

Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus

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Bovine respiratory disease (BRD) is the most costly disease affecting the cattle industry. The pathogenesis of BRD is complex and includes contributions from microbial pathogens as well as host, environmental and animal management factors. In this study, we utilized viral metagenomic sequencing to explore the virome of nasal swab samples obtained from feedlot cattle with acute BRD and asymptomatic pen-mates at six and four feedlots in Mexico and the USA, respectively, in April–October 2015. Twenty-one viruses were detected, with bovine rhinitis A (52.7%) and B (23.7%) virus, and bovine coronavirus (24.7%) being the most commonly identified. The emerging influenza D virus (IDV) tended to be significantly associated ($P=0.134$; odds ratio=2.94) with disease, whereas viruses commonly associated with BRD such as bovine viral diarrhoea virus, bovine herpesvirus 1, bovine respiratory syncytial virus and bovine parainfluenza 3 virus were detected less frequently. The detection of IDV was further confirmed using a real-time PCR assay. Nasal swabs from symptomatic animals had significantly more IDV RNA than those collected from healthy animals ($P=0.04$). In addition to known viruses, new genotypes of bovine rhinitis B virus and enterovirus E were identified and a newly proposed species of bocaparvovirus, *Ungulate bocaparvovirus 6*, was characterized. Ungulate tetraparvovirus 1 was also detected for the first time in North America to our knowledge. These results illustrate the

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Selected complete or partial genome sequences were submitted to GenBank/EMBL/DDBJ and accession numbers were obtained: BRBV strain MexB10, KU159357; BRBV strain MexB29, KU159358; BRBV strain USII/19, KU159359; BRBV strain MexB09, KU159360; BRBV strain MexB48, KU159361; BRAV strain BS16-2, KU159362; BRAV strain MexB15, KU159363; BRBV strain USII/02, KU159364; BVDV1 strain USII-S15, KU159365; BRSV strain USII/S1, KU159366; bovine enterovirus strain MexKSU/5, KU172420; UBPV6 strain USII/03, KU172421; UBPV6 strain Mex/S22, KU172422; UTPV1 (bovine hokovirus 2) strain BS-S13, KU172423; IDV (D/bovine/Mexico/S7/2015) segment 1 PB2 gene, KU710438; IDV (D/bovine/Mexico/S8/2015) segment 1 PB2 gene, KU710439; IDV (D/bovine/Mexico/S56/2015) segment 1 PB2 gene, KU710440; IDV (D/bovine/Mexico/S62/2015) segment 1 PB2 gene, KU710441; IDV (D/bovine/Mexico/S7/2015) segment 2 PB1 gene, KU710434; IDV (D/bovine/Mexico/S8/2015) segment 2 PB1 gene, KU710435; IDV (D/bovine/Mexico/S56/2015) segment 2 PB1 gene, KU710436; IDV (D/bovine/Mexico/S62/2015) segment 2 PB1 gene, KU710437; IDV (D/bovine/Mexico/S7/2015) segment 3 P3 gene, KU710426; IDV (D/bovine/Mexico/S8/2015) segment 3 P3 gene, KU710427; IDV (D/bovine/Mexico/S56/2015) segment 3 P3 gene, KU710428; IDV (D/bovine/Mexico/S62/2015) segment 3 P3 gene, KU710429; IDV (D/bovine/Mexico/S7/2015) segment 4 HE gene, KU171126; IDV (D/bovine/Mexico/S8/2015) segment 4 HE gene, KU171127; IDV (D/bovine/Mexico/S56/2015) segment 4 HE gene, KU171128; IDV (D/bovine/Mexico/S62/2015) segment 4 HE gene, KU171129; IDV (D/bovine/Mexico/S7/2015) segment 5 NP gene, KU710418; IDV (D/bovine/Mexico/S8/2015) segment 5 NP gene, KU710419; IDV (D/bovine/Mexico/S56/2015) segment 5 NP gene, KU710420; IDV (D/bovine/Mexico/S62/2015) segment 5 NP gene, KU710421; IDV (D/bovine/Mexico/S7/2015) segment 6 P42 gene, KU710430; IDV (D/bovine/Mexico/S8/2015) segment 6 P42 gene, KU710431; IDV (D/bovine/Mexico/S56/2015) segment 6 P42 gene, KU710432; IDV (D/bovine/Mexico/S62/2015) segment 6 P42 gene, KU710433; IDV (D/bovine/Mexico/S7/2015) segment 7 NS2 and NS1 gene, KU710422; IDV (D/bovine/Mexico/S8/2015) segment 7 NS2 and NS1 gene, KU710423; IDV (D/bovine/Mexico/S56/2015) segment 7 NS2 and NS1 gene, KU710424; and IDV (D/bovine/Mexico/S62/2015) segment 7 NS2 and NS1 gene, KU710425.

complexity of the virome associated with BRD and highlight the need for further research into the contribution of other viruses to BRD pathogenesis.

INTRODUCTION

Besides being the costliest disease affecting the beef industry, bovine respiratory disease (BRD) impairs the health and welfare of millions of cattle as well as their performance. It has been estimated that BRD causes productivity losses of \$23.60 per morbid calf (USDA, 2011). The economic impact due to BRD in the USA alone is estimated to exceed one billion dollars, annually (Griffin, 2006; Snowden *et al.*, 2007). The BRD complex accounts for approximately 70–80% of the morbidity in the USA (Hilton, 2014) and 84.5–99.9% of the morbidity in Mexican feedlot cattle (Villagomez-Cortes & Martinez-Herrera, 2013). BRD is responsible for widespread therapeutic and metaphylactic use of antibiotics in feedlots, which increasingly raises public health concerns of promoting antibiotic resistance (Nickell & White, 2010; Portis *et al.*, 2012; USDA, 2011). The etiology of BRD involves complex interactions between host, pathogen, environment and management factors. In feedlot cattle, disease pathogenesis is thought to initiate due to stress with animal transport followed by a primary viral insult often with secondary infection of resident bacteria (Mosier, 2014). Viral infection can cause increased susceptibility to bacterial infections by either immunosuppression or by damaging the epithelium of upper airways and injuring lung parenchyma that favours the migration and colonization of the lower respiratory tract by bacterial pathogens (Martin & Bohac, 1986). While disease outcomes are variable, higher morbidity and mortality are observed in the event of mixed viral and bacterial infections (Duff & Galyean, 2007; Irwin *et al.*, 1979).

Numerous pharmaceutical and biological products have been developed for controlling BRD, and these interventions have variable efficacy in reducing morbidity and mortality. Specifically, for decades, several commercial vaccines have been widely used to manage BRD in feedlots. While their composition varies, viral components include attenuated live strains of bovine viral diarrhoea virus types 1 and 2 (BVDV1 and 2), bovine herpesvirus type 1 (BHV1), bovine respiratory syncytial virus (BRSV) and bovine parainfluenza virus 3 (BPIV3), considered to be primary BRD viruses. Despite their widespread use, deaths attributed to BRD have steadily increased from 1994 to 2011 (Loneragan *et al.*, 2001; Snowden *et al.*, 2007).

