



Molecular surveillance of viral pathogens associated with diarrhea in pre-weaned Korean native calves

Ji-Hyoung Ryu^{1,2} · Seung-Uk Shin¹ · Kyoung-Seong Choi¹

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Abstract

Calf diarrhea causes severe economic losses in the cattle industry worldwide. This study investigated the prevalence of bovine coronavirus (BCoV), bovine norovirus (BNoV), bovine group A rotavirus (BoRVA), and bovine torovirus (BToV) in calves aged ≤ 60 days, regardless of diarrhea, across nine different regions in the Republic of Korea (ROK) and reported associations between these viruses and diarrhea. Fecal samples were collected rectally from 689 calves: normal ($n = 360$) and diarrheic ($n = 329$). BNoV (84/689, 12.2%) was the most prevalent regardless of diarrhea, followed by BCoV (37/689, 5.4%), BToV (15/689, 2.2%), and BoRVA (13/689, 1.9%). Although BCoV ($P = 0.032$) and BoRVA ($P = 0.007$) were significantly associated with diarrhea in pre-weaned calves, BNoV and BToV showed no association. Infection by the four pathogens had no relationship with calf age; BoRVA was detected only in calves aged < 30 days, but this finding was not statistically significant. Phylogenetic analysis revealed that BCoV isolates obtained in this study were distinct from the other known BCoVs, and all BNoV isolates belonged to GIII.2 genotype; genetic variations in BNoVs are present in the ROK. BoRVA isolates distributed in the ROK were assigned to G6P[5]. Within the P[5] genotype, our isolates were divided into two lineages: P[5]-III and P[5]-VIII. P[5]-VIII lineage was dominant in pre-weaned Korean native calves. Our BToV isolates were more closely related to a European isolate, B145, than to Japanese isolates. Here, BNoV was frequently identified in calves, suggesting its non-significant contribution to calf diarrhea, whereas BCoV and BoRVA were responsible for calf diarrhea in the ROK. Consequently, our results highlight the importance of rapid diagnosis against these viruses in calf diarrhea.

Keywords Calf diarrhea · Bovine coronavirus · Bovine norovirus · Bovine group a rotavirus · Bovine torovirus

Introduction

Calf diarrhea (CD) leads to severe economic losses in live-stock production worldwide owing to growth retardation and mortality of the affected young calves (Cho et al. 2013; Lyoo et al. 2018). CD can be attributed to multifactorial etiology, including various infectious agents as well as environmental and husbandry practices (Bendali et al. 1999; Meganck et al. 2015). Among numerous infectious agents causing diarrhea in

calves, bovine coronavirus (BCoV) and bovine rotavirus (BoRVA) are recognized as the most important viral pathogens (Athanasios et al. 1994). Moreover, bovine norovirus (BNoV) and bovine torovirus (BToV) are frequently associated with acute diarrhea in calves (Gebregiorgis and Tessema 2016; Mohamed et al. 2017).

BCoV is the causative agent for severe diarrhea in neonatal calves, winter dysentery (WD) in adult cattle, and respiratory diseases in cattle (Clark 1993; Heckert et al. 1990; Saif 1990). Enteric BCoV replicates in the epithelial cells of the gut and destroys the villi, thereby causing severe and often bloody diarrhea in calves; this could be life-threatening owing to the loss of electrolytes and malnutrition (Clark 1993). Disease in calves usually occurs within the first month of life. BCoV infection has a high morbidity but a low mortality. BCoV has four major structural proteins: nucleocapsid (N), membrane (M), hemagglutinin esterase (HE), and spike (S) (Spaan et al. 1988). BCoV is divided into three genotype groups: groups 1, 2, and 3 depending on N protein gene. Variations in the host

✉ Kyoung-Seong Choi
kschoi3@knu.ac.kr

¹ Department of Animal Science and Biotechnology, College of Ecology and Environmental Science, Kyungpook National University, Sangju 37224, Republic of Korea

² Present address: Foreign Animal Disease Division, Animal and Plant Quarantine Agency, Gimcheon 39660, Republic of Korea

range and tissue tropism of coronaviruses are attributed to the spike (S) glycoprotein (Bidokhti et al. 2012).

Noroviruses are known to cause epidemic and sporadic gastroenteritis in humans and animals (Ferragut et al. 2016). Phylogenetically, noroviruses are classified into six genogroups (GI–GVI) based on RNA-dependent RNA polymerase (RdRp) and capsid gene (Pourasgari et al. 2018). GI, GII, and GIV infect humans, whereas GIII and GV infect ruminants and rodents, respectively (Thomas et al. 2014). GVI is responsible for infections in canine (Vinje 2015). BNoVs are divided into two distinct genotypes: GIII.1 and GIII.2. Both subtypes have increasingly been detected in cattle with enteric or respiratory diseases worldwide (Turan et al. 2018). BNoV was initially identified in diarrheic calves aged < 7 days (Ando et al. 2000; Gunther and Otto 1987), but BNoV was subsequently found in the feces of both diarrheic and healthy animals (Di Martino et al. 2014; van der Poel et al. 2003).

