Article

Characterisation of the Upper Respiratory Tract Virome of Feedlot Cattle and its Association with Bovine Respiratory Disease

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Abstract: Bovine respiratory disease (BRD) is a major health problem within the global cattle industry. This disease has a complex aetiology, with viruses playing an integral role. In this study, metagenomics was used to sequence viral nucleic acids in the nasal swabs of BRD affected cattle. Viruses detected included those well known for their association with BRD in Australia (bovine viral diarrhea virus 1), as well as viruses known to be present but not fully characterised (bovine coronavirus) and viruses that have not been reported in BRD affect cattle in Australia (bovine rhinitis, bovine influenza D, and bovine nidovirus). Nasal swabs from a case control study were subsequently tested for 10 viruses and the presence of at least one virus was found to be significantly associated with BRD. Some of the more recently detected viruses had inconsistent association with BRD. Full genome sequences for bovine coronavirus, a virus increasingly associated with BRD, and bovine nidovirus were complete. Both viruses belong to the *Coronaviridae* family, which are frequently associated with disease in mammals. This study has provided greater insights into the viral pathogens associated with BRD and highlighted the need for further studies to elucidate more precisely the roles viruses play in BRD.

Keywords: bovine respiratory disease; virome; bovine nidovirus; bovine coronavirus; bovine herpesvirus 1; bovine viral diarrhea virus 1; bovine respiratory syncytial virus; case control; odds ratio

1. Introduction

Bovine respiratory disease (BRD) is the most significant health problem within the feedlot industry. Despite advances in veterinary medicine and improvements in control measures, BRD remains a major economic burden for the beef industry through reduced growth rates, mortality and organ condemnation, while increasing treatment and labour costs [1]. The pathogenesis of BRD is complex, with several viruses, bacteria, host and environmental factors contributing to its onset [2-11]. Viruses historically associated with BRD are bovine herpesvirus 1 (BoHV-1), bovine viral diarrhoea virus 1 (BVDV-1), bovine parainfluenza virus 3 (BPI-3) and bovine respiratory syncytial virus (BRSV) (Fulton, 2020). Bacterial infections (predominantly *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis*) are generally considered to be secondary pathogens, opportunistically colonising the respiratory mucosa following damage caused by primary viral infection or because of immunosuppression [9, 12, 13].

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Viral metagenomics, using next generation sequencing (NGS) technologies, has recently allowed for the rapid genetic characterisation of viral genetic material in clinical samples, the virome, and revealed the presence of both known and novel viruses in healthy and sick animals and people [14-17]. Unlike conventional diagnostics, this technology does not require prior knowledge of the genetic information of a pathogen for it to be detected, hence allowing for the unbiased assessment of clinical samples and the discovery of novel viruses. Although the pathogens listed previously are thought to be the principal pathogens associated with BRD, recent viral metagenomics studies suggest that the repertoire of viruses associated with BRD are more diverse, which may be a contributing factor to the failure to manage this disease adequately [14, 18].

The first aim of the current study was to characterise the virome present in the nasal swabs of feedlot cattle treated for BRD. The second aim of the study was to use virus specific-qPCR assays informed by the virome to determine the presence and absence of viral genetic material in nasal swabs taken from BRD affected and unaffected cattle to determine associations between these viruses and the risk of animals developing disease.

2. Materials and Methods

Viral Metagenomics

Nasal swabs used in this study were collected as part of the National Bovine Respiratory Disease Initiative (NBRDI), which was a nationwide prospective longitudinal study conducted in Australia to evaluate possible risk factors for BRD in feedlot cattle (Hay et al., 2014). Briefly, dry nasal swabs were collected from cattle treated for BRD with signs of respiratory disease. On receipt at the laboratory, the swabs were added to a 96 well-plate containing 500 µL phosphate buffered saline (PBS) containing 5× Antibiotic-Antimycotic (ThermoFisher Scientific, Waltham, United States of America). Samples were stored at -80^{0}C until required.

Six pools consisting of six nasal swab samples were prepared using 50 µL from each nasal swab sample. The 300 µL pooled samples were passed through a 200 nm filter (Merck) to remove eukaryotic cells, bacteria, and particulate debris. The resulting filtrate was incubated at 37^{0}C for 90 min in a cocktail of 14 U Turbo DNase (Ambion), 25 U Benzonase® (Sigma Aldrich) and 20 U RNase1 (ThermoFisher Scientific) to degrade host (bovine) or unprotected environmental nucleic acids. Viral RNA was isolated using the QIAamp MinElute Virus Spin kit® (Qiagen) according to the manufacturer's instructions.

Complementary DNA (cDNA) of RNA in the extract was prepared by reverse transcription using an oligonucleotide containing a specific nucleotide sequence (5′ residues 1 to 20) and a random sequence with 8Ns (residues 21 to 28) at the 3′ end (cDNA primer: 5′-CCTTGAAGGCGGACTGTGAGNNNNNNNN-3′) [14] using the Superscript III reverse transcription kit (ThermoFisher Scientific) according to the manufacturer's instructions. Second strand synthesis was performed using Klenow fragment DNA polymerase (New England Biolabs) and the cDNA primer so that the complementary strand of the cDNA also encoded the fixed portion of the cDNA primer at the 5′ terminus. The resulting double-stranded cDNA was PCR amplified using Platinum™ Taq DNA polymerase (ThermoFisher Scientific) and the oligonucleotide amplification primer: 5′-CCTTGAAGGCGGACTGTGAG-3′ [15]. The 50 µL reaction mix contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.2 µM of each primer and 1.0 U of polymerase. Amplification conditions were as follows: 95 °C for 5 min; 5 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min; 33 cycles of 95 °C for 20 sec, 59 °C for 20 sec and 72 °C for 1 min increasing by 2 sec per cycle; final extension of 72 °C for 7 min.

Amplicons were purified using Qiagen MinElute PCR Purification kit (Qiagen®) and then submitted to the Australian Genome Research Facility (AGRF) for library preparation and NGS. The nucleic acid was subjected to Nextera XT library preparation protocol (Illumina) and sequenced using Illumina's MiSeq platform to generate 300 nucleotide (nt) paired end reads.

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Sequence data was initially quality filtered to remove low quality sequences, reads less than 36 bp and Illumina-specific sequencing adaptors using the Trimmomatic program [19]. Trimmed reads were mapped to the host reference genome (*Bos taurus*: bosTau7) and the Illumina quality control template (PhiX174), and unmapped reads were retained for further analysis. Mapping was performed using Bowtie2 [20] with default parameter settings. De novo assembly was completed using Velvet Optimiser and performed using unmapped reads to generate contiguous sequences (contigs) [21, 22]. Sequence identity searches were performed with these resulting assembled contigs using BLASTN [23, 24] against selected databases. A custom database was constructed based on possible BRD associated viral sequences determined through the literature. The National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov) viral reference sequence database and The Nucleotide (nr/nt) database were also used.

Following BLAST analysis, contigs that were identified as viruses, were further analysed using the alignment and mapping programs within MEGA7 (Kumar et al., 2016) and Geneious 9 (http://www.geneious.com) [25]. Contigs were mapped to viral reference genomes to generate consensus sequences and to assess genome coverage of individual viruses. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 7 [26].

PCR and sequencing:

For bovine coronavirus (BCoV) and bovine nidovirus (BNV), near complete genome sequences were obtained following mapping of NGS reads to reference genomes. To generate sequence data to fill in the remaining gaps, oligonucleotides were designed encompassing regions for which no or poor sequence was obtained. The PrimerQuest software (Integrated DNA Technologies, Inc.) and the newly determined genome sequences were used to design these oligonucleotides. For BCoV, eight oligonucleotide pairs were designed with amplicons varying in size from 193 nt to 2596 nt. For BNV, oligonucleotides (12 pairs) were designed to facilitate amplification across the entire genome with amplicon sizes ranging from 1032 nt to 2518 nt (Supplemental Table S1).