Viral metagenomic sequencing has been used to investigate the virome associated with complex disease syndromes. Comparisons between the swine faecal virome from healthy and diarrhoeic animals identified large numbers of known and novel viruses, some of which are etiological agents of enteric disease (Shan *et al.*, 2011; Zhang *et al.*, 2014a). Metagenomic sequencing has also been used to identify and

characterize viruses associated with BRD in young dairy cattle. Utilizing a case-control format, the presence of bovine adenovirus 3 (BAdV3), bovine rhinitis A virus (BRAV) and influenza D virus (IDV) in nasopharyngeal and pharyngeal swab samples was significantly associated with BRD cases in 27–60-day-old hutch-housed dairy calves (Ng *et al.*, 2015). Surprisingly, viruses typically associated with BRD were not detected in these samples.

In the present study, we utilized metagenomic sequencing to characterize the virome of nasal swab samples collected from beef cattle with acute BRD and asymptomatic pen-mates at feedlot operations in Mexico and the USA from April to October 2015.

RESULTS

Sample collection

A total of 103 nasal swab samples, 63 and 40 from Mexican and USA steers, respectively, were collected. Steers originated from six feedlots from four states in Mexico (Baja California, Nuevo Leon, Zacatecas and Michoacán) and from four feedlots in the Midwest USA (Kansas). Five samples each were collected from diseased and asymptomatic cattle from a single pen for most feedlots. In one Mexican feedlot, 11 nasal samples were collected from diseased and two from asymptomatic animals, whereas at two USA feedlot sites, six and four samples from diseased and asymptomatic animals, respectively, were collected, per site. Samples from all sites were analysed individually except for the 11 nasal swabs from diseased animals from the Mexican site, which were distributed into two pools, whereas the two swabs from asymptomatic steers were pooled into one. Hence, 93 samples (47 from diseased and 46 from asymptomatic steers) were considered for analysis.

Virome of bovine nasal swabs

Ninety-three barcoded DNA libraries were generated from bovine nasal swabs collected from feedlots in Mexico and the USA and were sequenced on an Illumina MiSeq. A total of 77.4 million reads were generated from which ~40.5 million reads were derived from swabs of animals with acute respiratory disease and ~36.9 million reads were from swabs of asymptomatic pen-mates. Following *de novo* assembly, contigs were analysed by BLASTN and 21 viruses were identified (Table 1). Reference genomes were downloaded from GenBank for the best BLAST hits and the raw sequencing reads were mapped using templated assemblies. Assemblies were inspected manually and only where a minimum of 15 unique reads mapped to multiple regions of the viral genome was the virus considered detected and reads were

Table 1. Viruses detected by metagenomic sequencing of nasal swabs collected from cattle exhibiting acute clinical signs of BRD (symptomatic) and clinically normal pen-mates (asymptomatic) each from six feedlots in Mexico and four feedlots in the USA

Virus	Mexico			USA			Reads derived from symptomatic animals (%)
	No. of sites positive	Number of cattle positive (%)		No. of sites positive	Number of cattle positive (%)		
		Symptomatic	Asymptomatic		Symptomatic	Asymptomatic	
IDV	4/6	8/27 (29.6)	1/26 (3.8)	1/4	–	2/20 (10.0)	385 395 (99.9 %)
BRAV	4/6	5/27 (18.5)	7/26 (26.9)	4/4	18/20 (90.0)	19/20 (95.0)	2 185 274 (66 %)
BRBV	5/6	7/27 (25.9)	3/26 (11.5)	4/4	4/20 (20.0)	8/20 (40.0)	246 256 (62 %)
EVE	3/6	2/27 (7.4)	2/26 (7.7)	1/4	3/20 (15.0)	–	20 008 (35.4 %)
EVF	2/6	1/27 (3.7)	1/26 (3.8)	1/4	1/20 (5.0)	–	623 (16.6 %)
UBPV6	4/6	5/27 (18.5)	3/26 (11.5)	2/4	1/20 (5.0)	3/20 (15.0)	5731 (22.4)
UTPV1	1/6	–	1/26 (3.8)	2/4	5/20 (25.0)	1/20 (5.0)	220 520 (99.6 %)
BRSV	–	–	–	2/6	1/20 (5.0)	4/20 (20.0)	20 004 (70.5 %)
BVDV1	–	–	–	3/4	3/20 (15.0)	1/20 (5.0)	50 787 (93.7 %)
BVDV2	1/6	1/27 (3.7)	–	–	–	–	939 (100 %)
BCV	2/6	3/27 (11.1)	–	4/4	11/20 (55.0)	9/20 (45.0)	2 555 541 (70.8 %)
BNV	2/6	1/27 (3.7)	3/26 (11.5)	–	–	–	1098 (12.2 %)
BPV3	2/6	3/27 (11.1)	–	1/4	4/20 (20.0)	4/20 (20.0)	629 888 (97.8 %)
BAdV3	1/6	3/27 (11.1)	5/26 (19.2)	1/4	–	1/20 (5.0)	399 (0.26 %)
BAAV	1/6	3/27 (11.1)	5/26 (19.2)	–	–	–	47 (0.13 %)
BPIV3	–	–	1/26 (3.8)	1/4	–	1/20 (5.0)	–
BTv	1/6	1/27 (3.7)	–	–	–	–	304 (100 %)
BHV1	1/6	1/27 (3.7)	–	–	–	–	47 (100 %)
MdSGHV	2/6	4/27 (14.8)	–	–	–	–	1386 (100 %)
ssCDV	4/6	4/27 (14.8)	4/26 (15.4)	2/6	3/20 (15.0)	–	268 (50.9 %)
PBV	1/6	–	1/26 (3.8)	–	–	–	–

Abbreviations: BRBV, bovine rhinitis B virus; EVE, enterovirus E; EVF, enterovirus F; UBPV6, ungulate bocaparvovirus 6; UTPV1, ungulate tetraparvovirus 1; BCV, bovine coronavirus; BNV, bovine nidovirus; BPV3, bovine parvovirus 3; BAAV, bovine adeno-associated virus; BTv, bovine torovirus; MdSGHV, *Musca domestica* salivary gland hypertrophy virus; ssCDV, single-stranded circular DNA viruses; PBV, picobirnavirus.

tallied. Approximately nine million reads mapped to mammalian viral sequences. Seventy percent of total mammalian viral reads were recovered from the symptomatic animals while 30 % of the reads originated from asymptomatic steers. The overall percentage of detection of each virus separately from symptomatic and asymptomatic animals in Mexican and USA feedlots and the percentage of reads obtained from symptomatic animals are shown in Table 1.

Influenza D virus

IDV was detected in at least one nasal swab in four of the six Mexican and one of the four USA feedlots sampled (Table 1). Nearly all (99.7 %) viral reads were derived from diseased cattle ($n=8$). IDV was also detected in nasal swabs from three asymptomatic animals. As insufficient read coverage prevented assembly of complete genome sequences, the genome was amplified by PCR as previously described (Hause *et al.*, 2014) using RNA extracted from two IDV-positive nasal swabs each from two sites in Mexico, and subsequently sequenced. The predicted haemagglutinin esterase fusion (HEF) protein was 99 % identical to

that previously published for D/bovine/Kansas/13–21/2012 (GenBank accession no. AIO11632). Based on pairwise alignments of predicted HEF amino acid sequences, sequences collected from the same site were identical. Phylogenetic analysis of the HEF amino acid sequences found a close relationship between strains from Mexico and a clade of IDV characterized from the Midwestern USA (Fig. 1).