Group A rotavirus (RVA), a member of the *Reoviridae* family, is a major pathogen associated with severe, acute gastroenteritis in various host species worldwide (Manuja et al. 2008). RVAs have 11 genome segments of double-stranded RNA encoding six structural viral proteins (VP1–VP4, VP6, and VP7) and five or six non-structural proteins (NSP1–NSP6) (Estes and Cohen 1989; Pesavento et al. 2006). RVAs were classified into G (for glycoprotein) and P (for protease-sensitive) genotypes based on the two outer capsid proteins, VP7 and VP4, respectively (Matthijnssens et al. 2011). To date, based on genetic characterization, 32 G and 47 P genotypes have been identified in humans and animals (Dian et al. 2017). Among those, G6, G8, G10, P1, P5, and P11 were associated with most cases of diarrhea in cattle (Midgley et al. 2012).

Torovirus (ToV) is a genus belonging to the family *Coronaviridae*, order *Nidovirales* (Ito et al. 2016). ToVs cause enteritis and respiratory diseases and have been identified in humans, horses, cattle, and swine worldwide. BToV, formerly called Breda virus, was first detected in the USA during an outbreak of diarrhea in calves (Woode et al. 1982). Since then, BToV has been established as a causative agent of diarrhea in cattle and was identified in diarrheic calves across several countries (Gulacti et al. 2014; Kirisawa et al. 2007; Park et al. 2008). The prevalence of BToV has been detected in 2.9–36.4% of fecal samples obtained from diarrheic calves (Duckmanton et al. 1998; Park et al. 2008).

Although several studies have been conducted on the detection of viral pathogens associated with CD in the Republic of Korea (ROK) (Park et al. 2018; Park et al. 2007a; Park et al. 2011; Park et al. 2007b; Park et al. 2008; Ryu and Choi 2019), there is little available information about the association between diarrhea and viral pathogens. The objective of the present study was to investigate the prevalence of BCoV, BNoV, BoRVA, and BToV in calves aged 1–60 days with or without

diarrhea and the relationships between calf age and viral pathogens as well as to report any associations between these pathogens and diarrhea in calves.

Materials and methods

Sample collection

A total of 689 fecal samples were collected between March 2017 and October 2018 from pre-weaned Korean native calves for < 61 days. A veterinarian collected fecal samples rectally from individual calves. Depending on fecal condition, the obtained fecal samples were classified into normal ($n = 360$) and diarrheic ($n = 329$). Fecal samples were categorized according to age: 1–10 days ($n = 140$), 11–20 days ($n = 197$), 21–30 days ($n = 129$), 31–40 days ($n = 89$), 41–50 days ($n = 79$), and 51–60 days ($n = 55$). All samples were obtained from nine regions in the ROK. All fecal samples were placed in a 50-mL conical tube and subsequently transferred on ice in the Animal Immunology Laboratory at Kyungpook National University.

RT-PCR and sequencing

Total RNA was extracted from 200 μ L of fecal suspension using RNAisoPlus Reagent (Takara, Shiga, Japan) according to the manufacturer's instructions. RT-PCR was performed to amplify BCoV, BNoV, BoRVA, and BToV using the DiaStar One-Step RT-PCR Smart Mix (Solgent, ROK). PCR reaction volume was 20 μ L (10 μ L of $2 \times$ buffer, 1 μ L forward primer, 1 μ L reverse primer, 2 μ L of extracted RNA, and 6 μ L of RNase-free water). In negative controls, 8 μ L of RNase-free water without RNA was added. Nested-PCR assay for BToV was performed to increase sensitivity and specificity of amplification. Briefly, 2 μ L of RT-PCR product was subjected to nested PCR. PCR mixture consisted of 10 μ L of $2 \times$ EmeraldAmp GT Master Mix (Takara), 1 μ L of the nested-PCR forward primer, 1 μ L of the nested-PCR reverse primer, and 6 μ L DW. Primers used in this study are listed in Table 1. In case of BoRVA, RNA was denatured at 95 $^{\circ}$ C for 3 min and quenched on ice. Reverse transcription was conducted at 50 $^{\circ}$ C for 30 min; PCR activation was performed at 95 $^{\circ}$ C for 10–15 min followed by annealing at an appropriate temperature and time (Table 1); subsequently, extension was carried out at 72 $^{\circ}$ C for 1 min. PCR products of nested-PCR assay were separated by gel electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide. PCR products were purified using AccuPrep PCR Purification Kit (Bioneer, Daejeon, ROK) and subjected to direct sequencing (Macrogen, Daejeon, ROK).

Table 1 Information of primers used in this study

Virus	Target gene	Sequence (5'-3')	Amplicon size (bp)	Annealing temp (°C)	Reference
BCoV	Spike	TTGTAATTTTAATATGAGCAGCC TTCTGCCAACTATTATAATAAG	951	50	Tsunemitsu et al. (1999)
BNoV	RdRp	AGTTAYTTTTCCTTYTAYGGBGA AGTGTCTCTGTCAGTCATCTTCAT	532	51	Smiley et al. (2003)
BoRVA	VP7	GCCTTTAAAAGAGAGAATTTCCGTCT GG GGTCACATCATAACAATTCTAATCTAAG	1062	48	Isegawa et al. (1993)
	VP4	TTCATTATTGGGACGATTCACA CAACCGCAGCTGATATATCATC	863	48	
BToV	Spike	GTGTTAAGTTTGTGCAAAAAT TGCATGAAGCTATATGGTGT	741	55	Kirisawa et al. (2007)
		TGGATTAATTCAGGAGGTGCC CACTCTACATAGAGCGGTGTC	653		

