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Identification of a new bovine picornavirus (*Boosepivirus*) in the Republic of Korea

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ABSTRACT

Importance: Despite advancements in herd management, feeding, and pharmaceutical interventions, neonatal calf diarrhea (NCD) remains a major global health concern. Bacteria, viruses, and parasites are the major contributors to NCD. Although several pathogens have been identified in the Republic of Korea (ROK), the etiological agents of numerous NCD cases have not been identified.

Objective: To identify, for the first time, the prevalence and impact of *Boosepivirus* (BooV) on calf diarrhea in the ROK.

Methods: Here, the unknown cause of calf diarrhea was determined using metagenomics We then explored the prevalence of certain pathogens, including BooV, that cause NCD. Seventy diarrheal fecal samples from Hanwoo (*Bos taurus coreanae*) calves were analyzed using reverse transcriptase and quantitative real-time polymerase chain reaction for pathogen detection and BooV isolate sequencing.

Results: The complete genome of BooV was detected from unknown causes of calf diarrhea. And also, BooV was the most frequently detected pathogen (35.7%) among 8 pathogens in 70 diarrheic feces from Hanwoo calves. Co-infection analyses indicated that most BooV-positive samples were solely infected with BooV, indicating its significance in NCD in the ROK. All isolates were classified as BooV B in phylogenetic analysis.

Conclusions and Relevance: This is the first study to determine the prevalence and molecular characteristics of BooV in calf diarrhea in the ROK, highlighting the potential importance of BooV as a causative agent of calf diarrhea and highlighting the need for further research on its epidemiology and pathogenicity.

Keywords: Boosepivirus; neonatal calf diarrhea; *Hanwoo*; infectious pathogen

INTRODUCTION

Neonatal calf diarrhea (NCD) is a major health issue affecting calves globally and is associated with high rates of morbidity and mortality, often due to complications such as dehydration, acidosis, and electrolyte imbalance [1]. Despite improved herd management, NCD continues to be a leading cause of fertility decline and economic losses in the cattle

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Conflict of Interest
The authors declare no conflicts of interest.

Data Availability Statement
The data supporting the findings of this study are available from the corresponding author on reasonable request.

industry [2,3]. Among the various risk factors contributing to NCD onset, infectious pathogens have been consistently identified as a major cause [4]. Extensive research has been performed globally to understand the etiology of calf diarrhea, and various infectious agents, including certain bacteria, viruses, and parasites, have been reported to contribute to NCD development [4-7].

Similar to the findings of global studies, in the Republic of Korea (ROK), pathogens associated with calf diarrhea have been consistently identified. Bacteria such as *Escherichia coli* and *Salmonella* species (spp.); viruses including bovine rotavirus (BRV), bovine coronavirus (BCV), and bovine viral diarrhea virus (BVDV); and parasites such as *Cryptosporidium*, *Giardia*, and *Eimeria* spp. have been reported [2,6]. However, there have been several cases of NCD where no pathogen has been detected; the causes of these instances have been reported as "unknown" and are often disregarded despite their importance [2,4].

A new type of picornavirus, first reported in Japan in 2009 and identified in fecal samples associated with enteric diseases, was reclassified as boosepivirus (BooV) in 2020 [8,9]. The only countries that have reported BooV in calf diarrhea are China, Japan, and the United States [8-11]. The entire BooV genome species A and B has been analyzed [8-11]. However, the viruses have not been isolated for culture, and there is a lack of information in other countries. Therefore, research on the identification of BooV is limited. Since its identification in 2009, BooV has been classified into three species: BooV A, BooV B, and BooV C. Despite the growing global recognition of BooV, its incidence in calf diarrhea remains unknown in the ROK. This knowledge gap significantly limits our understanding of the epidemiological landscape of calf diarrhea in the country. Therefore, the primary objective of this study was to identify, for the first time, the prevalence and impact of BooV on calf diarrhea in the ROK.

METHODS

Sample description
This study was performed to determine the prevalence and molecular characteristics of BooV in NCD cases in the ROK. In this study, 70 diarrheic fecal samples (36 loose feces and 34 watery feces) of Hanwoo calves aged less than 60 days were randomly selected from those submitted to the laboratory in 2022. All samples were screened for BRV, BCV, BVDV, BooV, *C. parvum*, *Giardia* spp., and *Eimeria* spp., which are pathogens associated with NCD.

Total nucleic acid extraction
After transporting the diarrheic fecal samples to the laboratory, they were suspended in 0.01 M phosphate-buffered saline to prepare 30% fecal homogenates and centrifuged for 1 min at $100 \times g$. Total nucleic acids were extracted from the supernatants using the MagMAX™ Total Nucleic Acid Isolation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. All extracts were stored at –70°C until pathogen detection.