Bovine rhinitis A virus (BRAV)

BRAV was detected in at least one nasal swab in four of the six Mexican feedlot sites and at all USA sites in a total of 26/46 (57 %) asymptomatic and 23/47 (49 %) symptomatic individuals, respectively (Table 1). Twenty complete or near-complete genomes of BRAV were assembled. Phylogenetic analysis of the capsid (P1) amino acid sequences identified concurrent circulation of BRAV1 and BRAV2 both in the USA and in Mexico (Fig. 2a). Amino acid identities within BRAV1 and BRAV2 clades were greater than 97 % for P1 with the exception of BS16-2, which occupied an ancestral position to other BRAV1 strains and shared 95 % identity to other BRAV1 strains.

Bovine rhinitis B virus (BRBV)

BRBV was detected in all feedlot sites with the exception of one site in Mexico (Table 1). BRBV was identified in 24% (11/46) and 23% (11/47) of asymptomatic and symptomatic animals, respectively. Six and seven near-complete genome sequences were assembled from Mexican and USA samples, respectively. The P1 coding region showed 67–100% amino acid identity between strains. Phylogenetic analysis of this region identified two well-supported clades (Fig. 2b). Seven of the strains clustered with recent and historical BRBV references and shared greater than 89% identity. One strain, BRBV MexB09, shared 84% identity to the reference strain BRBV BSRI3 and less than 80% identity to other BRBVs. The remaining five strains formed a well-supported clade with a maximum of 71% identity to other BRBVs.

Enterovirus E and F (EVE and EVF)

Three EVE-positive samples originated from Mexican steers with the remaining positive sample collected in the USA (Table 1). Using *de novo* assembly, we obtained a 7332 base long contig from a Mexican nasal swab which by BLASTN analysis was most similar to EVE (GenBank D00214).

Interestingly, a ~850 base long region of the genome corresponding to the VP1 coding region of the genome failed to show significant similarity with EVE. Given the novelty of the virus (MexKSU/5), RACE was performed to complete the genome sequence (7451 bases). Genetic analysis identified a single large ORF comprising 6549 bases encoding a predicted 2182 aa polyprotein. The polyprotein of MexKSU/5 was most similar to EVE and had 83% identity to a reference genome (GenBank DQ092769). Analysis of predicted mature protein sequences found that while the capsid proteins VP2 and VP3 shared high identity to EVE, the VP1 of MexKSU/5 had only 55–57% identity to the VP1 sequence from both EVF (GenBank DQ092770) and EVE (GenBank X79369). Phylogenetic analysis was performed using the 3D polymerase (3Dpol) and P1 amino acid sequences (Fig. 3a and 3b, respectively). Analysis of 3Dpol found that MexKSU/5 clustered closely to EVE reference sequences; however, the P1 region failed to cluster with EVE or EVF.

A total of 3748 reads mapping to EVF were identified in samples from two Mexican and one USA feedlot sites, of which the majority (84%) originated from a single asymptomatic animal although EVF was also detected in two sick individuals (Table 1).

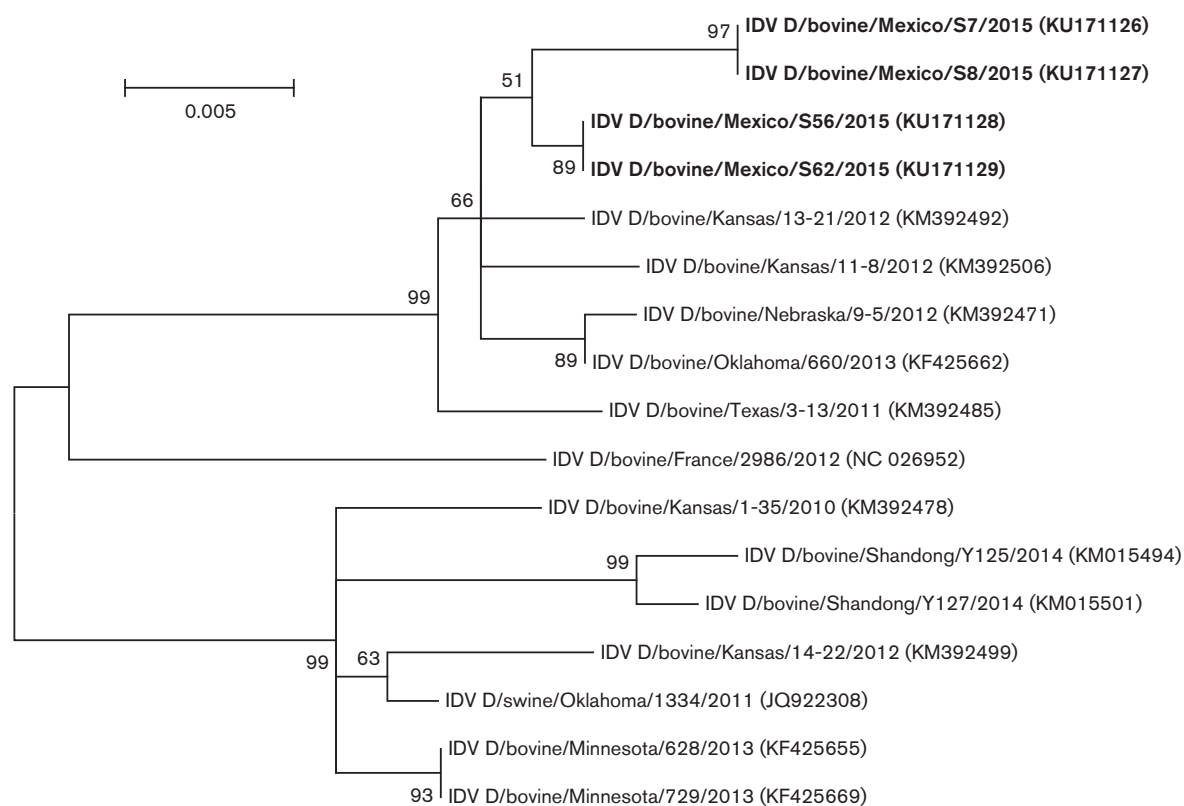


Fig. 1. Phylogenetic analysis of the amino acid sequences of IDV HEF protein. The phylogenetic tree was reconstructed by maximum-likelihood analysis with tree topology evaluated using 1000 bootstrap replicates. GenBank accession numbers are shown in parentheses. Numbers at nodes represent percentage bootstrap support. Sequences determined here are shown in bold type. Bar, substitutions per amino acid position.



Fig. 2. Phylogenetic analysis of predicted capsid P1 amino acids of (a) bovine rhinitis A virus (BRAV) and (b) bovine rhinitis B virus (BRBV). Phylogenetic trees were reconstructed by maximum-likelihood analysis with tree topology evaluated using 1000 bootstrap replicates. GenBank accession numbers are shown in parentheses. Numbers at nodes represent percentage bootstrap support. Sequences determined here are shown in bold type. Bar, substitutions per amino acid position.