Sample pools (cDNA) for which BCoV and BNV NGS sequence data was obtained were used as template for PCR. BCoV PCR was performed using Platinum® Taq HotStart DNA polymerase (ThermoFisher Scientific). The 25 µl reaction mix contained 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.8 µM of each primer and 1.0 U Taq DNA polymerase. Amplification parameters: initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min per kb. The reaction concluded with a 5 min extension time at 72°C.

For BNV, Phusion Green Hot-Start II High-Fidelity PCR Master Mix (ThermoFisher Scientific) was used according to the manufacturer's instructions with a 25 µL total volume. Amplification parameters were: initial denaturation at 98°C for 30 sec followed by 40 cycles of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 to 2 min depending on size the of the expected product. The reaction concluded with a 10 min extension time at 72°C.

Amplified products were run on a 1% agarose gel stained with Midori green and visualised with a UV transilluminator. Amplicons consistent with the expected sizes were excised from the gel and purified using the QIAquick® Gel Extraction Kit (Qiagen). Direct sequencing of each amplicon was performed using BigDye® Terminator v3.1 (Applied Biosystems™) according to the manufacturer's instructions and submitted to a commercial sequencing service for fragment analyses (Genetics Research Services, The University of Queensland, Australia).

Quantitative real-time PCR (qPCR):

Five sets of amplification oligonucleotides and corresponding dual-labelled hydrolysis probes were designed to detect four RNA viruses and one DNA virus that had not been reported in Australian cattle treated for BRD previously. Published studies have

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suggested the selected viruses may play a role in the development of BRD in feedlot cattle: BNV, bovine rhinitis A virus (BRAV), bovine rhinitis B virus (BRBV), influenza D virus (IDV) and ungulate bocaparvovirus 6 (UBPV6) [18, 27-29]. Briefly, in addition to using sequence data generated with NGS, available nucleotide sequences for these viruses were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/September 2017) and aligned using MEGA7 [30] (Kumar et al., 2016) to identify conserved regions suitable for oligonucleotide and hydrolysis probe design. The PrimerQuest software (Integrated DNA Technologies, Inc., Coralville, IA, U.S.A.) was used to design the oligonucleotide pairs and corresponding dual-labelled hydrolysis probes and their specificities were evaluated using the BLAST algorithm [31]. The dual-labelled probes had unique reporter dyes/fluorophores at their 5′ ends and Black Hole Quenchers® or Iowa Black®FQ at their 3′ ends. For BRBV, for which minimal sequence data was available across conserved regions, the primer-probe set from a published qPCR assay was used [32]. The nucleotide sequences, fluorophores and quenchers of the oligonucleotide pairs and dual-labelled hydrolysis probes are shown in Table 1.

Table 1. Oligonucleotide pairs and dual-labelled hydrolysis probe sequences used for quantitative real-time PCR detection of viruses.

Target Pathogen	Name	Primer/Probe sequence 5′-3′ [1]
Bovine nidovirus	BDV_Fwd	GTCAACTGGAGTAGGTCGAAAG
	BDV_Rev	TCAGCCTCATTCCTAACATCAC
	BDV_Probe	TEX615- AGGTACCATTACTATACTGAGCTGG-CAGC -BHQ-2
Bovine rhinitis A virus	BRVA_Fwd	AGGTACCCGGAGGTAACAA
	BRVA_Rev	GGTGCCTGATGAGACATAGAAG
	BRVA_Probe	6FAM-CCCAGGTCAGATCCAGAGTGTCAC-BHQ-1
Bovine rhinitis B virus	BRVB_Fwd	GCGATTGTGTCCTAGGGTTT
	BRVB_Rev	GCCACTGAGGTTAGCTTCTC
	BRVB_Probe	Cy5-CTGTCCTTTGCACGGCGTGG-BHQ-2 [2]
Influenza D virus	IDV_Fwd	GAGGAATGCTGATGGGAATGT
	IDV_Reverse	CTTTGTAGCCCAGTCCAGTAAC
	IDV_Probe	HEX-ATTACAGGGAGGAAGCATTGGCCA-BHQ-1
Ungulate bocaparvovirus 6	UBVP6_Fwd	GGGAAGAGTGGCTTCAGTTTAG
	UBVP6_Rev	GGCTCTTCTCCTTGTTCTTCTG
	UBVP6_Probe	HEX-TCCAGATACAATCAGAAGAAGCGCCA-ZEN/IABkFQ

[1] Fluorophores and quenchers are shown at the 5′ and 3′ termini respectively of each probe sequence.
[2] Primers and probe sequences from [32]

The specific assays were evaluated using the viral RNA pools and the individual viral RNA samples used for the pools. The exception was UBPV-6 for which the assay was optimised using synthetic double-stranded DNA fragment (gBlocks™ Gene Fragment -

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Integrated DNA Technologies, Inc.) as there was insufficient quantity of this virus in the RNA pools.

The assays were further tested using 60 nasal swabs extracts from cattle with BRD from feedlots (same feedlots that were used in pools). Viral RNA was extracted from these swabs using the QIAamp-MinElute Virus Spin kit (Qiagen) and qPCR performed as described below.

As part of assay evaluation each primer-probe set was also tested in a reaction with template containing viruses other than the ones they were targeting.

Case Control Study

The case-control analysis was part of a larger study to predict BRD outcome in feedlot cattle using latent class analysis which has previously been described [33]. Briefly, the study was conducted at a commercial feedlot in southern New South Wales, Australia, with cattle (*Bos taurus* castrated males, approximately 12-24 months old) sourced from saleyards or cattle backgrounding properties. Following induction, animals were checked daily by trained feedlot staff for visual signs of BRD. Animals were scored for visual signs of BRD in the pen using a modified version of the Wisconsin calf scoring chart which included assessment of seven visual signs: lethargy, head carriage, laboured breathing, cough, nasal discharge, ocular discharge, and rumen fill [34]. Each clinical sign was assigned a score from 0 to 3, with 3 the most severe. A case was defined as an animal with a score > 0 for at least one of the visual signs specific to BRD: nasal or ocular discharge, laboured breathing, or coughing. For each animal identified with BRD (case), an animal (control) exhibiting no visual signs of BRD (score 0 for all the seven visual signs) was removed from the same pen on the same day. Detailed information on the animals used, their management, BRD monitoring and clinical data collection was previously described [34].

A total of 288 nasal swabs from study cattle were collected for analysis (141 cases and 147 controls). On receipt in the laboratory, nasal swabs were resuspended in 500 µL PBS. Total nucleic acid was extracted from 200 µL nasal swab sample using the DNeasy 96 Blood and Tissue kit (Qiagen®) according to the manufacturer's instructions. The optional addition of RNaseA was omitted to permit the co-purification of RNA and DNA.

Quantitative real-time PCR (qPCR) was performed on these samples to test for the presence of genetic material for BoHV-1, BVDV-1, BRSV, BPI-3, BCoV, BNV, BRAV, BRBV, IDV and UBPV-6. The qPCR assay for the detection of BoHV-1, BCoV, BRSV and BPI-3 was performed as a multiplex reaction as described previously with BCoV replacing BVDV-1[35] (Horwood and Mahony, 2011). Detection of BRAV, BRBV and IDV was also performed as a multiplex reaction with primer and probe concentrations of 0.4 µM and 0.2 µM, respectively. BVDV-1 and BNV were detected using singleplex assays with primer and probe concentrations of 0.6 µM and 0.2 µM respectively. All viral RNA assays were performed using the QuantiTect Multiplex RT-PCR Kit (Qiagen) and Qiagen Rotor-Gene® Q machine. The reactions were performed as per manufacturer's instructions. For UBPV-6, qPCR was performed using IDT PrimeTime® Gene Expression Master Mix according to manufacturer's protocol and with primer and probe concentrations as per singleplex assays above. Samples were considered positive if the threshold cycle (Ct) value was ≤35.

Odds ratios (OR) were calculated to measure the association between virus detection and clinical signs of BRD. OR confidence intervals (CI) were used to estimate the precision of the OR and p values were also calculated from the CI. Statistical significance was defined as $p < 0.05$. The OR, its standard error, 95% CI and p value were calculated as described by [36, 37].