Metagenomic sequencing and analysis
The total RNA concentration was determined using Quant-IT RiboGreen (Invitrogen, USA). To assess the integrity of the total RNA, samples were run on a TapeStation RNA ScreenTape (Agilent, USA). A library was independently prepared with 0.5 µg of total RNA for each sample using Illumina Stranded Total RNA Library Prep with Ribo-Zero Plus (Illumina, Inc., USA). The first step in the workflow involved removing rRNA from the total RNA.

The remaining mRNA was fragmented into small pieces using divalent cations at high temperatures. The RNA fragments were copied into first-strand cDNA using SuperScript II reverse transcriptase (Invitrogen) and random primers. This step was followed by synthesis of second-strand cDNA using DNA polymerase I, RNase H, and dUTP. Next, these cDNA fragments were subjected to an end repair process, involving the addition of a single 'A' base and ligation of the adapters. The products were purified and amplified using polymerase chain reaction (PCR) to create a final cDNA library. The libraries were quantified using the KAPA Library Quantification kits for Illumina Sequencing platforms according to the qPCR Quantification Protocol Guide (KAPA BIOSYSTEMS, USA) and qualified using the TapeStation D1000 ScreenTape (Agilent Technologies, USA). The indexed libraries were then submitted to Illumina NovaSeq (Illumina, Inc.) for paired-end sequencing (2 × 150 bp). Quality checking and trimming of short reads were performed using Trim Galore (v.0.6.1) with a Q30 threshold, followed by extraction of viral reads from the dataset using Deconseq (v.0.4.3) with 70% query coverage and 90% identity. Subsequently, the viral reads were assembled using the SPAdes assembler (v.3.15.1), and the assembled contigs were annotated using Basic Local Alignment Search (BLAST)+ (v.2.10.1) against the National Center for Biotechnology Information (NCBI) viral database as of April 13, 2023, with a rank1 cutoff and an e-value threshold of $1 \times e\text{-}10$. Next, we calculated the alignment coverage with the genome of each viral species, and contigs covering 90% of the full sequences of the identified viruses were analyzed.

Detection of the pathogen causing calf diarrhea

Depending on the target pathogen, detection was performed using reverse transcriptase (RT)-PCR, quantitative real-time PCR (qPCR) or RT-qPCR. Specific primer/probe sets for each pathogen are listed in **Table 1** [12-16]. To detect BRV and BCV, reverse transcriptase (RT)-PCR was performed using the PrimeScript™ One-Step RT-PCR Kit Ver. 2 (Takara Bio Inc., Japan) according to the manufacturer-recommended protocols with a 25-µL solution of 2 µL of extracted template and 23 µL of reaction mixture containing 1 µL of PrimeScript™ 1 step Enzyme Mix, 12.5 µL of 2 × 1-Step Buffer, 1 µL of primer mixture, and 8.5 µL of RNase-free dH₂O. The final primer concentration was 0.4 µM. RT-PCR was performed at 50°C for 30

Table 1. Nucleotide sequences of PCR primers/probes used for pathogens associated with neonatal calf diarrhea

Classification	Microbial agent	PCR primer/probe sets	5'-nucleotide sequence-3'	References
Viruses (RT- PCR)	Bovine coronavirus	Forward	CTA GTA ACC AGG CTG ATG TCA ATA CC	[12]
		Reverse	GGC GGA AAC CTA GTC GGA ATA	
	Bovine rotavirus	Forward	TCA ACA TGG ATG TCC TGT ATT CCT	[13]
		Reverse	TCC CCC AGT TTG GAA TTC ATT	
	Boosepivirus	Forward	GAC CCT GAA TGC GGC TAA	This study
		Reverse	GCG AGT TYA GTC TCT TAT TTC CA	
Viruses (qRT-PCR)	Bovine viral diarrhea virus	Forward	CTC GAG ATG CCA TGT GGA C	[14]
		Reverse	CTC CAT GTG CCA TGT ACA GCA	
		BVD type 1 - Probe (FAM/BHQ1)	CAG CCT GAT AGG GTG CTG CAG AGG C	
		BVD type 2 - Probe (HEX/BHQ1)	CAC AGC CTG ATA GGG TGT AGC AGA GAC CTG	
Protozoa (q-PCR)	*Cryptosporidium parvum*	Forward	CAA ATT GAT ACC GTT TGT CCT TCT GT	[15]
		Reverse	GGC ATG TCG ATT CTA ATT CAG CT	
		Probe (HEX/BHQ1)	TGC CAT ACA TTG TTG TCC TGA CAA ATT GAA	
	Giardia spp.	Forward	CAT CCG CGA GGA GGT CAA	[16]
		Reverse	GCA GCC ATG GTG TCG ATC T	
		Probe (FAM/BHQ1)	AAG TCC GCC GAC AAC ATG TAC CTA ACG A	
	Eimeria spp.	Forward	AAA GGA TGC AAA AGT CGT AAC AC	[15]
		Reverse	TGC AAT TCA CAA TGC GTA TCG	
		Probe (FAM/BHQ2)	TGT TTC TAC CCA CTA CAT CCA AC	

PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; qRT-PCR, quantitative reverse transcription polymerase chain reaction; q-PCR, quantitative polymerase chain reaction.

min and 94°C for 2 min; 35 cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min; and a final extension at 72°C for 5 min. All PCR products were separated using gel electrophoresis on a 1.0% agarose gel stained with a commercial nucleic acid staining solution (RedSafe™ nucleic acid staining solution; Intron Biotechnology Inc., Korea) and subjected to direct sequencing using dideoxy termination with an automated sequencer (3730XL Capillary DNA Analyzer; Applied Biosystems, USA).

The qPCR and RT-qPCR were performed using the specific primer/probe sets for each pathogen (BVDV 1, BVDV2, *C. parvum*, *Eimeria* spp., *Giardia* spp.) (**Table 1**). The GoTaq One-Step RT-qPCR System (Promega, Madison, WI, USA) was used according to the manufacturer's protocols with a 10-μL reaction volume with 2 μL of extracted template and 8 μL of reaction mixture containing 5 μL of GoTaq qPCR Master Mix 2×, 0.2 μL of GoScript™ RT Mix for 1-Step RT-qPCR 50×, 2 μL of the primer-probe mixture, and 0.8 μL of RNase-free dH$_2$O. The final concentrations of the primer and probe were 0.3 and 0.2 μM, respectively. The qPCR and RT-qPCR was performed using the CFX Opus 96 Real-Time PCR System (Applied Biosystems). The cycling conditions were as follows: 1) RT for 10 min at 45°C (omitted for *C. parvum*, *Eimeria* spp., and *Giardia* spp.), 2) a 10-min activation step at 95°C, and 3) 40 cycles of 15 sec at 95°C and 60 sec at 60°C. After a 40-cycle reaction, samples with a quantification cycle below 35 were considered positive.

RT-PCR was performed to detect BooV using the PrimeScript™ One-Step RT-PCR Kit Ver. 2 (Takara Bio Inc.) according to the manufacturer's instructions, using the primers designed in this study (**Table 1**). A 25-μL reaction volume was prepared with 2 μL of extracted template and 23 μL of reaction mixture containing 1 μL of PrimeScript™ 1-step Enzyme Mix, 12.5 μL of 2× 1-Step Buffer, 1 μL of primer mixture, and 8.5 μL of RNase-free dH$_2$O. The final primer concentration was 0.4 μM. Finally, 336-bp products were amplified under the following conditions: 30 min at 50°C for reverse transcription, 2 min at 95°C for initial denaturation, 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, elongation at 72°C for 30 sec, and a final elongation at 72°C for 10 min. All PCR products were analyzed using gel electrophoresis on a 1.0% agarose gel stained with RedSafe™ nucleic acid staining solution (Intron Biotechnology Inc.).

Phylogenetic analysis

All positive samples were directly sequenced using dideoxy termination on an automatic sequencer (3730XL Capillary DNA Analyzer; Applied Biosystems). The obtained sequences were analyzed using NCBI's BLAST. The sequences were aligned using ClustalX (v. 2.0), and examined using a similarity matrix. Phylogenetic analysis was performed based on 3CD and P1-encoding sequences using the maximum likelihood method based on nucleotide alignment (**Fig. 1**). Bootstrap analysis was conducted with 1,000 replicates using MEGA version X.

Institutional review board statement

Ethical review and approval were waived for this study because this article does not contain any studies with live animals performed by any of the authors. All samples used in this study were received from field veterinarians who submitted them for diagnostic purpose.