BCoV: bovine coronavirus, BNoV: bovine norovirus, BoRVA: bovine group A rotavirus, BToV: bovine torovirus

Phylogenetic analysis

The obtained sequence data were applied to a Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database for homology analyses of BCoV, BNoV, BoRVA, and BToV genes. The sequence identities of all viruses were verified after comparing the sequences deposited in GenBank database using the BLAST software (<https://blast.ncbi.nlm.nih.gov/Blast>). The sequences were aligned using ClustalX (version 1.8) program. A phylogenetic tree was constructed using the neighbor-joining method based on nucleotide alignments. Bootstrap analysis was conducted with 1000 replicates using the MEGA version 6. Nucleotide sequences of BCoV, BNoV, BoRVA, and BToV obtained in this study were deposited in the GenBank database under the accession numbers MN650885–MN650894, MN156317–MN156366, MN650895–MN650906, and MN650907–MN650917, respectively.

Statistical analysis

The PCR results for each fecal sample were recorded either as positive or negative for each pathogen and categorized according to calf age (1–10, 11–20, 21–30, 31–40, 41–50, or 51–60 days) and fecal consistency (normal or diarrheic feces). The data were analyzed using the SPSS Statistics 25.0 software package for Windows (SPSS, Chicago, IL, USA). The associations between diarrhea and each pathogen were calculated for all ages together and for each age group separately using Pearson's chi-squared test or Fisher's exact test, as appropriate. *P* value of <0.05 was considered to be statistically significant.

Results

Prevalence of viral pathogens in pre-weaned Korean native calves

A total of 689 fecal samples collected from nine different regions were tested for BCoV, BNoV, BoRVA, and BToV using RT-PCR. BNoV was the most prevalent viral pathogen detected in 84 calves (12.2%) that originated from eight regions, followed by BCoV in 37 calves (5.4%) from eight regions, BToV in 15 calves (2.2%) from four regions, and BoRVA in 13 calves (1.9%) from three regions (Table 2). There were differences in viral pathogens detected according to regions. All viral pathogens were identified in the Gimje region; however, at least two viral pathogens were detected across the other seven regions, except in Anseong and Sangju regions wherein only one pathogen was found (Table 2).

The association between viral pathogens and fecal consistency (normal and diarrhea) was investigated (Table 3). Based on chi-squared analysis, BCoV was more frequently detected in diarrheic feces ($P = 0.032$) than in normal feces. The prevalence of BoRVA was significantly associated with diarrhea ($P = 0.007$). The detection rate of BoRVA was 5.5-fold higher in diarrheic calves than that in healthy calves. BNoV or BToV was detected in the feces of pre-weaned calves regardless of diarrhea. There was no statistically significant association between BNoV ($P = 0.796$) or BToV ($P = 0.662$) infection and diarrhea. As shown in Table 4, only four calves were co-infected with two viral pathogens, i.e., two calves were infected with BCoV + BNoV, one calf was infected with BCoV + BToV, and one calf was infected with BNoV + BToV. Co-infections with BCoV and BNoV were detected in one calf aged 31–40 days and in another calf aged 41–50 days. Co-infection with BCoV and BToV was detected in a diarrheic calf aged 1–

Table 2 Detection rate of viral pathogens in 689 fecal samples collected from nine different regions

Regions	BCoV	BNoV	BoRVA	BToV
Anseong (<i>n</i> = 39)	0	4 (10.3%)	0	0
Asan (<i>n</i> = 95)	5 (5.4%)	12 (12.7%)	1 (1.1%)	0
Geochang (<i>n</i> = 37)	2 (5.4%)	4 (10.8%)	0	1 (2.7%)
Gimje (<i>n</i> = 106)	6 (5.7%)	2 (1.9%)	9 (8.5%)	3 (2.8%)
Gyeongju (<i>n</i> = 6)	3 (33.3%)	1 (16.7%)	0	0
Mungyeong (<i>n</i> = 112)	3 (2.7%)	19 (15.6%)	0	9 (8.0%)
Samrye (<i>n</i> = 80)	4 (5.0%)	14 (17.5%)	3 (3.8%)	0
Sangju (<i>n</i> = 8)	1 (12.5%)	0	0	0
Yeoungju (<i>n</i> = 206)	14 (6.8%)	28 (13.6%)	0	2 (1.0%)
Total	37 (5.4%)	84 (12.2%)	13 (1.9%)	15 (2.2%)

BCoV: bovine coronavirus, BNoV: bovine norovirus, BoRVA: bovine group A rotavirus, BToV: bovine torovirus

10 days, whereas co-infection with BNoV and BToV was detected in a healthy calf aged 1–10 days. Co-infection with viral pathogens had no association with diarrhea.

