evaluate associations with BRD. However, bovine influenza D, bovine coronavirus, and bovine rhinitis B were shown to increase the risk of cattle having BRD by Zhang et al. (Zhang et al., 2019a) (Table 4). Two other viruses showing significant associations, albethey negative, with BRD risk by Zhang et al. (Zhang et al., 2019a), bovine nidovirus and ungulate bocaparvovirus-6 were also detected in this study (Table 4.10). Sample preparation, sequencing library construction and data methods were suggested as a possible reason for the lack of detection of some viruses regularly associated with BRD such as bovine herpesvirus 1 in this study.

Table 4.10 A summary of the viruses detected by next generation sequencing in nasal swabs of Australian feedlot cattle. Adapted from Ambrose and Gravel (Ambrose and Gravel, 2017).

Virus family	Species	Genome	Summary of results
Orthomyxoviridae	bovine influenza D	RNA	Four of seven genomics fragments detected. High sequence identity (>93%) to known viruses.
Coronaviridae	bovine coronavirus	RNA	Near complete genome. High sequence identity (>98%) to known viruses
Tobaniviridae	bovine nidovirus	RNA	Near complete genome. Comparatively low sequence identity (85%) to known viruses
Flaviviridae	bovine viral diarrhoea virus 1	RNA	Near complete genome. High sequence identity strains Bega (91%) and NADL (80%) from Australia & US respectively.
Picornaviridae	bovine rhinitis A	RNA	Partial genome sequence. Low sequence identity (80%) to known viruses
	bovine rhinitis B	RNA	Partial genome sequence. Low sequence identity (82%) to known viruses.
Parvoviridae	bovine parvovirus-1	DNA	Minor sequence. Low sequence identity (73%) to known viruses.
	bovine parvovirus-3	DNA	Minor sequence. High sequence identity (97%) to known viruses.
	ungulate bocaparvovirus-6	DNA	Minor sequence. High sequence identity (100%) to known viruses.
	bovine bosavirus	DNA	Near complete genome (78%). High sequence identity (>97%) to known viruses.
Polyomaviridae	bovine polyomavirus	DNA	Minor sequence. Medium sequence identity (83%) to known viruses.
Papillomaviridae	bovine papillomavirus 10	DNA	Minor sequence. High sequence identity (99%) to known viruses.
Circoviridae	bovine circular virus	DNA	Partial genome sequence. High sequence identify (85%) to dromedary circular virus, rather than bovine circular virus

The RNA virome of BRD Tissue samples

Analyses of the BRD metagenomic literature identified several RNA viruses that were of interest in the context of the tissues collected from fatal cases of BRD during B.FLT.0225. The samples were tested for the following RNA viruses, bovine influenza D virus, bovine nidovirus, bovine rhinitis virus A and bovine rhinitis virus B. Several samples tested positive for bovine influenza D virus and bovine nidovirus (Table 4.11). No bovine rhinitis virus A or bovine rhinitis virus B was detected (data not shown). As the prior metagenomic studies have identified bovine parvoviruses (DNA genome) in BRD samples, the BRD tissue samples were also test for this virus with no positives detected (data not shown).

The data clearly demonstrates the polymicrobial nature of BRD, as many of the samples tested positive for nucleic acids from one or more viruses (Table 4.11). In regard to the RNA virome of these BRD tissues, viruses with RNA genomes were detected in 52 of the tissues (n=126) analysed. BVDV-1 was the most commonly detected RNA virus with 39 samples testing positive to this virus. On the basis of the Ct values for BVDV-1, 14 of these animals were suspected BVDV-1 persistently infected (PI) animals. The next most frequently detected viruses were BRSV and bovine nidovirus both being detected in nine samples each. Several multiple infections were also detected, with seven and three samples testing positive for two and three RNA viruses respectively (Table 4.11). Interestingly, two of the three samples (samples 79 and 103) with three RNA viruses both contain BRSV and bovine nidovirus (Table 4.11). On the basis of the Ct values for BVDV-1, only sample 79 was suspected of being from a PI animal. Both of these samples were also positive for the DNA virus, BoHV-1, thus four viruses were detected in these tissue extracts (Table 4.11). When including the DNA viruses in these comparisons, five and two samples tested positive for three and four viruses respectively (Table 4.11).

Table 4.11 Summary of the detection of all pathogens where at least one virus was detected from tissue samples (n=126) collected from Australian feedlot cattle and died from BRD. Samples were test for; DNA viruses, Bovine herpesvirus 1 (BHV1), Bovine adenovirus (BAdV) and RNA viruses, Bovine respiratory syncytial virus (BRSV), Bovine viral diarrhoea virus 1 (BVDV1), Bovine influenza D virus (IDV), Bovine coronavirus (BCoV), Bovine Nidovirus (NidV), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm), *Mannheimia haemolytica* (Mh), and *Mycoplasam bovis* (Myb). Samples were also tested for bovine parvovirus (DNA) and bovine rhinitis virus A (RNA) bovine rhinitis virus B (RNA) (data not shown) with none of these detected. The qPCR result is shown for each pathogen with the Cycle Threshold (Ct) values coded as: + 45< Ct >35; ++ 34.9 < Ct >30; +++ 29.9 < Ct >25; ++++ 24.9< Ct >20; +++++ Ct<19.9. Samples testing positive to three or more RNA viruses are shaded. Total number of positives for each pathogen are shown.

Sample	BHV1	BAdV	BRSV	BVDV	BID	BCoV	NidV	Hs	Pm	Mh	Mb
3	+							+++	++		+++++
4		+++		++				++++			+++++
5	+++++							+			
8	+++			+				++	+++		+++++
9	+++				+			++	+		++++
10	+++				++			+	++	+++	+++++
11	++										++++
12	++++			++				+++	++	+	+++++
13				+++				++	++	+++	++++
15	++				+			+++	+		++
18	++++			++				++		++	+++++
19	++++							+++	+	+++	+++++
20	+			++				++	++	++++	+++
23	+++										++++
25	++++							++++	++	+	++++
26	+++							++		++	+++
28	++++			++					+++	+	+++++
29	++								+		++++
31	+++++							+++++	+++	++++	+++++
33				++				++	+	++	+++
34				+++				+++	+++	+++	+++++
35				+++				++	++	++	+++++
36				+++					+	++	++++
37				+++				++		+++	++
38				++				+++	+		+++++
41				+				+	+	++++	+++++
42			++	++++				++	++	+++++	+++++
43	++			++				++	+	+++++	+++
44	++++							+++	+	+++	+++++
45			++					+			+++++
47				++				+			++++
49	++									+	++
50	++++			+				+	+	++	+++
52	+++								++	++	+++++
53	++++							+++	++	+++	+++++
56	+++										+++

Sample	BHV1	BAdV	BRSV	BVDV	BID	BCoV	NidV	Hs	Pm	Mh	Mb
58				++++			++	++++	++	++++	++++
59	++++			++				++++	++	+++	+++++
60	++++							++	++	+	+++++
61				+++				++++			+++++
62	++							++		++	++++
64				++				+			++
67				+++				+++	++	+++	+++
68				++				+++	+	+++	++
69				++++				+++	+	++	++
70			++	++			+	++	++	++	+++
71			+	+				++	+	++	++
72		+		+				+++	+	++	++
73	+++			+				++	+	+++	++++
75	++++							+++	+	++	+++++
76	+++							+	++	++++	+++++
79	++		++	+++			++	+		++++	++
80	+++		+	++				++		++	+++
81	+++			+				+++	+	++	+++
82	++++							+++	+	++++	++++
83	+++										++
84		++++						+++	+	++	+++++
85	++++							++++	++		+++
86	++++							+++	++		+++
87	++++							+++++	++		+++
88	++++							+++	++	+++	+++
89	++++							+++	++	+	+++++
90	+++++							+++	++		+++++
91	++++			+			+	+++	++		+++++
92	++						++	+++++		+	+++
93	+++++				++		+	+++	+++		+++++
94				++++				+++	+		+++
95	+++			+				+	+		+++
96	+++							+++	+		+++
97	+++							++	+		+++
100	++++							+++	++		+++
102	++++							+++	++	+++	+++++
103	+++		++		++		+	+++	+	++	++++
104	+++							++	++		++++
105	++++		+					+++	++	++	+++++
106	+++++							++++		++++	++++
107				++					+	++	+++
113	++++							++	++	++++	+++++
114	++					++			++		+++++
115				+++					++	+	++++
116	++++		++	++++				+++	+++	+++	+++++
117						+++		++++	+	++	++++
118	++			+				++++	+++	+++	+++++

Sample	BHV1	BAdV	BRSV	BVDV	BID	BCoV	NidV	Hs	Pm	Mh	Mb
119	+++										++++
120							+	+	+++	++++	+++
121		+									+++
124								+++		++	++++
125						++		+		+++	++
Total	**58**	**4**	**9**	**39**	**5**	**3**	**9**	**104**	**82**	**76**	**121**

Discussion: As with previously published studies regarding the metagenomics of BRD, the analysis of the BRD tissues collected during B.FLT.0225 presents a complex scenario (Table 4.11). In contrast to the studies published from overseas, several of the viruses historically associated with BRD were the predominant viruses detected, with BoHV-1 and BVDV-1 identified in 58 and 39 samples, respectively. It is estimated that 14 of the BVDV-1 positive tissues came from persistently infected animals. The reason(s) for this finding is not readily apparent, for BoHV-1 it may be due to the published metagenomic studies using preparative methodologies which primarily target viruses with RNA genomes.

One of the interesting findings of the current study is the number of samples which contained genomic material for one or more viruses. The classical model for BRD development is a primary viral infection soon after feedlot induction which is more severe than expected due to a multitude of factors, predisposing cattle to severe secondary bacterial infections. Under this model it might be expected that analyses of tissues from cattle that have died from BRD would be largely free of virus, which is clearly not the case for the samples in this study. The exception to this would be cattle persistently infected with BVDV-1, as these animals would be positive to this virus throughout their lives.

Another interesting trend identified during the literature analysis, was that two viruses, bovine Nidovirus (RNA virus) and ungulate bocaparvovirus 6 (DNA virus), were negatively associated with BRD (Table 4). The authors of this study did not discuss this interesting finding (Zhang et al., 2019a). Testing of the Australian BRD tissue samples did detect bovine nidovirus (Table 4.11). This virus was also detected in samples which contain genetic material from at least two other viruses. The most commonly detected viruses with bovine nidovirus were BoHV-1 and BRSV. The Australian collection of BRD tissue samples have not been tested for ungulate bocaparvovirus 6. It was detected by Ambrose & Gravel during the metagenomic analysis nasal swabs from cattle pulled for BRD treatment in the same study (Ambrose and Gravel, 2017).

Bovine influenza D virus has recently emerged as a potential important respiratory pathogen of cattle (Ferguson et al., 2015). Several BRD metagenomic studies have detected bovine influenza D virus in the analysed swab samples (Ambrose and Gravel, 2017; Mitra et al., 2016; Ng et al., 2015; Zhang et al., 2019a). While of these studies reported a significant association between the detection of bovine influenza D virus and BRD (Ng et al., 2015). However this virus was not detected in a study which examined lung tissues of BRD affected cattle (Zhang et al., 2019b). Bovine influenza D virus was detected in the tissues analysed in the current study, albeit infrequently and at low levels (Table 6). To investigate the role of bovine influenza D virus in BRD, Zhang et al. (Zhang et al., 2019c) conducted cattle infection studies using a dual infection model with this virus and *M. haemolytica*. Calves infected with bovine influenza D virus five days prior to *M. haemolytica*, did not have exacerbated signs of disease or lung pathology compared to calves infected only with *M. haemolytica* (Zhang et al., 2019c).

As the experiment was conducted with healthy calves, the study does not rule a potential role of bovine influenza D virus in the pathogenesis of BRD under field conditions.

Of the RNA viruses detected in the tissues from BRD affected animals, bovine influenza D virus and bovine nidovirus warrant further investigation to determine their associations, if any, with BRD under Australian conditions.

4.5 New diagnostics for viruses identified in fatal BRD cases

4.5.1 A novel DNA virus diagnostic assay – Bovine adenovirus 3

Evaluation of the DNA polymerase (**Dpol) gene as a qPCR target**: Previously, a partial sequence was obtained for the Dpol gene from BAdV-3 gene using consensus primers to PCR amplify from BRD affected cattle. Fig. 1 illustrates the alignment of this sequences to the currently available BAdV-3 Dpol sequences in GenBank Dpol amplicon sequence. In comparison to these sequences the Australian sequence was 100% and 95.9% identical to BAdV-3 strains from Canada and China respectively. High levels of conservation between the Dpol gene sequences were expected as this gene plays a crucial role in the replication of the virus genome.

The second step in the design of a qPCR assay, is to identify segments of highly conserved sequence between 70 and 120 bp in length which is the optimal size of a qPCR amplicon. While there was a high level of identity between these Dpol gene sequences, there were no regions with 100% conservation longer than 64 bp (highlighted sequence in Fig. 4.4). As the length of this sequence was just below the optimal size for a qPCR assay it was evaluated. This evaluation suggested it was unlikely to be a good qPCR amplicon as it contain eight motifs (three to five bp each) of the same base (Fig. 4.4). In total these motifs accounted for 30 bp of the 64 bp of the potential amplicon. This low sequence complexity strongly suggested this amplicon was suitable for use in a qPCR assay, as it would be very difficult to identify sequences suitable for use as the two amplification primers and the dual-labelled probe. Moreover, the limited number of available sequences for the BAdV-3 Dpol gene lead to the conclusion this gene was unlikely to result in a qPCR assay that could be used with high confidence on field samples.

```
PCR amplicon AGAAGCCATCTATGTGTATGACATCTGCGGCATGTATGCCAGCGCCCTCACCCATCCTTT
WBR-1        ---------------------------------------------------------------------------
HLJ0955      ----------------------------------------------------T---------------C--------

PCR amplicon CCCAGCGGGAAAACCTTTGAACCCCTTTGATCGAGCGCTTGCAATTAAAAACTGGCAAGA
WBR-1        ---------------------------------------------------------------------------
HLJ0955      ---------------------------------------------------------------------------A-

PCR amplicon CCGCCTAACCCAGTTGCACAGACCCATTGACTATTTTGATCGCACCCTACTGCCCGCGAT
WBR-1        ---------------------------------------------------------------------------
HLJ0955      ----------GA-----------------------------------------------------G-----------C---

PCR amplicon ATTCACCATTGATGCAGACCCACCCCCAGAAGCATTCTTGGACGTTATACCGCCCTTCTG
WBR-1        ---------------------------------------------------------------------------
HLJ0955      ----------------------------------------------------A---T-----------------------

PCR amplicon CTCCAGAAAAGGAGGTCGCCTCTGCTGGACCAACGAGACACTACGGGGGGA
WBR-1        ------------------------------------------------------------------
HLJ0955      --------------------A-----------------------------------G---T--------
```

Fig. 4.4 Nucleotide sequence alignment of the DNA polymerase gene (Dpol) PCR amplicon from bovine adenovirus 3 (BAdV-3). The PCR amplicon was generated using consensus oligonucleotides for adenovirus Dpol genes. The sequence is aligned to the analogous sequences from the Canadian BAdV-3 strain WBR-1 (AF030154, 2011, complete genome) and the Chinese BAdV-3 strain HLJ0955

(JN381195, complete genome). Identical nucleotide residues in the PCR amplicon are shown as dashes (-). The shaded nucleotides were evaluated as qPCR amplicon.

Evaluation of the hexon gene as a qPCR target: Interrogation of the GenBank sequence database identified that the most widely characterised gene for BAdV-3 was the hexon gene, with four sequences identified. Nucleotide sequence alignment of these sequences indicated the nucleotide identity ranged from 99.8% to 93.1%. Compared to the American prototype BAdV-3 strain, isolated in 1965, the most divergent strain based on the hexon gene was the Chinese BAdV-3 strain HLJ0955 with 93.1% identity. The hexon sequence from the Canadian strain WBR-1 was most similar with 99.8% identity. A 327 bp region of the 2,735 bp hexon gene was identified which had high levels of sequence identity across the four available sequences (Fig 2). While there were the sequences analysed are only from four strains, they represented BAdV-3 isolates from 1965 to 2015, across a reasonably diverse geographic area.

Using qPCR assay designed tools, regions suitable for use as forward and reverse amplification primers and for a dual-labelled hydrolysis probe were identified (Fig 2; Table 4).

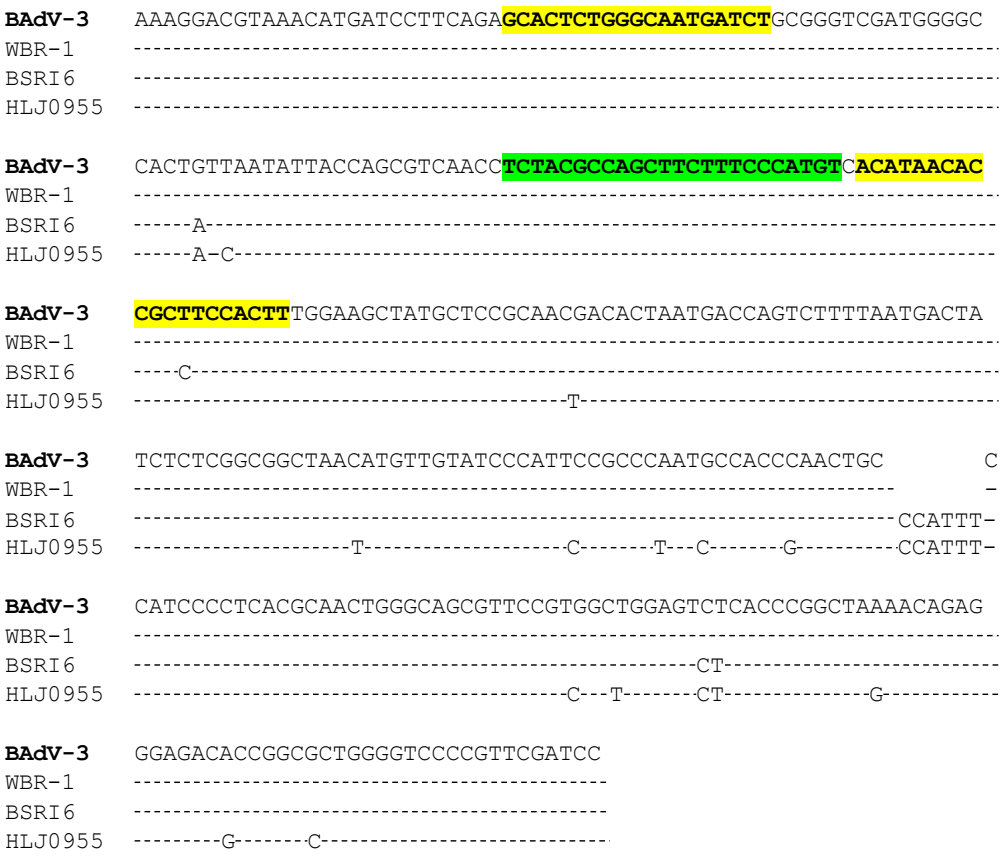

Fig. 4.5 Partial nucleotide sequence alignment of the hexon gene from the American prototype strain (Accession K01264) to the hexon sequences from other GenBank accessions; Canadian BAdV-3 strain WBR-1 (Accession AF030154, complete genome), the American strain BSRI6 (Accession KP264982) and the Chinese BAdV-3 strain HLJ0955 (JN381195, complete genome). Identical nucleotide residues in the PCR amplicon are shown as dashes (-). The shaded nucleotides were evaluated as qPCR oligonucleotides (yellow) and dual-labelled hydrolysis probe (green) components. Identical nucleotide residues in the PCR amplicon are shown as dashes (-).

Table 4.12 Nucleotide sequences of the oligonucleotides for the BAdV-3 qPCR assay

Oligonucleotide	Sequence (5' to 3')	Fluorophore (5')	Quencher (3')
BAdV3-Fwd	GCACTCTGGGCAATGATCT	-	-
BAdV3-Rev	AAGTGGAAGCGGTGTTATGT	-	-
BAdV3-Probe	TCTACGCCAGCTTCTTTCCCATGT	6FAM	BHQ-1

Evaluation of BAdV-3 qPCR assay: To evaluate the performance of the BAdV-3 qPCR assay, total nucleic acid extracts from tissue samples (n=125) generated during B.FLT.0225 were used as templates. The animals from which these tissues were collected were from different feedlots. Of the samples tested four returned positive results (Table 4.13). Samples 4 and 84 returned cycle threshold (Ct) values of 28.1 and 23.0 respectively in the qPCR assay. These Ct values suggest the presence of high levels of BAdV-3 genomic DNA in these samples. In contrast, samples 72 and 121 returned Ct values of 35.7 and 36.1 respectively in the qPCR assay. Suggesting that while these samples were also positive, there were only low levels of BAdV-3 genomic DNA in these samples.

Table 4.13 Summary of pathogen qPCR assays results for the BAdV-3 positive samples identified in this study. The qPCR assay results for each assay are shown as positive (+) or negative (-).

	Pathogen[1]								
Sample	BAdV-3	BCoV	BoHV-1	BRSV	BVDV-1	Hs	Pm	Mh	Mb
4	+	-	-	-	+	+	-	-	+
72	+	-	-	-	+	+	+	+	+
84	+	-	-	-	-	+	+	+	+
121	+	-	-	-	-	-	+	+	+

[1]Bovine adenovirus 3 (BAdV-3); bovine coronavirus (BoCV); bovine herpesvirus 1 (BoHV-1); bovine viral diarrhoea virus 1 (BVDV-1); *Histophilus somni* (Hs); *Pasteurella multocida* (Pm); *Mannheimia haemolytica* (Mh); *Mycoplasma bovis* (Mb).

Two of the samples testing positive for BAdV-3 were negative for all of the other viruses tested for (Table 4.13). The remaining BAdV-3 positive samples were negative for the other viruses of interest excepted BVDV-1.

Discussion:

The BAdV-3 qPCR assay described in this report performed well on the extracts from tissues of animals which died from respiratory disease. Two of the samples which reacted with the BAdV-3 qPCR were negative for the other viruses for which previous results were available. However, it is difficult to determine if BAdV-3 played an important role in the deaths of these animals as the qPCR results suggested contrasting levels of the virus in the samples, as one yield a low Ct value and the other a high Ct value.

Another interesting observation was the remaining two BAdV-3 positive samples were also positive for BVDV-1 (Table 4.13). The BVDV-1 Ct values for Sample 4 and 72 were 30.7 and 36.3 respectively. These Ct values are considered higher than what would be expect for cattle persistently infected (PI) with BVDV-1. Consequently, the presence of BAdV-3 and BVDV-1 in the tissues of these animals is probably due to co-infection, perhaps leading to fatal respiratory disease. Rather than a PI animal becoming infected with BAdV-3 and therefore increasing likelihood of severe disease.

Clearly, with the limited number of samples (four of 125 tested) it is difficult to make any conclusions regarding the role of BAdV-3 in BRD attributed to the death of these animals. Moreover, the role of viruses in fatal BRD is generally considered to be linked to primary infections leading to severe secondary bacterial infections. Consequently, it may be more informative to test nasal swabs taken from cattle undergoing treatment/assessment for BRD.

In the absence of a BAdV-3 isolate it is not possible to fully evaluate the performance of this assay due to limits on the quantity of template (four positive samples). We have elected to use this limited resource to further characterise the BAdV-3 genomes within the positive samples. This information will allow us to compare these sequences to available BAdV-3 sequences from overseas isolates to develop a more complete understanding of where the genetic diversity of Australian BAdV-3 viruses sit compared to other geographic regions. While it is possible to generate synthetic templates for use as controls in qPCR assays, we have generally not used this approach to prevent contamination issues. Moreover, these types of controls have limited application when testing field samples as they can give an unrealistic “best case scenario” of assay performance which cannot be matched in day to day testing.

The BAdV-3 qPCR assay appears to have very good specificity, as the samples it was tested on are very complex samples. All samples will contain bovine DNA and RNA, many have DNA or RNA from other BRD associated pathogens, e.g. bovine herpesvirus genome DNA (n=58), bovine viral diarrhoea virus 1 (N=40); *Histophilus somni* (n=104); *Pasteurella multocida* (n=82); *Mannheimia haemolytica* (n=76) and *Mycoplasma bovis* (n=121), as only four samples reacted in the assay it suggest little or no cross-reaction to these non-target organisms. In addition, the qPCR to identify samples, previously shown to contain BAdV-3 with the conventional PCR and amplicon sequencing suggest the assay it will be useful tool in BRD investigations.