To test the hypothesis that the Ct values for each virus detected in nasal swabs collected from animals with clinical signs of BRD is the same as the Ct values from samples collected from asymptomatic animals, a t-test was performed to compare the means of the two groups. Comparisons yielding $p < 0.05$ were considered statistically significant. This statistical method was used to test the hypothesis that the Ct values for IDV in animals

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co-infected with another virus is the same as the Ct values in animals infected with IDV alone.

3. Results

3.1 Viral Metagenomics

Nasal swabs collected from 36 animals treated for BRD were pooled into six pools of six animals and deep sequenced using the Illumina MiSeq platform. A total of 17,195,238 pair ended 300 nt sequence reads (average 2,865,873 per sample) were generated. Following quality control, de novo assembly was performed. BLAST searches of the resulting contigs against a custom BRD viral database, a viral reference sequence database (NCBI) and non-redundant sequence database (NCBI) identified several contigs in the experimental datasets with high identity to viruses in four of the six sample pools. The two pooled samples where no viral sequences were identified were not analysed further.

In the data from the remaining samples, sequences from viruses from the following families were identified: *Coronaviridae*; *Tobaniviridae*; *Flaviviridae*; *Orthomyxoviridae; Picornaviridae*; and *Parvoviridae*. Contigs were mapped to reference viral genome sequences using Geneious® (Version 9) which resulted in the generation of near complete and partial viral genome sequences for a subset of the viruses. Further analyses were undertaken, including phylogenetic analyses, to evaluate the relationships of these newly sequenced viruses to those viruses present in the databases.

3.1.1. Orthomyxoviridae

Viral sequences with high identity to IDV were identified in the data from one of the four pools. Sequence comparisons demonstrated that four of the seven genomic segments were represented in the dataset. Each of the four segments were assembled using the respective sequences from IDV strain D/bovine/Miyazaki/B22/2016 as guide templates. The lengths of each segment identified in the sequencing data and the coverage of the respective fragments compared to the reference strain are summarised in Table 2. Segment 1 was the most complete with a length of 1,054 nt, covering 44.6% of the analogous segment from the reference strain (Table 2). Overall, there was very high sequence identities and similarities at the nucleotide and amino acid levels, respectively (Table 2). These values were reduced for the segment 4 (encoding haemagglutinin-esterase), although it should be noted that it also had the lowest coverage of 12.1% (Table 2).

Maximum likelihood phylogenetic analysis of the partial nucleotide sequences of the IDV segment 1 (Fig. 1) demonstrated that this newly identified viral sequence clusters with other IDV strains and are distinct from other genera of the *Orthomyxoviridae* family. The inferred relationship to other IDV strains and tree topology were supported by high bootstrap scores.

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Table 2. Summary of the bovine influenza D virus partial genomic segment sequences identified in the current study compared to the strain IDV D/bovine/Miyazaki/B22/2016.

		IDV sequence data (current study)					IDV Reference	
Segment	Protein	Length (nt)	Coverage (%)	Nucleotide Identity (%)	Amino Acid Similarity (%)	GenBank	Length (nt)	Reference Accession
1	polymerase PB2	1054	44.6%	98.0	99.2	XXXXX	2,364	LC270265.1
2	polymerase PB1	929	39.9%	98.5	99.7	XXXXX	2,330	LC270266.1
3	polymerase 3 P3	918	41.0%	98.4	98.7	XXXXX	2,195	LC270267.1
4	haemagglutinin-esterase HE	248	12.1%	94.8	92.7	XXXXX	2,049	LC270268.1
5	nucleoprotein	NT[1]					1,775	LC270269.1
6	P42	NT[1]					1,219	LC270270.1
7	non-structural protein 2	NT[1]					868	LC270271.1

[1] Not detected.

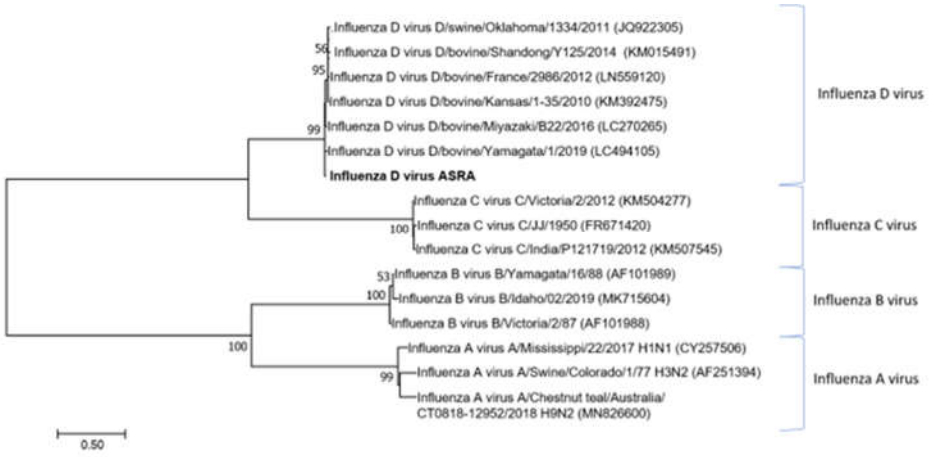

Figure 1. Phylogenetic tree of influenza D virus based on the polymerase PB2 gene. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Tamura-Nei model [38, 39]. Bootstrapping of 1000 replicates was performed. To determine the best model to use for phylogenetic analysis, model selection was performed which analysed the maximum likelihood fits of 24 different nucleotide substitution models. The trees are drawn to scale with the scale bar representing the number of nucleotide substitutions per site. Numbers at nodes represent percentage bootstrap support (values are indicated for each node >50%). The Australian sequences from this study are shown in bold. Isolate names and GenBank accession number for sequences used in the trees are shown.

3.1.2. Coronaviridae

Following *de novo* assembly, 255 contigs were identified in one sample pool with identity to the subfamily *Coronavirinae*. These contigs were mapped to the genome of BCoV-ENT (GenBank accession number NC_003045, [40] resulting in near complete genome coverage (data not shown). These regions were amplified by RT-PCR using the same sample pool extract used for the NGS library construction and directly sequenced using conventional dideoxy-terminator technology to complete the genome sequence. The first completed BCoV genome sequence from Australia (BCoV-Aus) was 30,999 nt in length with a G + C content of 36.9%. The BCoV-Aus genome sequence demonstrated greater than 98% nucleotide identity to other BCoV genomes in GenBank (data not

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shown). The genome organisation was typical of BCoVs and nucleotide identities between the BoCV reference genome sequence strain BCoV-ENT and buffalo coronavirus (Accession number KU558923 [41]) to which it had the highest nucleotide sequence identity are shown in Table 3. Phylogenetic reconstructions based on the ORF1ab gene demonstrated robust clustering with these viruses (Fig. 2). The BCoV genome deduced in this study encoded five putative accessory (non-structural) proteins characteristic of BCoVs [42]. In comparison to existing BCoV sequences, analysis of the BCoV sequence deduced in this study, identified that the open reading frames (ORFs) encoding the 4.8 kDa and 4.9 kDa non-structural proteins were truncated, resulting in smaller proteins than expected [40, 43]. The 4.9 kDa protein encoded by the BCoV characterised in the current study was 25 amino acids (aa) in length rather than the expected 29 aa [40]. Similarly, the 4.8 kDa protein was predicted to be reduced to 29 aa in length compared to the expected 45 aa. Similar changes to both these proteins were also evident in two buffalo coronaviruses (B1-28F and B1-24F) with which the BCoV in this study shows high identity. Both coronaviruses had shorter 4.9 kDa proteins (25aa) and the 4.8 kDa protein was 29 aa in length for B1-24F and 44 aa for B1-28F.

Table 3. Comparison of the Australian bovine coronavirus (BCoV-Aus) genome reconstructed in this study with next generation sequencing data to the BCoV reference strain BCoV-ENT and the buffalo coronavirus (BuCoV) strain B1-28F.