Prevalence of viral pathogens depending on calf age

The differences in the detection rates of viral pathogens according to calf age were also investigated (Table 5). Of all the viral pathogens detected, BNoV infection was the highest in calves aged 1–10 days, which gradually decreased with age; BNoV had a high infection rate in all age groups of pre-weaned Korean native calves. BCoV infections showed an even infection rate, except during 1–10 days, but the overall infection rate of BCoV was not higher than that of BNoV. BoRVA infection was detected only up to the age of 30 days and was not observed in calves aged ≥ 30 days. In pre-weaned Korean native calves, BoRVA infection rate was the lowest compared with the infection rate of other viral pathogens. Although BToV infection rate was high at the age of 21–30 days, it was detected in only three calves aged ≥ 30 days. Overall, there were no statistically significant differences between calf age and viral pathogens detected in pre-weaned calves.

Table 3 Detection rate of viral pathogens according to fecal consistency

Pathogen	No. (%) of positives among diarrheic calves (<i>n</i> = 329)	No. (%) of positives among normal calves (<i>n</i> = 360)	<i>P</i> value	Total (<i>n</i> = 689)
BCoV	24 (7.3%)	13 (3.6%)	0.032	37 (5.4%)
BNoV	39 (11.9%)	45 (12.5%)	0.796	84 (12.2%)
BoRVA	11 (3.3%)	2 (0.6%)	0.007	13 (1.9%)
BToV	8 (2.4%)	7 (1.9%)	0.662	15 (2.2%)

BCoV: bovine coronavirus, BNoV: bovine norovirus, BoRVA: bovine group A rotavirus, BToV: bovine torovirus

Table 4 Co-infections with two viral pathogens in 689 fecal samples

Variables	BCoV + BNoV	BCoV + BToV	BNoV + BToV
Fecal consistency			
Diarrhea	1	1	0
Normal	1	0	1
Age (days)			
1–10	0	1	1
11–20	0	0	0
21–30	0	0	0
31–40	1	0	0
41–50	1	0	0
51–60	0	0	0
Total	2	1	1

BCoV: bovine coronavirus, BNoV: bovine norovirus, BoRVA: bovine group A rotavirus, BToV: bovine torovirus

Phylogenetic analysis of BCoV, BNoV, BoRVA, and BToV

Of the 37 BCoV-positive amplicons, 10 good sequences were obtained and compared with the sequences of BCoV strains/isolates, including CD, winter dysentery (WD) as well as of respiratory BCoV (RBCoV), enteric BCoV (EBCoV), and avirulent strains. These 10 sequences showed 89.1–99.8% homology with each other. Phylogenetically, the nucleotide sequence of the S gene revealed no characteristic pattern of relatedness between all our isolates and the other known BCoVs (Fig. 1). Our isolates were distinct from the Korean CD and WD strains, but the HM573327 and MG518515 isolates identified from wild animals were included in the same cluster (Fig. 1).

All 84 amplicons of BNoV were sequenced, and 50 good sequences were obtained. These BNoV isolates shared 90.2–99.1% nucleotide sequence identity with each other. Nucleotide sequences detected in each farm were mostly identical, and we also included slightly different sequences depending on the region in our phylogenetic tree. Based on the partial RdRp region, all our isolates belonged to GIII.2 genotype and formed a separate clade from that of the previously detected Korean isolates (Fig. 2). The results demonstrated

Table 5 Prevalence of BCoV, BNoV, BoRVA, and BToV infections according to age group of calves

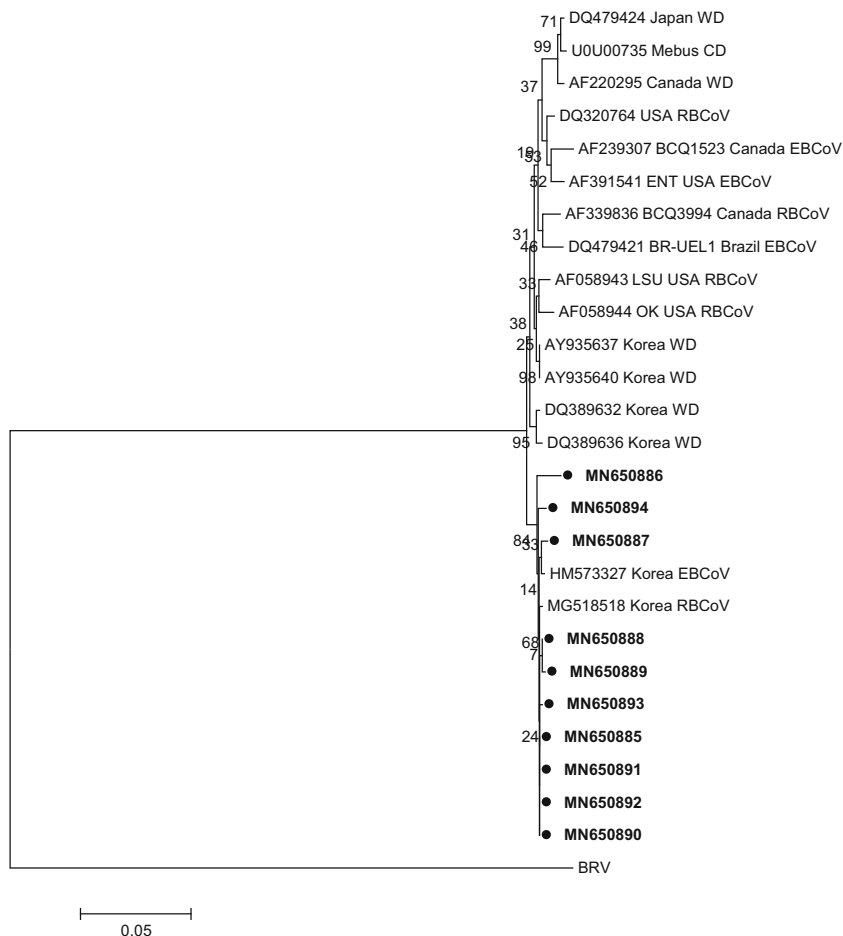
Age (days)	BCoV	BNoV	BoRVA	BToV
1–10 (<i>n</i> = 140)	10 (7.1%)	25 (17.9%)	5 (3.6%)	5 (3.6%)
11–20 (<i>n</i> = 197)	9 (4.6%)	21 (10.7%)	5 (2.5%)	1 (0.5%)
21–30 days (<i>n</i> = 129)	7 (5.4%)	16 (12.4%)	3 (2.3%)	6 (4.7%)
31–40 days (<i>n</i> = 89)	5 (5.6%)	10 (11.2%)	0	1 (1.1%)
41–50 days (<i>n</i> = 79)	4 (5.1%)	9 (11.4%)	0	1 (1.3%)
51–60 days (<i>n</i> = 55)	2 (3.6%)	3 (5.5%)	0	1 (1.8%)
<i>P</i> value	0.917	0.214	0.219	0.137
Total	37 (5.4%)	84 (12.2%)	13 (1.9%)	15 (2.2%)