4.6 Summary of Feedlot samples and Analyses

Figure 4.6.1 summarises the nasal swab samples collected during 2022 from the two participating feedlots. The number of samples analysed by each laboratory are shown, including the various methods used. Detailed descriptions of the analyses completed by the Microbiology Laboratory on samples collected from Feedlot A are described in Section 4.7. The remaining samples from Feedlot A and all samples from Feedlot B were analysed by the Virology Laboratory using qPCR and ONT sequencing. The results of the qPCR analyses are described in Section 4.8 and Section 4.9, for Feedlot A and Feedlot B, respectively. While the combined results for ONT sequencing for both feedlots are described in Section 4.10.

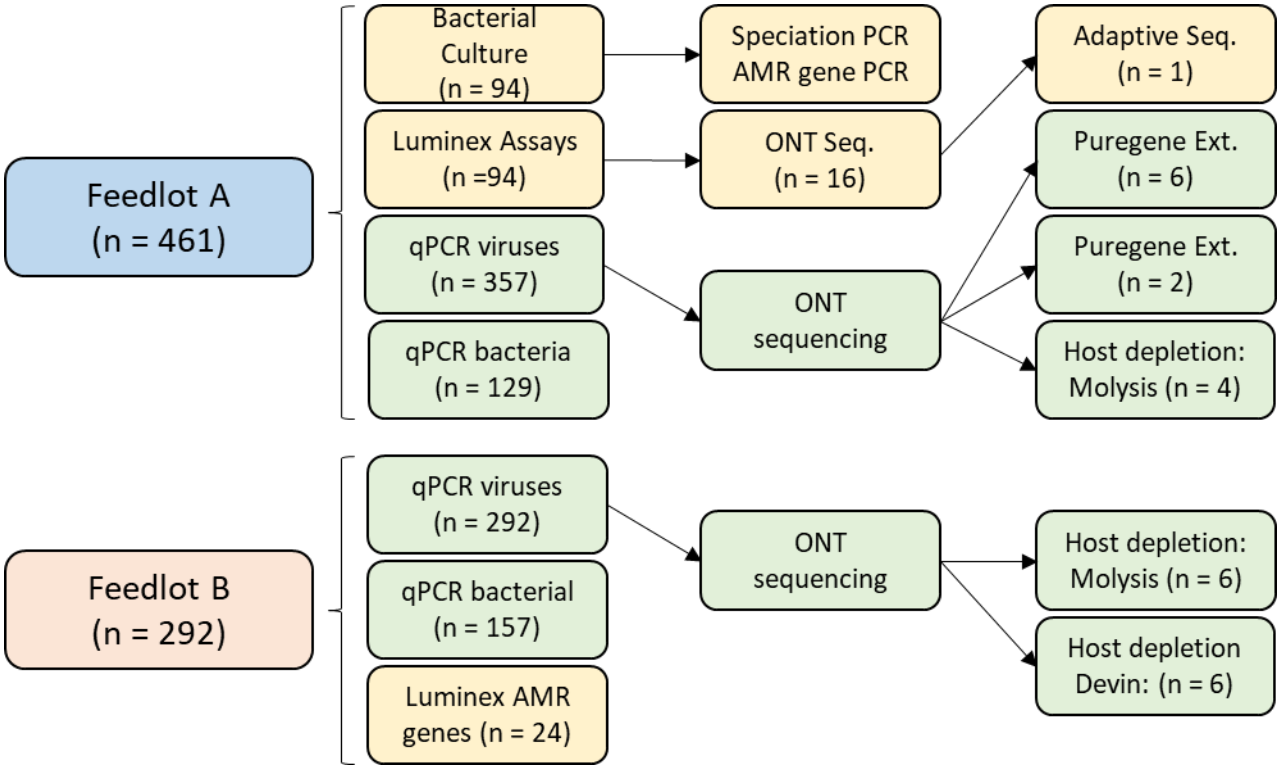

Figure 4.6.1. Schematic representation of the nasal swab samples collected from Feedlot A and Feedlot B over the course of 2022. Samples analysed by the Microbiology Laboratory (Yellow background) were subject to bacterial isolation, Luminex assays for bacterial species and antimicrobial genes. With selected samples also analysed using Oxford Nanopore Technology (ONT) sequencing. Samples analysed by the Virology Laboratory (green background) were subject to quantitative PCR (qPCR) analyses for viruses and bacterial associated with BRD. Selected samples were analysed using ONT sequencing using DNA extracted with various approaches, including strategies to deplete bovine DNA. The number of samples collected and analysed are shown in parenthesis.

4.7 Microbiology - Feedlot A

4.7.1 Isolation and detection of bacterial species associated with BRD

The multiplex assay developed in this project does not provide a confident species-specific result for *M. haemolytica*. Using the validation set of cultures, there were 17 isolates identified as *M. haemolytica* in the multiplex Luminex assay that failed to be confirmed as *M. haemolytica*. A total of 11 isolates were identified as *M. haemolytica* by both Luminex assays. It is clear that all positive reactions for *M. haemolytica* in the multiplex Luminex assay have to be re-confirmed by the single plex assay.

A total of 94 nasal samples were subjected to conventional bacteriological culture (followed by PCR identification of purified cultures), with a paired nasal swab analysed using the direct multiplex Luminex assays. The final results of the species identified from culture and Luminex are shown in the **Table 4.7.1**. Neither *M. haemolytica* nor *B. trehalosi* were isolated from any of the cultures.

The total number of pathogens detected in the 94 samples was greater by culture than by Luminex (Fig. 4.7.1). The difference between the number of pathogens detected between the two methods was statistically significant ($P < 0.05$).

A comparison of the number of the target pathogens detected by culture and Luminex is presented in Fig. 4.7.2.

Table 4.7.1. Summary of the final results of 94 samples from Feedlot A subjected to culture and colony PCR analyses to identify six bacterial species: *Pasteurella multocida, Histophilus somni, Truperella pyogenes, Mannheimia haemolytica*, *Bibersteinia trehalose* and *Mycoplasma bovis*.

Pathogen(s) cultured and identified (culture) or detected (Luminex assay)	**No. of animals by culture**	**No. of animals by Luminex**
No target pathogen detected	26	46
B. trehalose	0	1
H. somni	6	4
M. haemolytica	0	1
M. bovis	9	1
P. multocida	12	19
T. pyogenes	5	1
H. somni, M. bovis	8	0
P. multocida, B. trehalosi	0	2
P. multocida, H. somni	2	4
P. multocida, M. haemolytica	0	5

P. multocida, M. bovis	13	0
P. multocida, T. pyogenes	2	0
T. pyogenes, M. bovis	1	0
P. multocida, B. trehalose, M. bovis	0	4
P. multocida, H. somni, M. bovis	6	0
P. multocida, H. somni, T. pyogenes	1	0
P. multocida, M. haemolytica, M. bovis	0	1
P. multocida, M. bovis, T. pyogenes	2	0
P. multocida, B. trehalosi, H. somni, M. haemolytica,	0	1
P. multocida, B. trehalosi, M. haemolytica, M. bovis	0	3
P. multocida, B. trehalosi, M. bovis, T. pyogenes,	0	1
P. multocida, H. somni, M. bovis, T. pyogenes	1	0

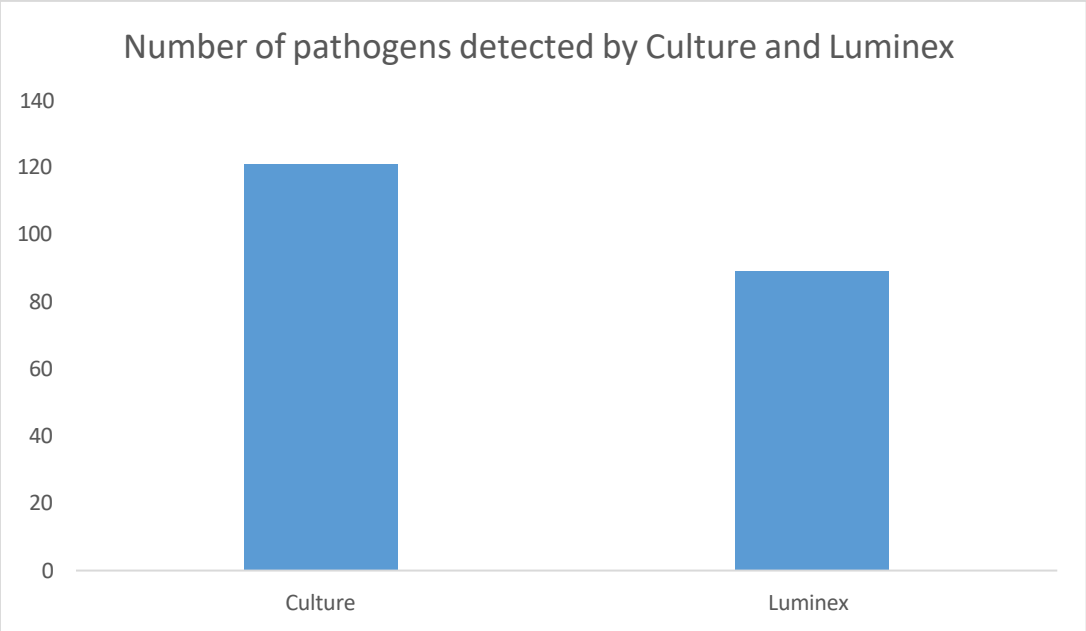

Figure 4.7.1. Comparison between the total number of bacterial pathogens detected by culture and the Luminex assay analyses using the 94 field samples from Feedlot A.

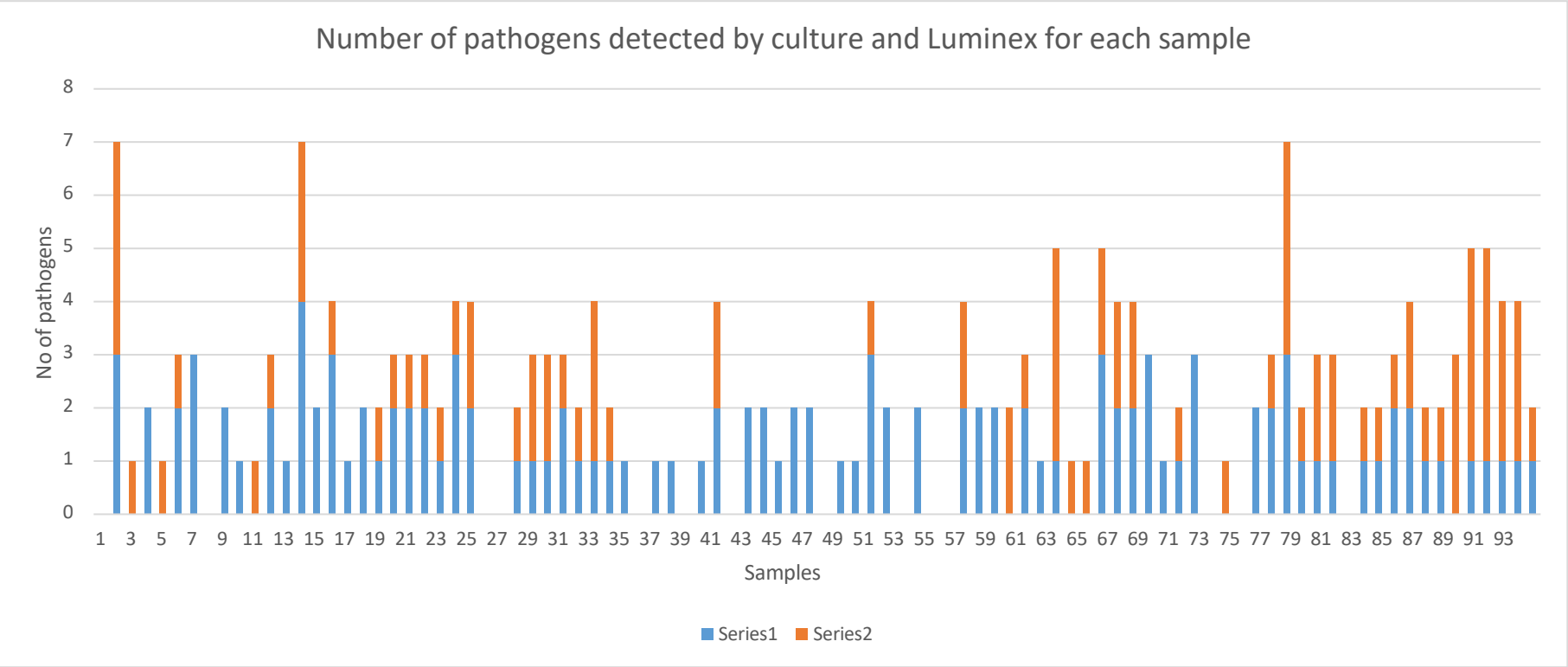

Figure 4.7.2. Number of pathogens detected by culture and Luminex for the samples (n = 94) analysed from each animal. Series 1 (Blue bars) represents the bacterial culture results and Series 2 (Orange bars) represents the Luminex results. The results are accumulative e.g in the first positive sample, the culture method yielded 3 pathogens while the Luminex method detected four pathogens.

The results from Luminex and culture assays for the 16 nasal swabs from week one of sampling were compared to that from ONT sequencing (Table 4.7.2).

Table 4.7.2. A comparison of the results for the 16 nasal samples subjected analyses by culture (with colony PCR identification), Luminex Assay and Oxford Nanopore (ONT) Sequencing for the six bacterial species of interest: *P. multocida* (PM), *H. somni* (HS), *T. pyogenes* (TP), *M. haemolytica* (MH), *B. trehalose* (BT) and *M. bovis* (MB).

	Bacterial species detected		
Sample	**Culture**	**Luminex**	**ONT**
1	Negative	Negative	BT, HS, MB
2	PM, HS, TP	PM, TP, BT, MB	PM, HS, TP, MB
3	Negative	TP	PM, MH, HS, TP, MB
4	PM, MB	Negative	MB
5	Negative	PM	Negative
6	PM, TP	HS	HS, MB
7	PM, HS, MB	Negative	MB
8	Negative	Negative	PM, MH, HS
9	PM, MB	Negative	MH, HS, TP
10	MB	Negative	MH, MB
11	Negative	HS	Negative
12	HS, MB	HS	HS
13	TP	Negative	PM, MB
14	HS, PM, TP, MB	PM, BT, MB	HS, MB
15	PM, MB	Negative	MB
16	PM, TP, MB	BT	PM, HS, MB

In Figures 4.7.3 to 4.7.6, direct comparisons of the results of the three analysis methods (Culture, Luminex, Nanopore) for *P. multocida, H. somni, T. pyogenes* and *M. bovis* used in this study are presented. Of the 16 samples, four *M. haemolytica* were identified with the Nanopore, yet culture and Luminex did not detect *M. haemolytica*. The culture of the 16 samples did not yield any *B. trehalosi* isolates, while the Luminex assay detected *B. trehalosi* in three samples and Nanopore sequencing identified the bacterium in one sample.

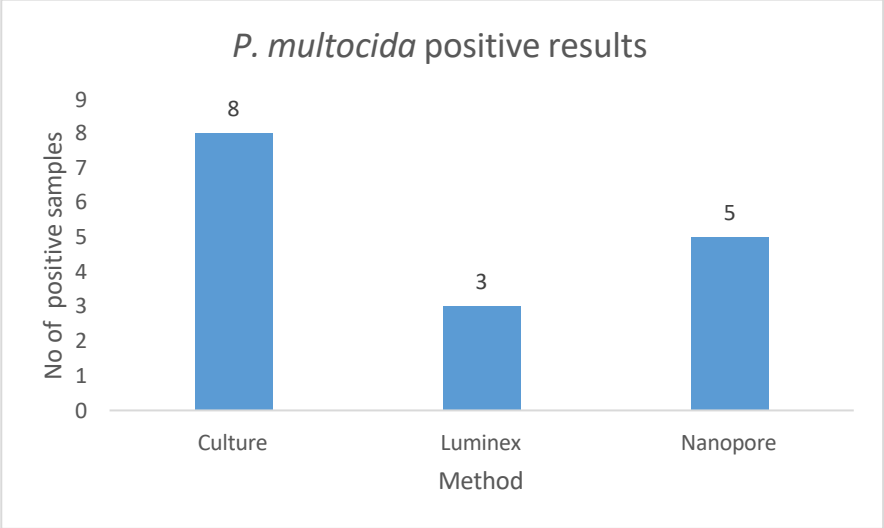

Figure 4.7.3. Comparison of the number of positive samples for bacterial isolation (culture), Luminex and Nanopore to culture for the detection of *P. multocida* in 16 nasal swab samples.

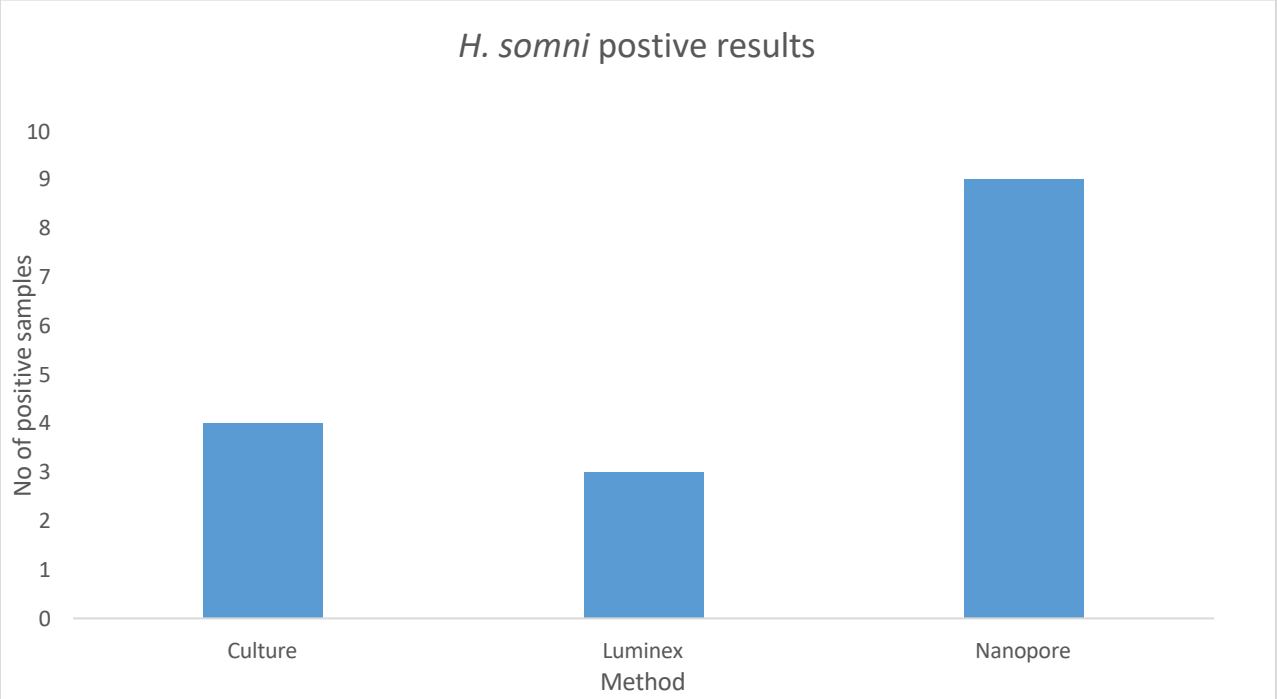

Figure 4.7.4. Comparison of the number of positive samples for bacterial isolation (culture), Luminex and Nanopore to culture for the detection of *H. somni* in 16 nasal swab samples.

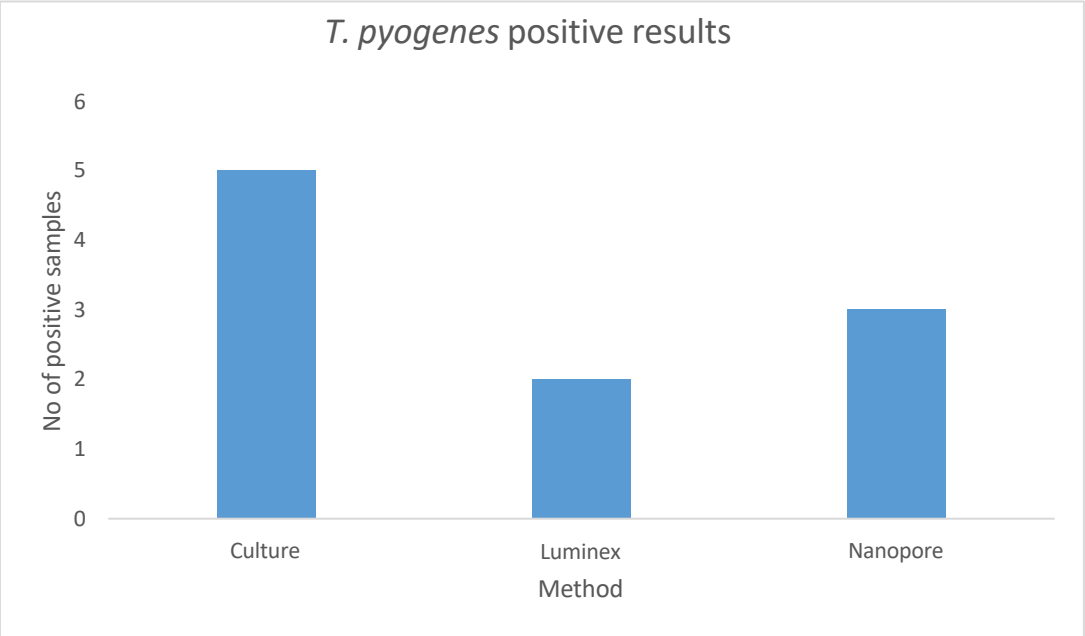

Figure 4.7.5. Comparison of the number of positive samples for bacterial isolation (culture), Luminex and Nanopore to culture for the detection of *T. pyogenes* in 16 nasal swab samples.

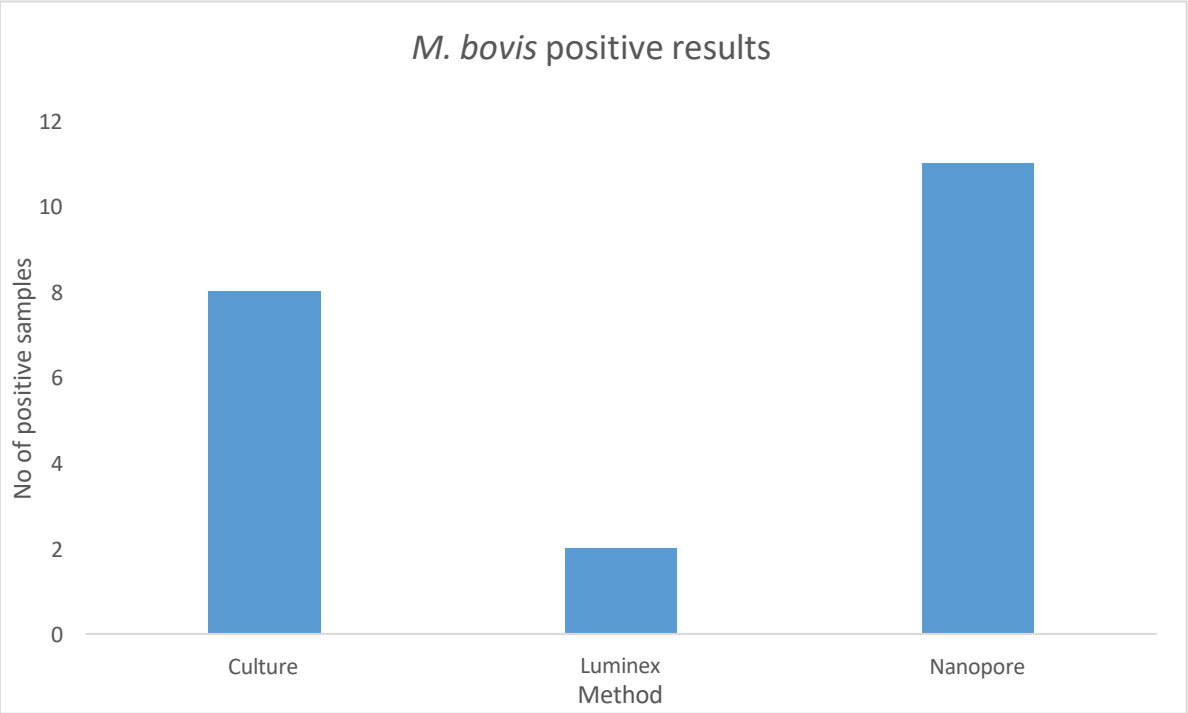

Figure 4.7.6. Comparison of the number of positive samples for bacterial isolation (culture), Luminex and Nanopore to culture for the detection of *M. bovis* in 16 nasal swab samples.

The results from the Luminex assays and Nanopore sequencing analyses for *P. multocida, H. somni, T. pyogenes* and *M. bovis* were compared with the gold standard of culture using the Cohen's kappa coefficient. The results are shown in Tables Table 4.7.3 to 4.7.7. Overall, the level of agreement between the gold standard test, culture and Luminex assays or Nanopore sequencing appeared to be dependent on the bacterial species of interest.