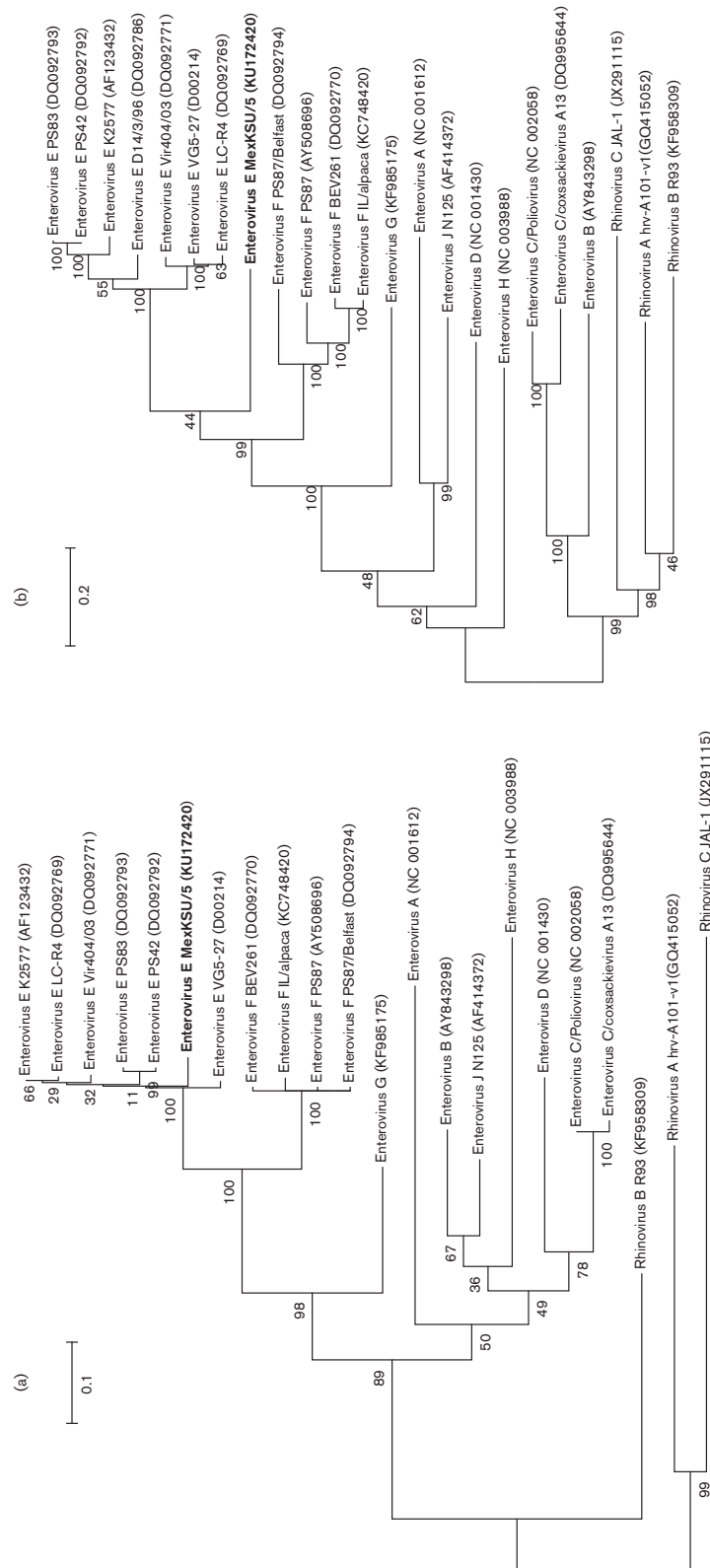


Fig. 3. Phylogenetic analysis of predicted amino acid sequences of (a) 3Dpol and (b) capsid P1 proteins of a novel enterovirus E. Phylogenetic trees were reconstructed by maximum-likelihood analysis with tree topology evaluated using 1000 bootstrap replicates. GenBank accession numbers are shown in parentheses. Numbers at nodes represent percentage bootstrap support. Sequences determined here are shown in bold type. Bar, substitutions per amino acid position.

Ungulate bocaparvovirus 6 (UBPV6)

A 5224 base long contig with only 69 % nucleotide identity to bovine parvovirus 1 (GenBank DQ335247) was identified following *de novo* assembly of host-subtracted reads from a nasal swab (USII/S3) originating from the USA. Genetic analysis identified three ORFs, consistent with bocaparvoviruses (Tijssen *et al.*, 2011). The larger ORF encoded a predicted 858 aa protein that shared 64 % identity to non-structural protein-1 (NS1) of bovine parvovirus 1 (BPV1) (GenBank DQ335247). The second ORF encoded a predicted 655 aa protein that was 60 % identical to the capsid protein VP1 of BPV1 Abinanti reference strain. A third ORF overlapping the NS1 and VP1 genes encoded a predicted 214 aa protein that was 65 % identical to the non-capsid protein (NP1) of BPV1.

A recent proposal suggested taxonomical changes to the family *Parvoviridae* such that all viruses in a genus should encode NS1 proteins with >30 % identity at the amino acid level and that all members of a species should encode NS1 proteins with >85 % amino acid sequence identity (Cotmore *et al.*, 2014). These requirements suggest assignment of this divergent parvovirus as a new species, *Ungulate bocaparvovirus 6* (UBPV6), in the genus *Bocaparvovirus*.

Another 5060 base long contig (near-complete genome) was additionally assembled from a Mexican bovine nasal swab (MexB/S22) that shared 98 % nucleotide identity with UBPV6. Using a UBPV6 reference, templated assembly of individual barcoded sequences identified its presence in at least one nasal swab in each of four of the six Mexican and two of the four USA feedlots sampled (Table 1). UBPV6 was identified in a total of six symptomatic and six asymptomatic individuals. Both UBPV6 isolates (USII/S3 and MexB/S22) clustered together in the phylogenetic analysis of the NS1 amino acid sequence and were most closely related to BPV1 (Fig. 4).

Ungulate tetraparvovirus 1 (UTPV1)

UTPV1, formally named bovine hokovirus (BHoV), is a ssDNA virus belonging to the genus *Tetraparvovirus* in the family *Parvoviridae* (Cotmore *et al.*, 2014). Metagenomic sequencing identified 221 343 reads of UTPV1 from samples from one Mexican and two USA sites in six asymptomatic and five sick individuals (Table 1). Two near-complete sequences of UTPV1 (5254 and 4994 bases, respectively) were determined from USA cattle samples. Both sequences shared 97 % identity to the reference sequence of BHoV2 (GenBank JF504698) at the nucleotide level and were 98 % identical to each other. Phylogenetic comparison of NS1 protein sequence showed that UTPV1 (BhoV2_BS-S13) clusters closest to BhoV1 and resides in a clade with other members of the genus *Tetraparvovirus* (Fig. 4). To our knowledge, this is the first report of UTPV1 in North American cattle; however, ungulate tetraparvovirus 2 was previously identified in ground beef samples by metagenomic sequencing (Zhang *et al.*, 2014b).

Bovine respiratory syncytial virus (BRSV)

BRSV was detected in nasal swabs collected from five animals (one sick and four asymptomatic) at two different sites in the USA (Table 1). We recovered two near-complete genome sequences (15 123 and 15 122 bases), one from each site. Both of these sequences shared 97 % nucleotide identity to a BRSV reference (GenBank AF295543). The stop codon of the glycoprotein (G) gene in both these sequences was shifted by 18 nt, and thus the G-protein is longer by 6 aa, i.e. 263 aa as opposed to 257 aa. This divergence of G length has previously been documented in some BRSV isolates (Furze *et al.*, 1997; Mallipeddi & Samal, 1993). The G of one isolate shared 86 % identity and that of the other shared 88 % identity to the reference sequence at the amino acid level.