BCoV: bovine coronavirus, BNoV: bovine norovirus, BoRVA: bovine group A rotavirus, BToV: bovine torovirus

that genetic variations exist within BNoVs prevalent in the ROK. Our isolates were divergent from Newbury2/1976/UK and Dumfries/1994/UK.

All 13 positive samples were sequenced, and 12 BoRVA isolates were obtained. The VP7 gene of our isolates belonged to G6 genotype (data not shown). A phylogenetic analysis revealed that the VP4 gene segment of 12 isolates was

Fig. 1 Phylogenetic tree of the S gene of CD, EBCoV, RBCoV, avirulent vaccine strains, Korean CD and WD strains, and BCoV strains/isolates obtained in this study. The phylogenetic tree was constructed using the neighbor-joining method in MEGA6. Bootstrap values are shown at branch nodes. The Korean isolates identified in this study are indicated by a circle symbol and in bold



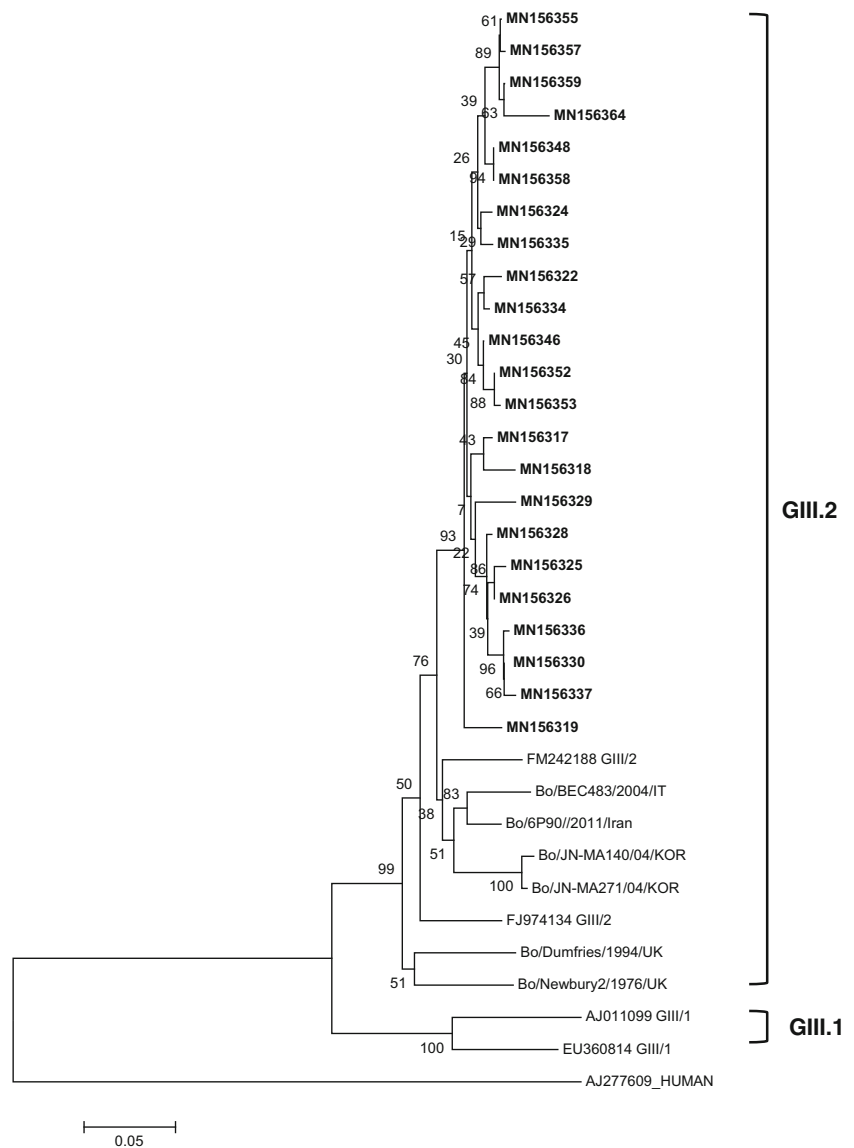
clustered in the P[5] genotype (Fig. 3). The combined G and P genotype of BoRVA isolates, which were distributed in the ROK regardless of diarrhea, was identified as G6P[5]. Within the P[5] genotype, our isolates were divided into two clades. While 11 P[5] isolates grouped together with the strains of the P[5]-VIII lineage, one other isolate belonged to the P[5]-III lineage in the phylogenetic tree (Fig. 3).