Using a suggested the Kappa value interpretive scale: values ≤ 0 as indicating no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (McHugh, 2012). There was no agreement between culture and the Luminex assays for any bacterial species. For Nanopore sequencing there was fair and moderate agreement with culture for *P. multocida* and *H. somni*, respectively (Table 4.7.3 and Table 4.7.4). There was higher agreement between culture and Nanopore sequencing for all of the bacterial species of interest, except for *T. pyogenes* (Table 4.7.5).

Table 4.7.3. Analysis by Cohen's kappa coefficient results for Luminex and Nanopore as compared to culture for *P. multocida*.

	Luminex		Nanopore	
	positive	negative	positive	Negative
Culture positive	2	6	2	6
Culture negative	1	7	4	4
Kappa value	**0.002**		**0.336**	

Table 4.7.4. Analysis by Cohen's kappa coefficient results for Luminex and Nanopore as compared to culture for *H. somni*

	Luminex		Nanopore	
	positive	negative	positive	negative
Culture positive	1	3	3	1
Culture negative	2	10	6	6
Kappa value	**0.090**		**0.452**	

Table 4.7.5. Analysis by Cohen's kappa coefficient results for Luminex and Nanopore as compared to culture for *T. pyogenes*

	Luminex		Nanopore	
	positive	negative	positive	negative
Culture positive	1	4	1	4
Culture negative	1	10	2	9
Kappa value	**0.131**		**0.021**	

Table 4.7.6. Analysis by Cohen's kappa coefficient results for Luminex and Nanopore as compared to culture for *M. bovis*

	Luminex		Nanopore	
	Positive	negative	positive	negative
Culture positive	1	7	6	2
Culture negative	1	7	5	3
Kappa value	**0.00**		**0.126**	

Table 4.7.7. Analysis by Cohen's kappa coefficient results for Luminex and Nanopore as compared to culture for *M. bovis*

	Luminex		Nanopore	
	Positive	negative	positive	negative
Culture positive	1	7	6	2
Culture negative	1	7	5	3
Kappa value	**0.00**		**0.126**	

To examine the level of agreement between bacterial culture and the Luminex assays the data for these methods were compared by Cohen's kappa coefficient comparison for the 94 samples. Briefly, if the same number of isolates were found by both method but different pathogens, then both methods did get a positive score for pathogen and the other method a negative for not the right pathogen (culture + and Luminex – and vice versa). If one method had more pathogens, then only that method was scored as positive (culture + and Luminex – or vice versa depending which method had more pathogens) and the same pathogen were scored as positive for both (culture + and Luminex +). Hence, using this approach 139 values were available for the analysis. With a Kappa value of -0.449, the analysis suggested there was no agreement between bacterial culture and Luminex assay based detection (Table 4.6.8).

Table 4.7.8 Analysis by Cohen's kappa coefficient comparison of the results for bacterial culture to Luminex assay detection for the 94 samples.

		Luminex Assays	
		Positive	Negative
Bacterial Culture	Positive	23	60
	Negative	42	14
		Kappa value -0.449	

4.7.2 Detection of antimicrobial resistance genes

In terms of antimicrobial resistance genes, 15 of the 94 samples showed the presence of either one or both *mphE* and *msrE* genes in the bacterial isolate obtained through culture. With the Luminex assay, 17 samples showed the presence of either one or both *mphE* and *msrE* genes (Table 4.7.9).

One of the samples (number 69) was positive for the presence of *erm42* gene when tested by Luminex assay. However, no bacteria were isolated from this sample, hence the presence of the *erm42* gene could not be tested via PCR. None of the isolates obtained via culture gave a positive result for the presence of the *erm42* gene when tested by PCR.

Table 4.7.9. The detection of macrolide resistant genes (*erm(42)*, *mphE* and *msrE*) detected specific PCR assays in cultured pathogens, *P. multocida* and *T. pyogenes*, compared to the detection by Luminex directly in samples. Not detected (ND).

		Genes detected	
Sample	Species	Culture/PCR	Luminex
1	ND	ND	ND
2	*P. multocida*	*msrE, mphE*	*msrE*
3	ND	ND	ND
4	*P. multocida*	*msrE, mphE*	ND
5 - 14	ND	ND	ND
15	*P. multocida*	*msrE, mphE*	ND
16	*P. multocida*	*msrE, mphE*	*msrE*
17	ND	ND	ND
18	*P. multocida*	*msrE, mphE*	
19 – 30	ND	ND	ND
31	*P. multocida*	*msrE, mphE*	ND
32 - 68	ND	ND	ND
69	ND	ND	*msrE, mphE, erm 42*
70	ND	ND	*msrE, mphE*
71 - 72	ND	ND	ND
73	ND	ND	*msrE, mphE*
74	ND	ND	ND
75	*P. multocida*	*mphE*	*msrE, mphE*
76	*T. pyogenes*	*msrE, mphE*	*mphE*

77	*P. multocida*	*msrE, mphE*	ND
78	*P. multocida*	*msrE, mphE*	*msrE*
79 - 81	ND	ND	ND
82	ND	ND	*mphE*
83	ND	ND	*mphE*
84	*P. multocida, T. pyogenes*	*msrE* (only TP)*, mphE*	ND
85	ND	ND	*mphE*
86	*P. multocida*	*msrE, mphE*	*msrE*
87	*P. multocida*	*msrE, mphE*	*mphE*
88	ND	ND	*mphE*
89 - 90	ND	ND	ND
91	ND	ND	*mphE*
92	*P. multocida*	*mphE*	*msrE*
93	ND	ND	ND
94	*P. multocida*	*msrE, mphE*	*msrE, mphE*

Table 4.7.10. Cohen's kappa coefficient results for the detection of macrolide genes by PCR in cultured bacterial isolates compared to Luminex assays from the same nasal swab samples.

	Luminex	
	positive	**negative**
Culture positive	8	14
Culture negative	10	72
Kappa value	**0.257**	

The beta-lactam resistance gene *blaROB*$_1$ was only identified via the Luminex from the swab of two animals (Number 28 and 29). The resistance sulphonamide gene *sul1* was only detected in a cultured *T. pyogenes* isolate (Number 78). The sulphonamide gene *sul2* was only detected in two cultured *T. pyogenes* isolates (Number 76 and 84).

The florfenicol resistant gene *florR* was found by Luminex in six isolates (Number 10, 58, 62, 76, 90 and 91) and by culture in two *T. pyogenes* isolates (Number 76 and 84), two *H. somni* isolates (Number 22 and 24) and two *P. multocida* isolates (Number 47 and 51).

The tetracycline resistant gene tet(Q) was only found in one animal sample (Number 16). The tetracycline resistance gene *tet(Y)* was not identified. The summary of the results for resistance genes *bla*$_{ROB}$*, sul1, sul2, tet(Q), tet(Y), florR* are shown in

.

Table 4.7.11. Summary of the resistant genes of selected beta-lactam, suphonomide and tetracycline detected by specific PCR assays in cultured pathogens, *H. somni, P. multocida* and *T. pyogenes*, compared to the detection by Luminex directly in nasal swab samples. Blank cells indicate negative results.

Sample Number	Species positive	Culture	Luminex
1-9			
10			florR
11-15			
16			tetQ
17-21			
22	H. somni	florR	
24	H. somni	florR	
25-27			
28			blaROB-1
29			blaROB-1
30-46			
47	P. multocida	florR	
48-50			
51	P. multocida	florR	
52-57			
58			florR
59-61			
62			florR
63-75			
76	T. pyogenes	florR, sul2	florR
77			
78	T. pyogenes	sul1	
79-83			
84	T. pyogenes	florR, sul2	
85-89			
90			florR
91			florR
92-94			

The results for the detection of antimicrobial resistance genes in the sample extracts using the Luminex assays are shown in Table 4.7.12. These results are of particular interest as none of the bacterial species typically associated with BRD were isolated from or detected in these samples. Overall genes involved in beta-lactam resistance were most frequently detected by the Luminex assays in these samples. AMR genes were from multiple antimicrobial classes were detected in several samples, with beta-lactams and tetracycline resistance genes being frequently detected together (Table 4.7.12). Five macrolide resistance genes were detected in Sample 69.

Table 4.7.12. Summary of the remaining antimicrobial resistance genes detected using the multiplex Luminex assays. The results are only shown for samples (n=36) where one or more resistance gens were detected. Blank cells indicate no gene no detected.

	Antimicrobial Resistance Gene Detected				
Sample Number	**Gene 1**	**Gene 2**	**Gene 3**	**Gene 4**	**Gene 5**
2	blaTEM		blaCTX-M-1		tet(M)
3			blaCTX-M-1		
5	blaTEM				
7	blaTEM				
8			blaCTX-M-1		
10					tet(D)
11			blaCTX-M-1		
12			blaCTX-M-1		
13			blaCTX-M-1		
21			blaCTX-M-1	tet(H)	tet(R)
23			blaCTX-M-1		
25			blaCTX-M-1		
29				tet(R)	
30	blaTEM	blaSHV		tet(H)	
31		blaSHV		tet(R)	blaCMY-2
32		blaSHV		blaKPC	blaCMY-3
33	blaTEM	blaSHV	blaCTX-M		blaCMY-4
34	blaTEM	blaSHV	blaCTX-M		blaCMY-5
35	blaTEM				blaCMY-6
36	blaTEM				blaCMY-7
37					
38		blaSHV			
43					tet(H)
44			blaCTX-M-1		
63				tet(R)	tet(W)
69	ermA	ermB	ermC	ermF	mefA
73				ermF	
74				ermF	
76				tetH	tet(R)
78					tet(R)
82				ermF	
90				ermF	
91	blaTEM			ermF	
92	blaTEM				
93	blaTEM				
94	blaTEM				

4.7.3 Metagenomic analysis using Oxford Nanopore Sequencing Platform

Total DNA extracted from dry swab samples from week 1 (MRG numbers 1-16) were extracted as described in DNA extraction from dry swabs section and the quality and quantity of the extracted DNA were checked using nanodrop and Qubit system. Library preparation was done using the ONT SQK-LSK109 kit with EXP-NBD104 kit used for PCR-free native barcoding (barcodes 1-12). Eight samples were pooled per run as displayed in **Table 4**.

Table 4. Summary of the parameters and sequencing outputs of nasal swab sample (n=12) extracts analysed by sequencing using Oxford Nanopore (ONT) technology.

ONT sequencing Strategy	Sample number	Size of raw fastq file	Total reads	Read Length N50	Non-host reads	Percentage of non-host reads (%)
Run 1 on the MK1C device						
	1	330 MB[1]	109238	6837	2 113	1.93
	2	122 MB	91022	1364	556	0.61
	3	625 MB	342181	2466	1647	0.48
	4	1.7 GB[2]	391055	7468	2244	0.57
	5	1.1 GB	414149	5252		4.08
	6	144 MB	87561	1841	665	0.76
	7	995 MB	812370	979	2065	0.25
	8	890 MB	318725	4939	2529	0.79
Run 2 on the MK1B device						
	9	427 MB	122795	5497	3981	3.24
	10	541 MB	112669	6868	2770	2.46
	11	219 MB	114360	1500	336	0.29
	12	181 MB	60396	3945	179	0.30
	13	404 MB	106551	5823	1101	1.03
	14	522 MB	143215	4725	520	0.36
	15	954 MB	481458	1150	1374	0.29
	16	110 MB	509819	1962	372	0.73
Adaptive sampling						
	12[3]	440 MB	556 836	683	2 969	0.53

[1]MB=MegaBytes. [2] GB=GigaBytes. [3] The library for adaptive sampling based sequencing was stored for over one week in the fridge prior to analysis as there were technical issues in the implementation of the adaptive sampling workflow. Hence the N50 was very low.

Two approaches were tested for ONT sequencing of the nasal swab extracts. The first was by pooling the barcoded sequencing libraries for eight samples, followed by loading the pools on a R9.4.1 flowcell and running the analyses on either a ONT MinION MK1C sequencing device (Run 1, **Table 4**) or a MK1B sequencing device (Run 2, **Table 4**).

The second approach was to analyse a sequencing library generated using one DNA sample (sample 12) on a R9.4.1 cell, with the sequencing analysis performed using a ONT MinION MK1B device connected to a Dell Precision-3650-Tower (Nvidia GeForce RTX 3060 GPU). Using MinKNOW V22.05.5

software with implemented Guppy v 6.1.5 was used to simultaneously run the sequencing and base calling programs, respectively. The reference bovine genome *Bos taurus* genome (GCA_002263795.2_ARS-UCD1.2) was used with minimap2 for indexing the genome. The bovine genome .bed file was downloaded from UCSC Table Browser and manually curated to reflect the chromosome names as described in the genome fasta file. The sequencing run started as normal with the adaptive sampling mode on. The flow cell with the loaded library was kept in the fridge for over one week as I was trouble shooting the adaptive sampling feature of the MinKNOW software, hence the library is likely to have degraded, resulting shorter reads than might have been expected compared to the other ONP sequence analyses (**Table 4**).

4.7.4. Post Sequencing data quality control and analyses

NanoPack suit was used for quality control, visualization and trimming of the long read sequences. BWA v 0.7.13 was used to map the filtered reads to the *Bos taurus* genome. Samtools v 1.3 was used to extract the proportion of the reads that did not align to provided bovine genome and convert them to fasta format. This data was then subjected to different downstream analysis. Kraken v2.1.2 with the inbuild database was used for the taxonomical assignment of the long reads. Diamond v2.0.15 with implemented blastx was used to compare the reads against the NCBI-nr protein database with the taxonomic content results visualised in MEGAN. Resfinder and CART as implemented in abricate/1.0.1 were used to search for antimicrobial resistance genes (**Figure 4.7.7**).

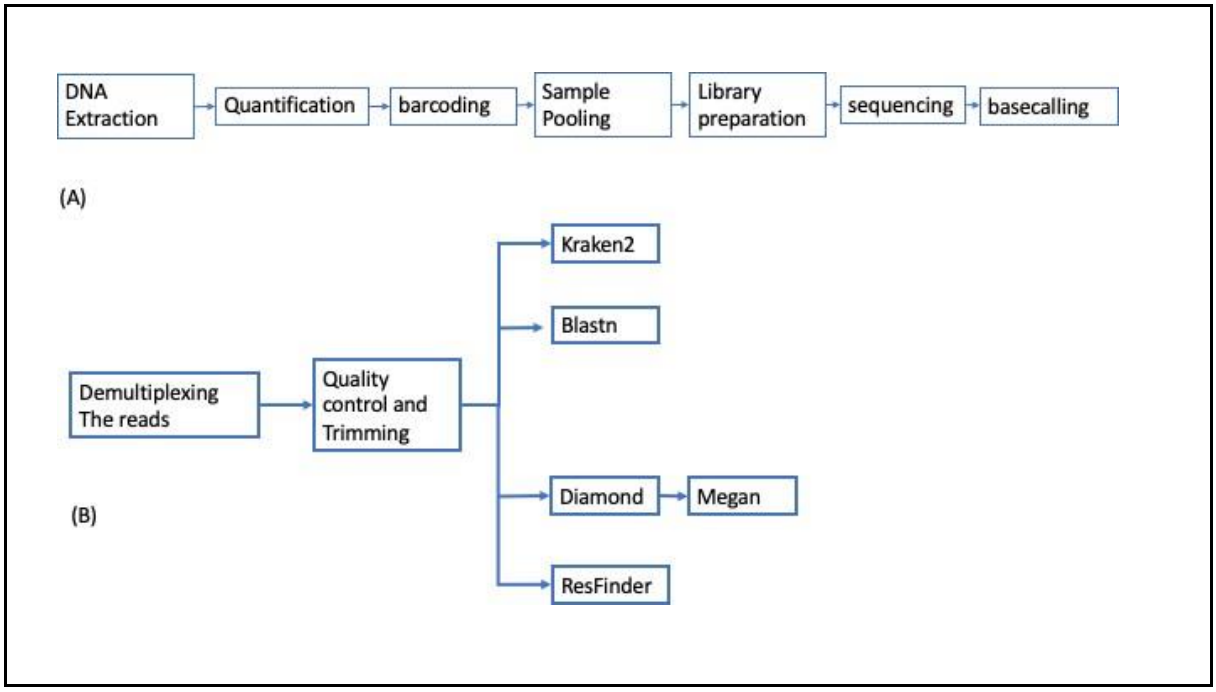

Figure 4.7.7. Schematic showing a summary of steps for sequencing (A) and bioinformatics analysis (B) of the sequenced samples.

4.7.5 Results for Nanopore sequencing

The results from the first two runs without adaptive sampling showed that more than 96% of the sequencing reads aligned to the host genome (**Table 4**). Panel A in Figure 2 shows the two different pathways that can be implemented when performing shotgun sequencing. Hence, at this stage, an effort was made to establish the adaptive sampling feature on the ONT MinKNOW software. With adaptive sample, the sequence of the DNA strand is determined simultaneously as it moves through the nanopore, as such the strand sequence is determined in real time. The software compares this nascent sequence to the provided reference genome for *B. taurus* as the bases are called. Sequences matching the bovine genome are ejected from the nanopore and the nanopore becomes available to accept another DNA strand (Figure 2). When implemented in this study, adaptive sampling for sample 12 changed the proportion of non-host DNA from 0.3% to 0.53% (**Error! Reference source not found.**). While the sequencing library used in the adaptive sequencing was likely to be degraded due to prolonged storage within the flowcell (>7 days) due to technical difficulties in implementing the analysis pipeline, there was still some evidence for the depletion of host genome sequencing reads in the dataset.

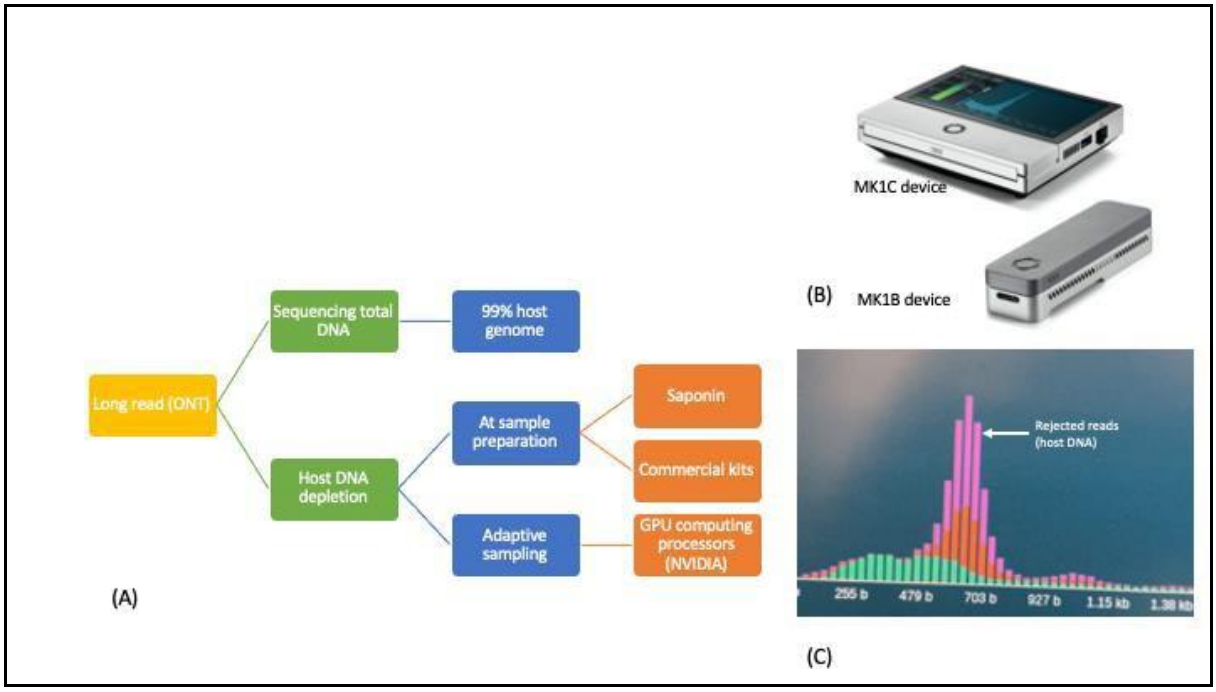

Figure 2. Schematic representation of the implementation of strategies to reduce the number of host read sequences for shotgun metagenomic sequencing analyses using Oxford Nanopore Technologies (ONT) sequencing. (A) Available ONT sequencing pipelines for reducing host sequencing reads. (B) ONT sequencing devices used in this study. (C) Example of the graphical representation of an ONT adaptive sampling run with the host reads identified and ejected from pores early in the strand sequencing at approximately 700 bases.

Table 4.7.14 summarises the three most frequently sequenced bacterial genera identified in the ONT sequencing datasets for each sample. The most frequently sequenced bacterial genus in most of the samples (13 of 16) were those that contain bacterial pathogens commonly associated with BRD (Table 4.7.14). However, when these samples were analysed at the species level, only six of the 12 samples were positive for a specific bacterial species commonly associated with BRD (Table 4.7.14). These results suggest that the repertoire of bacterial species associated with BRD could be wider than has been considered in the past.

Analysis of ONT sequencing data using the Diamond+Megan method as described by Bagci et al. (2021) increased the sensitivity of detecting the pathogenic bacterial species commonly associated with BRD (Table 4.7.15). For most samples, sequencing reads matching multiple species were identified, although *H. somni* (n=1) and *M. bovis* (n=3) were detected as the sole species in four samples (Table 4.7.15). Interestingly, the Diamond+Megan analysis identified sequencing reads corresponding to BoHV-1 in 12 of the 15 samples for which results were available. This highlights another advantage of sequencing based diagnostics that enable the simultaneous detection of bacterial and viral species in a sample. Noting that for the methods utilised in the current study for Oxford nanopore sequencing, only viruses with DNA genomes would be detected as the focus of these studies was to evaluate the feasibility of detecting AMR genes. With further development, for example including a complementary DNA synthesis step, it may be feasible to also detect RNA viral genomes. However, the inclusion of this step may limit the capacity to deploy ONT sequencing in the field.

Analysis of the data from these experiments identified four samples with sequence reads that matched AMR genes (Table 4.6.16). Of note in these results was Sample 10 for which three AMR genes were identified to two classes of antimicrobials, macrolide and tetracyclines (Table 4.7.16). Sample 10 was positive for *M. haemolytica* and *Mycoplasma bovis* by sequencing (Table 4.7.15), however at this time it is not possible to assign the AMR genes to either of these species. Similarly it is also plausible that these AMR genes may have been associated with the other bacterial genera and species detected in this sample (Table 4.6.14).

Sample 3 was positive for AMR gene, *lnu(C)*, and *P. multocida, H. somni, T. pyogenes, M. haemolytica,* and *M. bovis* (Table 4.7.15 and Table 4.7.16). The *lnu(C)* in *Streptococcus uberis* isolates associated with mastitis in Australian dairy cattle (Vezina et al., 2021). This was the first report of the *lnu(C)* AMR gene being detected in a bovine pathogen. Prior to this *lnu(C)*, had only been reported in isolates of *Streptococcus agalactiae* from humans (Achard et al., 2005; Gravey et al., 2013). While no *Streptococcus* spp. were detected in the ONT dataset for Sample 3, the detection of *lnu(C)* is important as it is a transposon associated AMR gene (Achard et al., 2005). Vezina et al. (2021) suggested that the detection of *lnu(C)* in their study may be the first example of an interspecies acquisition of an AMR gene by *S. uberis*,

Table 4.7.5. Summary of the Oxford Nanopore Technology sequencing results when analysed using the Diamond+Megan method (Bagci et al., 2021). The number of sequencing reads assigned to the species of interest are shown. The results are shown for *Pasteurella multocida* (PM)*, Histophilus somni* (HS)*, Truperella pyogenes* (TP)*, Mannheimia haemolytica* (MH, or *Mannheimia* species), *Bibersteinia trehalose* (BT), *Mycoplasma bovis* MB) and bovine alphaherpesvirus 1 (Bovine AH 1).

Sample ID	*B. trehalosi*	*P. multocida*	*M. haemolytica*	*H. somni*	*Truperella*	*M.bovis*	**Bobine AH1**	*bacterial species summary*
1	2	0	Mannheimia sp	1	0	1	2	BT,HS,Mb
2	0	8	0	2	1	24	56	PM,HS,TP,MB
3	0	5	1	6	1	24	4	PM,MH,HS,TP,MB
4	0	0	43 Mannheimia 1 Mh	0	0	3	19	MB
5	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
6	0	0	0	12	0	13	89	HS,MB
7	0	0	0	0	0	1	4	MB
8	0	1	14 Mannheimia sp and 1 Mh	0	0	0	1	PM,MH
9	0	0	6 Mannheimia sp and 2 Mh	1	0	0	4	MH,HS
10	0	0	142 Mannheimia sp and 2 Mh	0	0	3	2	MH,MB
11	0	0	mannheimia sp	0	0	0	0	
12	0	0	mannheimia sp	1	0	0	0	HS
12-adaptive	0	2	mannheimia sp	47	0	15	0	PM,HS,MB
13	0	1	37 mannheimia sp	0	0	2	4	PM,MB
14	0	0	1 Mannheimia sp	5	0	1	28	HS,MB
15	0	0	0	0	0	1	0	MB
16	0	6	2 Mannheimia sp	2	0	16	46	PM,HS,MB

Diamond results from Sample 5 not available.

