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BRIEF REPORT

Identification of boosepivirus B in U.S. calves

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Abstract

Bovine enteric disease has a complex etiology that can include viral, bacterial, and parasitic pathogens and is a significant source of losses due to morbidity and mortality. Boosepivirus was identified in calves with enteric disease with unclear etiology in Japan in 2009 and has not been reported elsewhere. Metagenomic sequencing and PCR here identified boosepivirus in bovine enteric disease diagnostic submissions from six states in the USA with 98% sequence identity to members of the species *Boosepivirus B*. In all cases, boosepivirus was identified as a coinfection with the established pathogens bovine coronavirus, bovine rotavirus, and cryptosporidia. Further research is needed to determine the clinical significance of boosepivirus infection.

Enteric disease is a significant health concern for bovine calves and a leading cause of morbidity and mortality early in life [1]. Clinical disease is often multifactorial, with viral, bacterial, and parasitic pathogens interacting with host and environmental factors. Bovine coronavirus (BCV) and bovine rotavirus (BRV) are etiologic agents of enteric disease; however, a multitude of viruses from diverse families have been identified in clinical specimens with unresolved clinical significance [2, 3]. For the family *Picornaviridae*, members of the genera *Enterovirus*, *Kobuvirus*, *Hunnivirus*, and *Bopivirus*, have been identified in cattle [4–9]. More recently, bovine picornavirus, reclassified as boosepivirus (BPV), was identified in fecal samples from calves with enteric disease in Japan in 2009 [10]. While two bovine species of BPV have been recognized, a member of a third species, *Boosepivirus C*, was identified in sheep (unpublished). To our knowledge, BPV has not been reported apart from its initial characterization in Japan in 2009.

Pooled intestinal tissue and fecal swabs were collected from 10-day-old calves in Missouri, USA, exhibiting prolonged diarrhea lasting for more than five days, resulting in death. Metagenomic sequencing was performed to identify possible pathogens as described previously [11]. A sample was clarified by centrifugation and digested with a nuclease cocktail, followed by reverse transcription and second-strand synthesis with barcoded primers containing a 3' random hexamer. Following purification, DNA was amplified using barcode-specific primers, followed by construction of a Nextera XT sequencing library. Sequencing was performed using a MiSeq sequencer with paired 151-base-pair chemistry. Reads trimmed of adapter sequences were assembled *de novo* using CLC Genomics software with contigs identified by BLASTx using BLAST2Go.

One of the 45 contigs assembled from the approximately 4.5 million reads was identified as BPV. The contig was 7,365 nucleotides (nt) in length and was assembled from 8,257 reads (0.18%). As open reading frame (ORF) analysis indicated that the 3' end of the single large ORF was incomplete, rapid amplification of cDNA ends was performed using a Roche 5'/3'-RACE Kit, 2nd generation, using primers designed to anneal near the 3' end of the genome, bovpic6615: 5'-GTCGCCACAGGTTCTGCTGTTG and bovpic6863: 5'-GGGTATGCCATCTGGAACTAGC. The remainder of the genome was amplified by amplification of overlapping approximately 1,000-base-pair (bp) segments. The final genome sequence was confirmed by Sanger sequencing. The sequence for BPV 21-0305 was submitted

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The genome sequence of boosepivirus B strain 21-0305 was deposited in the GenBank database under accession no. MZ052226.

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to the GenBank database under the accession number MZ052226.

The BPV genome sequence is 7,440 nt in length and is 86% identical to those of BPV isolates recovered from Japan in 2009, according to BLASTn analysis. The genome contains a single large ORF, encoding a protein of 2,341 amino acids that is 98% identical to that of BPV by BLASTp analysis. The 5' untranslated region, consisting of 314 nucleotides, was incomplete based on the length of 553 nucleotides reported for BPV.

A second contig 8,421 nt in length was also identified as a complete bovine kobuvirus (BKV) genome. This contig was comprised of 373,465 reads (8.3%). The BKV genome was 94% identical to that of bovine kobuvirus strain IL35164 (GenBank accession no. MN336260), which was identified in calves with diarrhea in the USA. [12]. The predicted 2,463-amino-acid protein encoded by the open reading frame was 98.2% identical to that of MN336260. No other mammalian viruses were identified.