Informed consent statement

Verbal consent for publication was obtained from the animal owners involved in this study at the time of sample submission. They have agreed to the use of anonymized data and any material for publication in this journal.
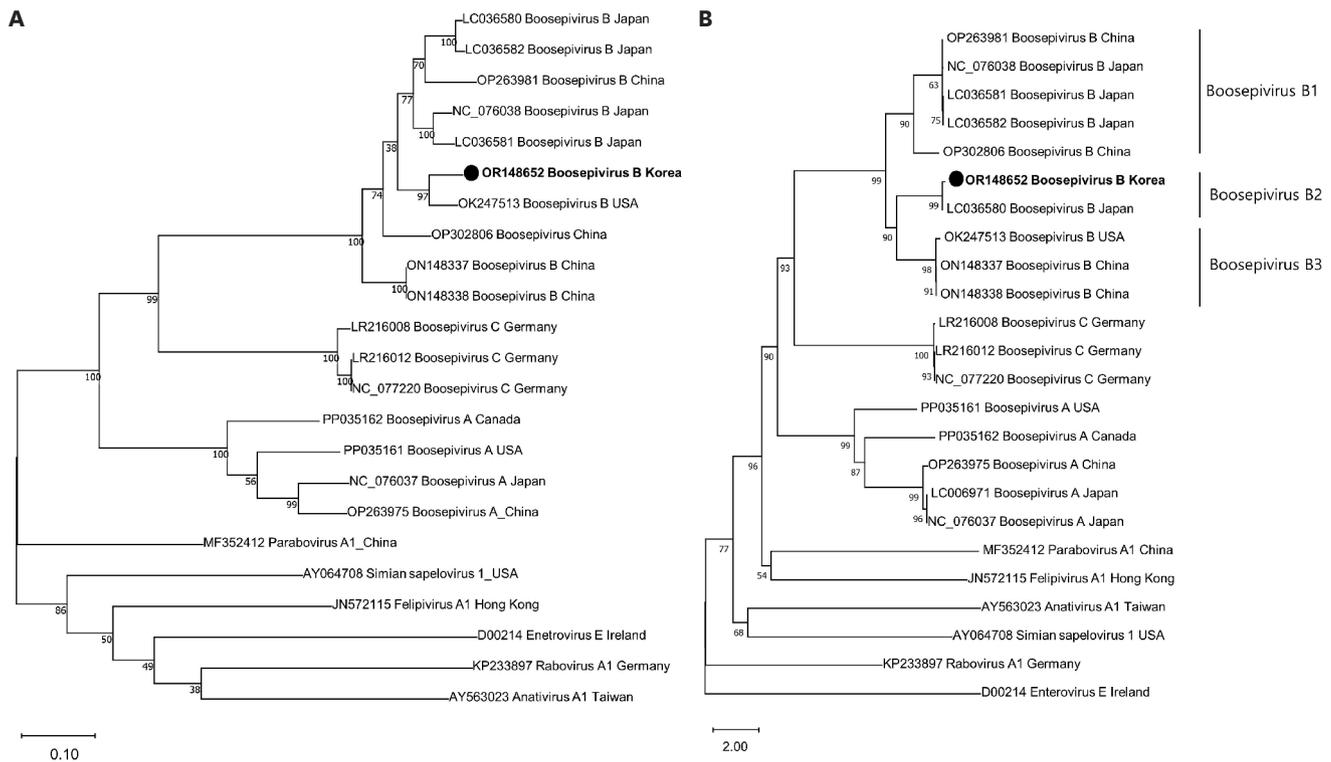
Fig. 1. Phylogenetic analysis of *Boosepivirus*es. Phylogenetic trees were constructed based on 3CD-encoding sequences (A) and P1-encoding sequences (B) using the maximum likelihood method with 1,000 bootstrap replicates in the MEGA X software. Black circles represent Boosepivirus isolates identified in this study.

RESULTS

Metagenomic sequencing

In September 2022, one diarrheic fecal sample from a 20-day old calf was submitted to the laboratory for testing enteric pathogens. RT-PCR, qPCR and qRT-PCR were performed to detect BRV, BCV, BVDV, BooV, *C. parvum*, *Giardia* spp., and *Eimeria* spp.; however, this sample was negative for all pathogens. To identify the RNA viral pathogens, metagenomic sequencing was performed. Metagenomic sequencing initially generated 70,678,702 reads, comprising a total of 7,138,548,902 bases. Following a quality trimming process that involved applying a Q30 threshold for accuracy, the data were refined to 69,960,258 reads, equaling to 7,013,383,042 bases. The assembly of these high-quality reads was then conducted using the RNAviral pipeline within the SPAdes software, resulting in the creation of 65,044 contigs. From these contigs, one contig had 7,622 nucleotides, and it was 85.8% identical to the sequences of BooV reported in Japan in 2009 (LC036580). This contig was submitted to the NCBI database and received accession number (SRA: SRR24891409, Genbank: OR148652). For the molecular characterization of the BooV in this study, homology with other BooV strains reported from other countries were compared; the BooV in this study was similar to BooV B2 (**Table 2**). The phylogenetic analysis based on 3CD and P1-encoding sequences showed that BooV in this study was clustered with the BooV B2 type (**Fig. 1**). Based on the sequences from the NCBI database and our sequences, PCR primer for 5′ UTR and L segment and conditions were designed to detect BooV (**Table 1**).