Table 4.7.16. The antimicrobial resistance genes (AMR) identified in the Oxford Nanopore Technology datasets of three nasal swab samples from Feedlot A. The AMR gene(s) identified in each sample are show, along with the percentage coverage and identity to the matching reference gene.

	Sample Number					
	3	**5**	**9**	**10**	**10**	**10**
AMR gene	*lnu(C)_1*	*erm(T)_2*	*erm(T)*	*msr(E)_1*	*mph(E)_1*	*tet(H)_1*
Percent Coverage (%)	98.38	79.32	97.96	96.88	95.59	96.01
Precent identity (%)	90.34	96.16	95.67	91.4	86.45	90.66

4.7.6 Discussion

Of all the bacterial pathogens targeted by the Luminex assays were detected in the nasal swab samples from Feedlot A. Of these, *P. multocida* was certainly the most prevalent pathogen. The discrepancy between the assays was pronounced. Some of it is explainable by the methods used in this study. As an example, for isolation of *T. pyogenes* plates had to be incubated for four days before colonies were visible. This long incubation time suggest low number of the bacterium in the collected samples that could explain why it was not detected as frequently with the corresponding Luminex assay. Similarly, *M. bovis* was infrequently by the specific Luminex assay, whereas isolation of this pathogen required a five day incubation for suspect colonies to test positive by a conventional PCR assay, indicating very low numbers of the pathogen on the original swab.

Why neither *M. haemolytica* nor *B. trehalose* were isolated by culture, yet both bacteria were detected by the Luminex assays and by ONT sequencing is difficult to interpret. There were no problems with the bacterial culture methods as the blood agar plates utilised readily supported growth of the control type strains for these species. The nasal swabs used for bacterial culture were collected into a specialised transport media for bacterial culture and were delivered to the laboratory on the same day as they were collected. In additional, *M. bovis* which lacks a protective cell wall, compared to *M. haemolytica* and *B. trehalose* was isolated from several samples in this study. This suggest there were no issues with the collection, transport and process of the samples that might have contributed to the inability to isolated *M. haemolytica* or *B. trehalose* in this study.

An interesting observation was that a several bacterial pathogens were found in combination with other bacterial pathogens. This is certainly not an unusual finding in the context of the BRD complex, where in combination with other factors, there are multiple pathogens that contribute to the development of disease. Some authors suggest that fatal BRD associated with viruses only contribute to 19% of risk of an animal dying. Suggesting that viruses are only a predisposing factor and that bacteria play a much larger role (Gagea et al., 2006; Griffin et al., 2010). Griffin et al. (2010) summed up the literature stating that an increase in *P. multocida* had been observed in fatal BRD cases, yet prior to this *M. haemolytica* was considered to be always linked to fatal BRD cases. Most of the BRD associated bacterial pathogens are commensal pathogen being opportunistic when the immune system is for example suppressed by *Mycoplasma bovis*. *Mycoplasma* species are known to be

synergistic and increase the risk of the invasion of other pathogens, as they destroy the cilia, which is the first line of defence. *M. bovis* is often associated with *P. multocida*, *M. haemolytica* and *H. somni* (Booker et al., 2008). This hypothesis is consistent with observations in the current study that *P. multocida* was the most prevalent bacterial pathogen, followed by *H. somni*.

When the Kappa coefficient was calculated the values for *P. multocida* was 0.002 and 0.336 for *P. multocida* detection by Luminex and Nanopore, respectively. When applying the cut offs given then there is no agreement of the *P. multocida* detection between the Culture and the Luminex assay. While the ONT fairs better and there seems a fair agreement. However, the suggested guidelines by Landis and Koch (1977) were not accepted by everybody, with Fleiss (1981) suggesting that a Kappa coefficient over 0.75 would be excellent, while 0.4 to 0.75 was between fair and good, however a coefficient below 0.4 deemed to be poor. Other studies have suggested that a Kappa value below 0.60 indicates inadequate agreement among the raters and concluded that results should be considered with very little confidence (Montes de Oca et al., 2017).

The Kappa coefficient for *H. somni* was also very low for both tests, 0.090 and 0.250 for Luminex and ONT, respectively, compared to culture. Again there was higher agreement between ONT and culture, albeit still poor agreement by all categorisation schemes. This trend of poor agreement was also identified for *T. pyogenes* and *M. bovis*. This trend had nothing to do with small sample size, as even with 94 sample the Kappa was actually in the negative.

By culture significantly more pathogens were detected compared to the Luminex assays. This result could be due to the small numbers of the target pathogenic bacteria in the nasal swab samples. Prior to plating of the samples onto agar to isolated individual bacterial colonies, an amplification step was performed over several days in liquid culture. Consequently, this approach to culture may overestimate the number of pathogens present in the original sample. In additional, the entire original sample was used for culture amplification step, while for Luminex based diagnostics an aliquot of the original sample was used for extraction to prepare DNA template for the analysis. Therefore for pathogens present in low numbers, stochastic distribution of the pathogens in the sample may decrease the likelihood of successful detection by Luminex.

The macrolide resistant genes were present in many of the samples compared to the other AMR genes included in the assay. *P. multocida* isolates were the main source of the macrolide genes *msrE* and *mphE* amplification. *T. pyogenes* also harboured these two macrolide resistant genes. The macrolide resistant gene *erm42* was only detected in one isolate by Luminex. The *msrE* and *mphE* genes have recently been reported from feedlots in Australia in *P. multocida* and *M. haemolytica* (Alhamami et al., 2021; Alhamami et al., 2022). To date, there have been no reports of these genes being detected in *T. pyogenes*.

The florfenicol resistant gene *florR* was detected in isolates of *P. multocida*, *H. somni* and *T. pyogenes* in the current study. This resistant gene was not reported in the most recently published study of antimicrobial resistance in feedlots (Alhamami et al., 2021; Alhamami et al., 2022). This gene can be transferred between among bacteria of the same species, different species or genera if it is encoded on a plasmid (da Silva et al., 2017; Lu et al., 2018).

The genes *blaROB-1* and tet(Q) were only detected by Luminex analyses. *P. multocida*, *H. somni* and *T. pyogenes* do not carry these AMR genes, suggesting that these genes were in other bacteria species of the microbiome. Determining associated host of these AMR genes would be an important step to determine the overall risk they pose to BRD treatments.

Based on the repertoire of AMR genes detected by the Luminex assays, there might be a lot more AMR genes circulating in Australian feedlots than previously reported (Alhamami et al., 2021; Alhamami et al., 2022). This suggest that it would be prudent to monitor AMR using a method that is not dependent on prior assumptions of the bacterial species and/or AMR genes present in the population of intertest. For example, the application of Luminex technology requires knowledge of the AMR genes and sequences to enable the design assay components. In contrast a sequencing based approach, such as ONT sequencing, has the potential to provide a comprehensive analysis of the bacterial species and AMR genes present in a sample. In this study the results from the ONT sequence analysis of some samples clearly demonstrated this principle. Some samples that where negative by culture for the bacterial pathogens of interest and AMR genes, were positive for AMR genes by ONT sequencing. As commensal bacteria can drive AMR, the AMR genes circulating in a feedlot population of respiratory pathogens might be influence by the ARM genes of the commensal organisms of the microbiome. One such gene that has been found by ONT sequencing in this study was the transposon- associated lincosamide resistance gene *Inu(C)* (Achard et al., 2005).

Improved microbiome assessments based on ONT sequencing results using adaptive sampling methods to reduce the proportion of host genome sequences in the final dataset has been reported for the bovine reproductive tract (Ong et al., 2022). Therefore, the optimisation of the ONT workflows is an important step to towards the application of sequence based analyses of nasal swabs taken from cattle being treated for BRD.

4.8 Feedlot A

4.8.1 Summary of collected samples

To evaluate the potential of ONP sequencing to nasal swabs were collected from Feedlot A from early March 2022 to late October 2022 with a total of 367 samples collected (Table 4.8.1). The criterion for sampling cattle was they were deemed to required treatment by the feedlot hospital staff, this was typically based on observed clinical signs by pen riders and confirmation of a rectal temperature >39.5◦C at the hospital pen. In some instances, cattle undergoing a "retreat" for BRD were also sampled.

The feedlot supplied the hospital session records for the days that sampling occurred, with the key parameter Days on Feed (DOF) summarised in Table 4.8.1. Of particular note is a very wide range for DOF for cattle undergoing BRD treatment as it ranged from 5 to 520 days.

Table 4.8.1 Summary of the samples collected from Feedlot A during 2022. The date of sample collection, number of animals treated for BRD on that day, number of animals sampled are shown. The range and average number of days on feed (DOF) at the time of BRD treatment are also shown.

Sampling Date	BRD treated	Sampled	DOF Range	DOF Ave.
15/03/2022[$]			11 to 42	
24/03/2022	22		20 to 44	30.7
29/03/2022	38	19	5 to 63	29.6
07/04/2022	29	15	7 to 50	27.2
13/04/2022	28	22	9 to 121	32.5
20/04/2022[$]	7			
27/04/2022	11	2	14 to 44	25.3
04/05/2022	15*	21	14 to 75	34.3
12/05/2022	30	33	3 to 520	48.1
18/05/2022	25	36	6 to 205	43.8
01/06/2022	16	21	14 to 142	26.9
09/06/2022	10	10	10 to 70	33.8
21/06/2022	42	26	12 to 118	35.8
05/07/2022	24	26	13 to 50	29.4
28/07/2022	40	18	8 to 80	30.8
23/09/2022	31	46	8 to 45	33.5[#]
28/09/2022	34	15	26 to 105	35.7
07/10/2022	16[^]	20	22 to 359	68.8
12/10/2022	17	28	15 to 48	31.1
26/10/2022	19	9	8 to 77	41.8
Summary	**447**	**367**	**3 to 520**	**35.6**

[$] All samples processed by the Microbiology Laboratory only.

* One animal treated as "Honker" after 51 DOF.

[#] Two animals listed as being treated at -96 DOF on hospital report were excluded from the average DOF.

[^] One animal treated as "Honker" after 29 DOF.

4.8.2 Summary of qPCR detection of viruses

All samples received from Feedlot A by the Virology Laboratory were tested for four viruses that have been historically associated with BRD with only two of these viruses being detected (Table 4.8.2). Briefly, bovine herpesvirus (BoHV-1) was the most frequently detected virus with 64.3% of samples testing positive. While 4.9% of samples were positive for bovine coronavirus (BCoV).

Table 4.8.2 Summary of the qPCR detection of bovine herpesvirus 1 (BoHV-1), bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV) and bovine parainfluenza virus 3 (BPIV3) in extracts from nasal swabs collected at Feedlot A.

		Number of qPCR Positives			
Sampling Date	**Sample Number**	**BoHV-1**	**BCoV**	**BRSV**	**BPIV3**
15/03/2022[$]		ND	ND	ND	ND
24/03/2022					
29/03/2022	19	2	0	0	0
07/04/2022	15	2	0	0	0
13/04/2022	22	19	0	0	0
20/04/2022[$]		ND	ND	ND	ND
27/04/2022	2	0	0	0	0
04/05/2022	21	12	0	0	0
12/05/2022	33	23	0	0	0
18/05/2022	36	34	1	0	0
01/06/2022	21	10	0	0	0
09/06/2022	10	9	1	0	0
21/06/2022	26	25	0	0	0
05/07/2022	26	22	0	0	0
28/07/2022	18	18	3	0	0
23/09/2022	46	19	5	0	0
28/09/2022	15	4	2	0	0
07/10/2022	20	14	3	0	0
12/10/2022	28	15	2	0	0
26/10/2022	9	8	1	0	0
Summary	**267**	**236**	**18**	**0**	**0**

4.8.3 Evaluation of high loads of BoHV-1 (IBR virus)

As cattle entering Feedlot A received a dose of Rhinogard™ at feedlot induction, the time elapsed from induction to testing positive to BoHV-1 is of interest to ensure the positive result is not residual vaccine. In our experience with the use of Rhinogard™ in pen trial studies we would expect cattle to clear the vaccine within 10 to 12 days from administration. Similarly, we would expect the result of the Ct value be above 25. As it can be difficult to assess the importance of a single qPCR result in the context of a viral infection the Ct based criterion was strengthened to include only those values less than 20, 21 samples were identified with Ct values less than 20. The characteristics of the animals from which these nasal swabs were collected are summarised in Table 4.8.3. The range of BoHV-1 Ct values was 12.44 to 19.99 for these samples, with five samples yield values less than 15 (Table 4.8.3). These later Ct values are exceptionally low, as we have not recorded values less than 15 in pen trial challenge experiments or even when growing BoHV-1 in laboratory cell culture.

The range of days on feed (DoF) for this group of animals was 21 to 60 (Table 4.8.3), strongly suggesting that the BoHV-1 detected is not the vaccine strain. Also of interest was that four samples were collected well outside the expected peak of BRD (days 14 to 28), at either 45 DoF or 60 DoF (Table 4.8.3).

Attempts were made to isolate the BoHV-1 strains from animal ID 2212558 (Ct = 12.44) and animal ID 2212578 (Ct = 17.62) to enable further characterisation of these viruses. These experiments were not successful.

The extracts for Animal ID 2212558 (Ct = 12.44) and Animal ID (Ct = 17.62) were analysed by ONT sequencing (Section 4.10.2; Table 4.10.2). The results of this sequencing experiment are described in detail in Section 4.10.2. Briefly, the vast majority of sequencing reads for the libraries from these samples corresponding to the host genome, this prevented the further characterisation of the BoHV-1 strains. It may be possible to develop approaches that would enable the sequencing of these BoHV-1 strains. As an example we are currently developing “capture” based ONT sequencing strategies in other projects. This approach uses a deactivated form of the gene editing protein, dCRISPR-Cas9 and specific guide RNA to bind DNA molecules of interest (van Haasteren et al., 2021). The dCRISPR-Cas9 in combination with a BoHV-1 specific guide RNA(s), would bind with the copies of the BoHV-1 genome in the samples of interest. The complexes could then be purified from the sample, once the viral genomes are released they could then be used to generate virus specific sequencing libraries.

Table 4.8.3 The qPCR results of selected samples showing high loads of bovine herpesvirus 1 (BoHV-1). The Days on Feed (DoF), date of sample collection and animal identifier are shown with the qPCR results (Ct values) for BoHV-1. Selected samples were also analysed by qPCR for *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh). Ct values less than 15 are highlighted yellow.

DoF	Date	ID	BoHV-1	Mb	Hs	Pm	Mh
21	13/04/2022	2212578	17.62	25.41	27.09	36.06	>40
21	13/04/2022	2212558	12.44	22.64	19.35	27.36	>40
28	04/05/2022	2214365	19.99	ND	ND	ND	ND
33	12/05/2022	2214440	18.34	ND	ND	ND	ND
NK	12/05/2022	2214456	15.57	ND	ND	ND	ND
36	18/05/2022	2215784	16.24	ND	ND	ND	ND
NK	18/05/2022	2215792	17.33	ND	ND	ND	ND
15	01/06/2022	2220A376	18.25	ND	ND	ND	ND
28	09/06/2022	2219A731	12.69	ND	ND	ND	ND
36	21/06/2022	2220A180	14.38	ND	ND	ND	ND
NK	21/06/2022	2214T309	16.67	ND	ND	ND	ND
36	05/07/2022	2222186	17.21	ND	ND	ND	ND
36	05/07/2022	2222146	17.33	ND	ND	ND	ND
36	05/07/2022	2222895	17.57	ND	ND	ND	ND
21	05/07/2022	2224523	17.79	ND	ND	ND	ND
30	28/07/2022	2226A060	17.35	32.84	28.94	NA	>40
30/31	28/07/2022	2226AXXX	13.75	ND	ND	ND	ND
45	23/09/2022	2232B861	16.57	27.14	27.65	32.23	>40
45	23/09/2022	2232B862	13.08	35.24	28.85	34.61	>40
44	07/10/2022	2234626*	16.78	35.56	32.13	33.61	>40
60	07/10/2022	2232A622	16.01	>40	>40	>40	>40

*2234626 - Retreat; first treat 28/09/2022 34 DOF no sample

4.9 Feedlot B

4.9.1 Summary of samples collected 2022

Sample collection at Feedlot B was conducted by the animal health crew. Initially, staff were requested to collected samples on a daily basis for one week based on the estimated number of BRD cases. However, the estimate on BRD cases proved to be inaccurate and consequently they were requested to sample once a week. A summary of the samples provided are shown in Table 4.9.1.

Table 4.9.1 Summary of the samples provided by Feedlot B.

Collection dates	Sample month	Sampling days	Samples
29/06/2022	June	2	24
28/07/2022	July	11	78
		No date	2
18/08/2022	August	12	60
07/09/2022			
07/09/2022	September	15	84
06/10/2022		No date	2
15/11/2022	October	9	32
		No date	5
15/11/2022	November	4	6
		Total Samples	**293**

4.9.2 Summary of qPCR detection of viruses

All of the samples (n=293) were tested using the same virus specific qPCR assays used for the Feedlot B samples. Once again, BoHV-1 was the most frequently detected virus, with 107 (36.6%) of samples testing positive. Evaluating the BoHV-1 results with respect the Ct value, 16 and 8 samples yield values less than 25 and less than 20, respectively. While 10 (3.8%) and 14 (4.8%) samples were positive for BCoV and BRSV, respectively. The results are summarised in Table 4.9.2 on a monthly basis.

A subset of these samples (n = 160) were also tested by qPCR for four the bacteria that have frequently been associated with BRD. *Histophilus somni* was the most frequently detect bacterial species with 76 (47.5%) testing positive. The remaining bacteria were detected at similar frequencies (Table 4.9.2).

Table 4.9.2 Summary of the testing of extracts from nasal swabs provided by Feedlot B by qPCR for selected viruses and bacteria frequently associated with BRD. Results are shown for of bovine herpesvirus 1 (BHV-1), bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV) and bovine parainfluenza virus 3 (BPIV3) in collected for *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh). The number of samples analysed are shown for viruses and bacteria are shown, along with the number testing positive to each pathogen of interest.

	Viral qPCR results					Bacterial qPCR results				
Month	***n***	**BHV1**	**BCoV**	**BRSV**	**BPIV3**	***n***	**Mb**	**Hs**	**Pm**	**Mh**
June	24	7	0	0	0	15	4	11	0	1
July	80	6	3	0	0	50	9	28	1	10
August	60	23	2	4	1	43	7	19	17	12
Sept	86	52	2	1	0	9	2	5	9	7
Oct	37	18	3	9	0	37	9	10	10	5
Nov	6	1	0	0	0	6	1	3	1	0
Total	**293**	**107**	**10**	**14**	**1**	**160**	**32**	**76**	**38**	**35**

4.9.3 Detection of AMR genes

Samples (n = 25) from Feedlot B were evaluated using the multiplex Luminex assays for AMR genes (Table 4.9.3). All of the samples tested were positive for the macrolide resistance genes, *msrE* and *mphE*, along with the sulphonamide AMR gene *sul2*. High levels of the tetracycline AMR genes, *tetH* and *TetR*, were also detected with, 24 and 34 samples testing positive, respectively. *Histophilus somni* was the most frequently detected bacterial species detected with 18 samples testing positive, with seven of these also positive for *M. bovis*. Five samples were positive for multiple AMR genes, but negative for the four bacterial species of interest by qPCR. This included the sample from Animal ID 222404071, that was positive for nine AMR genes, with at least one gene from each antimicrobial class. All of the samples tested were negative for *P. multocida*.

Table 4.9.3. Summary of the antimicrobial resistance genes from various classes detected in nasal swab samples (n =25) from Feedlot B using the multiplex Luminex assays. Only positive (Pos) Luminex results are shown. The qPCR results for the detection of bacterial species commonly associated with BRD are also shown, *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), and *Mannheimia haemolytica* (Mh).

	Macrolides			Beta-lactams				Tetracyclines						Sulphonamides		
Animal ID	***msrE***	***mphE***	***mefA***	***blaTEM***	***blaCTX-M1***	***blaKPC***	***cfxA***	***tetB***	***tetH***	***tetY***	***tetR***	***tetM***	***tetW***	***sul1***	***sul2***	**qPCR**
22240179	Pos	Pos					Pos		Pos			Pos	Pos	Pos	Pos	Mb
222404071	Pos	Pos		Pos	Pos	Pos	Pos		Pos		Pos				Pos	
222402019	Pos	Pos							Pos		Pos				Pos	Mb, Hs
221802061	Pos	Pos							Pos		Pos				Pos	Hs
221704049	Pos	Pos						Pos	Pos	Pos	Pos	Pos	Pos		Pos	
222204009	Pos	Pos					Pos		Pos	Pos	Pos	Pos	Pos		Pos	Mb, Hs
221504017	Pos	Pos						Pos	Pos	Pos	Pos				Pos	Mb, Hs
222702096	Pos	Pos							Pos	Pos	Pos				Pos	Hs
221802087	Pos	Pos							Pos		Pos		Pos		Pos	Hs, Mh
221802089	Pos	Pos							Pos	Pos	Pos				Pos	Hs
221801059	Pos	Pos			Pos		Pos		Pos	Pos	Pos		Pos		Pos	
222206136	Pos	Pos						Pos	Pos	Pos	Pos			Pos	Pos	
222204113	Pos	Pos							Pos				Pos		Pos	Mb, Hs
221704129	Pos	Pos		Pos					Pos	Pos	Pos				Pos	Hs
221802076	Pos	Pos							Pos	Pos	Pos				Pos	Mb, Hs
222001036	Pos	Pos							Pos		Pos				Pos	
222508013	Pos	Pos					Pos		Pos		Pos		Pos		Pos	Hs
222503136	Pos	Pos							Pos		Pos				Pos	Hs
221802091	Pos	Pos							Pos		Pos		Pos		Pos	Hs
221802081	Pos	Pos	Pos				Pos		Pos		Pos		Pos		Pos	Hs
222201020	Pos	Pos						Pos	Pos	Pos	Pos	Pos			Pos	Hs, Mh
222201098	Pos	Pos							Pos		Pos				Pos	Hs
222202006	Pos	Pos			Pos		Pos		Pos		Pos				Pos	Mb, Hs
222702048	Pos	Pos							Pos		Pos				Pos	Hs
222504060	Pos	Pos									Pos		Pos		Pos	
Total Pos	**25**	**25**	**1**	**1**	**3**	**1**	**7**	**4**	**24**	**10**	**23**	**4**	**10**	**2**	**25**	

4.10 Oxford Nanopore Sequence Analyses 2022

The aim of this component of the project was to evaluate the capacity of ONT sequencing to be used by feedlot staff to monitor the pathogens and/or presence of antimicrobial resistance (AMR) genes. There are two major elements that underpin this potential application of ONP technology. The first is sample preparation and the second is the preparation of the sequencing library to be loaded on to the ONP flow cell. Ideally, both of these steps would require minimal specialised laboratory equipment and involve a minimal number of manipulations.

Four sample extraction methods were evaluated. The first method was the Puregene kit that is currently used in combination with ONT sequence for major sequencing projects, such as sequencing of the bovine genome and other large genomes. This approach is known to yield high molecular weight and highly purified DNA from the sample of interest. The construction of several sequencing libraries using total DNA isolated from nasal swabs yielded good quantities of sequencing data. However, as might be expected the majority of the DNA sequenced was bovine.

4.10.1 ONT Sequencing with PrepMan™ Sample Extraction

The simplest method use for extraction DNA from nasal swabs was the PrepMan™ reagent. This approach was highly considered attractive for field applications as it is a rapid (5 min) three step process (mix, boil, spin) before the sample is ready for DNA based analysis. This reagent has been successfully used for the detection of viruses associated BRD in previous studies. The PrepMan™ extracts were initially testing using qPCR assays for four viruses and four bacteria associated with BRD (Table 4.10.1). Bovine herpesvirus 1 was detected in three samples and at least one bacterial species was detected in all samples. An ONP sequencing library was constructed using these extracts, however they did not yield any sequencing data (Table 4.10.1).

It is not readily apparent why the PrepMan™ extract libraries did not yield any sequencing data. However, the most likely cause is high levels of impurities in the extracts either interfering DNA accessing the pores or inactivating the pores on the flow cell. While it may have been possible to construct usable libraries from these extracts by re-extracting (eg with Puregene), this would have negated the any benefits of using this reagent, as result this approach was not investigated further.