To investigate the evolutionary history of BPV 21-0305, the region of the genome encoding the 3D protein was identified by alignment with reference sequences. Reference sequences for picornavirus 3D nucleotides were downloaded from the *Picornaviridae* resources website from the International Committee of Taxonomy of Viruses [13]. Sequences were aligned by ClustalW as implemented in MEGA X. Phylogenetic analysis was next performed by the maximum-likelihood method using the best-fitting GTR+G+I model of evolution. Tree topology was evaluated using 1,000 bootstrap replicates. Boosepivirus strain 21-0305 formed a sister clade to one formed by multiple strains of BPV recently reclassified as members of the species *Boosepivirus B* (Fig. 1). This, along with the 98% identity of the polyprotein of strain 21-0305 to members of *Boosepivirus B*, suggests that strain 21-0305 belongs to the species *Boosepivirus B* [4]. Together, the boosepiviruses formed a well-supported monophyletic clade, supporting their designation as members of a separate genus. Apart from other boosepiviruses, strain 21-0305 was similar to unclassified canine and bat picornaviruses.

A TaqMan assay was designed targeting the 3D region of BPV, using the primers Forw, 5'-TTTCCATTCCTC ATACACCCTG, and Rev, 5'-TCTCTTCCGTTGTGCCAT G, and the probe 5'-FAM-ATTCCTCGACCAGCGAAT GCTCTC. Next, RNA was isolated from a collection of 48 bovine enteric disease diagnostic submissions submitted for diagnostic testing. Real-time reverse transcription PCR was performed using TaqMan Fast Virus 1-Step Master Mix according to the manufacturer's instructions. Six of the 48 (12.5%) samples were positive for BPV, including the original sample in which the virus was detected. Cycle threshold values ranged from 18.1 to 34.2 (Table 1). The six positive samples originated from diseased calves from four Midwestern states as well as California. The 12.5% prevalence, combined with detection in multiple states across a broad geographic area, suggests that BPV infections are common in U.S. cattle.

As enteric disease is often multifactorial, we explored coinfections of BPV with significant enteric disease pathogens in the six BPV-positive samples. Bovine coronavirus and BRV were detected by RT-PCR, while cryptosporidia were detected microscopically (Table 1). Enteric pathogenic bacteria were identified by aerobic culture. All BPV-positive cases were also positive for at least one etiologic pathogen of enteric disease. These results suggest that BPV is commonly found in coinfections with known enteric pathogens. Interestingly, for cases 1 and 4, the Ct value for BPV was considerably lower than for BCV and BRV, suggesting that BPV was present in the clinical samples at higher titers than BCV and BRV.

Routine metagenomic sequencing of a diagnostic submission here identified BPV for the first time since its original and only description from Japan in 2009. While BPV was originally identified in clinical samples from calves with enteric disease that tested negative in routine tests for BCV, BRV, and bovine torovirus, here, BPV was always found in association with BCV and BRV infections. Interestingly, the initial description of BPV noted that, for three of the five samples positive for BPV, BKV was also present. We also identified BPV and BKV coinfection. Bovine kobuvirus has been detected in calves with enteric disease in many countries. In China, BKV was significantly associated with calf diarrhea [14]. In the USA, BKV infection was associated with histologic lesions consistent with enteric disease [12]. In the BPV-positive sample here, BKV accounted for 8.3% of the total reads, while only 0.18% of the reads mapped to BPV, suggesting that BKV was present at much higher levels in our enteric disease sample. Further research is needed to determine the clinical significance of boosepivirus infection.