Bovine viral diarrhea virus 1 and 2 (BVDV1 and BVDV2)

BVDV1 was detected at three USA sites with 54 173 reads, of which a majority of the reads (93.7 %) were derived from three sick animals (Table 1). Three near-complete and one partial sequence of BVDV1 were recovered. These sequences shared 91–96 % nucleotide identity and 95–98 % polypeptide amino acid identity to the reference sequences.

BVDV2 was identified in a single symptomatic animal at one Mexican feedlot site. A total of 939 reads spanned 81.7 % of the genome that had 86 % nucleotide identity to the reference genome (GenBank FJ527854).

Bovine coronavirus (BCV)

Sequencing revealed a total of 3 611 613 reads mapping to bovine coronavirus together from nasal swabs collected from two Mexican and all four USA sites (Table 1). Nine complete or near-complete BCV sequences were assembled and were 99 % identical to the reference nucleotide sequence. The predicted spike proteins for all the BCV genomes determined in the present study were also 99 % identical to the reference sequence.

Bovine nidovirus (BNV)

BNV was recently identified and characterized from cattle with respiratory disease (Tokarz *et al.*, 2015). Reads mapping to BNV were detected in samples from two Mexican feedlots (Table 1). A total of 9037 reads were collectively generated from three asymptomatic and one sick animals that spanned 20 016 bases in length sharing 94 % nucleotide identity to the only reference sequence (KM589359) available in GenBank.

Bovine parvovirus 3 (BPV3)

BPV3 is classified to species *Ungulate erythoparvovirus 1* (Cotmore *et al.*, 2014). Four near-complete viral genomes closely related to BPV3 (GenBank AF406967) were

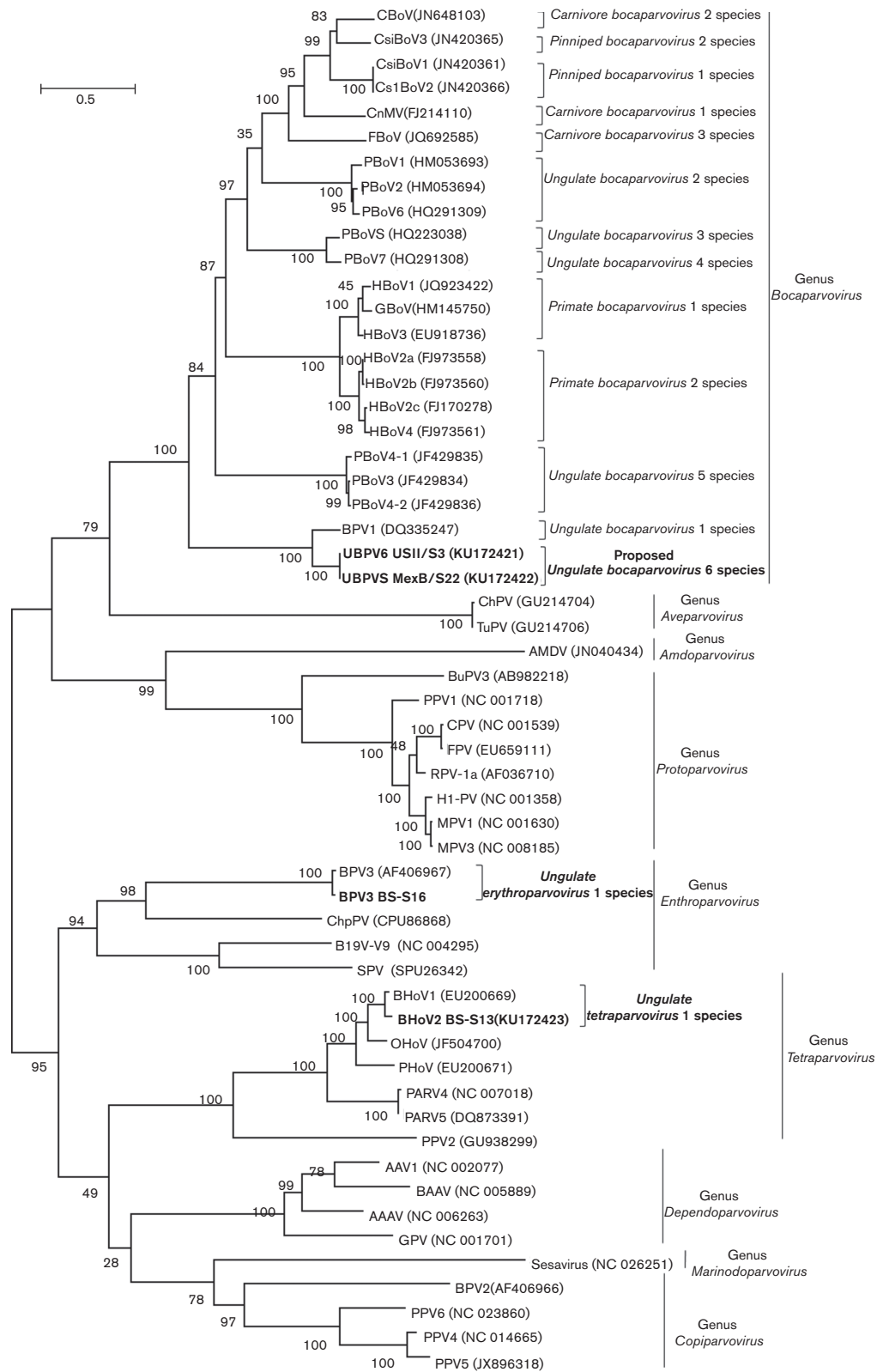


Fig. 4. Phylogenetic analysis of NS1 protein sequences of subfamily *Parvovirinae*. The phylogenetic tree was reconstructed by maximum-likelihood analysis with tree topology evaluated using 1000 bootstrap replicates. GenBank accession numbers are shown in parentheses. Numbers at nodes represent percentage bootstrap support. Sequences determined here are shown in bold type. Bar, substitutions per amino acid position.

assembled from metagenomic sequencing reads. A total of 644 094 reads mapped to a BPV3 reference genome, of which 97.8 % were derived from diseased cattle ($n=6$) samples (Table 1). BPV3 was also identified in four asymptomatic animals. Predicted NS1 protein sequences had 99–100 % amino acid identity to the reference species (GenBank AF406967) and clustered along with it in the phylogenetic analysis (Fig. 4).

Bovine adenovirus 3 (BAdV3)

A total of 153 841 reads mapped to a BAdV3 reference genome were derived from samples from eight animals originating in a single site in Mexico (five asymptomatic, three sick) and one asymptomatic animal at a USA site (Table 1). A near-complete 34 005 bp sequence was obtained that was 99 % identical to the reference sequence (GenBank JN381195) at the nucleotide level.

Bovine adeno-associated virus (BAAV)

We identified BAAV in the same samples from Mexican cattle (Table 1) from which BAdV3 was recovered, supporting previous work that BAAV requires selected proteins derived from bovine adenovirus for its replication (Handa & Carter, 1979; Myers *et al.*, 1980). A near-complete 4589 base sequence was assembled from 37 433 reads that shared 98 % nucleotide identity to the reference sequence (GenBank AY388617).