Table 2. Nucleotide identities of the Boosepivirus in this study (OR148652) compared to other types of *Boosepivirus*

Type of BooV (NCBI No.)	Nucleotide identity (%)												
	Complete genome	L	VP4	VP2	VP3	VP1	2A	2B	2C	3A	3B	3C	3D
A (LC006971)	46.4	-	-	58.4	60.3	55.6	-	-	61.8	-	-	59.5	61.3
B1 (LC036579)	83.0	88.0	75.1	77.6	80.5	72.7	85.2	83.6	83.7	86.8	91.3	88.6	87.7
B2 (LC036580)	85.8	83.1	85.6	84.3	87.2	86.2	84.4	84.2	84.8	86.8	91.3	89.9	87.3
C (LR216006)	47.2	-	58.9	60.3	63.4	55.3	-	48.1	58.9	-	-	62.1	60.6

BooV, bovine boosepivirus; -, less than 25%.

Description of diarrheic samples

Seventy diarrheic fecal samples were collected from Hanwoo (*Bos taurus coreanae*, Korean indigenous cattle) calves aged less than 60 days from 13 Hanwoo farms in the ROK. The farms are located in four provinces: Gyeonggi-do (Anseong), Chungcheongnam-do (Dangjin, Yesan, Gongju, and Cheongyang), Jeollabuk-do (Buan, Jeongeup, and Gimje), and Gyeongsangnam-do (Sancheong).

Results of analysis of infectious pathogens associated with NCD

The detection rate of each pathogen associated with NCD is presented in **Table 3**. BooV (25/70, 35.7%) was the most detected, followed by BRV (13/70, 18.6%), BVDV 2 (4/70, 5.7%), BCV (3/70, 4.3%), and BVDV1 (1/70, 1.4%). *C. parvum*, *Giardia* spp., and *Eimeria* spp. were not detected. At the farm level, BooV was the most prevalent, detected in 10 out the 13 farms (76.9%). This was followed by BRV, which was found on eight farms (61.5%). BVDV2 was detected on three farms (23.1%), BCV was present on two farms (15.4%), and BVDV1 was identified in only one farm (7.7%).

To determine the relationship between BooV and other pathogens, the co-infection rates of BooV and other pathogens were analyzed (**Table 4**). Of the 25 BooV-positive fecal samples, 20 (80.0%) were infected with BooV alone, 4 (16.0%) were co-infected with BRV, and 1 (4.0%) was co-infected with BVDV2.

Table 3. Detection frequency of pathogens associated with neonatal calf diarrhea from 70 Hanwoo calves from 13 farms in the Republic of Korea

Pathogens	Number of positive calves (positive rates, %)	Number of positive farms (positive rates, %)
Bovine rotavirus	13 (18.6)	8 (61.5)
Bovine coronavirus	3 (4.3)	2 (15.4)
Bovine boosepivirus	25 (35.7)	10 (76.9)
Bovine viral diarrhea virus type 1	1 (1.4)	1 (7.7)
Bovine viral diarrhea virus type 2	4 (5.7)	3 (23.1)
C. parvum	0 (0.0)	0 (0.0)
Giardia spp.	0 (0.0)	0 (0.0)
Eimeria spp.	0 (0.0)	0 (0.0)

Table 4. Pathogens co-infected with BooV from 70 Hanwoo calves in the Republic of Korea

Pathogens	Number of positive calves	Prevalence (%)
BooV	20	80
BooV+BRV	4	16
BooV+BVDV2	1	4
Total	25	100

BooV, bovine boosepivirus; BRV, bovine rotavirus; BVDV2, bovine viral diarrhea virus type 2.

Phylogenetic analysis of BooV

Twenty-five BooV-positive amplicons were obtained to determine the molecular characteristics of BooV in Hanwoo calves. All sequences were submitted to the GenBank database under accession numbers OR467506–OR467530. All sequences in this study were compared with those previously reported for BooV A, BooV B, and BooV C. These sequences showed 83%–100% homology with our isolates. The phylogenetic analyses showed that all isolates in the present study belonged to BooV B (**Fig. 2**).