Of the 15 amplicons, 11 BToV isolates were sequenced and compared with other BToV isolates/strains. Our isolates showed 98.3–99.8% identity with each other. Phylogenetic analysis based on the partial S gene revealed that BToVs were divided into three clusters. All our BToV isolates clustered with B145 formed a separate cluster with Japanese isolates, and diverged from Croatian BToV isolates. BNoV isolates belonging to each of the three clusters were distant from the Breda 1 strain (Fig. 4).

Discussion

In this study, we investigated the prevalence of BCoV, BNoV, BoRVA, and BToV infections regardless of diarrhea in pre-weaned Korean native calves from nine different regions in

Fig. 2 Phylogenetic tree constructed based on a portion of the RdRp region (532 bp) of BNoVs using the neighbor-joining method in MEGA6. Bootstrap values are shown at branch nodes. The Korean isolates identified in this study are indicated by a circle symbol and in bold

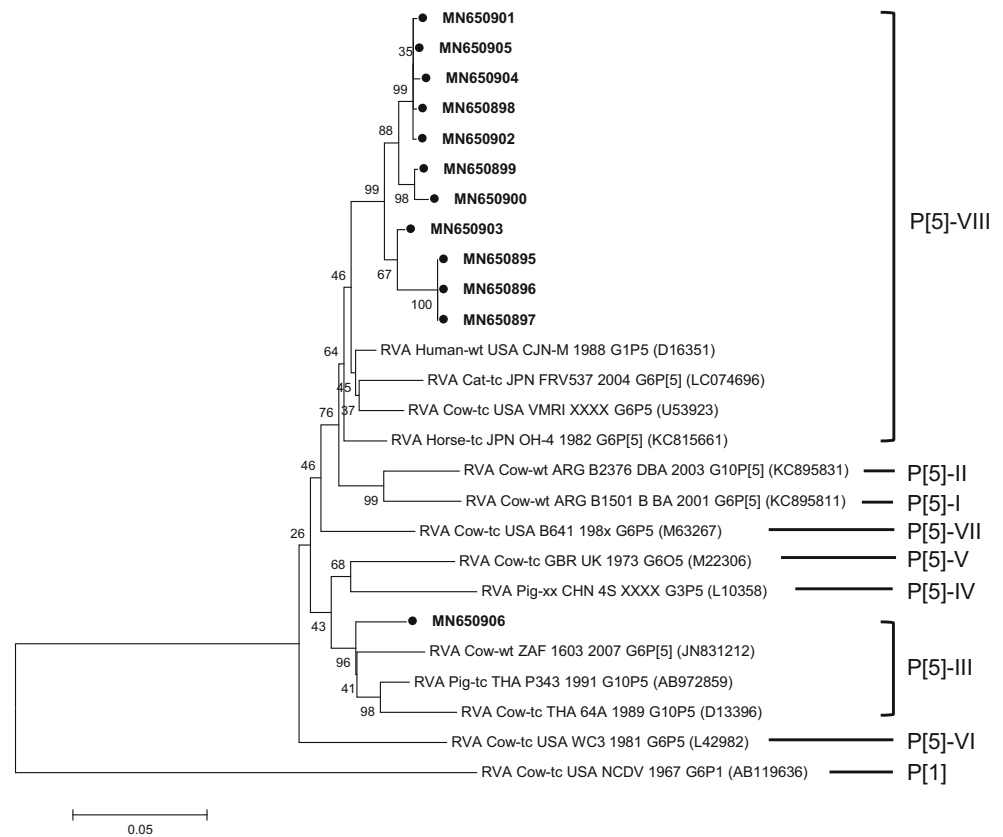


the ROK and performed genetic characterization of the identified viral pathogens. In our samples, BNoV was the most frequently detected pathogen; while BNoV and BToV were detected regardless of diarrhea, BCoV and BoRVA were mainly detected in diarrheic feces, suggesting an association with diarrhea. Moreover, the identified viral pathogens had no relationship with calf age. Our present finding that BCoV infection was associated with diarrhea was consistent with the result of a previous study conducted by our group (Park et al. 2018). However, the results of the present study did not reveal whether BCoV infection causes diarrhea in calves of only a certain age. Additionally, a possibility of the presence of other pathogens, including other bacteria, viruses, and protozoa, could not be ruled out in the fecal samples in which the abovementioned viral pathogens were not detected.