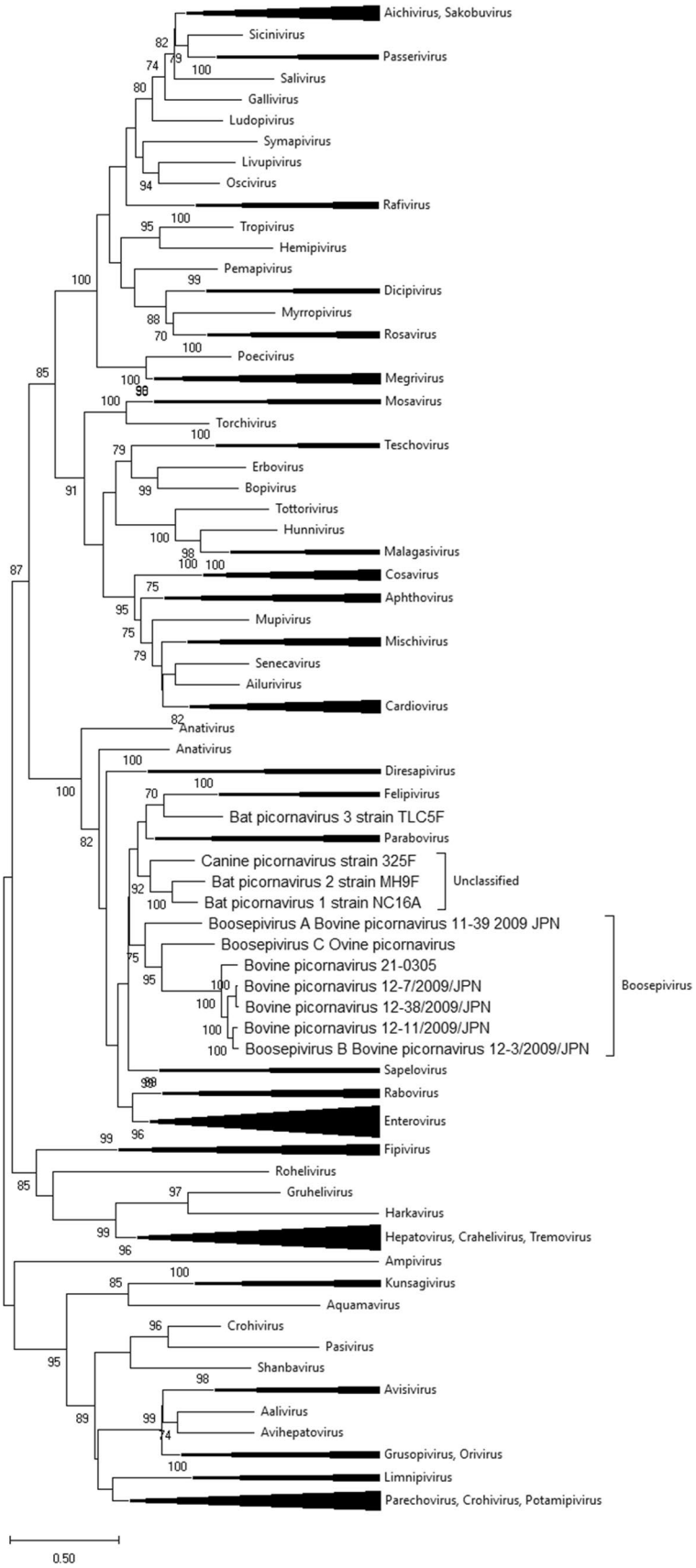

Fig. 1 Phylogenetic analysis of the genome region encoding the 3D protein of bovine picornavirus. Sequences were aligned using ClustalW, and a phylogenetic tree was constructed by the maximum-likelihood method using the GTR + G + I model of evolution with 1,000 bootstrap replicates. Bootstrap values 0.70 or greater are shown

Table 1 Bovine enteric disease diagnostic test results for boosepivirus (BPV)-positive diagnostic submissions

Case	State	BPV	BRV	BCV	Cryptosporidium	Aerobic culture
1	South Dakota	20.9	35.7	29.8	Negative	Negative
2	Nebraska	28.6	18.2	31.8	Positive	Negative
3	South Dakota	21.5	14.5	Negative	Positive	Negative
4*	Missouri	18.1	27	Negative	NT	Negative
5	California	28.9	29.4	36.1	NT	NT
6	Minnesota	34.2	Negative	25.2	Positive	Negative

Bovine rotavirus (BRV) and bovine coronavirus (BCV) were detected by quantitative RT-PCR, and the cycle threshold value for positive samples is shown. The presence (positive) or absence (negative) of cryptosporidium is indicated as well as significant organisms detected by aerobic culture

NT not tested

*Original case where BPV was detected by metagenomic sequencing

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Availability of data and material The genome sequence for boosepivirus B strain 21-0305 was submitted to GenBank under accession no. MZ052226.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

Ethics statement The animal specimens used in this study were sourced from samples submitted for diagnostic testing by licensed veterinarians as part of their professional responsibilities. As such, no specific institutional animal care and use committee approval was required.

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