Bovine parainfluenza virus 3 (BPIV3)

A total of 9991 reads obtained from a single asymptomatic animal in the USA enabled assembly of a near-complete genome sequence (15 370 bases) sharing 99 % nucleotide identity to the BPIV3 genotype C reference genome (GenBank KJ647287). Another 502 reads were obtained from a single asymptomatic Mexican animal which yielded 82.8 % coverage of the genome. This sequence shared 97 % nucleotide identity to the BPIV3 genotype B reference sequence (GenBank KJ647284). The matrix (M) protein from both of these sequences shared 100 % identity to the reference sequences at the amino acid level.

Bovine torovirus (BTV)

BTV was detected in one diseased Mexican animal (Table 1). Only a partial sequence of the S (spike) gene was assembled, which had 97 % and 98 % identity to the reference nucleotide and reference protein sequences, respectively.

Bovine herpesvirus 1 (BHV1)

Reads ($n=47$) mapping to BHV1 were identified only in one sick animal from a feedlot in Mexico.

***Musca domestica* salivary gland hypertrophy virus (MdSGHV)**

MdSGHV is an unassigned circular, dsDNA virus which replicates in salivary glands of adult flies, inhibits egg production in infected flies and is characterized by causing salivary gland hypertrophy (Prompiboon *et al.*, 2010). To our knowledge, this virus has never been reported in animals; however, in the present study, MdSGHV was detected in four sick animals located at two feedlots in Mexico. Approximately 21 % of the genome was assembled from a single sample and had 95 % nucleotide identity to a reference genome (GenBank EU522111).

Circular DNA viruses and picobirnavirus

Numerous diverse, small, single-stranded, circular viruses belonging to the family *Circoviridae* have been previously shown to infect farm animals (Li *et al.*, 2010). Likewise, the family *Picobirnaviridae*, consisting of a bi-segmented dsRNA genome, are genetically diverse and have been shown to infect a range of animals but are relatively poorly characterized (Ganesh *et al.*, 2014; Ng *et al.*, 2015). For single-stranded circular DNA viruses (ssCDV), a total of 623 reads were found in samples from seven sick and four asymptomatic animals originating from four Mexican and two USA sites. Picobirnavirus (PBV) was detected in only one asymptomatic animal from a Mexican site (Table 1). As only a relatively small number of reads mapped to these diverse viral families, further annotation was not pursued.

Association between virus detection in nasal swabs and clinical disease

None of the random intercepts in the generalized linear mixed models fitted to determine associations between the presence of viral genome (based on detection of sequence reads) and clinical disease accounted for any of the variability in the outcome. Regardless, random effects were kept in the models to account for the design structure of the study. Moreover, none of the variables pertaining to virus DNA sequence read numbers were normally distributed. After several transformations were attempted, the dichotomous forms were chosen. Categorization based on quartiles or other cut-offs was not feasible for most of the variables given the limited distribution of sequence reads among enumerable samples (those above the detection limit). Variables pertaining to BHV1 and BTV were not included in the analysis given their small effective sample size (i.e. only one sample had ≥ 15 sequence reads for these viruses). Table 2 depicts odds ratios (OR) and their 95 % confidence intervals (CI), model-adjusted means and their 95 % confidence intervals, as well as the corresponding P values. None of the virus sequence read variables, modelled as dichotomous predictors (presence of ≥ 15 sequence reads), were significantly associated at the 5 % significance level ($P < 0.05$) with the probability of being a BRD case. However, having at least one sequence read for IDV (OR=2.94, OR 95 %

CI=0.71–12.13; $P=0.134$), or BRSV (OR=0.18, OR 95 % CI=0.02–1.64; $P=0.126$), was significantly associated, at the 15 % level ($P<0.15$), with the probability of animals having BRD clinical signs.

As IDV was the only virus positively (OR>1) associated with clinical signs of BRD with marginal statistical significance ($P<0.15$), we performed IDV-specific quantitative reverse transcription PCR (qRT-PCR) on the original samples. Identical to results determined using metagenomic sequencing, eight samples from symptomatic animals and three samples from asymptomatic animals tested positive. The C_t values for samples derived from symptomatic animals (C_t range=20.3–36.6) were significantly lower than those derived from asymptomatic animals (C_t range=34.6–37) using the Wilcoxon test ($P=0.04$).

DISCUSSION

BRD has long been recognized as the most frequent and economically important disease affecting cattle and has a multifactorial etiology that involves complex interactions amongst pathogens, host and environmental factors. The beef industry has widely used numerous commercial multivalent vaccines of variable viral composition including BHV1, BVDV, BRSV and BPIV3 viruses to control clinical disease. A number of other viral pathogens have also been reported in cattle exhibiting BRD, including two species of bovine rhinitis virus (Bogel & Bohm, 1962; Hause *et al.*, 2015; Yamashita *et al.*, 1985), bovine reovirus (Ellis, 2009), bovine enterovirus (Dunne *et al.*, 1974) and BCV (Decaro *et al.*, 2008); however, these viruses are not frequently targets of routine diagnostic testing or intervention strategies. In the present study, 21 distinct viruses were detected in samples from this population of steers, including established BRD viral aetiological agents such as BVDV and BRSV (Gershwin *et al.*, 2000; Ridpath, 2010). Detection rates of BHV1, BVDV, BRSV, BCV and IDV in symptomatic animals (2.1 %, 8.5 %, 2.1 %, 29.8 % and 17.0 %, respectively) were similar to those determined by the Kansas State Veterinary Diagnostic Laboratory as part of routine BRD diagnostic testing in 2015 by multiplex qRT-PCR (3.8 %, 10.4 %, 10.4 %, 27.9 %, 7.0 %, respectively; unpublished data). Interestingly, statistical analysis of the association between virus detection in asymptomatic and acutely ill animals found that only IDV was a risk factor of moderate ($P<0.15$) significance. Despite sequencing 93 samples and sample pools, the relatively low specific virus detection rate in nasal swabs resulted in a small effective sample size, which prohibited achieving stronger statistical significance. It is important to note that given the observational nature of this study and the use of a purposive sampling, we can evaluate associations between potential exposures and acute BRD in the study population, rather than support causal inferences. Further analysis of the variance components of the random effects included in the analyses of these data indicated that most of the variability occurred at the sample rather than at the feedlot or state

levels, thus a larger number of samples would have likely increased our ability of detecting significant differences. The observations made in the present study, which can inform the generation of hypotheses, can be further investigated to broaden our understanding of the virome with an aetiological role in BRD.

Initially isolated and characterized from swine, IDV has since been frequently identified in bovines, which has been suggested as a possible reservoir with worldwide distribution (Chiapponi *et al.*, 2016; Ducatez *et al.*, 2015; Hause *et al.*, 2014). However, little is known about the pathogenesis in its native host. Further analysis of IDV C_t values found significantly lower C_t values, indicative of higher viral replication, in acutely ill animals compared with asymptomatic pen-mates. While not conclusive, these results suggest IDV may play an aetiological role in BRD pathogenesis. Higher viral loads for influenza A virus were found in humans with pneumonia as compared with those with less severe upper respiratory tract infections (Li *et al.*, 2010). Previously, IDV was also identified at a higher incidence (29.1 %) in calves with BRD as compared with healthy animals (2.4 %) (Ferguson *et al.*, 2015). A recent metagenomic survey of a large calf ranch experiencing acute respiratory disease found a significant correlation between detection of IDV, BRAV and BAdV3, and clinical disease (Ng *et al.*, 2015). Apart from IDV, our results do not support association of BRAV and BAdV3 with BRD. Differences in type of cattle (dairy vs beef) and study design, including the number of production sites and of animals surveyed, in addition to differences in animal age and management conditions, as well as viral strains and co-infections, could account for the differences observed.