DISCUSSION

Conventional methods for identifying pathogens in clinical samples often require prior knowledge about possible infectious agents, making diagnosis difficult without such
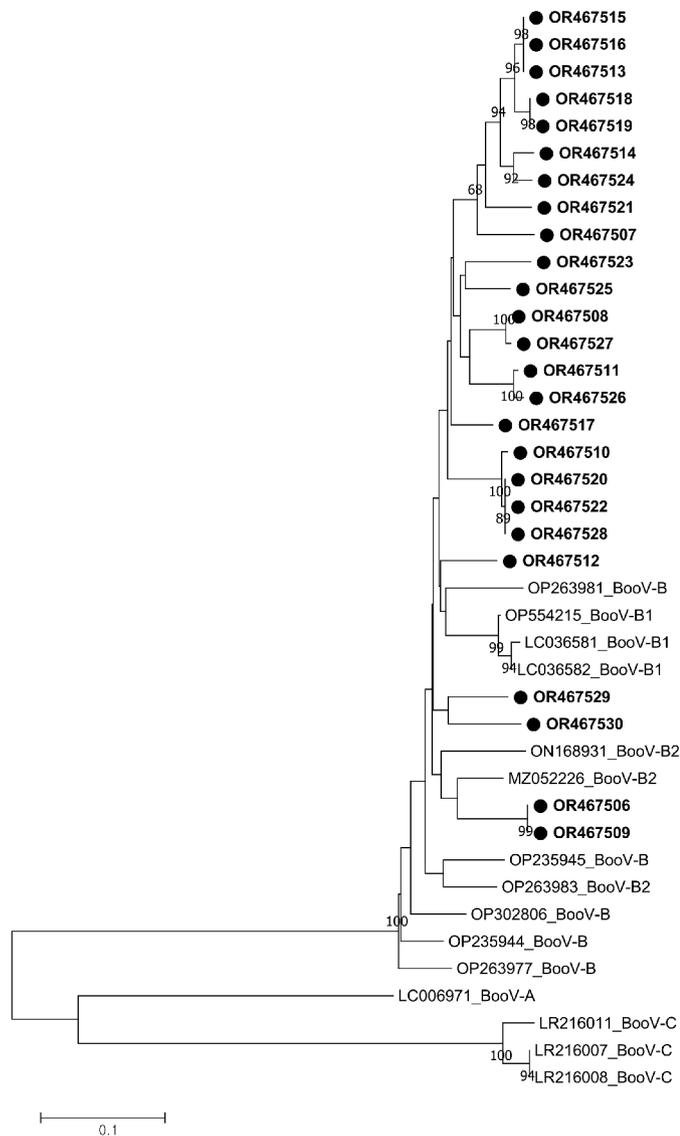

Fig. 2. Phylogenetic analysis based on partial sequences of the 5′ UTR and L segment of *Boosepivirus*es. Phylogenetic trees were constructed using the maximum likelihood method with 1,000 bootstrap replicates in the MEGA X software. Black circles represent the Boosepivirus isolates identified in this study.

information. Recently, the metagenomic approach has become a crucial technology for overcoming this obstacle. While there are several challenges, including the high cost of next generation sequencing (NGS) and the possibility of errors during experiments and data analysis, numerous studies have demonstrated the effectiveness of NGS as a novel approach for detecting previously unidentified pathogens. In this study, we found a complete genome sequence of BooV using NGS from the sample that were initially considered non-infectious because the causative agents were not identified. We examined the prevalence of BooV in the ROK and its co-infection with other pathogens (BRV, BCV, BVDV1, BVDV2, *C. parvum*, *Giardia* spp., and *Eimeria* spp.) related to NCD and performed genetic characterization of BooV.

In the present metagenomics analysis, a complete genome sequence of BooV was identified in diarrheic feces. BooV has been associated with diarrhea in cows and calves, and BooV B has been reported only in cows, consistent with the findings of the present study, where the detected isolate was identified as BooV B. To identify the subfamily and genotype of BooV B, further typing of BooV B was performed through phylogenetic analysis based on the 3CD and P1 coding genes, which have been recently proposed to distinguish subfamilies and genotypes of picornaviruses [17]. As a result, the BooV isolates in this study were identified as BooV B2. According to the classification of the International Committee on Taxonomy of Viruses, BooV is currently delineated into two subtypes, BooV B1 and B2. However, our study, leveraging previously reported sequences from China and the United States, revealed a distinct cluster separate from BooV B2 for the differentiation of subfamily and genotype. Notably, the sequence divergence of 3CD between reported BooV B1 (NC_076038) and BooV B2 (LC036580) is approximately 90%. Additionally, BooV B sequences reported in the United States (OK247513) and China (ON148337 and ON148337) exhibit 86%–88% differences from both BooV B1 and B2, forming a separate cluster. Hence, they were designated as BooV B3.