Open reading frame	BCoV-Aus Bases	BCoV-ENT Bases; identity (%)	BuCoV B1-28F Bases; identity (%)
Complete genome	30,999	31,028; 98.9	30,985; 98.5
orf 1ab polyprotein	21,278	21,284; 99.1	21,284; 98.4
32 kDa non-structural protein	837	837; 98.7	837; 98.1
haemaglutinin esterase (HE)	1,275	1,275; 99.1	1,275; 97.7
spike structural protein (S)	4,092	4,092; 98.3	4,092; 98.8
4.9 kDa non-structural protein	89	90; 92.2	78; 100
4.8 kDa non-structural protein	142	138; 85.6	135; 90.2
12.7 kDa non-structural protein	330	330; 98.5	330; 99.4
small membrane protein (E)	255	255; 100	255; 99.6
matrix protein (M)	693	693; 98.7	693; 100
nucleocapsid protein (N)	1,347	1,347; 98.7	1,347; 99.5
internal protein (I)	624	624; 98.7	624; 99.5

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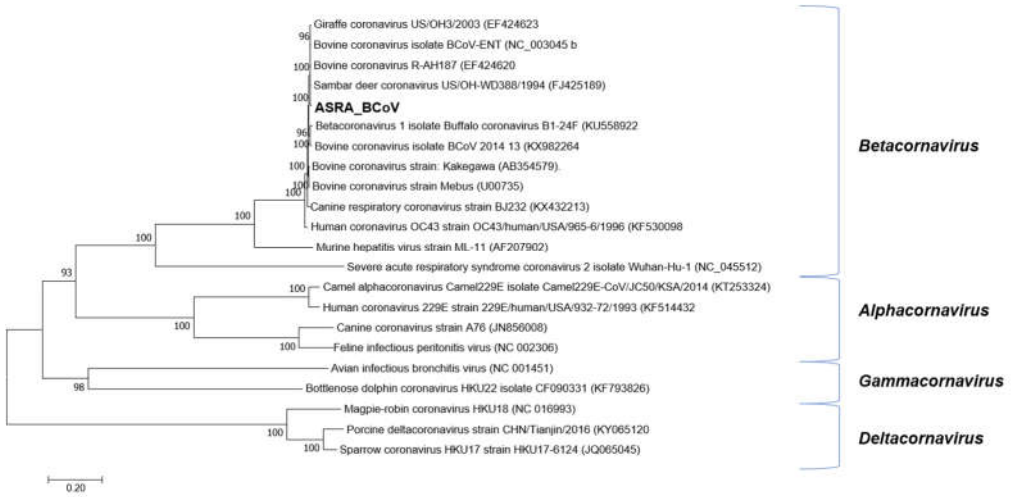

Figure 2. Phylogenetic tree of *Coronaviridae* based on the ORF1ab gene**.** The phylogenetic tree was constructed using the Maximum Likelihood method based on the General Time Reversible model. Bootstrapping of 500 replicates was performed. Phylogenetic analysis of predicted nucleotide sequences determined in this study. To determine the best DNA model to use for phylogenetic analysis, model selection was performed which analysed the maximum likelihood fits of 24 different nucleotide substitution models. The trees are drawn to scale with the scale bar representing the number of nucleotide substitutions per site. Numbers at nodes represent percentage bootstrap support (values are indicated for each node >50%). The Australian sequences from this study are shown in bold. Isolate names and GenBank accession number for sequences used in the trees are shown.

3.1.3. Tobaniviridae

Following *de novo* assembly, 612 contigs were identified with identity to BNV. At the time of this analysis, there was one full genome sequence available in GenBank for comparison, BNV strain TCH5 (GenBank Accession NC_027199, [27]. Oligonucleotide pairs were designed to amplify PCR amplicons spanning putative gaps in the viral genome, identified after mapping of the contigs to the reference sequence. The addition of these amplicon sequences to the genome assembly and mapping to the reference genome sequence, resulted in a full-length genome sequence that was 20,262 nt in length. The NGS derived genome sequence has 85.9% identity to the reference BNV genome [27].

Genome annotation revealed genome organisation consistent with the BNV previously reported, with a large replicase polyprotein and several shorter downstream ORFs. Nucleotide identities for genes ranged from 75.6% for the glycoprotein G2 to 94.8% for the hypothetical protein (Table 4). Further sequencing and analysis of more Australian BNV isolates is required to elucidate the genome sequence more accurately.

Multiple sequence alignment and phylogenetic analysis clearly demonstrated that this virus groups with the BNV TCH5 as a member of the *Bostovirus* genus within the subfamily *Remotovirinae* (Fig. 3).

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Table 4. Comparison of the Australian bovine nidovirus (BNV-Aus) genome sequence to the reference BNV genome sequence of strain TCH5.

Characteristic	BNV-Aus	BNV TCH5	Nucleotide Identity (%)	Amino acid Similarity (%)
complete genome	20,262	20,261	85.9	
replicase polyprotein (pp1a/b)	15,323	15,332	90.5	
glycoprotein S (S)	1,686	1,689	87.2	
membrane protein 1 (M1)	696	696	81.9	83.5
nucleocapsid (N)	534	537	91.8	
glycoprotein G2 (G2)	1,371	1,368	87.2	
hypothetical protein	267	267	85.8	86.5
			64.1	
			75.6	
			92.0	
			94.8	

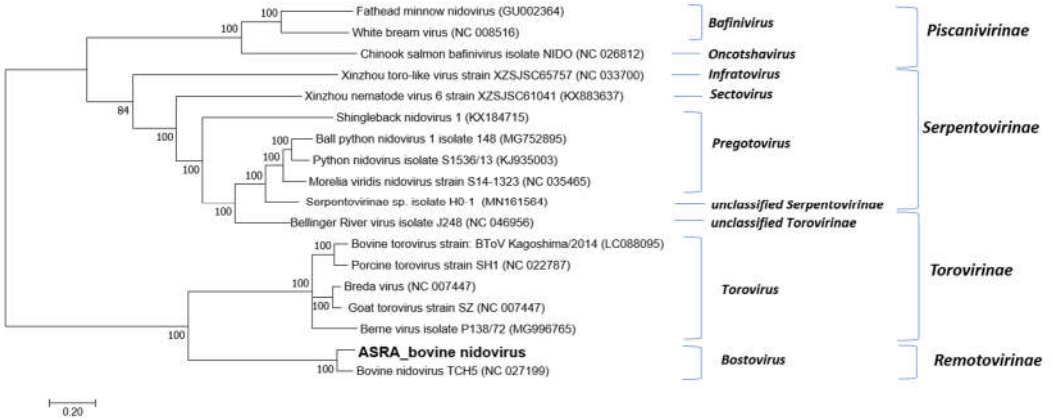

Figure 3. Phylogenetic tree of family *Tobaniviridae* based on the replicase polyprotein (pp1a/b) gene. The phylogenetic tree was constructed using the Maximum Likelihood method based on the General Time Reversible model. Bootstrapping of 500 replicates was performed. Phylogenetic analysis of predicted nucleotide sequences determined in this study. To determine the best DNA model to use for phylogenetic analysis, model selection was performed which analysed the maximum likelihood fits of 24 different nucleotide substitution models. The trees are drawn to scale with the scale bar representing the number of nucleotide substitutions per site. Numbers at nodes represent percentage bootstrap support (values are indicated for each node >50%). The Australian sequences from this study are shown in bold. Isolate names and GenBank accession number for sequences used in the trees are shown.

3.1.4. Flaviviridae

There were 520 BVDV-1 contigs identified in the analysed samples. A consensus sequence was generated following mapping of these contigs to the Australian Bega isolate (Accession number KF896608). The consensus sequence covered more than 98% of the Bega isolate genome (a non-cytopathogenic strain) with a sequence identity of 91%. Phylogenetic analysis demonstrated that this genome sequence clusters with other BVDV-1c isolates (Fig. 4). The 1c genotype is the most reported genotype in the Australian cattle population [44, 45].