Table 4.10.1 Summary of the qPCR analyses of nasal swab extracts prepared using the PrepMan™ reagent. The results are shown for bovine herpesvirus 1 (BHV1), bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV), and bovine parainfluenza virus 3 (BPIV3), *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh). The cycle threshold values are shown, with values >40 being deemed negative.

	Viruses				Bacteria			
Animal ID	**BHV1**	**BCoV**	**BRSV**	**BPIV3**	**Mb**	**Hs**	**Pm**	**Mh**
2237096	37.65	>40	>40	>40	>40	>40	>40	>40
2237196	>40	>40	>40	>40	>40	27.91	>40	>40
2237A186	34.08	>40	>40	>40	>40	32.16	38.54	>40
2234729	>40	>40	>40	>40	30.59	32.64	34.66	>40
2236B254	37.25	>40	>40	>40	>40	20.4	>40	>40

4.10.2 ONT Sequencing with Puregene Sample Extraction (n=6)

The standard approach for the isolation of genomic DNA for ONT applications is the used of the Puregene extraction kit. Total DNA was isolated from six nasal swab samples collected from Feedlot A and sequencing library constructed using the Rapid Barcoding kit. The selected samples had been tested by qPCR for the presence of genomic DNA from viral and bacterial pathogens frequently associated with BRD. Table 4.8.2 summarises and compares the results of the ONP sequencing and the qPCR results. Overall the ONP sequencing generated high numbers of sequencing reads. However the vast majority of sequencing reads aligned to the bovine genome, the most extreme example being ID 2211038 for which 106965 reads aligned to the bovine genome while 2,513 reads did not (Table 4.10.2). Despite this bias towards the host genome, there was some agreement between the sequencing data and the qPCR with both detecting *H. somni.* Interestingly, other bacteria were also identified by the ONP sequencer. For example, *Clostridium* accounted for at least 67.4% of the sequence reads in the five positive samples. As cattle are routinely vaccinated and boosted with multivalent clostridial vaccines it is somewhat surprising that these species dominated the sequencing results. Also of note was the detection of *Moraxella* as *Moraxella bovis* is well known as the causative agent of pinkeye (infectious bovine keratoconjunctivitis) in cattle. *Mycoplasma bovis* and *P. multocida* were detected in all samples by the respective qPCR assays, however they were not detected in the ONP datasets (Table 4.10.2).

With respect to viruses, all samples were also positive to BoHV-1 in the qPCR analyses. However, BoHV-1 was only two of the six samples by ONP sequencing. The BoHV-1 samples positive by sequencing were two of the samples described previously as having extremely high virus loads (Ct values <20), with one of these being the highest viral load (Ct = 12.44) detected in this study (Table 4.10.3 and Table 4.10.2). The samples with BoHV-1 Ct values of 17.62 and 12.44 only account for 6.5% and 4.1% of the ONP sequencing reads, respectively. While in the remaining samples, with Ct values ranging from 22.51 to 30.72, no sequencing reads were identified that aligned to BoHV-1.

It was also observed that in four of the six samples, sequence reads corresponding to the human genome were detected in the filtered ONP sequencing data with 14.8% to 23.6% of sequencing reads being classed into this category. This was unexpected as the datasets had been filtered to remove sequences that align to the bovine genome. It is suspected that these sequencing reads are actually bovine sequences that did not match with sufficient identity during the initially bioinformatic filtering processes. They may represent as yet uncharacterised variants of the bovine genome or some form of artefacts from the library construction process that masks their identify to the bovine genome.

Overall, while there was some agreement between the ONP sequencing and the qPCR assays it was concluded that the high levels of bovine DNA in the extracts resulting high representation of this genome in the sequencing data, potentially limiting the capacity to detect the pathogens of interest.

Table 4.10.2 Summary of the Oxford Nanopore sequencing results for six nasal swab extracts from Feedlot A extracted with the Puregene kit and the sequencing library prepared using Rapid Barcoding. Taxonomic assignments were made at the genus level by aligning (Align) non bovine sequences. The fraction of ONP sequences assigned to each taxon are show. The qPCR results for detection of bovine herpesvirus 1 (BHV1), ungulate bocaparvovirus 6 (UBPV6), *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh) are also shown. Agreement between the ONP assignments and qPCR results are highlight blue.

	ONP Seq			Taxonomic group assignments						qPCR Results					
ID	**DoF**	**Align**	**bovine**	**Clostridium**	**Bacillus**	**Moraxella**	**Histophilus**	**Homo**	**Varicellovirus**	**BHV1**	**UBPV6**	**Mb**	**Hs**	**Pm**	**Mh**
2211038	30	2513	106965	0.67362	0.12883	0.0319	0.0135	0.14847		29.02	>40	35.77	23.02	38.89	>40
2212578	21	2404	49110	0.67678	0.07916	0.01583		0.16359	0.06464	17.62	34.94	25.41	27.09	36.06	>40
2212584	21	1721	49110	0.75514		0.05761		0.18519		23.09	>40	31.51	27.12	39.24	>40
2212455	21	951	33081	0.69565		0.06522		0.23551		30.72	>40	25.03	25.29	29.61	>40
2212558	21	2342	21981	0.78551		0.17188			0.04119	12.44	>40	22.64	19.35	27.36	>40
2211874	??	471	0							22.51	20.11	28.66	24.91	37.11	>40

4.10.3 ONT Sequencing with Puregene Sample Extraction (n=2)

In an attempt to increase the likelihood of detecting the BRD associated pathogens using ONP sequencing. Two of the previously analysed samples were re-examined. By reducing the number of sequencing libraries added to the flow cell, it was hypothesised that an increased number of sequencing reads would be obtained (increased sequencing depth), thus increasing the likelihood of improving the agreement between the sequencing and qPCR results. The results of this experiment are summarised in Table 4.10.3. The hypothesis was partial proved as reducing the number of libraries added to the ONP flow cell did increase the yield of sequencing reads (compare Table 4.10.2 and Table 4.10.3). This was particularly true for the library of ID2211874, where the total yield of sequencing reads increased from 471 (Table 4.10.2) to 34,336 (Table 4.10.3). However, there was no improvement in the agreement between the ONP sequencing and the qPCR results for the pathogens of interest. In fact the level of agreement actually decreased. In the first analysis of the extract from ID2212578, 6.5% of the non-bovine sequencing reads aligned to the genome of BoHV-1, in agreement with the qPCR Ct = 17.62. In contrast, the second sequencing analysis of this sample did not yield any reads corresponding to BoHV-1, despite a 7.8 fold increase in sequencing reads. The increased depth of sequencing resulting in the generation of reads corresponding to a wider range of bacteria (Table 4.10.3). However, with this increased range the list of most frequently detected genera also changed. For example, the initial sequence of ID2212578, *Clostridium* and *Moraxella* were the first and third most frequently detected bacteria (Table 4.10.2). In contrast, in the re-sequencing, *Clostridium* and *Moraxella* were the sixth and first most frequently detected bacteria (Table 4.10.3). Also of interest was the detection of *M. haemolytica* in the second analyses of this sample, as the bacterium was not detected by qPCR (, Clostridium and Moraxella were the first and third most frequently detected bacteria (Table 4.10.3). For both samples, the increase in sequencing depth also resulted in an increase in samples, initially screened and discarded as bovine genome and well as the proportion of sequences deemed to be human in the secondary analysis.

The results of this experiment suggest that increasing the depth of sequencing may not necessarily increase the likelihood of detecting bacterial and/viral pathogens that have historically been associated with BRD. Rather increased depth of sequencing is likely to increase the diversity of bacteria detected.

Table 4.10.3 Summary of the Oxford Nanopore sequencing results for two nasal swabs from feedlot A extracted with the Puregene kit (Qiagen). The sequencing library was constructed using the ONP Rapid Barcoding kit. Taxonomic assignments were made at the genus level by aligning (Align) non bovine sequences. The fraction of ONP assigned to each taxon are show. Taxonomic grouping assigned are shown, Moraxella (Mx), Acinetobacter (Acinet), Psychrobacter (Psych), Mannheimia (Mann), Escherichia (Esch), Clostridium (Clost), Bacillus (Bac), and Homo (human). The qPCR results for detection of bovine herpesvirus 1 (BHV1), ungulate bocaparvovirus 6 (UBPV6), *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh) are also shown. Agreement between the ONP assignments and qPCR results are highlight blue.

		ONP Seq		Taxonomic group assignments								qPCR Results					
ID	**DoF**	**Align**	**Bovine**	**Mx**	**Acinet**	**Psych**	**Mann**	**Esch**	**Clost**	**Bac**	**Homo**	**BHV1**	**UBPV6**	**Mb**	**Hs**	**Pm**	**Mh**
2212578	21	2137	400612	0.18889	0.01296	0.01019	0.04907	0.03241	0.2463	0.05185	0.40463	17.62	34.94	25.41	27.09	36.06	>40
2211874	NK	208	34128						0.79592		0.20408	22.51	20.11	28.66	24.91	37.11	>40

4.10.4 ONT Sequencing with Host Depletion 1 - MolYsis Basic5

The previous analyses suggest that presence of bovine DNA in the nasal swab extracts was reducing the capacity to detect pathogen DNA. This is considered unavoidable as the collection of the nasal swab required the swab to contact the mucosal surfaces and therefore the collection of bovine cells. When extracted and used for ONP library preparation of the size of the bovine genome compared to bacterial and viral genomes further exacerbates this issue, as fragmentation is used in these processes, meaning the final number of bovine DNA molecules are likely to outcompete the pathogen DNA molecules when it comes to the frequency of entering pores on the flow cell to enable sequencing.

To address this issue, sample extraction protocols that have been shown to remove host DNA and increase the proportion of bacterial DNA in final extracts were evaluated in this study. The first approach to be tested was the MolYsis Basic5, while the specifics of how this approach works is proprietary manufacturer knowledge, the basic principles are the preferential lysis of host cells, then enzymic degradation of released host DNA. Following inactivation of the enzymes, the microbial DNA can be safely extracted. It is likely that the use of this approach may limit the capacity to detect some pathogens of interest. For example, as *M. bovis* is not a true bacterium as it lacks are cell wall it may be susceptible to lysis. Similarly, as BoHV-1 is an enveloped virus it may also be lysed by this approach. However, it is possible that BoHV-1 could be still detectable via sequence as the viral capsid may protect the viral DNA from degradation. A similar process to this has been used for several years to reduce host contamination in the preparation of BoHV-1 genomic DNA for sequencing applications.

Sequencing libraries were prepared for four samples from Feedlot A following extraction with the MolYsis kit combined with the Puregene extraction kit. Three of the four libraries yield usable amounts of sequencing data. Overall there was good depletion of bovine sequences from the resulting sequencing data, with two of the three libraries yield higher numbers of sequence reads that did not align to the bovine genome compared to those that did (Table 4.10.4a). Analyses of the non-bovine sequences also increased the number of bacterial species identified for three of the four samples analysed. However none of the bacterial species identified via ONP sequencing corresponded to the qPCR results for these samples. As shown in Table 4.10.4b, all of the samples tested positive by qPCR to *H. somni*, however this bacterium was not detected in the ONP dataset.

Table 4.10.4a Summary of the Oxford Nanopore sequencing results for four nasal swabs from Feedlot A extracted with the MolYsis kit and the Puregene kit (Qiagen). The sequencing library was constructed using the ONP Rapid Barcoding kit. Taxonomic assignments of sequencing reads were made at the genus level. The fraction of ONP assigned to each taxon are shown. Taxonomic grouping assigned are shown as, *Acinetobacter* (Acin), *Moraxella* (Mx), *Jeotgalicoccus* (Jeot), *Staphylococcus* (Staph), *Escherichia* (Esch), *Psychrobacter* (Psyc), *Klebsiella* (Kleb), *Streptococcus* (Strep), *Lactobacillus* (Lacto), Enterococcus (Enter), *Bacillus* (Bac), *Turicibacter* (Turic), *Chryseobacterium* (Chrys) and Homo (human).

	ONP Seq		Taxonomic group assignments												
ID	**Align**	**Bovine**	**Acin**	**Mx**	**Jeot**	**Staph**	**Esch**	**Psyc**	**Kleb**	**Strep**	**Lacto**	**Enter**	**Bac**	**Turic**	**Chrys**	**Homo**
2232B821	2100	411	0.252	0.095		0.066	0.215		0.069				0.040			0.252
2236A876	2563	216	0.037	0.415		0.022	0.166	0.030	0.137	0.030						0.159
2235A006	824	2109		0.083	0.206	0.142	0.145		0.037	0.142	0.037	0.034		0.043	0.055	0.062
2235A019	33	0					1									

Table 4.10.4b Summary of the qPCR results for four nasal swabs from Feedlot A extracted with the MolYsis kit and the Puregene kit (Qiagen). Animal identifiers are shown with the qPCR results (Ct values) for BoHV-1. The samples were also analysed by qPCR for *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh).

ID	BHV1	Mb	Hs	Pm	Mh
2232B821	29.85	35.15	35.27	32.15	31.3
2236A876	38.4	>40	31.73	33.26	>40
2235A006	32.7	>40	34.26	>40	>40
2235A019	>40	32.1	17.98	35.59	35.19

4.10.5 ONT Sequencing with Host Depletion 2 - MolYsis Basic5

Host depletion was further evaluated using the MolYsis Basic5 kit, six samples from Feedlot B were extracted and subject to ONP sequencing and qPCR sequencing analyses. Overall excellent host depletion was achieved with these samples with less than 7% of sequencing reads aligning to the bovine genome for all sequencing libraries (Table 4.10.5). When compared to the qPCR results there was some agreement with the sequencing analyses. For example, two of the three qPCR positives for *M. bovis*, were also positive by ONP sequencing (Table 4.10.5).

Three of the sequencing libraries yield high numbers of sequencing reads, an order of magnitude higher than the other two (ID224102043 and ID224102043) (Table 4.10.5). It is noted that in general for these libraries that yield higher amounts of sequencing data, agreement with the qPCR results were more concordant compared to the libraries that yielded less data.

However, three samples that were positive for BoHV-1 and *H. somni* were all negative by ONP sequencing. Similar to the first set of samples extracted using the MolYsis Basic5 from Feedlot A, none of these qPCR positives had low Ct values. However all the Ct values were less than 35 and would therefore be considered positive for the purposes of other disease investigations. As with the first application of this host depletion technology, these data suggest that the overall representation of some pathogens of interest may decrease when this approach is used.

It was also noted that genus *Haemophilis* spp. was well represented in four of the six libraries (Table 4.10.5). While this bacterium is not generally associated with BRD, it is associated with respiratory disease in other species.

Another interesting observation was in comparing the bacterial genera identified in each library, *Acinetobacter* spp was well represented in all sequencing libraries (Appendix D). On the basis of sequencing numbers its highest ranking was 2 for ID221403096 and lowest was 11 for ID224102043. This bacterium has not been considered in the context of BRD. However, *Acinetobacter baumannii* is considered of importance in human medicine as it associated with morbidity and mortality due to its highly drug resistant nature (Mateo-Estrada et al., 2022). In contrast, bovine isolates tend to have high antimicrobial susceptibility. To date there do not appear to be any studies which have investigated the potential role of this bacterium in BRD.

Table 4.10.5 Summary of the Oxford Nanopore sequencing results for six nasal swabs from Feedlot B extracted with the MolYsis Basic5 for host depletion. The sequencing libraries were constructed using the ONP Rapid Barcoding kit. The ONP data are shown as number of reads aligned to bacteria (Align), the number of bovine genomic reads (Bovine) and the percentage of all reads aligning to the bovine genome (B%). Taxonomic assignments of sequencing reads were made at the genus level. The fraction of ONP assigned to each taxon are show. Taxonomic assignments were made at the genus level. Taxonomic groupings assigned are shown as, *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Haemophilis* (Haem), *Pasteurella multocida* (Pm), *Mannheimia haemolytica* (Mh) and *Bibersteinia* (Biber). The fraction of ONP assigned to each taxon are show. The qPCR results for the detection of bovine herpesvirus 1 (BHV1), Mb, Hs, Pm and Mh are also shown. Agreement between the ONP assignments and qPCR results are highlighted in blue.

ONP Data				Taxonomic Assignments							qPCR Results				
ID	**Align**	**Bovine**	**B%**	**Genera**	**Mb**	**Hs**	**Haem**	**Pm**	**Mh**	**Biber**	**BHV1**	**Mb**	**Hs**	**Pm**	**Mh**
223205031	631464	10034	1.6	411	0.00011		0.00019	0.00003	0.0001	0.0002	34.2	32.3	30.2	>40	>40
224102043	15723	697	4.2	19							>40	>40	31.7	>40	>40
223801069	377185	24360	6.1	205			0.00005	0.00004	0.00208		>40	>40	34.9	>40	>40
221403096	159352	781	0.5	165	0.00047		0.00034		0.0006		34.8	39.2	>40	32.8	30.7
224104006	13866	243	1.7	15							26.9	>40	>40	>40	31.0
222405113	149345	570	0.4	213			0.00039				>40	36.1	>40	>40	33.9

4.10.6 ONT Sequencing with Host Depletion 3 – Devin kit

To further explore the capacity of host depletion to improve the detection of bacterial species in the nasal swab samples, a second technology Devin kit extraction kit was utilised in combination with the ONP Rapid Barcoding kit using samples from Feedlot B. The results of the ONP sequencing data analyses are summarised and compared to the qPCR results for six samples in Table 4.10.6. The Devin kit facilitated excellent host depletion in the majority of samples, with five of the six libraries yielding <4% bovine genome reads. Overall there was a dramatic increase in the number of bacteria genera detected in these ONP libraries, with between 232 and 106 different types of bacteria detected in the various samples (Table 4.10.6). When compared to the qPCR data, the ONP sequencing was more sensitivity for the detection of *M. bovis* and *M. haemolytica*. The two samples that tested positive for *H. somni* and *P. multocida* by qPCR were also positive by ONP sequencing.

While four of the six samples were positive for BoHV-1 by qPCR, the virus was not detected in the sequencing data (Table 4.10.6). While these samples were qPCR positive for BoHV-1, the Ct values for three were >37 suggesting very low levels of the virus in the samples. While the Ct value for the fourth sample had a modest Ct value of 26.99, it would appear that even with host depletion this level of virus is not sufficient to be detected by ONP sequencing. This is consistent with the experiment summarised in Table 4.8.2 with only samples with qPCR Ct values less than 20 yielding ONP sequencing reads.

Of note in these sequencing datasets is the broad range of bacterial species identified across the samples. As shown in Table 4.10.6, while there was detection of several species the bacteria associated with BRD, the number of sequencing reads for these bacteria were not high. For example, ID224001152 yielded 365,390 sequencing reads, of which 0.01% were *M. bovis* equating to an estimated 37 specific reads. However, with the sequencing reads 50.4% of reads were aligned to *Pseudomonas* (Appendix E). This a good reminder that analyses of samples via ONP or any other high throughout sequencing technologies provides information on the complete microbiome of the sample being analysed. As a consequence, while a bacterium of known pathogenicity maybe present in the sample, it does not necessarily follow the expected paradigm and be the dominant species within the microbiome. In conventional disease investigations, pathogenic bacterial species are often referred to as fastidious (i.e., hard to isolate and/or grow in the laboratory), as a consequence highly selective processes are often employed to enable isolation these pathogens which removes them from the context of the microbiome in subsequent interpretations.

As identified in the previous analysis, *Haemophilus* spp. and *Acinetobacter* spp. were present, being detected in five and six of the samples, respectively. *Acinetobacter* was not ranked as highly in the frequency of identified reads, ranging from four for ID223107093 to 30 for ID224001105 (Appendix E).

Overall, the Devin approach to host depletion was highly successful, not only was there strong agreement between the ONT sequencing and qPCR results this was achieved in the background of high numbers of bacterial genera (Table 4.10.6). These results suggest that the Devin kit could be ideal for host depletion while retaining the microbial diversity with the original sample.

Table 4.10.6 Summary of the Oxford Nanopore sequencing results for six nasal swabs from Feedlot B extracted with the Devin kit for host depletion. The sequencing libraries were constructed using the ONP Rapid Barcoding kit. The ONP data are shown as number of reads aligned to bacteria (Align), the number of bovine genomic reads (Bovine) and the percentage of all reads aligning to the bovine genome (B%). Taxonomic assignments of sequencing reads were made at the genus level. The fraction of ONP assigned to each taxon are show. Taxonomic assignments were made at the genus level. Taxonomic groupings assigned are shown as, *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Haemophilis* (Haem), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh) The fraction of ONP assigned to each taxon are show. The qPCR results for the detection of bovine herpesvirus 1 (BHV1), Mb, Hs, Pm and Mh are also shown. Agreement between the ONP assignments and qPCR results are highlighted in blue.

	ONP Data			Taxonomic Assignments						qPCR Results			
	Align	**Bovine**	**B%**	**Genera**	**Mb**	**Hs**	**Haem**	**Pm**	**Mh**	**BHV1**	**Mb**	**Hs**	**Pm**	**Mh**
224001105	186415	345	0.19	198	0.00047 (192)			0.00024 (138)		>40	>40	>40	>40	>40
221403157	121401	790	0.65	232	0.00017 (229)			0.0002 (137)	0.0005 9 (136)	>40	>40	>40	36.31	>40
224001152	365390	14978	4.10	219	0.0001 (210)	0.0001 (35)	0.00012 (34)		0.0000 8 (36)	37.34	>40	27.8	>40	>40
220402037	114643	683	0.60	221	0.00035 (217)			0.0002 (127)	0.0003 3 (126)	37.25	>40	>40	>40	>40
224104006	79973	1434	1.79	164			0.00024 (25)			26.99	>40	>40	>40	31.01
223107093	38262	67319	63	106						37.21	>40	35.69	34.23	>40

4.10.7 ONT Sequencing for Detection of AMR genes

To evaluate the capacity of the ONT sequencing to detect antimicrobial resistance genes (AMR), the various datasets were analysed using ABRicate. Briefly, ABRicate is a compilation of multiple databases (NCBI, CARD, ARG-ANNOT, Resfinder, MEGARES, EcOH, PlasmidFinder, Ecoli_VF and VFDB) that enables screening of sequences for AMR genes (and virulence genes). The results of these analyses are summarised in Table 4.10.7. Overall the results suggest the main factor influencing the likelihood of detecting AMR genes, not unexpectedly, is the total number of sequencing reads generated. Similarly with increasing number of sequence reads, the confidence that samples are truly free of AMR genes also increases. As an example, the sequencing library for ID224001152 yielded 365,390 sequences, none of which match any AMR genes in ABRicate. As this was one of the highest number of sequences obtained any of the samples, it is considered to be AMR gene free with high confidence (Table 4.10.6).

It should be noted that the AMR gene sequences in ABRicate appear to be redundant. While the data shown in Table 4.10.7 is for individual sequences in each dataset it is possible that the reads may correspond or be aligned to the same AMR gene. Consequently, the number of sequencing reads matching an AMR gene should not be considered to equate to the number of AMR genes in a given sample.

Similar to the results from the multiplex Luminex assays, there was a great diversity of AMR genes detected by ONT sequencing on Feedlot B compared to Feedlot A (Table 4.10.7 and Table 4.10.8). While 67 AMR genes were detected across all of the ONT sequencing datasets from both feedlots only three (blaOXA-427, msr(E) and erm(F)) were detected at both (Figure 4.10.1A). The AMR genes detected at Feedlot A were dominated by those from the beta-lactam class of antimicrobials, followed by macrolide resistance genes (Table 4.10.7; Figure 4.10.1). On Feedlot B, aminoglycoside (n=12), beta-lactams (n =9), macrolide (n = 9), polyketide/tetracycline (n = 10) resistance genes were detected at similar frequencies.

When antimicrobial classes were considered, macrolide and beta-lactam AMR genes were detected on both feedlots (Figure 4.10.1B). While resistance genes to antimicrobials from the sulfonamide, aminoglycoside, polyketide, fluoroquinolone, and diaminopyrimidine classes were detected exclusively on Feedlot B (Figure 4.10.1B).

These analyses confirm that the detection of AMR genes using ONP sequencing technologies is highly plausible. However, it may be necessary to develop guidelines that specify the required depth of sequencing.

Table 4.10.6 Summary of the number of reads matching antimicrobial genes (AMR) in the Oxford Nanopore datasets generated in this study when compared to the ABRicate compiled database. The Animal ID numbers are shown, Days on Feed (DoF), number of sequencing reads used to identify bacterial taxons for each sample (Align), number of sequencing reads matching the bovine genome (Bovine), number of Align reads that match AMR genes (Reads AMR), the percentage of these AMR reads (%) and method of host sequence depletion (Host Dep.).