BCoV is considered a major viral pathogen causing CD (Torres-Medina et al. 1985). Here, BCoV was the second most

prevalent viral pathogen detected in 5.4% of all fecal samples. BCoV infections were found in eight of the surveyed regions, reflecting a broad distribution of BCoV among pre-weaned Korean native calves. Although BCoV is more prevalent in calves aged 1–10 days compared with the calves of other age groups, our results demonstrated no association between BCoV infection and calf age. Several studies have reported no association between BCoV infection and CD (Bartels et al. 2010; Uhde et al. 2008). However, the present study showed that BCoV infections were more commonly identified in diarrheic feces than in normal feces, indicating that BCoV was associated with diarrhea. A phylogenetic analysis based on the S gene revealed that BCoV isolates detected in this study diverged from CD and WD isolates reported previously in the ROK and were clustered into a separate branch. Interestingly, our isolates were more closely related to the isolates from wild animals. This suggests that genetic

Fig. 3 Phylogenetic tree of VP4 gene with a 741-bp amplicon of the P[5] genotype lineage, assigned from P[5]-I to P[5]-VIII. The tree was constructed using the neighbor-joining method in MEGA6. Bootstrap values are shown at branch nodes. The Korean isolates identified in this study are indicated by a circle symbol and in bold



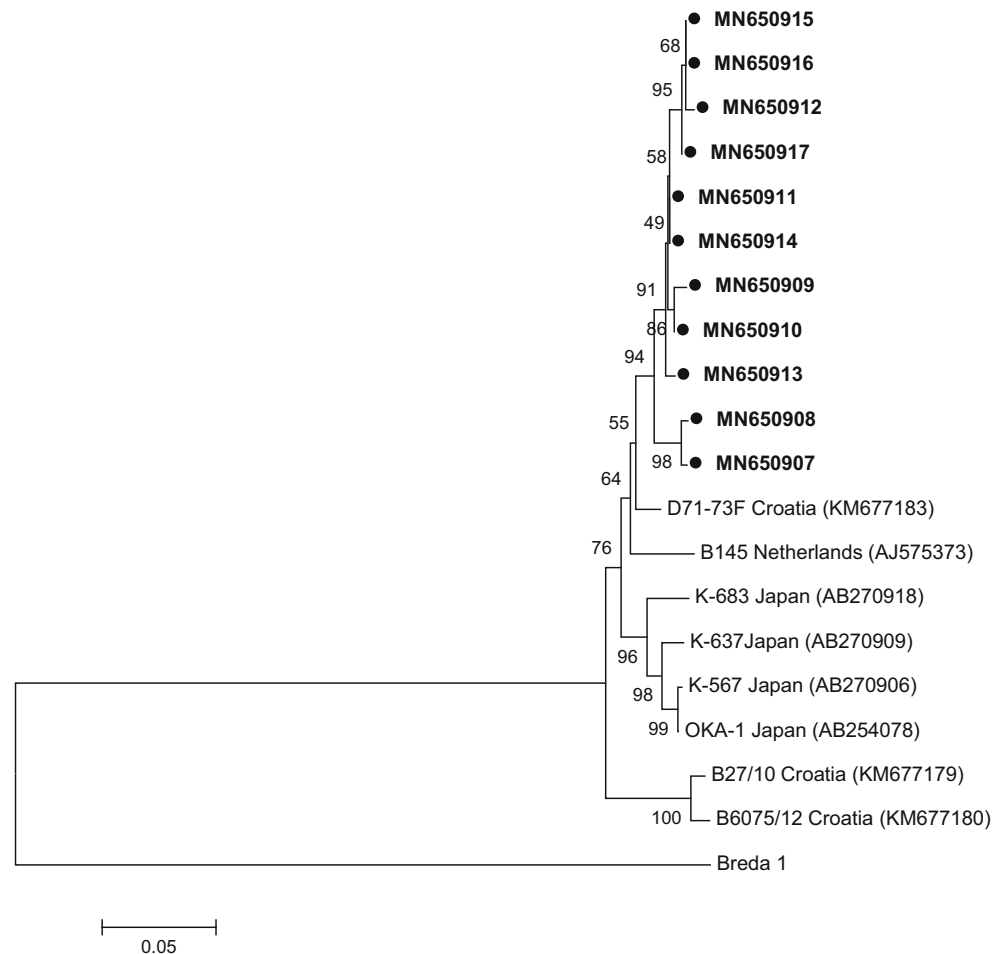
variations exist within BCoV isolates prevalent in the ROK. Therefore, the relationship between genetic variations and pathogenicity should be established in future studies.