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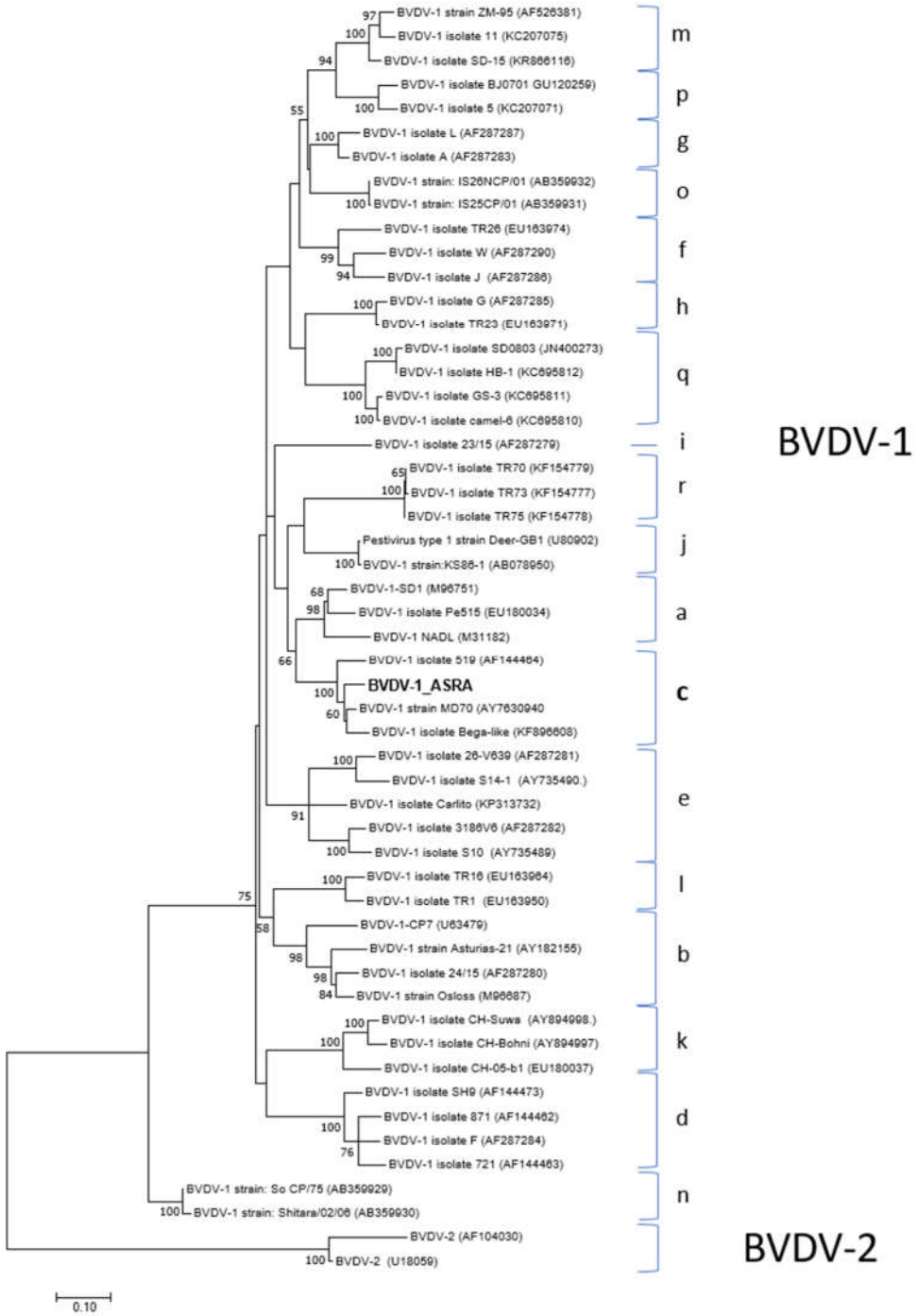

Figure 4. Phylogenetic tree of Bovine Viral Diarrhoea Virus 1 based on the N^{pro} gene. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Kimura 2-parameter model [26]. Bootstrapping of 1000 replicates was performed.

3.1.5. Picornaviridae

Several contigs (22) with sequence identity to bovine rhinitis A virus (BRAV) and bovine rhinitis B virus (BRBV) were detected. Alignment of these contigs to representatives of the BRAV and BRBV genomes (~ 7500 nt in length) produced consensus sequences covering 50.8% and 23.7% of the BRAV and BRBV genomes, respectively (data not shown). Although there was only 80% nucleotide identity with the reference BRAV and BRBV genomes, phylogenetic reconstruction strongly supported that the viral sequences identified in this study cluster with their respective viruses in the *Apthovirus* genus (Fig. 5). The genetic clustering and overall phylogenetic tree topology was supported by high

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bootstrap scores. Further work is required to obtain more comprehensive sequence data for these viruses in the Australian cattle population.

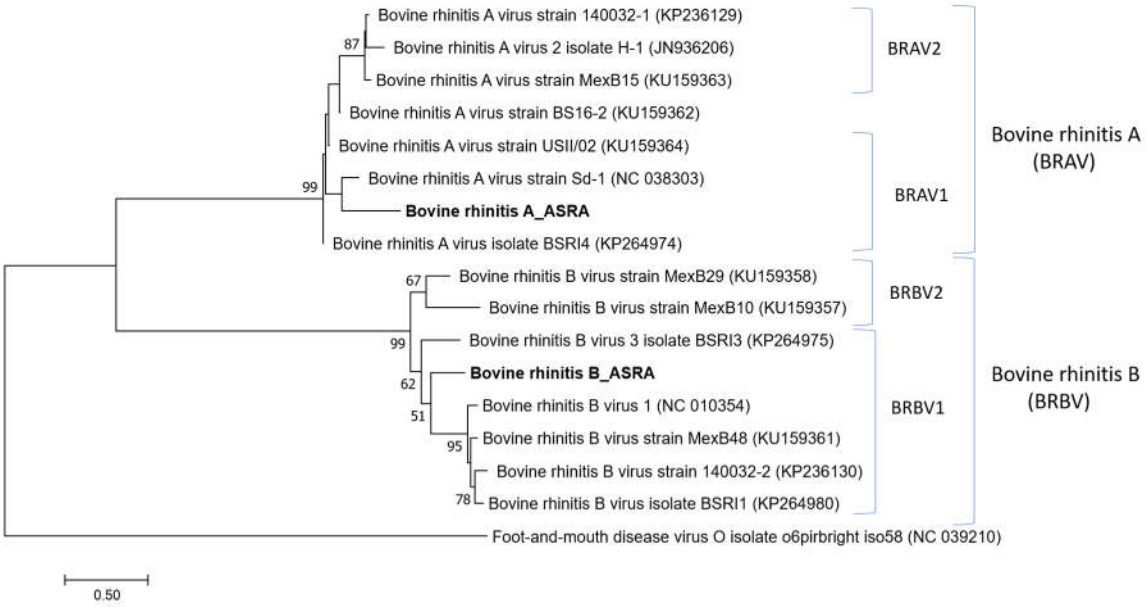

Figure 5. Phylogenetic tree of *Aphthovirus* based on the polyprotein gene. The phylogenetic tree was constructed using the Maximum Likelihood method based on the Kimura 2-parameter model [26]. Bootstrapping of 1000 replicates was performed.

3.1.6. Parvoviridae

Although sample preparation was optimised for the preferential sequencing of RNA viruses, several viruses with small DNA genomes belonging to the *Parvoviridae* family were also detected. This may be due to a small amount of viral DNA remaining post DNase treatment which was subsequently amplified prior to library preparation. Alternatively, the sample extracts may have contained transcripts from these viruses. Several sequences corresponding to three viral genera within this family were identified.