Section	ID	DoF	Align	Bovine	Reads AMR	%	Host Dep.
4.8.2	2211038	30	2513	106965	0	0.00	None
	2212578	21	2404	49110	0	0.00	None
	2212584	21	1721	49110	0	0.00	None
	2212455	21	951	33081	0	0.00	None
	2212558	21	2342	21981	0	0.00	None
	2211874	?	471	0	0	0.00	None
4.8.3	2212578	21	2137	400612	17	0.00	None
	2211874	?[1]	208	34128	0	0.00	None
4.8.4	2232B821	?[1]	2100	411	34	1.35	Molysis
	2236A876	?[1]	2563	216	33	1.19	Molysis
	2235A006	?[1]	824	2109	17	0.58	Molysis
	2235A019	?[1]	33	0	7	21.21	Molysis
4.8.5	224001105	?[1]	186415	345	1	0.00	Molysis
	221403157	?[1]	121401	790	7	0.01	Molysis
	224001152	?[1]	365390	14978	0	0.00	Molysis
	220402037	?[1]	114643	683	1	0.00	Molysis
	224104006	?[1]	79973	1434	7	0.01	Molysis
	223107093	?[1]	38262	67319	12	0.03	Molysis
4.8.6	223205031	?[1]	631464	10034	1678	0.26	Devin
	224102043	?[1]	15723	697	4	0.02	Devin
	223801069	?[1]	377185	24360	1150	0.29	Devin
	221403096	?[1]	159352	781	17	0.01	Devin
	224104006	?[1]	13866	243	0	0.00	Devin
	222405113	?[1]	149345	570	102	0.07	Devin

[1] Unknown.

Table 4.10.7. Summary of the antimicrobial resistance genes (AMRG) detected in the Oxford Nanopore Technology (ONT) sequence datasets from Feedlot A using the Abricate compiled database. The nasal swabs used for total DNA were extracted for sequencing the Molysis kit combined with DNeasy purification. All extracts were prepared for sequencing using the ONT Rapid PCR barcoding protocol. The number of sequencing reads matching the listed ARMG are shown. The total number of sequencing matching AMRG for each animal are also shown.

	Animal Identification Number					Antimicrobial	
AMRG	**2232B821**	**2236A876**	**2235A006**	**2235A019**	**2212578** [1]	**Class**	**Example**
blaTEM-112	1		1			beta-lactam	Ampicillin
blaTEM-116	1					beta-lactam	Ampicillin
blaTEM-122	1	1				beta-lactam	Ampicillin
blaTEM-146	1	1				beta-lactam	Ampicillin
blaTEM-150	2	1		1	6	beta-lactam	Ampicillin
blaTEM-156	9	4	2	1		beta-lactam	Ampicillin
blaTEM-168	5	1			4	beta-lactam	Ampicillin
blaTEM-171					2	beta-lactam	Ampicillin
blaTEM-181	1		1			beta-lactam	Ampicillin
blaTEM-183	1	1	3		2	beta-lactam	Ampicillin
blaOXA-427					1	beta-lactam	Ampicillin
blaTEM-1A	11	8	1	3		beta-lactam	Ampicillin
blaTEM-1C				1		beta-lactam	Ampicillin
blaTEM-231	1	2			2	beta-lactam	Ampicillin
blaTEM-79				1		beta-lactam	Ampicillin
blaTEM-81		1				beta-lactam	Ampicillin
blaTEM-90		1				beta-lactam	Ampicillin
erm(C)	1		3			macrolide	Erythromycin
erm(F)			6			macrolide	Erythromycin
erm(T)	1	1				macrolide	Erythromycin
lnu(A)		1				macrolide	Lincomycin
msr(E)		10				macrolide	Erythromycin
Total Reads	**36**	**33**	**17**	**7**	**17**		

[1] The nasal swab from this animal was extracted using the Puregene kit.

Table 4.10.8 Summary of the antimicrobial resistance genes (AMRG) detected in the Oxford Nanopore Technology (ONT) sequence datasets from Feedlot B using the Abricate compiled database. The nasal swabs used for total DNA were extracted for sequencing using either the Molysis kit combined with Puregene (E1) or the Devin kit (E2). All extracts were prepared for sequencing using the ONT Rapid barcoding protocol. The number of sequencing reads matching the listed ARMG are shown. The total number of sequencing matching AMRG for each animal are also shown.

	Animal[1] – Extraction method												Antimicrobial	
AMRG	**A - E1**	**B - E1**	**C - E1**	**D - E1**	**E - E1**	**F - E1**	**G - E2**	**H - E2**	**I - E2**	**J - E2**	**K - E2**	**L - E2**	**Class**	**Example**
aac(6')-Ib-cr			1										fluoroquinolone	Ciprofloxacin
aac(6')-Ib			3										aminoglycoside	Amikacin
aadA1	89		5								1		aminoglycoside	Streptomycin
aadA15	1												aminoglycoside	Streptomycin
aadA2	2												aminoglycoside	Streptomycin
aadA9				1									aminoglycoside	Streptomycin
ant(2'')-Ia			27										aminoglycoside	Gentamicin
ant(3'')-Ia	103		2	1		1					1		aminoglycoside	Streptomycin
aph(3')-Ia	236		14										aminoglycoside	gentamicin
aph(3'')-Ib	259	1	140	1		12							aminoglycoside	Streptomycin
aph(3')-IIa	1												aminoglycoside	
aph(6)-Ic	1												aminoglycoside	Streptomycin
aph(6)-Id		1	125	1		12							aminoglycoside	Streptomycin
blaCARB-5												1	beta-lactam	Ampicillin
blaCARB-8			52									1	beta-lactam	Ampicillin
blaCARB-10				2									beta-lactam	Ampicillin
blaCGB-1			1										beta-lactam	Ampicillin
blaFONA-5						2							beta-lactam	Ampicillin
blaOXA-347						3	1						beta-lactam	Ampicillin
blaOXA-284			1										beta-lactam	Ampicillin
blaOXA-363			2										beta-lactam	Ampicillin
blaOXA-427											3		beta-lactam	Ampicillin
catB3			82										phenicol	Chloramphenicol
cmx			116										phenicol	Chloramphenicol

dfrA1			6										diaminopyrimidine	Trimethoprim
dfrA17			77										diaminopyrimidine	Trimethoprim
dfrA5			1			4							diaminopyrimidine	Trimethoprim
erm(F)				1		4				1			macrolide	Virginiamycin
lnu(B)				1									macrolide	Lincomycin
mef(A)				1									macrolide	Erythromycin
mef(C)						1							macrolide	
mph(B)						1							macrolide	Erythromycin
mph(E)	16		92	3		7						2	macrolide	Erythromycin
mph(G)	2					1							macrolide	
mph(N)						1							macrolide	Erythromycin
msr(E)	8		58			5						1	macrolide	Erythromycin
sul1	21		91	1		4					1		sulfonamide	Sulfamethoxazole
sul2	294	1	60	1		12						3	sulfonamide	Sulfamethoxazole
tet(33)	26			1								2	polyketide	Tetracycline
tet(39)	1											1	polyketide	Tetracycline
tet(42)				1							1		polyketide	Tetracycline
tet(A)								3					polyketide	Tetracycline
tet(G)	2		99			2		1					polyketide	Tetracycline
tet(H)	5		61										polyketide	Tetracycline
tet(M)	1												polyketide	Tetracycline
tet(X)	315	1	34	1		26		3				1	polyketide	Tetracycline
tet(Y)						4							polyketide	Tetracycline
tet(Z)	7												polyketide	Tetracycline
Total Reads	**1678**	**4**	**1150**	**17**	**0**	**102**	**1679**	**7**	**0**	**1**	**7**	**12**		

[1] Letters represent the following animal identification numbers: A ID 631464; B 1572; C 377185; D 159352; E 13866; F 149345; G 186415; H 121401; I 365390; J 114643; K 79973; L 38262. The sequencing data for Animals A to L.

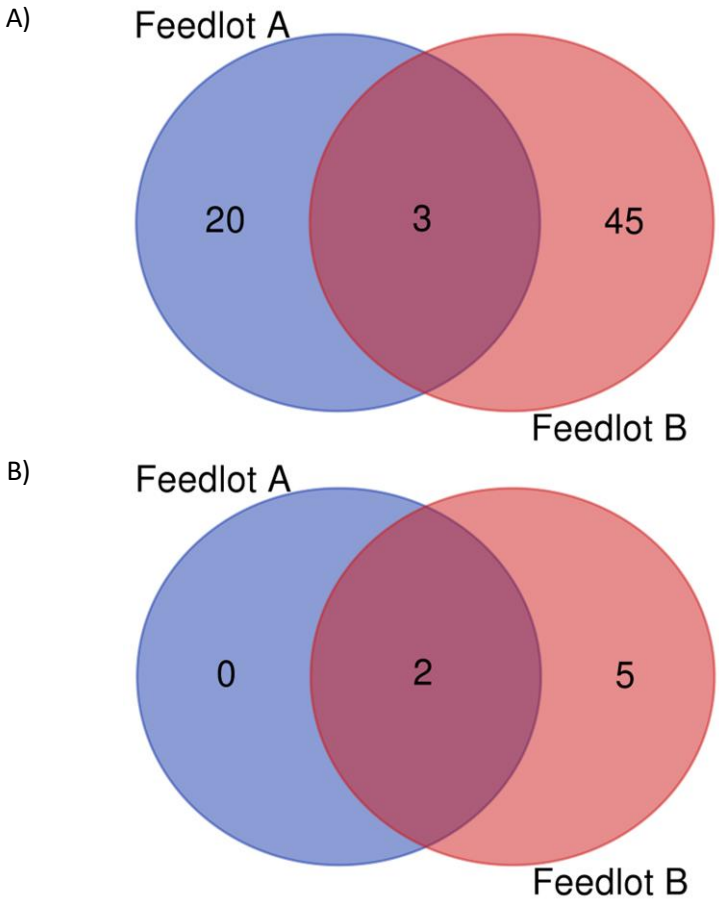

Figure 4.10.1. Comparison of the antimicrobial resistance (AMR) genes detected by Oxford Nanopore Technology sequencing in nasal swab samples collected from Feedlot A and Feedlot B. Panel A) The ARM genes detected on Feedlot A (n = 20, blaTEM-181, blaTEM-231, blaTEM-122, blaTEM-90, blaTEM-146, blaTEM-150, lnu(A), blaTEM-1A, erm(C), blaTEM-168, erm(T), blaTEM-183, blaTEM-1C, blaTEM-156, blaTEM-116, blaTEM-112, blaTEM-171, blaTEM-79, blaTEM-81) only, on feedlot B (n = 45 aadA2,, mph(N), mph(G), sul1, tet(33), aadA1, tet(X), mph(B), aac(6')-Ib-cr, lnu(B), blaOXA-347, blaCARB-8, aac(6')-Ib, tet(G), cmx, tet(42), mef(A), blaCGB-1, tet(M), blaCARB-5, tet(39), blaFONA-5, tet(A), aph(3'')-Ib, blaOXA-284, aadA9, aph(6)-Id, dfrA17, ant(2'')-Ia, dfrA1, mph(E), blaOXA-363, tet(Z), catB3, tet(Y), mef(C), sul2, aph(3')-Ia, blaCARB-10, aph(6)-Ic, aadA15, tet(H), aph(3')-IIa, dfrA5, ant(3'')-Ia) only, and those detected on both feedlots (n = 3, *blaOXA-427, msr(E),* and *erm(F)*). Panel B) The classes of AMR genes detected on Feedlot A (n = 0) only, on feedlot B (n = 5; sulfonamide, aminoglycoside, polyketide, fluoroquinolone, and diaminopyrimidine) only and those detected on both feedlots (n = 2, macrolide and beta-lactams).

4.10.8 ONT Sequencing by Feedlot A Staff

A preliminary investigation was undertaken to evaluate the potential of feedlot staff undertaking sample collection, sample preparation and extraction, ONT sequencing library preparation, loading of the library onto a flowcell and initiating sequencing data acquisition. Briefly, feedlot staff collected nasal swab samples (n =15) from cattle undergoing treatment for BRD. Two of the samples were extracted using the Puregene kit by the feedlot staff member under the direct supervision. The sample extracts were then used to construct ONT sequencing libraries using the Rapid Barcoding kit. The sequencing library was subsequently loaded onto a ONT flowcell operated by the MK1C device (Fig. 4.7.8). Puregene extracts of the samples were also analysed by qPCR for the presence of the bacteria commonly associated with BRD. The samples were also tested by qPCR for the presence of the viruses associated with BRD that have DNA genomes, as these could be detected via the ONT sequencing analysis.

Table 4.10.7 summarises the results of these analyses. Consistent with previous experiments using Puregene extracts, the majority of the sequencing reads matched the bovine genome. Consistent with other ONT sequencing analyses the bacterial genera, *Clostridium*, *Moraxella*, and *Acinetobacter* were detected in both sequencing libraries. There was some agreement between the ONT sequencing and the qPCR results in this analysis. Both samples were positive for *H. somni* using both technologies (Table 4.10.7). While both samples were positive to *Mannheimia* by ONT sequencing, *M. haemolytica* was not detected by qPCR. A possible explanation for this is that while the organism(s) detected by ONT sequencing belonged to the genus *Mannheimia* they were not *M. haemolytica*. No sequence reads were identified for the three DNA viruses detected by qPCR. The Ct values for the virus positive samples were high (lowest 28.3 for UBPV6) indicating low amounts of the viral DNA, explaining why they were not detected in the ONT sequencing data (Table 4.10.7).

The sequencing datasets were also analysed for AMR genes by comparison to the Abricate database. Two AMR genes were identified in the sequencing data for Animal ID 2304A316, with the number of sequencing reads matching AMR genes *mph(E)* and *msr(E)* were 16 and 12, respectively. While for animal ID 2304044 reads (n = 6) corresponding to the *msr(E)* AMR gene six sequence reads were identified. All of the AMR genes identified in these samples have the capacity to confer resistance to antimicrobials in the macrolide class.

Also of interest with respect to these samples, was the number positive for BoHV-1, as previous samples from Feedlot A show high prevalence (64.5%) and high quantities of this virus in some samples (Table 4.8.2). Consistent with previously analyses, 80% (n = 12) of the samples were positive for BoHV-1, including the two analysed by ONT sequencing (Table 4.10.7). However, none of the BoHV- 1 Ct values were suggestive of high viral loads as the Ct values ranged from 31.4 to 34.8, which likely underpins the lack of any sequencing reads corresponding to this virus in either dataset.

This section confirm the feasibility of feedlot staff setting up an ONT sequencing workflow. The results presented here were generated from a “one off” training activity with direct supervision by an experienced ONT user. With additional practice feedlot staff would become proficient in these processes and be able to execute them independently with a high degree of success. However, currently the workflows for data analyses are computationally intensive and require specialised bioinformatics skills to complete. Thus to complete the final stages of the ONT pipelines it is likely that continued expert support would be required. Although as ONT matures, desktop analyses tools that enable persons with less bioinformatics skills to complete the required analyses are likely to become available.

Table 4.10.7. Summary of the Oxford Nanopore sequencing results for two nasal swab extracts from Feedlot A extracted with the Puregene kit and the sequencing library prepared using Rapid Barcoding performed by Feedlot A Staff. Taxonomic assignments were made at the genus level by aligning (Align) non bovine sequences. The fraction of ONP sequences assigned to each taxon are show. The qPCR results for detection of bovine herpesvirus 1 (BHV1), *Mycoplasma bovis* (Mb), *Histophilus somni* (Hs), *Pasteurella multocida* (Pm) and *Mannheimia haemolytica* (Mh) are also shown. Agreement between the ONP assignments and qPCR results are highlight blue. The cycle threshold (Ct) values for the qPCR are shown. Empty cells indicate a negative qPCR result.

	Sequencing reads		ONP Seq Taxonomic group assignments[1]									qPCR Results				
ID	**Align**	**bovine**	**Clostri**	**Morax**	**Mann**	**Bacill**	**Myco**	**Shiga**	**Acinet**	**Histop**	**Psych**	**BHV1**	**Mb**	**Hs**	**Pm**	**Mh**
2304A316 [2]	31620	634146	0.613	0.108	0.025	0.024	0.002	0.005	0.003	0.002	0.002	31.4	31.8	24.0	30.7	
2304044 [3]	42980	1021967	0.692	0.054	0.003	0.044		0.002	0.004	0.026	0.002	34.6	33.6	22.5		
2302928													25.0	29.3		26.5
2301536												32.7	29.6	29.0	28.6	
2304A241													18.8		19.6	
2304953 [4]												34.2	30.0	25.0		
2304954													34.4	33.4		
2250916												34.1	27.1		27.0	
2302A870												32.6	24.2	19.4	28.1	
2304034												34.8	34.0	34.6		
2307A199												34.6	33.3		33.4	
2304920												34.6	32.7			
2304A310												34.2	27.3			
2304A178												32.7	32.3	24.3		
2304040 [5]												32.9	33.3		33.6	26.6

[1]Microbial Taxonomic group abbreviations: Clostri, Clostridium; Morax, Moraxella; Mann, Mannheimia; Bacill, Bacillus; Myco, Mycoplasma; Shiga, Shigella; Acinet, Acinetobacter; Histop, Histophilus; Psych, Psychrobacter.

[2]Additional Microbial Taxonomic groups identified in the sample from this animal by ONT sequencing: Chryseobacterium, 0.00235.

[3]Additional Microbial Taxonomic groups identified in the sample from this animal by ONT sequencing: Corynebacterium, 0.00266; Streptococcus, 0.00392, Methanobrevibacter, 0.0011.

[4]Sample also positive for bovine adenovirus (Ct = 30.2) by qPCR. This virus has a DNA genome therefore detection by ONT sequencing plausible.

[5]Sample also positive for ungulate bocaparvovirus 6 (Ct = 28.3) by qPCR. This virus has a DNA genome therefore detection by ONT sequencing plausible.

5. Conclusions

5.1 Key findings

The key findings of this report are:

Putative biomarkers were identified for the two virus (bovine herpesvirus 1 and bovine viral diarrhea virus 1) that are commonly associated with BRD in Australia. However, due the complexity of BRD, it is likely that a panel of biomarkers will be required to support the diagnosis of this disease.

The repertoire of viruses detected in the tissues of Australian feedlot cattle that have died of BRD are similar to those detected in other published studies. Of particular interest was the detection of bovine influenza D virus in five samples, as this has been reported as an emerging virus with respect to associations with BRD.

In contrast to several published studies, three of the viruses commonly referred as the “big four”, bovine herpesvirus 1, bovine viral diarrhea virus 1 and bovine respiratory syncytial virus were frequently identified with in the viral populations of the tissues analysed. The reasons for this finding are not readily apparent. One possible explanation is it may be a result of widespread adoption of vaccination against these viruses in other countries, thus reducing the likelihood of these viruses being detected in those studies.

The genome wide analyses determine an estimate for the heritability of BRD resistance of 0.20 ± 0.04, this supports further investigations to improve this estimate. Using the same approach that was utilised in the final stages of this project, collaborating with Angus Australia, may prove to be a highly effective way to progress this aspect of improving BRD resistance in the Australian cattle population.

The samples from one of the feedlots samples, BoHV-1 was detected with unexpectedly high frequency (65%). While in some of these animals very high levels of the virus were detected. As the feedlot utilises Rhinogard™, the results suggest that further research is required to understand the drivers of this result. There are several factors that might contribute to these results, such as, changes in BoHV-1 field strains, over reliance on vaccination to control BRD, or improper administration of the vaccine (noting that no evidence has been identified for any of these factors), without further investigation the problem may persist or become more widespread in the sector.

Oxford Nanopore technology sequencing proved to be highly amenable to detecting a wide range of bacteria and also in nasal swabs collected from cattle being treated for BRD. The application of strategies to remove bovine DNA from samples prior to analyses is essential to ensure robust detection of the range of bacteria present. The generated datasets also enable the detection of antimicrobial resistance genes in nasal swabs of cattle being treated for BRD. It was also demonstrated feedlot staff were able to successfully complete the experimental components of the ONT sequencing pipeline. With further development ONT based approaches have the potential to be an important component of future antimicrobial stewardship programs.

5.2 Benefits to industry

The capacity to objectively confirm BRD cases through the use of biomarkers would reduce the number of false BRD cases being administered treatment. Thus provided a saving to feedlot operators through reduced treatment costs.

Knowledge of the viruses identified in tissues from cattle that have died from BRD will inform the development of new diagnostics (this project) and new vaccines. These tools will provide industry with the capacity to better management BRD, delivery increased profitability through less BRD.

Improved management of BRD will also have wider benefits to the red meat industry as it will underpin treatment decisions (biomarkers) and/or reduce the use antimicrobials (diagnostics/vaccines) to treat affected cattle. These outcomes contribute to industry stewardship on the therapeutic use of antimicrobial in livestock, an essential component in global efforts to combat antimicrobial resistance.

6. Future research and recommendations

6.1 Future R&D

The study has provided two estimates for the heritability of BRD resistance/susceptible. The first was derived from using DNA extracted from blood clots that were collected during an industry funded project that aimed to improve the diagnosis of BRD. A genomic heritability for BRD resistance was estimated to be 0.52±0.2 (n = 192). The second estimate utilised data for animals that were genotyped as part of another industry funded project. Based on the phenotype, the genomic heritability of an animal being treated for BRD was 0.2 ± 0.04. As the estimate was derived from a larger number of animals (n = 1666) it was more accurate. It is sufficiently accurate for inclusion in applicable breeding plans. Increasing the number and type/breed of animals used this analysis would improve the accuracy of this estimate and potential make it applicable across multiple breeds. This could be achieved by setting up the dedicated sampling of the optimal number of feedlot cattle to give the desired precision in the heritability estimate. While this would likely be successful, it would be resource intensive strategy require at least 10,000 animals to be sampled. In contrast, the opportunistic build of a more comprehensive dataset using genotyping data from existing or future research projects, similar to the approach used in this project, could be an effective strategy. Though it is likely that it would take longer to accumulate the require number of datapoints. An emerging approach that could revolution the genotyping of livestock is referred to as genotyping by whole-genome skim-sequencing. Genotyping essentially determines relative location of single nucleotide polymorphisms in the genome of animals and how these correlated with phenotypes. The same outcome could be achieved by completely sequencing the genome of the animal of interest as it would yield all variations. However, due to the cost and time required this is not feasible in most cases. In comparison whole-genome skim-sequencing strategies have emerged that enable genotypes to be imputed from very low sequencing coverage of the genome of interest (Adhikari et al., 2022; Malmberg et al., 2019). This dramatically reduces the time and cost of generating useful genotypes of the animals of interest. As demonstrated in the current project, one of the issues with the detection of pathogens and AMR genes was large amounts bovine DNA sequence generated. Although the yield of bovine sequence data was highly variable and considered insufficient to enable whole-genome skim-sequencing based genotyping. As these technologies mature it will likely be plausible to combine animal genotyping, pathogen detection and AMR gene detection in one analysis as recently reported for cattle using ONT sequencing (Lamb et al., 2020; Lamb et al., 2021). However, to make this a cost-effective approach there will be a need for high level multiplexing of samples to reduce the cost per animal.

One aspect of the genetics of BRD which has not been investigated is epigenetic regulation. In this context epigenetics refers to the modification of specific areas of the genome resulting the alternation of gene expression, this occurs through methylation and demethylation of genetic elements for increased and decreased activity, respectively. It is well recognised that environmental stimuli can influence an animal's risk of developing BRD. Similarly, an animal's genome can be methylated or and demethylated resulting in changes in gene expression in response to environmental stimuli. The role of epigenetics changes in response to environmental stimuli in the development of asthma in children is the subject of intense research (Solazzo et al., 2020; Yang et al., 2015).

There is clearly a need to investigate further what appear to be highly virulence stains of BoHV-1 detected in one of the feedlots in this study. There are three aspects of importance in this respect. Firstly, the cattle with these high viral loads had been vaccinated. Secondly, some of the BoHV-1 positive animals were outside what might be considered the typical window for BRD. Thirdly, the levels of virus detected in some animals were extremely high, exceeding the amounts of BoHV-1 produced in experimentally infected cattle and also in laboratory cells. If these strains truly have such increased virulence capacity they represent a real risk to the wider feedlot sector. If these strains were to become dominant within the Australian cattle population, the gains made through the development and adoption of vaccines maybe lost.

There is also a need to improve the overall performance of the ONP sequencing technology. It is likely this will occur as the technology matures, indeed ONP are in the process of rolling out their next version of the sequencing flow cells. As the technology improves its capacity to be used in more challenging conditions will be enabled.