BRAV and BRBV are classified in the genus *Aphthovirus* in the family *Picornaviridae* (Hollister *et al.*, 2008). BRAV is known to have two serotypes, BRAV1 and BRAV2, while BRBV is recognized to have a single serotype (BRBV1). Here, BRAV and BRBV were present in 52.7 and 23.7 % of the swabs, respectively. Both serotypes of BRAV were identified. In addition to strains mapping to historical BRBV references, five strains formed a well-supported clade with a maximum of 71 % identity to other BRBVs. As known serotypes of BRAV (BRAV1 and BRAV2) share ~87 % identity in P1 and form well-defined clades, it is likely that BRBV strains MexB29, USII/19, USII/05, MexB10 and MexB39 represent a novel serotype of BRBV, proposed as BRBV2. Bovine rhinitis viruses are established aetiological agents of BRD and we recently reported evidence of their common detection in BRD diagnostic submissions (Betts *et al.*, 1971; Ide & Darbyshire, 1972; Mohanty *et al.*, 1969; Hause *et al.*, 2015). Despite being amongst the most prevalent viruses detected in the present study, positive samples were essentially equally distributed between asymptomatic and symptomatic animals. It is unclear if the lack of association between BRV detection and clinical disease is due to differing strain pathogenicity, the need for coexistence of other factors, or a reflection of the limited sample size. As previously discussed, detection of

Table 2. Two-way tables of frequency, measures of association and *P* values of univariable associations between presence of viruses (modelled as dichotomous predictors) with the probability of an animal having clinical signs of acute BRD

Virus	Seq. reads	BRD status		OR*	OR* 95% CI	Mean† (%)	Mean† 95% CI (%)	P value‡
		Yes ('sick') <i>n</i>	No ('asymptomatic') <i>n</i>					
BRAV	≥15	23	26	0.74	0.32–1.69	46.94	33.36–60.99	0.466
	0	24	20	–	–	54.55	39.65–68.67	
UTPV1	≥15	5	6	0.79	0.22–2.86	45.45	19.99–73.54	0.721
	0	42	40	–	–	51.22	40.35–61.97	
BPV3	≥15	6	4	1.54	0.40–5.97	60.00	29.35–84.42	0.529
	0	41	42	–	–	49.40	38.68–60.17	
BAAV	≥15	3	5	0.56	0.12–2.55	37.50	12.31–71.95	0.448
	0	44	41	–	–	51.76	41.07–62.30	
BAdV3	≥15	3	6	0.46	0.10–1.98	33.33	10.91–67.12	0.290
	0	44	40	–	–	52.38	41.60–62.95	
BRBV	≥15	11	10	1.10	0.41–2.95	52.38	31.56–72.40	0.848
	0	36	36	–	–	50.00	38.49–61.51	
IDV	≥15	8	3	2.94	0.71–12.1	72.73	40.95–91.11	0.134
	0	39	43	–	–	47.56	36.88–58.47	
EVE	≥15	5	2	2.62	0.47–14.6	71.43	32.12–92.96	0.265
	0	42	44	–	–	48.84	38.33–59.45	
EVF	≥15	2	1	2.00	0.17–23.7	66.67	14.89–95.81	0.579
	0	45	45	–	–	50.00	39.67–60.33	
UBPV6	≥15	6	6	0.98	0.29–3.34	50.00	24.08–75.92	0.968
	0	41	40	–	–	50.62	39.71–61.46	
BVDV	≥15	4	1	4.19	0.44–40.3	80.00	30.20–97.37	0.212
	0	43	45	–	–	48.86	38.47–59.36	
BPIV3	≥15	0	2	–	–	–	–	–
	0	47	44	–	–	–	–	
BNV	≥15	1	3	0.31	0.03–3.22	25.00	3.24–76.82	0.324
	0	46	43	–	–	51.69	41.23–62.00	
MdSGHV	≥15	4	0	–	–	–	–	0.984
	0	43	46	–	–	48.31	38.00–58.77	
BCV	≥15	14	9	1.74	0.66–4.62	60.87	39.94–78.44	0.259
	0	33	37	–	–	47.14	35.65–58.95	
BRSV	≥15	1	5	0.18	0.02–1.64	16.67	2.21–63.87	0.126
	0	46	41	–	–	52.87	42.26–63.24	
	Total <i>n</i>	47	46					

The number of sequence reads was categorized in two levels: ≥15 = at least one sequence read; 0=0 sequence reads (below detection limit of the assay).

*Odds ratio.

†Model-adjusted means.

‡From generalized linear mixed models.

BRAV, IDV and BAdV3 correlated strongly with BRD at a single farm (Ng *et al.*, 2015).

Despite numerous reports of its isolation from animals with clinical disease (McClenahan *et al.*, 2013; Moll & Ulrich, 1963; Phillip & Darbyshire, 1971), an aetiological role for enteroviruses in BRD has not been established. While in our study EVE was more commonly identified in symptomatic animals, this result was not statistically significant. This study identified an EVE strain (MexKSU/5) with a highly divergent VP1. As the enterovirus capsid proteins VP1, VP2

and VP3 show genetic variability and segregate strains into genotypes/serotypes, it is likely MexKSU/5 represents a new serotype of EVE. In China, recently, a novel EVE was isolated from cattle with severe respiratory and enteric disease, where mutations were largely localized to the VP1 and VP4 capsid regions of the genome (Zhu *et al.*, 2014). Similar to the EVE identified in our study, which contains a divergent VP1 gene and unusual cleavage sites, it is unclear if this novel EVE played an aetiological role in clinical disease, illustrating our incomplete understanding of the role of EVE infecting cattle (Zhu *et al.*, 2014).

Three different species in the family *Parvoviridae* were identified in bovine swabs here, including a proposed new species, *Ungulate bocaparvovirus 6*. UBPV6 was present in 12.9% of the nasal swabs, equally distributed between asymptomatic and sick animals. UBPV6 was most similar to ungulate bocaparvovirus 1 (originally bovine parvovirus 1), a virus capable of causing a wide range of symptoms including gastrointestinal and respiratory tract disease as well as reproductive failure (Abinanti & Warfield, 1961; Barnes *et al.*, 1982; Weiblen *et al.*, 1983). Three other bovine parvoviruses, ungulate copiparvovirus 1 (BPV2), BPV3 and UTPV1/BHoV, have been identified in bovines. Similar to UBPV6, BPV2, BPV3 and UTPV1 have not been established as aetiologic agents of disease (Allander *et al.*, 2005; Lau *et al.*, 2008; Tse *et al.*, 2011). In addition to UBPV6, BPV3 and UTPV1 were detected in nasal swabs from both asymptomatic and sick animals in the USA and Mexico and were not found to be statistically associated with disease.

A majority of the nasal swabs ($n=82$) tested positive for at least a single virus. A mean of 1.9 and 2.1 viruses were detected per swab from asymptomatic and sick animals, respectively; however, this difference was not significant. For most viruses, a lack of effective sample size hindered statistical significance between individual virus detection and the probability of disease. Analysis of additional samples collected at different times throughout outbreaks of respiratory disease, or inoculation challenge studies may increase the likelihood of demonstrating more conclusive disease associations.