In the present analysis of identification of the prevalence of pathogens causing calf diarrhea, BooV was the most frequently detected pathogen among the seven investigated, with an infection rate of 35.7%. Despite various reports on calf pathogens in the ROK, the high detection rate of BooV compared to that of other pathogens underscores its potential significance as a pathogen associated with NCD in the ROK [2,6,18]. NCD is a multifactorial disease; therefore, in this study, we investigated the co-infection of BooV with other known pathogens that cause calf diarrhea. We found co-infections of BooV with viruses such as BRV, BVDV1, and BVDV2, but most cases were found to be single infections of BooV. Despite the limited number of samples, the fact that most BooV-positive cases were single-infection cases implied that BooV plays a notable role in causing diarrhea in calves. Further research with larger samples is needed to confirm these initial findings and to fully understand the effects of BooV on NCD. However, not only single infection, co-infection with BRV, BVDV1, and BVDV2 were also observed in this study. Co-infections involving *Cryptosporidium* or bovine kobuvirus, distinct from the pathogens identified in our study, have also been reported in the United States [9,19]. These findings suggest that BooV in calves might interact synergistically with other enteric pathogens, which might result in increased complexity and severity of NCD. Unfortunately, we were unable to isolate these viruses to determine their pathogenicity. Therefore, further studies are required to determine the intricacies of their pathogenesis, fully understand the mechanisms driving the disease process, and assess the clinical implications of BooV infection. However, we could not distinguish the BooV B subfamily. The distinct sequences forming the same clusters in this study showed 84%–90% homology with the reference sequence of BooV B1 and 79%–86% homology with the reference sequence of BooV B2. The nucleotide homology of the target sequences of BooV B1 (LC036581, LC036582,

and OP554215) and BooV B2 (MZ052226, ON168931, and OP263983) was 88%–90%, which was higher than that observed in the separately clustered groups in this study. Considering that the virus has only been reported recently in very few countries [8-11], further research on the evolution of the virus and typing of its clusters is needed.

The family *Picornaviridae*, to which BooV also belongs, includes Aichi viruses B and D, enteroviruses E and F, and parechoviruses, which are associated with calf diarrhea, as well as bovine astrovirus, which can cause neurological and respiratory symptoms in cows [20-23]. Picornaviruses have highly variable tissue tropism and can easily cross interspecific barriers [10]. Furthermore, as spillover of BooV B has been reported in other animals such as sheep, additional research on other livestock species or symptoms in the ROK might be necessary [10].

In this study, the prevalence and molecular characteristics of seven pathogens associated with NCD, with special focus on BooV, were investigated in Hanwoo calves in the ROK. BooV was the most frequently detected pathogen in 70 Hanwoo calves, and co-infection with other pathogens was identified. According to the results of the phylogenetic analysis, all BooV isolates discovered in this study were classified under BooV. While certain isolates clustered with previously reported BooV types B1 and B2, others constituted separate clusters exclusively comprising isolates discovered in the present study. This is the first study to explore the prevalence and molecular characteristics of BooV in the ROK.

REFERENCES

1. Cho YI, Yoon KJ. An overview of calf diarrhea - infectious etiology, diagnosis, and intervention. *J Vet Sci*. 2014;15(1):1-17. **PUBMED** | **CROSSREF**

2. Lee SH, Kim HY, Choi EW, Kim D. Causative agents and epidemiology of diarrhea in Korean native calves. *J Vet Sci*. 2019;20(6):e64. **PUBMED** | **CROSSREF**

3. Wei X, Wang W, Dong Z, Cheng F, Zhou X, Li B, et al. Detection of infectious agents causing neonatal calf diarrhea on two large dairy farms in Yangxin County, Shandong Province, China. *Front Vet Sci*. 2021;7:589126. **PUBMED** | **CROSSREF**

4. Cho YI, Han JI, Wang C, Cooper V, Schwartz K, Engelken T, et al. Case-control study of microbiological etiology associated with calf diarrhea. *Vet Microbiol*. 2013;166(3-4):375-385. **PUBMED** | **CROSSREF**

5. Alkan F, Karayel İ, Catella C, Bodnar L, Lanave G, Bányai K, et al. Identification of a bovine enteric calicivirus, Kırklareli virus, distantly related to neboviruses, in calves with enteritis in Turkey. *J Clin Microbiol*. 2015;53(11):3614-3617. **PUBMED** | **CROSSREF**