One aspect that has emerged from the results of this project is the complexity of the microbiomes that are present in cattle being treated for BRD. While the array of viral and bacterial pathogens could be identified in many nasal swab samples using conventional pathogen specific molecular assays (Luminex and qPCR), in many cases these were not detected in the ONT sequencing datasets. This lack of agreement can be partially explained by the large amounts of host DNA in many sample extracts, as the application of host-depletion strategies increased the diversity of bacteria identified. However, while the diversity increased the likelihood of detected what might be considered a pathogen in the context of BRD did not. This suggest that prior research may have been too focused on bacteria that were perceived as being pathogenic. The subsequent development of diagnostics to detect these organisms may have biased microbial associations with BRD. Add to this that is often suggested that pathogenic bacteria are fastidious with respect culture in the laboratory. Consequently, specific media have been developed to increase the likelihood that these pathogens are isolated if they are present in the sample being analysed. The advent of ONT sequencing and sequencing technologies that enable nonbiased evaluation of the microbiomes associated with cattle being treated for BRD presents a new problem, namely– what does all this data mean? Nonbiased interpretation of these data could be highly amenable to artificial intelligence and/or machine learning approaches to identify the patterns in these complex datasets. It is likely that a well-defined BRD learning dataset would be required to develop the artificial intelligence and/or machine learning algorithms for these purposes.

As an example, the genus *Acinetobacter* was identified the majority of the ONT sequencing datasets in the current study. It is well recognised as an adventitious pathogen in human medicine, particularly with respect to AMR, but has not been considered in the context of BRD. Previous studies BRD metagenomic studies have reported the detection of Acinetobacter in their datasets (Holman et al., 2015; Holman et al., 2017; Zeineldin et al., 2017). In a recent study, Mateo-Estrada et al. (2022) characterised the genomes Acinetobacter baumannii isolates from cattle (n=8) and pigs (n=8), the main species of interest with respect to human medicine. The isolates were obtained from animals at the time of slaughter for human consumption and were not reported to be exhibiting clinical signs at

this time (Hamouda et al., 2011). Multilocus core genome based phylogenetics demonstrated the animal isolates were genetically distinct from clinical reference isolates (Mateo-Estrada et al., 2022). The animal isolates formed a single phylogenetic clade. The pig faecal isolates (n=8) formed a single cluster, a second cluster contain bovine faecal isolates (n =2) and a third cluster contained bovine faecal isolates (n = 4) with bovine nasal isolates (n = 2). The authors also reported the livestock isolates had distinct AMR gene and virulence profiles compared to clinical isolates (Mateo-Estrada et al., 2022). As the study was focused on how these isolates were related to human clinical isolates there was no discussion regarding the importance of the isolates with respect to veterinary medicine. Similarly, as the animals were sampled at slaughter it is assumed that they were free of any signs of disease.

Included in these functions could be strategies to link the detected bacterial genera/species with detected AMR genes. The linking of an AMR gene to specific bacterial species should be feasible for those genes that are encoded withing the bacterial chromosome. Indeed this a key advantage of using ONT sequencing compared to a specific molecular assay for AMR gene detection. In most cases a sequence read with an AMR gene will contain the additional flanking sequences. These flanking sequences could be used in some instances to link the gene to a specific bacterial species. In contrast, it would be difficult to design a molecular assay with this capacity. However, many AMR genes are encoded within mobile genetic elements and linking these to a specific bacterium or group of bacteria can more problematic and likely to require the development of more complex tools to enable this.

There is also the emerging concept of moving from analysing the microbiome to analysing the resistome within the sample of interest. As the name suggests the resistome refers to the “population” of AMR genes present in the sample of intertest. While several studies on antimicrobial resistomes have been published for cattle they have largely focused on the intersection between livestock protection, the environment and human medicine, consequently these studies have focused on the intestinal antimicrobial resistomes (Lawther et al., 2022; Liu et al., 2021). However, (Noyes et al., 2016) examined the antimicrobial resistomes of beef cattle from feedlot entry through to slaughter. The study reported a reduction in diversity in the resistomes from entry to slaughter to loss of AMR genes not associated with the antimicrobials used to treat study animals, suggesting that selection had occurred. Importantly, the resistomes were not detected in the produces of the study animals. Combining knowledge of the microbiome and the resistome of BRD affected cattle could in form vaccine development (targeting pathogens associated with disease) and treatment (choice of antimicrobial selection) decisions towards improved antimicrobial stewardship.

The datasets generated in the current study, clearly demonstrate the capacity of ONT sequencing to detected AMR genes and therefore suggest the technology could be used to enhance antimicrobial stewardship in the Australian feedlot sector. However the nonbiased capacity of the technology also means that repertoire of AMR genes detected, similar to the bacterial species detected, could be more extensive than might be anticipated. It might be expected that as macrolides are used in the sector, that macrolide resistance genes would be detected, and this was certainly the case in the current study. However, as the majority of the cattle were undergoing their first treatment for BRD, it is not known if that were treated with or exposed to macrolides prior to arrival at the feedlot. This suggest that once an animal has been sampled and treated there is a need to conduct follow up tracing and testing of the animal to assess the outcome of treatment from several perspectives. Firstly, to determine if the treatment was effective. Feedlots would currently have quite detailed information the outcome of BRD treatments, as retreats are closely monitored in their systems. Secondly, repeat sampling of animals would enable assessments to be made as to whether or not any detected AMR genes have contributed to the failure of the first treatment. This might be detected in the ONT sequencing by significant increases in the data points matching the AMR gene identified in the first

sample that match the antimicrobial use for treatment. Thirdly, repeat sample would enable assessments to be made with respect to what changes have occurred, if any, in the microbiome of cattle as a potential consequence of treatment. Again it might be expected that the proportion of some bacterial species identified in the first sample would increase. Though the possibility of other unanticipated changes in the microbiome cannot be excluded as the antimicrobial treatment is likely to impact on all susceptible bacterial species. Ideally, any repeat sampling and testing should also include animals for which the first treatment is successful. Of particular interest in these animals would be the identification of microbiome components that are indicative of successful treatment. It would also be of interest to determine how the microbiome of these animals differ from those that require additional treatments. Building these components into a machine learning algorithm might enable the identification of factors (e.g., specific AMR genes, bacterial species etc) for which a specific crush-side test could be developed to either directly inform treatment, if bacterium A is present treat with antimicrobial X, or predict treatment outcome, if bacterium B is present, the likely outcome of a successful treatment with antimicrobial Y is 85% or with antimicrobial Z is 95%. The ideal experimental design for examining this approach would be to sample animals at feedlot induction, at first treatment, and prior to any additional retreats. Including samples of animals from the same cohort at similar times that are not treated and do not require retreating would likely improve the predictive power of the algorithm.

It should be noted that the current study was restricted to the analysis of nasal swabs from cattle undergoing treatment for BRD. Thus linking what is detected in these swabs may not necessarily directly reflect what is happening with respect to disease in other parts of the respiratory tract. One partial solution to this issue would be to sample and analyses the microbiomes along the respiratory tract of cattle that have died from BRD. While it was planned to sample lung tissues as part of the current study, there were no opportunities to collect these samples. However, sampling tissues from BRD deaths could be misleading as these are animals at the extreme end the disease spectrum. To fully understand the changes in microbiome with respect to clinical diagnosis and recovery it would be necessary to sample the respiratory tract of animals at the time of treatment to enable linking of the microbiomes. Similarly, the effectiveness of antimicrobial treatment would also need to be examined in a similar manner for those that recover, those that require retreats and those that become chronically affected. However, these types of studies where cattle are required to be killed for sampling have always been difficult to garner industry support for, even with compensation for lost animals on offer. The only alternative would be sampling under anaesthesia, though this is considered impracticality in a commercial feedlot setting. Add to this such a dramatic intervention could seriously compromise the performance of any animals involved, suggesting little benefit from the perspective of saving the animal.

6.2 Practical application of the project insights

Knowledge of the virome associated with BRD offers two methods of practical application to reduce the impact of this disease in Australian feedlots. The first application is in diagnostic assay development (multiplex assays for bacteria, AMR genes and two viral assays, developed in this project). Currently, there is limited capacity to investigate BRD cases and outbreaks. Applying new

diagnostic tools for an extended range of pathogens will improve our understanding of the role they play in BRD development. This information can then be used to develop targeted management strategies. The second, application of improved understanding of the role of pathogens is to inform the development of new vaccines for BRD.

The application of ONP sequencing proved to be highly amenable to detecting a wide range of bacteria and AMR genes in nasal swabs collected from cattle being treated for BRD. The application of strategies to remove bovine DNA from samples prior to analyses is essential. A key advantage of this approach is it is not limited by preconceived ideas about what pathogens may or may not be present. However, as demonstrated by this study, the technology generates so much data that clarifying the roles of some pathogens or potential pathogens associated with BRD is difficult. Consequently, the current application of ONP technology in the feedlot industry would need to be frame by very specific questions such as: is this pathogen present? Or is there any evidence for resistance to a specific antimicrobial? This would enable more targeted analysis pipelines to be developed.

A detailed description of the methods utilised in this project are described in Appendix F. This protocol will enable the replication processes reported in Section 10 of this report. The methods described the approaches take with respect to the host DNA depletion strategies undertaken to increase the proportion of sequencing data that was specific for the microbial components of the nasal swab extracts.

6.3 Development and adoption activities

The project results will be communicated to targeted groups. The first group is the State veterinary laboratories to determine their interest adding new diagnostic tests to their existing panels. The second group is the feedlot consulting veterinarians to inform them of the advances in our understanding of the range of viral pathogens that are potentially associated with BRD. This group will also be essential in improving clinical investigations of BRD and use of new diagnostics. The third group is veterinary pharma, as this group are crucial in driving the development of new BRD vaccines.

The feedlot sector will be engaged directly to inform them of these important outcomes with respect to BRD. This engagement will take place at various industry forums such as animal health workshops, BeefEx, and other similar opportunities which become available.

The project results will be published in peer-reviewed journals to inform the research community of the latest developments in BRD, pathogens associated with this disease, AMR genes present, and the potential of ONT sequencing to enable improved monitoring of these aspects of BRD.

The project team have engaged closed with the feedlots involved in the study. A staff from one of the feedlots has visiting our laboratory to go through the experimental processes of preparing nasal swab samples for ONP sequencing analyses. While it now beyond the scope of this project we will also visit at least one of the feedlots and complete an ONT analyses sample pipeline. One of the feedlots remains very keen to undertake this activity.
The participating feedlots have been provided with detailed reports of the results of the analyses of their respective samples. The project team has also meet with feedlot representatives to discuss the experimental results, potential implications of these results and how the results could be utilised to contribute to antimicrobial stewardship activities. Similar presentations could also be given to other audiences throughout the red-meat sector for those who are interested in outcomes of this project.

7. Appendices

7.1 Appendix A: Keyword search: Biomarkers, Bovine, Respiratory

Total references: 72
References high rank: 28
References included: 32

Treatment, Pathogen or Effect	Study design	Methodology	Sample	Marker - Up regulated	Marker - Down regulated	Reference / comments
Bovine viral diarrhoea virus 2	Cattle infection	NGS Samples Day 0, 4, 9, 16	Serum	Bta-miR-339a Day 9 Bta-miR-486 Day 9 Bta-miR-30e-5p Day 4, 9, 16 Bta-miR-185 Day 4, 9 Bta-miR-92a Day 4, 9	Bta-let-7c Day 4, 16 Bta-miR-2284x Day 4, 16	Taxis et al. (2017) The latest study in the area. Unclear if any of these miRNAs will be differentially expressed in response to other viral infections or even for BVDV-1 Also of interest with respect to these miRNAs are their potential targets and what pathways those targets are involved in. The targets might be easier to detected in field test.
Alleviated the effects of transport with meloxicam	Cattle transported		Blood breath	24hr Hp-MMP-9 Tumour Necrosis Factor α Plasma protein Total carbon dioxide	24hr cortisol lymphocytes	Van Engen et al. (2014) Transport ,1316 km 16hr More on stress than BRD

				neutrophil, platelet, monocyte, white blood cell, and red blood	
BRD genetics	Dairy and beef cattle	GWAS			Van Eenennaam et al. (2014) Identified SNPs which explain 30% of BRD variation
BRD genetics	Japanese black cattle				Tsuchida et al. (2010) SNP in CXCR1 gene associated with WBC and platelet counts in disease animals. Based on CXCR1 SNP associations with disease in other cattle lines (mastitis) for IL8 expression. See Rambeaud et al. (2006) *Could see if CXCR1 mutations are in the 3-UTR for possible miRNA interactions.*
Mannheimia haemolytica	Black heifers (240kg)	various	blood	MH calves blood biomarkers pain (substance P) on d 0.5 Stress (cortisol) on d 0.5 and 1 haptoglobin on d 0.5, 1, 2, 3, 7 Metalloproteinase on d 1, 2, 3	Theurer et al. (2013) Investigate the impacts of MH challenge at high ambient temperature >/= 32oC – higher temp increases severity of clinical signs. Also monitored behaviour (Accel/Pedom/GPS) with Mh group moving less & lying down more.
Respiratory disease	dairy calves		serum	Haptoglobin (S-Hp)	Svensson et al. (2007)

	(833)					S-Hp or fever; S-HP and fever Potential as a herd-level indicator of disease
Enzootic bronchopneumonia	Calves		peripheral blood neutrophils	alpha(4)-integrin (on PNGs) haptoglobin alveolar macrophages; PMN, polymorphonuclear neutrophilic granulocytes (PNG)		Soethout et al. (2003) *Could look for alpha(4)-integrin 3-UTR and miRNA interactions.*
BRD	162 healthy beef calves 1-2 days after weaning and transporta tio		bronchoalveolar lavage fluid (BALF)		Annexin A1, annexin A2, peroxiredoxin I, calcyphosin, superoxide dismutase, macrophage capping protein and dihydrodiol dehydrogenase 3.	Senthilkumaran et al. (2013) Compared proteomics of 7 health and 7 that developed BRD Conclude annexins may help minimise lung inflammation *Could look for annexin A1 and A2 3UTR miRNA interactions.*
Bovine herpesvirus 1 & M. haemolytica	bovine bronchial epithelial cells BBEC	qPCR		rapid increase in IL-1, IL-8 and TNF-alpha mRNA in BBEC Adherence of polymorphonuclear neutrophils (PMN) to infected BBEC Increased expression of LFA-1 expression and susceptibility to M. haemolytica LKT		Rivera-Rivas et al. (2009) BHV-1 infection of BBE cells triggers cytokine expression that contributes to the recruitment and activation of neutrophils, and amplifies the detrimental effects of M. haemolytica LKT

						Could look for gene 3UTR miRNA interactions in the upregulated genes.
Bovine herpesvirus 1 & M. haemolytica	bovine peripheral blood mononuclear cells (MNC)			BHV-1 infection increased LFA-1 expression leading to enhanced LKT binding & cytotoxicity BHV-1 infection increased CD18, IL-1beta, and IFN-gamma mRNA expression by MNCs		Leite et al. (2004) Similar findings to Rivera-Rivas et al. (2009) except different cell type. *Could look for gene 3UTR miRNA interactions in the upregulated genes.*
BRD to Day 42 Castration status	Beef bulls (n=588) Beef steers (n=591)	CBC variables	blood	RBC counts	Eosinophil count	Richeson et al. (2013) BRD for bulls were 3.32 times the odds of BRD for steers
Acute respiratory disease experimentally induced by Chlamydia psittaci. haptoglobin (Hp), lipopolysaccharide binding protein (LBP) C-reactive protein (CRP), and lactoferrin (Lf)	Calves (n=13)	acute phase proteins	Broncho-alveolar lavage fluid (BALF)	BSA, Hp, LBP, CRP and Lf in BALF Absolute concentrations of LBP and Hp in BALF correlated significantly with the respiratory score. The quotient [LBP]/[BSA] in BALF peaked significantly in acutely infected animals (4 dpi) showed a time-dependent decrease during the recovery phase (9-14 dpi), and was significantly higher compared to healthy controls.	Concentrations of Hp and Lf in BALF as well as [Hp]/[BSA]--and [Lf]/[BSA]-quotients decreased during the study in infected animals, but were never higher than in healthy controls.	Prohl et al. (2015) LBP in blood and BALF The quotient [LBP]/[BSA] in BALF perfectly paralleled the clinical course of respiratory illness after infection. Hp and Lf as suitable local biomarkers of respiratory infections in cattle

BVDV & PI Genetics	Brahman x Hereford	312 microsatellites				Neibergs et al. (2011) BVD-PI dams with unaffected calves detected associations with BVD-PI for all markers tested on BTA2 3 of 7 markers on BTA26 *Could try cross referencing these markers for miRNA clusters.*
Expression of serum amyloid A (SAA) and haptoglobin (Hp)		qPCR	Multiple tissues	Highest SAA was found in thyroid Also in pancreas and submandibulary gland	Hp highest in pancreas and submandibulary gland	Lecchi et al. (2012) Need to understand source of SSA and Hp if using as a biomarker. Examined expression of seven reference genes (ACTB, GAPDH, HMBS, SDHA, YWHAZ, SF3A1, EEF1A2) and three genes, namely SF3A1, HMBS and ACTB *Maybe important if trying to use saliva to measure an APP.*
calm and temperamental Brahman bulls in response to handling and transportation.	Brahman	innate immune and blood parameters	Blood	neutrophil:mononuclear cell ratios were greater in temperamental bulls compared to calm bulls at 24h 48hr calm bulls had elevated neutrophil L-selectin expression, and phagocytic and oxidative burst	24h, expression of peripheral neutrophil beta(2)-integrin decreased among all bulls	Hulbert et al. (2011) Data suggest calm bulls are better able to resist microbes 96hr following transport. *Could look for miRNA interactions with peripheral*

				48hr temperamental bulls greater glucose and cortisol 48hr calm bulls had elevated neutrophil L-selectin expression, and phagocytic and oxidative burst activity 48hr PMBC stimulated with endotoxin higher levels of TNF, no difference between bull types. 96hr calm bulls increased neutrophil phagocytosis, oxidative burst, and cell adhesion molecule expression	*neutrophil beta(2)-integrin and TNF.*
intravenous lipopolysaccharide (LPS) Hp-MMP 9 to the serum acute phase protein response	Jersey calves	physiologic parameters, peripheral blood cell counts and serum cortisol (C), Hp-MMP 9, Hp, alpha1-acid glycoprotein (AGP), serum amyloid A (SAA) were obtained starting 24 hours	Blood	1hr rapid onset of depression, tachypnea, leukopenia, neutropenia and lymphopenia 1hr Cortisol increased Serum Hp-MMP 9 complexes were detectable in serum by 0.5 hours and peaked at 16hr Total serum Hp increased by 12hr 8hr Serum amyloid A 16hr AGP 96hr Hp, SAA and AGP	Hinds et al. (2014) Hp-MMP 9 complexes appear sooner and decline more rapidly than other APPs Hp-MMP9 is stored pre-formed, it provides information specifically addressing the LPS-induced activation of bovine neutrophils. Contributions of Hp-MMP 9 to the serum APP response may provide useful information, independent of hepatic responses, in diagnosis of acute inflammation.

		before to 96			
Bibersteinia trehalosi and Mannheimia haemolytica	cross bred dairy calves (n=35)		Serum Hp and Hp- MMP 9 concentrations increased by day 7 in Mh Serum Hp- MMP 9 concentrations increased by day 3 in Bt		Hanthorn et al. (2014) Can detected early inflammation due to infection and perhaps subclinical inflammation
Bovine Parainfluenza Virus Type 3 BPI3 challenge		proteomics serum	BPI3V Phosphoprotein P and host T-complex Protein 1 subunit theta were found to be accumulated at the latter stages of infection within bovine fibroblasts		Gray et al. (2017) Different protein levels dependent on animal vaccination status however only T-complex Protein 1 subunit theta to be detectable in animal sera
Vaccination Pfizer Rispoval(R) PI3 + RSV intranasal vaccine		UPLC-MS metabolomic	First vaccination taurodeoxycholic acid and propionylcarnitine Second vaccination glycocholic acid, N-[(3alpha,5beta,12alpha)-3,12-Dihydroxy-7,24-dioxocholan-24-yl] glycine, uric acid biliverdin	Second vaccination hippuric acid	Gray et al. (2015) Could be used to assess successful vaccination or tailoring of vaccines to give the most appropriate and/or better responses.
BRD	Cattle	Western blot / ELISA	haptoglobin		Godson et al. (1996) Maybe first paper to link haptaglobin as a BRD marker.

						So why not adopted in the field as yet??
Respiratory disease	Cattle	Acoustics of coughing				Ferrari et al. (2010) possible to discriminate cough sounds from other sounds and that cough sound can be used as a non-invasively diagnostic tool
stress responses among newly received calves	Cattle	leukocyte heat shock protein (Hsp) response, selected neutrophil-related gene expression and oxidative stress		1 & 5hr cortisol 1 h, 5 h and 1 day Hsp60 and Hsp70A1A 7 days beta-glycan levels correlated with lack of adhesions at slaughter Oxidative stress responses, measured through the oxidation products of the exogenous linoleoyl tyrosine (LT) marker, revealed that hydroperoxidation and epoxidation of membranes		Eitam et al. (2010) LT oxidation products and levels of beta-glycan Predict model for cattle at risk of BRD at Day 7 nearing 100% *No follow-up studies and only one citing paper.*
BRD and Mycobacterium avium subsp. paratuberculosis	Cattle	SNP analysis				Casas et al. (2011) SNPs in the ANKRA2 and CD180 genes with BRD and presence of MAP
BRD	Cattle	Breath analysis Haptaglobin	evaluate exhaled N(2)O (eN(2)O), exhaled CO (eCO)	Ratio of eN(2)O:eCO(2) lowest at arrival intermediate at 1st and 2nd treats Greatest at the 3rd treat	Lower body weight – 3rd treat	Burciaga-Robles et al. (2009) Ratio of eN(2)O:eCO(2) & level haptoglobin are arrival not predictive of BRD

			serum	greater at times of treatment cf. arrival haptoglobin greatest at 1st treat lowest at 2nd & 3rd treats Intermediate arrival.		Although parameters might be useful as part of the diagnostic process.
BRD BRD incidence in the cohort was 38%	Feedlot steers (n=232)	L-lactatemia Temperament scores	Blood	Stoic or very excited calves showed x2.2 more likely to have BRD Temperament score of 2 (average temperament) every 1-log unit increase of lactatemia at processing x1.9 odds of BRD case Dynamics of L-lactatemia was associated with the hazard of death – 1-log increase X 36.5 risk of death		Buczinski et al. (2015) impact of L-lactatemia differed by temperament strata. Includes cost benefit analysis of metaphylactic treatment. Determined level of 5.0mmol/L would minimise the costs associated with treating non-BRD cases. In a previous study the authors have utilised crush-side testing (Boulay et al., 2014).
Transportation stress in bulls 9h trucking	Aberdeen Angus (12); Friesian (12) Belgian Blue x Friesian,	metabolic, inflammatory, and steroid variables, and total leukocyte counts	Blood/plasma	Kinase Total leukocyte counts plasma cortisol cortisol:dehydroepiandrosterone ratio (P < 0.001 progesterone	Albumin Globulin Total protein dehydroepiandrosterone haptoglobin and fibrinogen testosterone	Buckham Sporer et al. (2008) effect of breed for all variables except plasma urea, creatine kinase, and testosterone, Sampled at-24, 0, 4.5, 9.75, 14.25, 24, and 48 h relative to the initiation of transportation (0 h)

BRD Utilised the BRD model Viral challenge followed by bacterial challenge after 4 days.	Cattle	AAP Metabolomics Elemental	Blood	day 4 postviral infection: haptoglobin apolipoprotein AI (not linked to fatal BRD outcome) viral infection - glucose, LDL, valine, phosphorous, and iron Disease outcome - lactate, glucose, iron	*Overall number of changes in parameters, that could be measured, suggestive of "stress" – although no specific links to disease risk* Aich et al. (2009) Proteomic and metabolite profiles did not discriminate between animals that survived or died based on Day 0 sample serum elemental profiles prior to infection was, however, predictive of BRD outcome Combining all three analyses of the Day 4 (prior to bacterial challenge) samples was predictive of BRD outcome. *Demonstrates the feasibility of complex analyses to discriminate between animals who are likely to have a poor BRD outcome. However, clearly such complicated analyses are not suitable for crush side testing.*
Respiratory disease and oxidative stress	Holstein-Friesian calves	Reactive oxygen metabolites (ROMs) and biological	Blood and exhaled breath condensate (EBC)		Ranade et al. (2014) The results indicate that antioxidative defence not only increases with time in the plasma of new-born calves but

			antioxidant potential (BAP) were Oxidative stress index (OSI), where OSI = ROMs/BAP x 100. Plasma concentrations of antioxidant barrier (OXY), thiol antioxidant barrier (SH), advanced oxidation protein products (AOPP), albumin, and non-esterified fatty acids (NEFA)				also that it is related to protein oxidation processes. Furthermore, the data support the use EBC H2O2 as a novel biomarker to assess respiratory OS in calves. *Very young calves would need to assess these measures in much older cattle.*
Respiratory and diarrhoeal disease	Calves	Antibody levels to Resp Path	Blood	Increasing alpha-2 globulins s	IgG's below 7.5g/L		Pardon et al. (2015) Seronegative BCV at risk of diarrhoea

					Seronegative for BRDV at risk of respiratory disease Increasing alpha-2-globulins and low Ig decreasing ADG *Very young calves, as study concludes on the importance of providing sufficient colostrum. While not directly related to Aust feedlot sector, does demonstrate the importance of prior exposure. Be it via natural exposure or vaccination.*
Respiratory disease	Calves	Gene expression	blood	Alpha(4)-integrin Correlated with haptoglobin	Soethout et al. (2003) Role of Alpha(4)-integrin in pulmonary inflammation via neutrophil infiltration. *As with other genes described previously could investigate the tole of miRNA in regulation*
Histophilus somni (H. somni) related disease					Tiwari et al. (2009) Role of platelet endothelial cell adhesion molecule-1 (PECAM-1) and endothelial nitric oxide synthase (eNOS) in neutrophil migration/infiltration

7.2 Appendix B Keywords: biomarker, saliva, respiratory

References total 95
References high ranking 11
References included 15

Treatment/Pathogen/Effect	Study design	Methodology	Sample	Marker - Up regulated	Marker - Down regulated	Reference / Comments
chronic inflammatory lung diseases	Human patients	Mass Spec	Saliva	Human alpha-defensins (human neutrophil peptide (HNP)1, HNP2, HNP3) and three C-terminal amidated peptides, one of which is phosphorylated on serine		Terracciano et al. (2011) Use of silica bead to fractionate sputum samples Differentiate between asthma and chronic obstructive pulmonary disease *Need to check if there are any similarities between COPD and BRD.*
"Field" diagnosis of respiratory disease	Human (n = 273)	Antibody bead based capture	Saliva	human vascular endothelial growth factor (VEGF), interferon gamma-induced protein 10 (IP-10) interleukin-8 (IL-8), epidermal growth factor (EGF), matrix metalloproteinase 9 (MMP-9)		Nie et al. (2014) Fully automated analysis, can also be utilized in point-of-care diagnostics six inflammatory protein biomarkers that potentially correlate with asthma and cystic fibrosis in 70 minutes The current settings of the platform allow simultaneous analysis of up to 10 different proteins in a single assay.

				interleukin-1 beta (IL-1*β*). The current settings of the platform allow simultaneous analysis of up to 10 different proteins in a single assay		Nie et al. (2013) – initial study which identified biomarkers. *Either test saliva from BRD cases and controls for known biomarker. Alternatively compare proteomics of BRD cases and controls using saliva.*
Pneumonia Cases N = 15 Controls N = 16	Children		Saliva & serum	Ca, P, and Mg concentrations Serum - white cell counts & neutrophils	Salivary flow rate reduced Uric acid concen. by 60% Serum - sodium	Klein Kremer et al. (2014) Compared the results in saliva to serum. Overall the changes detected were in agreement but more profound in the salvia Serum – 3-80% Saliva – 42-275% *Note reduced flow of saliva in cases, whereas increase salivation is reported in some BRD cases.*
pulmonary tuberculosis disease (n=32) and other respiratory diseases (n=72)	Humans	Bio-Plex platform - multiplex cytokine platform	Saliva	MCP-1	IL-15 granzyme A IL-1b IL-9 MIP-1β IL-10 SAA Ferritin	Jacobs et al. (2016b) The study was looking ways to monitor the success of TB treatment Evaluated 33 host markers of inflammation Some of the differences are looking at success of TB treatment. *Evaluation of these biomarkers in cattle could be good starting point. Several have been identified in the standard approaches as potential BRD biomarkers.*
Tuberculosis Disease	Humans					Jacobs et al. (2016a)

Various diseases – including respiratory	Pigs	ADA activity	Saliva Pigs chewed a sponge	Total adenosine deaminase activity were significantly elevated in animals with local inflammation, gastrointestinal disorder and respiratory disorder

Similar to the latter study above but used 69 host markers.