- *Bocoparvovirus*

Six contigs (206 to 351 bases in length) were identified that were most closely related to viruses in the *Bocaparvovirus* genus. One contig was 100% identical over a 205 nt region of the ORF2 structural protein of the reference ungulate bocaparvovirus 6 (UBPV-6) genome (Accession number NC_030402, [18]. The remaining contigs were 73-79% identical to both bovine parvovirus-1 and UBPV-6 (data not shown). The taxonomy of this viral family has changed with bovine parvovirus-1 being placed within the *Ungulate bocaparvovirus 1* species grouping and UBPV-6 being designated as a separate species within the *Bocaparvovirus* genus [46].

- *Erythroparvovirus*

A 343 nt contig corresponding to the putative capsid protein of bovine parvovirus 3 was identified as having 97.3% nucleotide sequence identity to the reference bovine parvovirus-3 isolate (Accession number AF406967). This virus belongs to the *Ungulate erythroparvovirus 1* species [46].

- *Unclassified*

There were 46 contigs with nucleotide sequences with highest nucleotide identity to Bosavirus MS-2016a, an unclassified parvovirus (data not shown). There is only one corresponding sequence available for comparison in GenBank for this virus (Accession number KY019139, [47]. Approximately 88% of the viral genome (total length 5371 nt) was assembled with overall 92.5% sequence identity to the reference genome. The only other

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published genome sequence for bosavirus came from US calf sera with no further information available with respect to its origin [47].

3.1.7 Other viruses

Several viral sequences, with contigs greater than 200 nt in length were identified in this study which were most closely related to bovine polyomavirus 2a (*Polyomaviridae)*; bovine papillomavirus 10 (*Papillomaviridae)*; dromedary stool associated circular virus (*Circoviridae)* and bovine enterovirus E (*Picornaviridae)*. As these viruses were considered unlikely to play important roles in BRD development they were not analysed further.

3.2. Case control study of virus detection: BRD cases versus Control Animals

Before the case-control study was conducted some preliminary information on the frequency of detection of each of the RNA viruses identified in the NGS dataset was obtained from 60 nasal swab samples from cattle with BRD. These swabs were collected as part of the of the NBRDI [7]. Of these samples, 23 (38%) were positive for BNV, 17 (28%) were positive for BRAV, one (1.7%) sample was positive for BRAB, and three (5%) samples were positive for IDV. The newly designed primers and probe demonstrated no cross reactivity with other pathogens such as BoHV-1, BVDV-1, BPI3, BCoV and BRSV (data not shown).

To further evaluate the potential associations between the viruses detected in the NGS analysis and BRD, extracts from nasal swabs collected from cattle with clinical signs of BRD (n=141) and from matched, healthy cattle (n=147) were tested for the presence of genomic material of ten viruses using qPCR assays [34]. A summary of the positive and negative qPCR results for each virus of interest is shown in Table 5. A complete list of the qPCR results for the cases and controls are provided in supplemental Tables S3 and S4, respectively.

In total, viruses were detected 113 times in samples from 96 animals. When all animals were considered (cases and controls), BNV was the most frequent virus detected, accounting for 25.6% of viruses detected, followed by IDV (23%) and BoHV-1 (16.8%). In cases, a positive virus result was obtained 73 times in samples from 61 animals. For cases, BoHV-1 was the most frequently detected virus, representing 26% of all viruses detected, followed by IDV (22%) and BNV (20.5%). Among the controls, a virus was detected 40 times in 35 animals. BNV was the predominant viral pathogen detected (35%) followed by IDV (25%) and BRAV (22.5%) in the control group.

At least one virus was detected in 33.3% (96) of animals: 43.3% (61/141) of BRD cases; and 23.8% (35/147) of controls. To evaluate if there were any associations between animals testing positive for the viruses of interest and BRD, odds ratios and their 95% confidence intervals were estimated (Table 5). The presence of at least one virus was significantly associated with clinical signs of BRD ($p = 0.0005$). Analyses of how each virus affected the risk of an animal being diagnosed with BRD showed that BoHV-1 was the only virus significantly associated with this disease. Animals testing positive for BoHV-1 were 47 (2.8-785.8 95% CI, $p = 0.007$) times more likely to diagnosed with BRD (Table 5). The presence of more than one virus was observed in 16 animals (5.5%), representing 7.8% of cases (n = 11) and 3.4% of controls (n = 5) (Supplemental Table S3 and S4).

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Table 5. Summary of the viral risk factors in the BRD case/control study. The qPCR based detection of ten viruses in nasal swabs from cattle diagnosed with BRD (cases) and healthy cattle (controls) are summarised. Estimated odds ratios (OR) for the effect a positive qPCR result on the risk of cattle being diagnosed with BRD. The 95% confidence intervals (95% CI) are shown for risk factor, along with p values with values <0.05 indicating a significant association.

Risk factor	qPCR Result	Cases (%)	Controls (%)	OR	95% CI	p value
infected with one or more virus	Positive	61 (43.3)	35 (23.8)	2.4	1.5-4.0	0.0005
	Negative	80 (56.7)	112 (76.2)			
bovine herpesvirus 1	Positive	19 (13.5)	0 (0)	47	2.8-785.8	0.0074
	Negative	122 (86.5)	147 (100)			
bovine coronavirus	Positive	2 (1.4)	3 (2.0)	0.7	0.1-4.2	0.7
	Negative	139 (98.6)	144 (98.0)			
bovine respiratory syncytial virus	Positive	6 (4.3)	2 (1.4)	3.2	0.6-16.2	0.2
	Negative	135 (95.7)	147 (98.6)			
bovine parainfluenza virus	Positive	1 (0.7)	0 (0)	3.1	0.1-78	0.5
	Negative	140 (99.3)	147 (100)			
bovine viral diarrhoea virus 1	Positive	3 (2.1)	0 (0)	7.5	0.4-145.6	0.2
	Negative	138 (97.9)	147 (100)			
influenza D virus	Positive	16 (11.3)	10 (6.8)	1.8	0.8-4.0	0.2
	Negative	125 (88.7)	137 (93.2)			
bovine rhinitis A virus	Positive	3 (2.1)	9 (6.1)	0.3	0.1-1.3	0.1
	Negative	138 (97.9)	138 (93.9)			
bovine rhinitis B virus	Positive	0 (0)	0 (0)	Not done	-	-
	Negative	141 (100)	147(100)			
bovine nidovirus	Positive	15 (10.6)	14 (9.5)	1.1	0.5-2.4	0.8
	Negative	126 (89.4)	133 (90.5)			
ungulate bocaparvovirus 6	Positive	8 (5.7)	2 (1.4)	4.4	0.9-20.9	0.07
	Negative	133 (94.3)	145 (98.6)			

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To compare the relative amounts of virus detected between cases and controls, Ct values (indicative of the amount of virus in a sample) between the two groups were compared. No significant differences were identified between the mean Ct values from samples collected from cattle with clinical signs of BRD in comparison to asymptomatic cattle for any of the viruses (Supplemental Table S5). For all viruses, except BoHV-1, BVDV-1 and BPI-3 (for which no virus was detected in health control animals), overlap was observed between the ranges of Ct values between BRD cases and control animals. Additionally, when IDV was detected, there was no significant differences in the mean IDV Ct values for animals with co-infections or those infected with IDV alone for BRD cases, control or for all animals (Supplemental Table S6).

4. Discussion

Bovine respiratory disease is a major health problem for the beef cattle industry around the world causing severe economic losses [1, 48, 49]. The disease has a complex aetiology with the interaction between multiple pathogens, host, management, and environmental factors all contributing to the risk of disease. The availability of NGS based viral metagenomics in recent years has provided a powerful tool for the large-scale and unbiased detection of known and discovery of unknown viruses in BRD affected animals [14, 18, 50]. This study was no exception, with viruses from a number of different families detected in the nasal swabs of feedlot cattle affected by BRD. Several of these viruses have not been detected previously in Australian cattle, such as IDV, BRAV, BRBV, BNV and UBPV-6. The detection of these viruses agrees with other virome studies of cattle with BRD [14, 18, 50, 51].