In this study, BNoV was the most prevalent viral pathogen in pre-weaned Korean native calves. BNoV was detected in eight farms, barring one (Sangju). BNoV alone was identified in Anseong farm, which was cleaner and more spacious than the other farms (Table 2). It is speculated that norovirus is a zoonotic pathogen; thus, it is transmitted to calves through humans. According to our results, BNoV had an infection rate of $\geq 10\%$, except in the Gimje farm (Table 2). Our study reported a BNoV prevalence rate of 12.2%, which was higher than that reported previously in the ROK (Park et al. 2007a). A previous study reported that BNoV infection is endemic in diarrheic calves in the ROK (Park et al. 2007a), whereas our results revealed that BNoV was found regardless of diarrhea and that BNoV infection had no association with diarrhea. Consequently, we cannot make a definite conclusion that BNoV infection is a direct cause of CD. Moreover, BNoV prevalence was higher in calves aged 1–10 days and gradually decreased with age (Table 5). We found no correlation between BCoV and calf age. BNoV occurred as a co-infection with BCoV or BToV, but not with BoRVA. Owing to few co-infection samples, it was not possible to demonstrate that those viruses were associated with diarrhea. Based on the partial RdRp region, 50 BNoV isolates were classified into

GIII.2 genotype and were divergent from the previously reported Korean isolates (Fig. 2). The results suggest that GIII.2 is the dominant genotype of BNoV circulating in the ROK, with genetic variations occurring in this strain. Further studies are warranted to investigate the pathogenicity and pathogenesis of BNoV.

BoRVA is the primary cause of acute diarrhea in neonatal calves (Reynolds et al. 1986; Snodgrass 1986; Uhde et al. 2008). Here, the prevalence of BoRVA was considerably higher in diarrheic feces than that in normal feces. The present study revealed that BoRVA infection was significantly associated with diarrhea. BoRVA infection was observed only in calves aged < 30 days, indicating that BoRVA causes diarrhea in young animals. Here, the prevalence of BoRVA was very low, whether the infection rate of BoRVA is originally low or whether it varies according to different groups of rotaviruses, such as group B and group C, remains unclear to date. A previous study revealed that group C rotavirus caused diarrhea in Korean native calves (Park et al. 2011). Another possibility is that vaccination for BoRVA has been well implemented in the ROK, which has caused a reduction in BoRVA infection rate among calves. This reflects the importance of vaccination. Several reports have indicated that the most prevalent genotypes of BoRVA are G6, G8, and G10 in combination with P[1], P[5], P[7], and P[11], with G6P[5] being the most prevalent in cattle among several countries (Alfieri et al. 2004;

Fig. 4 A phylogenetic tree of the S gene of BToV strains/isolates and Korean isolates constructed using the neighbor-joining method in MEGA6. Bootstrap values were shown at branch nodes. The Korean isolates identified in this study are indicated by a circle symbol and in bold



Badaracco et al. 2013; Collins et al. 2014; Fukai et al. 2004; Park et al. 2011). All our isolates detected in this study were assigned to G6P[5]. Moreover, to our knowledge, the P[5]-III and P[5]-VIII lineages have been identified for the first time in the present study, and the P[5]-VIII lineage is dominant in pre-weaned Korean native calves. Molecular characterization of RVA strains prevalent among animals is of great importance in terms of both animal and public health. Although the prevalence of BoRVA was low in pre-weaned calves, BoRVA should be continuously surveyed to determine BoRVA genotypes circulating in Korean native calves because of interspecies transmission and reassortment events.

BToV was originally isolated from diarrheic calves (Lojkic et al. 2015) and is now distributed worldwide (Gulacti et al. 2014; Hoet et al. 2002; Koopmans et al. 1991; Park et al. 2008). Our results revealed that the prevalence of BToV was 2.2%, which was slightly lower than that previously reported in the ROK (Park et al. 2008). This could be attributed to the different sample collection regions and the different target genes used for detecting BToV as well as calf age. BToV infection was high in Mungyeong farm compared with the other farms. The pathogens detected varied according to the farms from which samples were collected (Table 2), likely

owing to the difference in hygiene levels and management systems in farms. BToV alone acts as a primary enteric pathogen in calves aged < 30 days (Aita et al. 2012). However, our result showed that BToV infection was more prevalent in calves aged ≤ 30 days, but this prevalence was not statistically significant ($P = 0.137$) and was also not associated with diarrhea ($P = 0.662$). Our findings were inconsistent with those of a previous study (Aita et al. 2012). Here, BToV was found only in co-infections with BCoV or BNoV (Table 4), and these co-infections did not cause diarrhea. To date, there is little available information regarding BToV infection causing diarrhea in the ROK. Phylogenetic analysis revealed that our isolates were more closely related to Japanese strains than to the Breda 1 strain (Fig. 4). Although BToV infection has been reported in the ROK, owing to differences in the target gene, we could not reveal genetic diversity between the isolates identified in this study and the previously reported Korean isolates. Further studies involving large-scale epidemiologic investigation of several genes are warranted to identify the prevalence of BToV and its association with diarrhea.

The results of this study revealed that BNoV is most frequently detected in pre-weaned Korean native calves. Of the viral pathogens examined, BCoV and BoRVA appear to be the

major viral pathogens contributing to CD in the ROK, and the prevalence of BCoV is higher than that of BoRVA. The prevalence of BCoV, BNoV, BoRVA, and BToV was not significantly associated with calf age. The association of BNoV and BToV with the pathogenesis of diarrhea in pre-weaned calves remains inconclusive. Therefore, monitoring and surveillance of BCoV and BoRVA in cattle populations are recommended for preventing and controlling diarrhea in calves.

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Compliance with ethical standards

This study was approved and performed in accordance with the ethics guidelines and procedures of the policy of the review to Kyungpook National University Animal Care and Use Committee. Each farmer also provided their written informed consent prior to inclusion in our study.

Conflict of interest The authors declare that they have no conflict of interest.

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