6. Chae JB, Kim HC, Kang JG, Choi KS, Chae JS, Yu DH, et al. The prevalence of causative agents of calf diarrhea in Korean native calves. *J Anim Sci Technol*. 2021;63(4):864-871. **PUBMED** | **CROSSREF**

7. Nagai M, Omatsu T, Aoki H, Otomaru K, Uto T, Koizumi M, et al. Full genome analysis of bovine astrovirus from fecal samples of cattle in Japan: identification of possible interspecies transmission of bovine astrovirus. *Arch Virol*. 2015;160(10):2491-2501. **PUBMED** | **CROSSREF**

8. Nagai M, Omatsu T, Aoki H, Kaku Y, Belsham GJ, Haga K, et al. Identification and complete genome analysis of a novel bovine picornavirus in Japan. *Virus Res*. 2015;210:205-212. **PUBMED** | **CROSSREF**

9. Wang L, Lim A, Fredrickson R. Genomic characterization of a new bovine picornavirus (boosepivirus) in diarrheal cattle and detection in different states of the United States, 2019. *Transbound Emerg Dis*. 2022;69(5):3109-3114. **PUBMED** | **CROSSREF**

10. Ji C, Zhang Y, Feng Y, Zhang X, Ma J, Pan Z, et al. Systematic surveillance of an emerging picornavirus among cattle and sheep in China. *Microbiol Spectr*. 2023;11(3):e0504022. **PUBMED** | **CROSSREF**

11. Savard C, Wang L. Identification and genomic characterization of bovine *Boosepivirus* A in the United States and Canada. *Viruses*. 2024;16(2):307. **PUBMED** | **CROSSREF**

12. Kim EM, Cho HC, Shin SU, Park J, Choi KS. Prevalence and genetic characterization of bovine coronavirus identified from diarrheic pre-weaned native Korean calves from 2019 to 2021. *Infect Genet Evol*. 2022;100:105263. **PUBMED** | **CROSSREF**

13. Solberg OD, Hasing ME, Trueba G, Eisenberg JN. Characterization of novel VP7, VP4, and VP6 genotypes of a previously untypeable group A rotavirus. *Virology*. 2009;385(1):58-67. **PUBMED | CROSSREF**

14. Letellier C, Kerkhofs P. Real-time PCR for simultaneous detection and genotyping of bovine viral diarrhea virus. *J Virol Methods*. 2003;114(1):21-27. **PUBMED | CROSSREF**

15. Guy RA, Payment P, Krull UJ, Horgen PA. Real-time PCR for quantification of *Giardia* and *Cryptosporidium* in environmental water samples and sewage. *Appl Environ Microbiol*. 2003;69(9):5178-5185. **PUBMED | CROSSREF**

16. Thanthrige-Don N, Lung O, Furukawa-Stoffer T, Buchanan C, Joseph T, Godson DL, et al. A novel multiplex PCR-electronic microarray assay for rapid and simultaneous detection of bovine respiratory and enteric pathogens. *J Virol Methods*. 2018;261:51-62. **PUBMED | CROSSREF**

17. Zell R, Knowles NJ, Simmonds P. A proposed division of the family Picornaviridae into subfamilies based on phylogenetic relationships and functional genomic organization. *Arch Virol*. 2021;166(10):2927-2935. **PUBMED | CROSSREF**

18. Ryu JH, Shin SU, Choi KS. Molecular surveillance of viral pathogens associated with diarrhea in pre-weaned Korean native calves. *Trop Anim Health Prod*. 2020;52(4):1811-1820. **PUBMED | CROSSREF**

19. Hause BM, Nelson E, Christopher-Hennings J. Identification of boosepivirus B in U.S. calves. *Arch Virol*. 2021;166(11):3193-3197. **PUBMED | CROSSREF**

20. Zhu L, Xing Z, Gai X, Li S, San Z, Wang X. Identification of a novel enterovirus E isolates HY12 from cattle with severe respiratory and enteric diseases. *PLoS One*. 2014;9(5):e97730. **PUBMED | CROSSREF**

21. Wang L, Fredrickson R, Duncan M, Samuelson J, Hsiao SH. Bovine Kobuvirus in calves with diarrhea, United States. *Emerg Infect Dis*. 2020;26(1):176-178. **PUBMED | CROSSREF**

22. Oba M, Obinata S, Takemae H, Kazama K, Oguro M, Ito K, et al. Prevalence and genetic diversity in bovine parechovirus infecting Japanese cattle. *Arch Virol*. 2023;168(3):91. **PUBMED | CROSSREF**

23. Zhu Q, Li B, Sun D. Bovine astrovirus: a comprehensive review. *Viruses*. 2022;14(6):1217. **PUBMED | CROSSREF**