A five-marker biosignature comprising of IL-1beta, IL-23, ECM-1, HCC1 and fibrinogen diagnosed TB disease with a sensitivity of 88.9% (95% CI,76.7-99.9%) and specificity of 89.7% (95% CI, 60.4-96.6%) after leave-one-out cross validation, regardless of HIV infection status. Eight-marker biosignatures performed with a sensitivity of 100% (95% CI, 83.2-100%) and specificity of 95% (95% CI, 68.1-99.9%) in the absence of HIV infection.

As above exploring these biomarkers in cattle could be a good starting point. Also the Se/Sp estimates were affected by HIV status, need to explore further the impact of co-infections which are of course relevant to BRD.

Gutierrez et al. (2017)

Total adenosine deaminase activity correlated to acute phase proteins - haptoglobin and C-reactive protein

Could be a highly sensitive assay given it is based on enzyme activity so likely to amplify detection compared to detecting "protein" alone.

porcine reproductive and respiratory syndrome virus	Pigs	immunofluorometric assays	serum, saliva and meat juice	porcine reproductive and respiratory syndrome virus
Review of technologies for emerging technologies				

Apparently, there are also promising results for human applications – so might need to specifically review that literature
Perhaps if there was a colour substrate for the enzyme activity??
Amount of sample might be an issue given the pigs chewed on the sponge. Ideally want to have test perform on minimal amounts of saliva.

Gomez-Laguna et al. (2010)
Seems to be from the same group as Gutierrez et al. (2017)

Not readily apparent how the salvia was collected. Use Salivette tubes (Sarstedt), which have a "chewable" insert which is then spun to recover saliva. Based on the size though it seems the most likely scenario would be the pigs would eat them! Not sure if they would be suitable of post-mortem collection.

Overall looks promising. Difficult to tell from the figures in the manuscript, but it looks as though saliva was the most variable sample.
Dixon et al. (2016)
Have reviewed point of care diagnostics from an 18 month period for COPD. Published in March 2016, so perhaps from Mid 2014 to end of 2015.

Review of point of care diagnostics for protein biomarkers.				Eighty technologies evaluated, of which 25 were considered promising. Includes biomarkers in saliva/sputum and wearable technologies. Barbosa and Reis (2017)
respiratory and digestive diseases and diabetes 1026 patients and 553 apparently healthy individuals.	Humans	Immunodiffusion assays	Saliva	lactoferrin Sukharev et al. (2009) Seems promising though article in Russian. Could try cross referencing lactoferrin & respiratory. No pubmed citations of this specific article though.
psychological and physiological indicators of stress – implicated in the inflammatory process.	humans		Saliva	calcium-binding protein spermatid-specific 1 (CABS1) is expressed in the human submandibular gland Ritz et al. (2017) Positive association with cortisol due to both acute and academic stress *Could check for bovine homologues in saliva samples. Overall low conservation between bovine and human protein sequences – 65% identity & 77% similarity, so may not be good cross between antibodies, if there are no bovine specific reagents.*

pulmonary tuberculosis and patients with other nonspecific respiratory diseases	Humans		Serum & saliva	Serum lactoferrin with pulmonary tuberculosis Ferritin level can serve as an indicator of tissue destruction during inflammation and of the course of rehabilitation processes		Kuznetsov et al. (2013) Can probably used both types of samples but serum is more consistent. *Probably not that surprising given serum would be expected to be the most consistent sample.*
tuberculosis	Humans		Serum & saliva	Saliva had higher levels of GM-CSF and VEGF Serum had higher levels of MIP-1a, b, TNF-a, G-CSF and IFN-g		Namuganga et al. (2017) Serum levels of IL-6, VEGF and TNF-a were significantly different between participants with active TB disease and those with other respiratory diseases. Authors conclude that salivary biomarkers are worthy of further exploration
Upper respiratory tract infections – mainly the common cold	Humans	Multiple	Saliva and tears		tear SIgA concentration decreased after exercise (-57%, $P < 0.05$) in line with the "open-window theory" but was unaffected by dehydration. Saliva flow rate decreased and saliva SIgA concentration increased	Hanstock et al. (2016) One of many studies which have explored the relationship between exercise and increased susceptibility to infection. Given there are some links to dehydration there may be some potential relevance to BRD, for this and other related studies. Although, might require a specific review of the exercise physiology related literature.

		after exercise and during dehydration	For the most part it although the outcomes are similar – ie respiratory disease – the underlying causes are dissimilar, stress/exposure versus exercise. Perhaps the source of stress is unimportant from a physiological outcome point of view. Other recent examples in this space: Gillum et al. (2017) Bellar et al. (2017)

7.3 Appendix C Keywords: microRNA, bovine, disease

References total	63
References high ranking	11
References included	15

Pathogen	Study design	Methodology	Sample	Up regulated	Down regulated	Reference
FMDV	Cattle infection	qPCR Array miScript	Serum	Acute infection miR-22-5p (+2.73); miR-497 (+26.16); miR-369-3p (+28.57); miR-34a (+28.57); miR-144 (+28.78); miR-146a (+34.36); miR-17-5p (+35.88) Persistence infection iR-22-5p +2.17 miR-147 +5.28 miR-1224 +5.69 miR-144 +23.78 miR-154a +24.05 miR-497 +24.62 miR-17-5p +40.84 miR-205 +41.22 miR-31 +43.37 Convalescent miR-455-3p +68.17	Acute infection miR-26b (−3.09); miR-1281 (−2.50) let-7g (−1.96) Persistence infection miR-1281 (−3.41) miR-181b (−2.77) miR-23b-5p (−2.44) Convalescent miR-150 -1.65	Stenfeldt et al. (2017)
FMDV			Cell culture			Gutkoska et al. (2017)

						Role of miR-203a-3p and miR-203a-5p in FMDV virus replication. Use miRmap to identify targets http://mirmap.ezlab.org/app/
BVDV-2	Cattle infection	NGS Samples Day 0, 4, 9, 16	Serum	Bta-miR-339a Day 9 Bta-miR-486 Day 9 Bta-miR-30e-5p Day 4, 9, 16 Bta-miR-185 Day 4, 9 Bta-miR-92a Day 4, 9	Bta-let-7c Day 4, 16 Bta-miR-2284x Day 4, 16	Taxis et al. (2017)
BVDV			Cell culture		bta-miR-17 bta-let-7 Technically not down-regulated but due to specific sequestering	Scheel et al. (2016) Role of bta-miR-17 and bta-let-7 in BVDV replication, miR sequestered by virus leading to changes in host gene expression for virus replication Mir-17 interaction with 3'-UTR enhances viral translation. Fu et al. (2015) Over expression of bta-miR-29b reduced NADL replication
Bluetongue virus	Sheep	NGS Qpcr	Primary testis cells	oar-let-7d, oar-miR-29b novel-mir-29 novel-mir-61	oar-let-7f, oar-mir-10b, oar-miR-369-5p, novel-mir-158	Du et al. (2017)
Mycoplasma bovis	Beef cattle	NGS Also analysed expression relative to	Serum	bta-let-7b bta-miR-24-3p	bta-miR-92a bta-miR-423-5p	Casas et al. (2016) Potentially some crossover with heat stress?

		season & age dependent increases				
BoHV-5			Brain tissue	bta-miR-155 specific		Oliveira et al. (2017) mir-155 regulation of TLR genes
Mastitis	Dairy cattle		tissue	Bta-miR-2898 +6.25-f		Wang et al. (2014)
Mastitis	Dairy cattle					Li et al. (2012) Altered regulation of HMGB1 by bta-miR-223 due to SNP in 3' UTR
Streptococcus uberis,	Cultured cells			2 hpi bta-mir-29e bta-mir-708 4 hpi bta-let-7b bta-miR-98 bta-miR-let-7c bta-miR-708 6 hpi bta-let-7b bta-mir-200c bta-mir-210 bta-mir-24-2 bta-mir-128-2 bta-mir-let-d bta-mir-128-1 bta-let-7e bta-mir-185	 bta-miR-29b-2 bta-miR-193a, bta-miR-130a bta-mir-29e bta-miR-29b-2 bta-miR-29c bta-miR-29e bta-miR-100 bta-miR-130a ENSBTAG000000	Lawless et al. (2014)

				bta-mir-652 bta-mir-494 bta-mir-2342		
Streptococcus uberis	Holstein	NGS	Milk purified exosomes	bta-miR-223 bta-miR-142-5p x263 bta-miR-1246 x196 bta-miR-183 x6 bta-miR-99a-5p x3 bta-miR-101 x-3 bta-miR-10a x15 bta-miR-99b x3	bta-miR-296-5p bta-miR-502b x−13 bta-miR-378b x−10 bta-miR-2285 g-3p x−4 bta-miR-181b x−3 bta-miR-2419-5p x−3	Sun et al. (2015) Milk exosome study collected 48 hr post infection bta-miR-223 and bta-miR-296-5p specific for infected and uninfected respectively. Exosomes purified via sucrose gradients Detailed description of miRNA analyses.
Mycobacterium bovis	Holstein –Friesian	Agilent custom array & TaqMan qPCR	Stimulated PBMC	Unvaccinated bta-miR-188 (W0, x6.7) bta-miR-155 (W2, x2.5) bta-miR-188 (W11, x6.4) bta-miR-155 (W11, x3.3) bta-miR-664 (W11, x2.5 Vaccinated bta-miR-188 (W-4, x3.2 bta-miR-188 (W7, x2.4) bta-miR-155 (W11, x2.2) bta-miR-188 (W11, x3.9)		Golby et al. (2014) Potential DIVA application bta-miR-155 Differentially expressed between BCG vaccinated cattle and unvaccinated cattle following challenge – a time dependent manner. Challenged at Week 0 (W0)
Mycobacterium avium subspecies paratuberculosis (MAP)	Holstein-Friesian 3 to 6 weeks old	NGS	Serum (1mL)	Infected–TP0 vs 6 months bta-miR-205 2.14 Control–TP0 vs 6 months bta-miR-205 2.23	bta-miR-432 -2.23 bta-miR-432 -2.07	Farrell et al. (2015) More variation in expression based on age/development

			bta-miR-126-5p 1.67 bta-miR-143 1.59 bta-miR-27a-3p 1.55 bta-miR-92b 1.54 bta-miR-10b 1.51	bta-miR-127 -1.78		than infection controls of the 6 month period. Infected selected based on IFN levels.
Mycobacterium avium subspecies paratuberculosis (MAP)			bta-mir-6517 bta-mir-7857	bta-mir-19b bta-mir-19b-2 bta-mir-1271 bta-mir-100 bta-mir-301a bta-mir-32		Malvisi et al. (2016) Complicated analyses of exposed, positive (based on IFN) and unexposed animals. Appears to be minimal correlation with Farrell et al. (2015)
Mycobacterium avium subspecies paratuberculosis (MAP)	NGS	Stored sera miRNeasy	bta-miR-29a bta-miR-92b			Shaughnessy et al. (2015) Possible use of bta-miR-345-3 constant expression over time.
Early pregnancy	Qiagen PCR arrays	plasma	Day 24 bta-miR-26a 1.76			Ioannidis and Donadeu (2016b)
Oestrous		plasma	Day 16 Oestrous let-7f bta-miR-125b bta-miR-145 bta-miR-99a-5p			Ioannidis and Donadeu (2016a)
Embryonic viability			bta-miR-25 bta-miR-16b bta-miR-3596			Pohler et al. (2017)

Heat Stress	Holstein	qPCR	Isolates PBMC	bta-miR-181a (inhibitor)		Differential expression (n=27) between pregnant and embryonic death Chen et al. (2016) Suggests down regulation of bta-miR-181a can limit cellular damage in response to heat stress.
Heat stress	Holstein	NGS qPCR	Serum (3mL)	miR-19a, miR-19b, miR-27b, miR-30a-5p, miR-181a, miR-181b, miR-345-3p, and miR-1246	miR-26a, miR-146a, miR-146b, and miR-199a-3p	Zheng et al. (2014)

7.4 Appendix D Complete Results ONP sequencing – Host depletion Devin

See Attached Excel file.

7.5 Appendix E Complete Results ONP sequencing – Host depletion MolYsis Basic5 2

See Attached Excel file.

7.6 Appendix F Protocol for the Processing and Analysis of Nasal Swabs using ONT sequencing

BRD feedlot testing – nasal swab extraction, host depletion and ONT sequencing

7.6.1 Nasal swab extraction – no host depletion

Adapted from Gentra Puregene protocol for buccal brush (Qiagen, 2011 handbook, p.28 available at https://www.qiagen.com/us/Resources/ResourceDetail?id=a9e6a609-4600-4b03-afbd-974318590ce5&lang=en)

1. On day of collection, nasal swabs were transferred to 1.5mL microcentrifuge tubes containing 300µL cell lysis solution and 1.5µL proteinase K solution, vortexed on high for 20 seconds and incubated on heatblock overnight at 55^{o}C.
2. The following morning, swabs were carefully discarded from solution using tweezers and squeezing maximise the recovery of the resuspension. The swab was then discarded.
3. Protein precipitation solution (100µL) was added to resuspension, followed by vortexing on high vigorously for 20 seconds. Samples were then incubated on ice for 5 minutes.
4. Samples were centrifuged at 16,000 x *g* for 3 minutes in benchtop centrifuge to pellet protein and cell debris. Supernatant was transferred to new 1.5ml microcentrifuge tubes containing 300µL isopropanol.
5. Samples were mixed by gently inverting 50 times. Samples were centrifuged again at 16,000 ×*g* for 5 minutes.
6. The supernatant was carefully aspirated and discarded. The tube was drained on paper towel to remove any excess liquid.
7. The DNA pellet was washed DNA by adding 300µL freshly made 70% ethanol, gentle inversion 3 to 5 times and centrifugation at 16,000 ×*g* for 1 minute.
8. The supernatant was carefully aspirated and discarded. Excess fluid was drained of on paper towel. The DNA pellet was air-dried for 5 minutes at room temperature.
9. DNA pellet was dissolved by adding 50µL DNA hydration solution, gently flicking to mix, and incubating on heatblock at 65^{o}C for 1 hour.
10. The DNA samples were stored at 4^{o}C until required.

7.6.2 Nasal swab extraction – Molysis™ host depletion

Adapted from Molysis Basic5 Bacterial enrichment kit Protocol 1: Small size sample DNA isolation (Molzym GmbH & Co., Germany).

1. On day of collection, nasal swabs were transferred to 5ml collection tube containing 1ml 1× PBS containing 1 × antibiotic-antimycotic (ThermoFisher Scientific) and stored at 4^{o}C until extraction.
2. The liquid (750µL) from nasal swab resuspension was added to a 2mL microcentrifuge tube and 250µL of Buffer SU added. Buffer CM (250µL) was added and the sample vortexed on high for 15 seconds.
3. The sample was incubated at room temperature for 5 minutes.
4. Buffer DB1 (250µL) and 10µL of MolDNase B were added to the lysate, followed by vortexing on high for 15 seconds. The sample was then incubated at room temperature for 15 minutes.
5. The sample was centrifuged at 12,000 ×*g* for 10 minutes. The supernatant was carefully aspirated and discarded.
6. Buffer RS (1mL) was added, followed by vortexing on high and centrifugation at 12,000 ×*g* for 5 minutes. The supernatant was carefully aspirated and discarded.
7. Buffer RL (80µL) was added, and the sample resuspended by vortexing on high.
8. BugLysis solution (20µL) and β-mercaptoethanol (1.4µL) were added and vortexed on high for 15 seconds.
9. The sample was then incubated in at 37^{o}C for 30 minutes.
10. Following the addition of 300µL cell lysis solution and 1.5µL proteinase K solution from the Puregene Kit, DNA was extracted as described in Section 7.6.1
11. Samples were stored at 4^{o}C until required for analysis.

7.6.3 Nasal swab extraction – Devin™ host depletion

Nasal swab samples were prepared for ONP sequencing using the Devin™ Microbial DNA Enrichment Kit (Product Number MEK01-24, Micronbrane Medical) as described below.

1. Nasal swabs were added to 3mL of 1×PBS supplemented with 1 × antimicrobial and antimycotic (ThermoFisher Scientific) and stored at 4◦C until required.
2. Three microfuge tubes (1.5mL) were labelled, “MB”, “WB1” and “WB2”, respectively. Transferred all the beads from well A4 into Tube MB. Transferred all the buffer from wells A2 + A3 into Tube WB1. Transferred all buffers from wells A5 + A6 into Tube WB2. Repeated process for each nasal swab to be extracted.
3. Tube MB was placed onto a magnetic rack and the supernatant transferred into Tube WB2.
4. Tube MB was removed from rack, and 500µL of buffer from Tube WB1 added to it, followed by brief vortexing.
5. Nasal swab resuspensions (2.75mL) were sterile filtered using filters provided by in the kit.
6. The filtrate was centrifuged at 16,000 ×*g* 15min and the supernatant aspirated and discarded.
7. The pellet was resuspended in 200µL of Incubation buffer containing 20µL Proteinase K and 20µL Lysozyme and thoroughly mixed.
8. The sample was incubated at 60◦C for 10 minutes, with vortexing for 10 seconds every 4 minutes.
9. Ethanol (300µL of 100%) was added to the sample, followed by vortexing and centrifugation.
10. Tube MB tube was placed on the magnetic rack, and the supernatant transferred into Tube WB1.
11. Tube MB was removed from rack and the sample transferred to it.
12. Tube MB (containing beads and sample) was vortexed 5mins and briefly centrifuged. The tube was then returned to magnetic rack. The supernatant was aspirated and discarded.
13. The beads and sample in Tube MB were washed with 750µL of WB1 and vortexing for 30sec. After brief centrifugation the tube was returned to magnetic rack. The supernatant was aspirated and discarded.
14. The beads and sample in Tube MB were washed with 750µL of WB2 and vortexing for 30sec. After brief centrifugation the tube was returned to magnetic rack. The supernatant was aspirated and discarded.
15. With the lid open, Tube MB was incubated at 45◦C in a heating block for 10 minutes to dry the beads.
16. Elution buffer (50µL) was added to Tube MB, and the solution mixed by pipetted up and down.
17. Tube MB was returned to the heating block to 45^{o}C, with mixing by pipetting up and down five times at 1 minute intervals for 5 minutes.
18. The tube was returned to magnetic rack, the supernatant carefully aspirated and transferred to a nuclease free microfuge tube.
19. The purified DNA was stored at 4◦C until required.

7.6.4 Sequence Library Preparation by Rapid Barcoding and ONT sequencing

Adapted from the Oxford Nanopore Technologies Rapid Barcoding Sequencing (SQK-RBK004) instructions.

1. Nasal swab nucleic acid extract (7.5µL) was combined with 2.5µL of Fragmentation Mix, one of Rabid Barcodes RB01 to RB06 for each of the six samples to assign a unique barcode to each. The tubes were mixed by gently flicking and briefly spun.
2. The samples were incubated with the barcode mixes in a thermocycler at 30^{o}C for 1 minute. Followed by incubation at 80^{o}C for 1 minute and then rapidly quenched on ice.
3. The Barcoded samples were pooled into a 1.5mL microcentrifuge tube and an equal volume of AMPure XP beads (60µL) added to the tube, followed by gentle flicking to mix.
4. Samples were incubated at room temperature for 5 minutes, with gentle flicking to mix the beads approximately every 20 to 30 seconds.
5. Samples were briefly centrifuged and placed on a magnet to pellet beads. With the tubes remaining in contact with the magnet, the supernatants were carefully aspirated and discarded.
6. Freshly prepared 70% ethanol (200µL) was used to wash the beads, while keeping the sample in contact with the magnet. The supernatant was carefully aspirated and discarded.
7. Sample was briefly centrifuged and returned to the magnet. Residual ethanol was aspirated.
8. The tube was removed from magnet and the pellet allowed to air dry briefly.
9. Pellet was resuspended in 10µL of 10mM Tris-HCl (pH 7.5) containing 50mM NaCl and incubated at room temperature for 2 minutes.
10. Sample tube was returned to the magnet to pellet beads and using wide bore tips, the eluate was transferred the to a clean 1.5ml microcentrifuge tube.
11. RAP solution (1µL) was added to the 10µL barcoded DNA, followed by gentle mixing by flicking and brief centrifugation.
12. The sample was incubated at room temperature for 5 minutes.
13. In preparation for sequencing, a quality control check was performed on the MinION flow cell as per the manufacturer's instructions.
14. Sequencing Buffer, Flush Tether, and Flush Buffer were thawed.
15. Prepared by added Flush tether buffer (30µL) was added to a fresh tube of Flush buffer, followed by mixing by pipetting.
16. The MinION flow cell was primed by using P1000 pipette to remove any air bubbles from the priming port and 800µL of flush buffer mix added.
17. The flow cell was then incubated at room temperature for 5 minutes.
18. The Sample library (Step 12) was prepared for sequencing by adding 34µL sequencing buffer, 25.5µL loading beads and 4.5µL nuclease-free water, followed by gentle mixing.
19. The sample port of the MinION flow cell was opened and a further 200µL of flush buffer mix added to the priming port.
20. The sequencing library was mixed with mix loading beads by pipetted up and down. The library sample was then loaded onto the cell through the sample port in a dropwise manner.
21. The priming and sample ports were closed, and the flow cell incubated at room temperature for ten minutes prior to commencing the sequencing run and data acquisition as per the manufacturer's instructions.

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