Our results demonstrate that the virome of nasal swabs collected from animals with BRD from different environments is extremely diverse and variable. While BRD viral preventative efforts largely focus on inclusion of BVDV, BRSV, BHV1 and PI3, our results demonstrate that many additional viruses are present in the bovine upper respiratory system. Further research is needed to determine the individual contributions of these viruses to BRD in cattle in North America; however, this work demonstrates the utility of metagenomic sequencing for the unbiased detection of viruses in cattle.

METHODS

Sample collection. A sample of 103 feedlot steers, weighing approximately 227–363 kg (500–800 lb), from a total of 10 feedlots, six in Mexico and four in USA, was utilized. Nasal swab samples were collected (one per animal) from 10 animals per pen (for a total of one pen per feedlot): five samples were collected from diseased and five from asymptomatic steers. Cattle exhibiting clinical signs of acute respiratory disease (i.e. rhinorrhoea, cough, laboured breathing, depression and ocular discharge) were considered as diseased (symptomatic) as assessed by a veterinarian. Swabs were also collected from asymptomatic (i.e. absence of clinical signs of respiratory disease) cattle located in the same pens as their symptomatic counterparts. Nasal swabs were collected from the nasopharyngeal region using a sterile polyester-tipped swab (Becton Dickinson). Swabs were placed in a tube containing 3 ml universal viral transport medium (BDUVT, BD Diagnostics) and shipped on ice to the Veterinary Diagnostic laboratory at Kansas State University for processing and testing.

All samples were collected by veterinarians as part of diagnostic surveillance activities.

Sample preparation. Swab samples were clarified by centrifugation (14 000 g, 5 min) and 180 μ l of the supernatant from each sample was treated with 20 μ l of a cocktail of nucleases for 90 min at 37 °C to degrade any host nucleic acids (Neill *et al.*, 2014). Viral nucleic acids were extracted using a viral purification kit (QIAamp MinElute virus spin kit, Qiagen) as per the manufacturer's instructions and eluted in 30 μ l nuclease-free water. First-strand synthesis was carried out with a known primer (FR26RV-N; GCCGGAGCTCTGCAGATATC NNNNNN; Allander *et al.*, 2005) using a Superscript III First-Strand synthesis kit (Life Technologies). Following inactivation of reverse transcriptase and denaturation of dsDNA by heating at 95 °C for 5 min and rapid cooling to 4 °C, the RNA–DNA hybrid was denatured by RNase H digestion followed by second-strand synthesis using Sequenase DNA polymerase (Affymetrix). cDNA size selection and purification was carried out using Agencourt AMPure XP beads (Beckman Coulter) as per the SPRIselect protocol to isolate cDNA fragments greater than 250 bp. Purified cDNA was subsequently amplified (40 cycles) using primer FR20RV (Allander *et al.*, 2005; GCCGGAGCTCTGCAGATATC). Amplified cDNA was purified by Agencourt AMPure XP beads (Beckman Coulter) and quantified on a Qubit 2.0 fluorometer (Invitrogen) using the Qubit dsDNA high sensitivity reagent kit (Thermo Fisher Scientific) before proceeding to NexteraXT library preparation.

NexteraXT library preparation (Illumina) and sequencing. From each sample, 5 μ l DNA at 0.2 ng μ l⁻¹ concentration was used for library preparation as per the NexteraXT library preparation (Illumina) protocol using MiSeq v2 kit reagents. A volume of 24 μ l of pooled barcoded DNA libraries was mixed with 576 μ l of HT1 (hybridization buffer), incubated at 96 °C for 2 min, chilled on ice and loaded into a thawed MiSeq Reagent cartridge for a sequencing run on an Illumina MiSeq instrument using paired 150 bp reads.

Metagenomic data assembly and analysis. Paired-end reads for each sample or sample pool were imported into CLC Genomics Workbench 8.0 software (Qiagen) in separate folders. Reads were mapped to the host reference genome (*Bos taurus*) and unmapped reads were collected and *de novo* assembled into contigs using default parameters. Consensus sequences were extracted from contigs of ≥ 200 bp and analysed against the non-redundant nucleotide database (nr/nt) of NCBI using the BLASTN algorithm. The viral genomes with the lowest expectation values (E -values $\leq 10^{-5}$) were used as references for templated assemblies using the non-host subtracted paired reads in order to tally reads mapping to each virus. ORFs were extracted from either reference-based or *de novo*-assembled contigs. Assembled viral nucleotide sequences and ORF-translated peptides were searched against the non-redundant database with BLASTN and BLASTP algorithms to find percent-identity.

RACE. The missing 5' and 3' ends of selected viruses were confirmed by RACE using the 5'/3' RACE Kit, 2nd Generation (Roche Applied Science) followed by amplification with TaKaRa rTaq DNA polymerase (Clontech). Sequences were assembled and edited manually to produce complete sequence of the viral RNA genome containing 5' and 3' extremities.

Real-time PCR. Total nucleic acid was extracted from 200 μ l of selected clarified nasal swab samples using a Qiagen QIAamp MinElute virus spin kit. Quantitative reverse transcription real time PCR for IDV was performed as previously described (Hause *et al.*, 2013) in order to further characterize IDV on the subset of samples where sequence reads for this virus were identified.

Phylogenetic analysis. Nucleotide and derived amino acid sequences from related viruses were aligned separately using MUSCLE (Edgar, 2004) with default settings. The best-fitted model of evolution was identified for each alignment using the Bayesian Information Criterion in MEGA 6 (Tamura *et al.*, 2013) and maximum-likelihood trees were reconstructed using 1000 bootstrap iterations to evaluate the strength of branching.

Statistical analysis. Associations between the number of DNA sequence reads for different viruses and the probability of an animal having clinical signs of BRD were analysed using generalized linear mixed models in SAS 9.4 (SAS Institute), with each virus being modelled independently (only univariate models were fitted). Models were fitted using a binary distribution, logit link, maximum-likelihood estimation and random intercepts for feedlots within states and of states within countries to account for the hierarchical structure of the study, using Proc Glimmix. The outcome consisted of the probability of a animal showing clinical signs of acute BRD (as defined by the feedlot veterinarian), modelled as a dichotomous variable (1=clinical signs of BRD vs 0=asymptomatic), and the predictor variables consisted of the number of DNA sequence reads for several respiratory viruses obtained from nasal swab samples using viral metagenomics. Descriptive statistics (e.g. mean, median, SD, range) were computed for all predictor variables, on the original scale, by outcome status (BRD or asymptomatic). Linearity between each of the predictors and the outcome, on the logit scale, was assessed using locally weighted regression smoothing plots. Variables not meeting the linearity assumption were log transformed (i.e. base 10 logarithm, where 0 values were assigned values of 0.1 to allow log transformation) or categorized (as dichotomous predictors: 1: DNA sequence reads ≥ 1 ; and 0: DNA sequence reads=0). Odds ratios, model-adjusted means and their 95 % confidence intervals were estimated for the association between each respiratory virus and the probability of BRD. The Mann–Whitney U-test (ranksum command in Stata 12; StataCorp), non-parametric test (Wilcoxon, 1945), was performed to test the hypothesis that the cycle threshold (C_t) values for IDV in nasal swabs collected from animals with BRD clinical signs is the same as C_t values from samples collected from asymptomatic animals. Values of $P < 0.05$ were considered statistically significant.

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