At least one virus was detected in 33.3% of cattle (43.3% of cases and 23.8% of controls) and the presence of one or more viruses in an individual was shown to be significantly associated with BRD, supporting the important role viruses play in the pathogenesis of this complex disease. The three most common viruses detected in cases were BoHV-1, IDV and BNV. Two of these viruses, IDV and BNV, in addition to BRAV, were the most frequently detected viruses in control animals.

In the case/control study, BoHV-1 was the only virus found to be significantly associated with BRD, with BoHV-1 positive animals being 47 times more likely to be diagnosed with the disease (Table 5). The OR estimate was very imprecise suggesting the association between BoHV-1 and BRD risk was highly confounded. An important confounder of this result is that cattle were vaccinated with a modified live intranasal BoHV-1 vaccine on entry to the feedlot [34, 52]. There is insufficient data to determine whether the detected BoHV-1 is the vaccine or a field strain of the virus. Considering all animals were vaccinated at the same time, and case and control animals were matched, that there were no BoHV-1 positive animals among the controls, suggests the animals had cleared the vaccine at the time of BRD diagnosis. Other viruses were unlikely to have contributed to clinical signs in the BoHV-1 positive animals as only three of the 19 BoHV-1 positive animals were co-infected with another virus (all with Ct values greater than 33 (Supplemental Table S3 and S4). Exclusion of these BoHV-1 positive animals from the dataset marginally reduced the BRD risk (data not shown). Similar to the current study, Hay et al. [3] also reported that cattle vaccinated at feedlot entry with the same BoHV-1 vaccine were at increased risk of developing BRD, OR = 6.0 (0.6-24.4 95% Credible Interval) [3]. While Hay et al. [3] had a large study population (n = >35,000), the authors suggested the OR estimate was confounded by highly clustered application of the vaccine within the study population at the feedlot (n = 14) level. As feedlots in the study either used the live vaccine or did not there was insufficient statistical power to further investigate this effect. The authors suggested randomised controlled trials were required to examine this effect. The current study provides further weight to the need for such trials as do other studies. Zhang et al. [53] reported that animals testing positive for BRSV had a greatly increased risk of BRD (OR = 13.422, 1.454–123.885 95%CI, p = 0.022) compared to healthy animals. These animals were vaccinated at induction with modified live vaccines for BoHV-1, BVDV, BRSV and BPI3. Similar to the study of [6], the imprecise risk estimate suggests

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high levels of confounding that require further investigation to elucidate the underpinning mechanisms.

BNV was the most frequently virus detected in this study (10.1% of all animals; 25.6% of all viruses). This virus was first reported in cattle with BRD in a US feedlot in 2013 [27]. In the current study, a full-length genome was assembled and, although only 85.9% identical to the reference genome, phylogenetic analysis revealed that it clustered with the reference isolate in the *Tobaniviridae* family (subfamily *Remotovirinae*, genus *Bostavirus*). As BNV is a recently emerged virus, its clinical significance is yet to be clearly defined. In the initial report, there was no conclusive data to associate the virus with illness as it was not the only viral agent identified and healthy cattle were not available for comparison [27]. Interestingly, BNV positive cattle were found to be 12.8 times (OR = 0.078, 95%CI 0.021–0.288, p = 0.000) less likely to be diagnosed with BRD in a Canadian feedlot [50]. A similar trend was observed in Mexican feedlots, albeit in fewer animals, where 3.7% and 11.5% of BRD cases and controls tested positive for BNV, respectively [18]. In the current study, despite being the most prevalent virus detected, no association with BRD was detected, with positive samples being evenly distributed among the case (10.6%) and control animals (9.5%). Co-infections with BNV were also detected in this study with six of the 15 cases and two of the 14 control cases. No difference in the average Ct values between cases or controls infected with BNV was observed, suggesting the lack of association with disease was not due to virus titre at the time of sampling (Supplemental Table S5).

It is evident that several aspects of BNV and its association with BRD warrant further investigation. Given the limited amount of sequence data available for this virus, further sequencing of positive samples is required to accurately characterise its genome sequence and organisation. Additionally, more data on the relationship of this virus with clinical signs of BRD is required, particularly due to observed association with reduced risk of disease reported in a previous study [50] and the comparatively high number of positive samples from control animals in the current study.

Parvoviruses are recognised as important pathogens in various groups of mammals, however, there are few published studies with respect to the clinical significance of these viruses in cattle. Two members of the family *Parvoviridae* from the genus *Bocaparvovirus*, bovine parvovirus 1 and UPBV-6, were detected in the current study. Bovine parvoviruses have been reported to be associated with respiratory and gastrointestinal diseases in cattle [54]. Despite this, the role of UBPV-6 in the pathogenesis of BRD remains unclear as it has been detected with high frequency in healthy cattle [18, 50]. However, in the present study, BRD cases tended to be 4.4 times (p = 0.07) more likely to have the virus than controls animals. Moreover, Zhang et al. [50] reported that cattle positive for UBPV-6 were 3.4 times (OR = 0.296, 95%CI 0.108–0.814, p = 0.019) less likely to be diagnosed with BRD. In the current study, UBPV-6 was detected in more cases (5.6%) than controls (1.4%), although this positive association with BRD was not statistically significant. Additional viruses from the *Parvoviridae* family were also detected in this study. BPV3 and bosavirus are usually observed as a contaminant of commercial bovine serum, although BPV3 has also been detected in cattle in Brazil, however there was no evidence to support its association with clinical disease [55, 56].

It would seem implausible that either BNV or UBPV-6 provide specific protection from BRD, rather their association with reduced risk of disease may represent an unperturbed state of the respiratory microbiota, where in a healthy animal, the presence of some viruses is, if not commensal, benign. This hypothesis is consistent with the changing paradigm that mucosal surfaces are not sterile, suggesting that research should be equally focused on characterising the microbiomes of healthy animals as well as diseased animals to better understand the pathogenesis of complex diseases such as BRD.

IDV is the most recently discovered member of the *Orthomyxoviridae* family and is the first influenza virus to be associated with cattle, the species considered to be the natural reservoirs of this virus [57]. IDV sequences were identified in both cases (11.3%) and controls (6.8%) in this study. Six of the 16 cases with IDV infection were co-infected with one or two other viruses (BRSV once, UBPV-6 twice, BNV twice, BRSV and BNV once) whilst in

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the controls two of the five animals were co-infected (BRAV and BNV). This virus is being increasingly detected around the world, although there are conflicting reports with respect to its association with BRD. IDV is found predominantly in the upper respiratory tract of cattle and is generally associated with mild to moderate respiratory disease [18, 58]. It has also been reported in asymptomatic animals which was observed in the current study [18, 51]. This could be attributed to the fact that cattle, being the natural reservoirs of this virus, may be more likely to carry the virus without displaying clinical signs of disease.

It has been proposed that IDV may contribute to BRD through exacerbating the effects of co-infecting pathogens because of changes it induces in the upper respiratory tract [14, 58-60]. IDV has been more commonly detected in cattle co-infected with other pathogens [60, 61], and higher IDV loads have also been reported in symptomatic cattle in which multiple viruses were detected in comparison to those infected with IDV alone [58, 59]. In the current study, there were no associations between IDV viral loads or co-infections and Ct values, suggesting no significant differences between animals co-infected or solely infected with IDV (in cases, controls and all animals, data not shown). Additionally, no difference in Ct values was observed between IDV detected in cases and IDV detected in controls.

Rhinitis viruses, BRAV and BRBV, were also detected in this study. These viruses have not been reported before in Australian cattle although they are being reported more frequently in published studies [14, 18, 28, 50, 51]. Rhinitis viruses have also been found to have an inconsistent association with BRD and it has been suggested that other factors may be required for disease to develop in cattle infected with these viruses [14, 18, 50]. At least two serotypes for rhinitis A have been reported which may be a contributing factor to the reported differences in pathogenicity. In this current study, although BRAV was detected more frequently in controls than cases (6.1% vs 2.1%) this was not a statistically significant association. Further research is required to determine if there are any associations between these viruses and BRD in feedlot cattle.

There were other viruses detected in this study that were considered unlikely to play important roles in BRD development. Similarly, to bosavirus and BPV3, bovine polyomavirus is usually considered to be a contaminant in tissue culture serum. However, recent studies have implicated bovine polyomavirus 1 and 2 in kidney and non-suppurative encephalitis in cattle, respectively [62, 63]. Bovine papilloma virus 10 has been associated with cutaneous papillomas in cattle [64].

As with previous reports, the current study has identified a wide repertoire of viruses in both BRD affected and unaffected cattle. Collectively, the BRD virome studies highlight the power of applying NGS as an unbiased diagnostic tool to detect the presence/absence of known and unknown viruses. These studies also highlight that detection of a virus or viruses does not equate to causality with respect to the disease of interest. Viruses, particularly BNV, BRAV and IDV, were detected in 23.8% of asymptomatic cattle in the current study. To date, these three viruses have been reported to have variable associations with BRD [14, 18, 50, 51] and therefore associations with disease when detected in symptomatic cattle remains problematic, when current paradigms suggests viruses are pathogens that cause disease. The detection of virus in asymptomatic animals could also be due to subclinical infections; the detection of the virus in the disease incubation period before the onset of clinical signs; or continued shedding of virus once clinical signs have resolved. Asymptomatic carriers potentially pose a significant risk to a herd with respect to transmission to susceptible animals. It is also difficult to draw conclusion with respect to these emerging viruses, as information on the role they play in the pathogenesis of BRD and other diseases, if any, is yet to be defined.

With the increasing use of NGS technologies, viruses, known, emerging and novel, are being detected and identified more frequently in healthy people and animals. Therefore, consideration should also be given to a potential commensal, or at least non-clinical, role(s) for these viruses in the respiratory system [65]. Of particular note are the associations of BNV and UBPV-6 with BRD warrant further investigation due to their significant

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association with reduced risk of disease reported in previous studies and the higher number of positive samples for these viruses in the control animals from the current study [50]. Clearly, more data on the relationship of these viruses in animals without BRD are required. It would seem improbable that BNV or other viruses provided specific protection from BRD, rather their association with reduced risk of disease may represent an unperturbed state of the respiratory virome, where in a healthy animal, the presence of these and perhaps other viruses is, if not commensal, is benign. The interaction of viruses with commensal microbiota (particularly bacteria but also fungi) in addition to the composition of the commensal microbiota at time of infection, may influence disease outcomes in individuals. The commensal microbiota is known to influence the health of the host. Preliminary research has examined the role viruses have on the commensal microbiota with both positive and negative outcomes documented including both the exacerbation and suppression of viral infections [66]. This work has been predominantly conducted in humans with a focus on the gut microbiome although there is some evidence to support similar interactions between bacteria and viruses within the human respiratory tract too [65]. These findings would be expected to occur in animals too, but research is required to evaluate these interactions. This suggests that research should be equally focused on characterising the viromes and microbiomes in healthy animals to better understand the pathogenesis of complex diseases such as BRD.

Hick et al. [67] reported the detection and isolation of BCoV in Australian cattle affected by BRD. While BCoV has also been associated with BRD mortality in Australian feedlot cattle [68]. The current study reports the first complete genome sequence for BCoV from the Australian cattle population. Previous studies have reported that specific genotypes of other BRD associated virus, BoHV-1, BVDV-1, and BPI3 circulate within this population, perhaps a consequence of this country's strict quarantine controls [45, 69-71]. The phylogenetic analyses completed in the current study demonstrated the robust clustering of the ORF1ab gene sequence with homologous BCoV sequences from other countries with no evidence for specific linages detected (Fig. 1b). Unlike many other viruses with RNA genomes (e.g. BVDV-1), the coronavirus genome replication complex has proof reading capacity that is likely to contribute to stable genome replication overtime without external selection factors, such as vaccination [72].

5. Conclusions

In conclusion, the results of the present study are similar to those reported by other studies confirming the complexity of the virome in cattle with and without BRD and highlight the need for further research to clearly define the roles, if any, of a suite of emerging viruses in the pathogenesis of BRD. Future research should also aim to elucidate the importance of the presence of viruses in healthy animals as this may provide insights into the dysbiosis which leads to disease. Improved knowledge of the viruses involved with BRD in cattle will inform the implementation of management and preventative strategies including informing the development of diagnostic tests and vaccines aimed at reducing the impact of this economically important disease within the intensive finishing sectors of the global beef industry.

Supplementary Materials:

Supplemental Figure S1. Schematic representation of the annotated bovine coronavirus genome sequence determined in this study. Predicted coding sequences (CDS) are shown and named using the nomenclature of the polypeptides they encode are illustrated.

Supplemental Figure S2. Schematic representation of the annotated bovine nidovirus genome determined in this study. Predicted coding sequences (CDS) are shown and named using the nomenclature of the polypeptides they encode are illustrated.

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Supplemental Table S1. Oligonucleotide pairs used for PCR amplification and direct amplicon sequencing to resolve gaps and/or regions of low sequence coverage in the bovine coronavirus (BCoV) genome following the assembly of next generation sequencing data.

Supplemental Table S2. Oligonucleotide pairs used for PCR amplification and direct amplicon sequencing to resolve gaps and/or regions of low sequence coverage in the bovine Nidovirus (BNV) genome following the assembly of next generation sequencing data.

Supplemental Table S3. Quantitative real-time PCR threshold cycle (Ct) values for cattle diagnosed with bovine respiratory disease (BRD cases, BC) for the case/control study. The CT values are shown for the respective viruses where the value was ≤35 was deemed positive. Blank cells indicate a negative result.

Supplemental Table S4. Quantitative real-time PCR threshold cycle (CT) values for cattle not diagnosed with bovine respiratory disease (controls, C) for the case/control study. The CT values are shown for the respective viruses where the value was ≤35 was deemed positive. Blank cells indicate a negative result.

Supplemental Table S5. Comparison of the threshold cycle (Ct) values from the quantitative real-time PCR analyses of extracts from nasal swab from feedlot cattle diagnosed with bovine respiratory disease (case) and health cattle (control). The results for bovine coronavirus (BCoV), bovine respiratory syncytial virus (BRSV), influenza D virus (IDV), bovine rhinitis A virus (BRAV), bovine nidovirus (BNV), and ungulate bocaparvovirus 6 (UBPV6) are shown.

Supplemental Table S6. Comparison of the threshold cycle (Ct) values from the quantitative real-time PCR analysis for influenza D virus (IDV) of extracts from nasal swab from feedlot cattle diagnosed with bovine respiratory disease (case) and health cattle (control). Comparison of the extract Ct values in cattle with viral co-infections and those with IDV alone are shown.

Author Contributions: For research articles with several authors, a short paragraph specifying their individual contributions must be provided. The following statements should be used "Conceptualization, R.K.A. and T.J.M; methodology, R.K.A., J.L.G. and T.J.M; formal analysis, R.K.A. and T.J.M.; investigation, R.K.A., J.L.G. and T.J.M; resources, R.K.A., C.B.H., L.A.G. and T.J.M; data curation, R.K.A. and J.L.G.; writing—original draft preparation, R.K.A. and T.J.M; writing—review and editing, R.K.A., C.B.H., J.L.G., L.A.G., and T.J.M; supervision, L.A.G. and T.J.M; project administration, L.A.G. and T.J.M.; funding acquisition, R.K.A., J.L.G., L.G. and T.J.M. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The collection of the samples utilised in the viral metagenomics study was approved by the Production and Companion Animal Ethics Committee, University of Queensland (Approval # SVS/383/07/MLA, SVS/495/08/MLA, and SVS/125/10/MLA. All methods were carried out in accordance with the relevant guidelines and regulations.

The collection of the samples utilised in the case-control study was approved by Animal Ethics Committee of Research Integrity and Ethics Administration, The University of Sydney (Approval # 1118). All methods were carried out in accordance with the relevant guidelines and regulations.

Informed Consent Statement: Not applicable.

Data Availability Statement: All primary data is available from the corresponding author on request.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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