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 ScienceDirect

Veterinary Microbiology 130 (2008) 371–379

**veterinary
microbiology**

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Short communication

Molecular detection and characterization of unclassified bovine enteric caliciviruses in South Korea

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Received 26 October 2007; received in revised form 30 January 2008; accepted 31 January 2008

Abstract

The unclassified bovine enteric calicivirus (BEC) is a new bovine enteric calicivirus that is different from bovine norovirus, and causes diarrhea and pathologies in the small intestine of calves. This virus includes Nebraska (NB)- and Newbury agent 1 (NA1)-like strains. The prevalence of this BEC and its genetic characterization has only been reported in the UK and the USA. This study examined the prevalence and genetic diversity of these BECs in diarrheic calves in South Korea. Among a total of 645 diarrheic fecal specimens obtained from 629 cattle herds, these unclassified BECs were detected in 59 (9.1%) diarrheic fecal samples from 57 herds (9.3%) by either RT-PCR or nested PCR. Sequence and phylogenetic analyses of the partial RdRp gene showed that all the Korean BECs clustered together and were closely related to the NB-like viruses (80.9–88.1% nucleotide and 84.5–98.4% amino acid) but not to the NA1-like viruses (75.8–78.4% nucleotide and 79.7–82.8% amino acid). Although these viruses could not be classified into NA1- and NB-like viruses from the sequence and phylogenetic data of the entire capsid gene, all the Korean BECs clustered together on a branch separate from the other known BECs. These results show that these BEC infections are endemic in diarrheic calves in South Korea. The infecting strains are genetically closer to the NB-like viruses but have a distinct evolutionary pathway.

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Keywords: Unclassified bovine enteric calicivirus; Calves; Prevalence; Genetic diversity

1. Introduction

Caliciviruses are small, non-enveloped viruses that are 27–38 nm in diameter and possess a single-

stranded, plus-sense RNA genome of 7.3–8.3 kb in length and a major structural protein (Green et al., 2001; Sosnovtsev et al., 1998). The family *Caliciviridae* is classified into four genera: *Norovirus* (NoV), *Sapovirus* (SaV), *Vesivirus* and *Lagovirus* (Green et al., 2000) with a new proposed genus, Nebraska (NB)-like (Smiley et al., 2002). Caliciviruses have been identified in a number of animal species in

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association with respiratory, vesicular and hemorrhagic and enteric diseases. In humans, NoVs and SaVs have been associated with enteric diseases only, and are the most common cause of food- and water-borne, acute, non-bacterial gastroenteritis in humans worldwide (Green et al., 2001). Animal SaVs and NoVs also cause gastroenteritis in swine, calves and mink (Bridger, 1990; Guo et al., 2001; Saif et al., 1980).

In addition to bovine noroviruses (BNoV), another bovine enteric calicivirus, Newbury agent-1 (NA1), was reported to be associated with calf diarrhea in the UK in 1978 (Woode and Bridger, 1978). Electron microscopy, animal cross-protection experiments and solid-phase immune electron microscopy (Bridger et al., 1984; Dastjerdi et al., 1999; Woode and Bridger, 1978) revealed NA1 to be unrelated to the bovine enteric calicivirus, Newbury agent-2, which is the prototype BNoV. Genomic data were recently obtained from two virus strains, Bo/Nebraska/80/US and Bo/Newbury1/76/UK (Oliver et al., 2006; Smiley et al., 2002). Smiley et al. (2002) reported that the NB-like bovine enteric calicivirus is genetically most similar to SaVs and lagoviruses, and causes pathology in the small intestine of gnotobiotic calves. Oliver et al. (2006) showed that NB and NA1 viruses form a distinct clade that is independent of the 4 recognized genera. The evidence for classifying the NA1 and NB viruses into the same genus was their identical genome organization, identical composition and location of predicted ORF1 protease cleavage sites as well as the almost identical capsid proteins (Oliver et al., 2006). Until now, this potentially new genus, including NB- and NA1-like unclassified bovine enteric caliciviruses (BECs), has not been named by the International Committee on Viral Taxonomy. In this study, therefore, we use the name of these viruses as “unclassified BECs” with “NB- and NA1-like viruses” to designate the strains.

Determination of the antigenic relationship between genogroups and genotypes of enteric caliciviruses is difficult because most viruses, with the exception of Cowden porcine SaV, do not grow in a cell culture (Flynn and Saif, 1988; Han et al., 2004; Parwani et al., 1991). Therefore, molecular studies have relied upon the availability of stool samples collected during outbreaks or from human volunteers and experimental animal studies. Similar to the genomes of lagoviruses and SaVs, unclassified BECs have two predicted open

reading frames (ORFs) that encode non-structural and contiguous capsid proteins (ORF1), as well as a basic protein that is characteristic of the family *Caliciviridae* (Oliver et al., 2006; Smiley et al., 2002). The NA1 and NB viruses can be assigned into two separate genotypes, based on the genetic divergence in the RNA-dependent RNA polymerase (RdRp) gene (Oliver et al., 2006).

The prevalence of the unclassified BECs and its genetic characterization has only been reported in the UK (Oliver et al., 2006; Woode and Bridger, 1978) and the USA (Han et al., 2004; Smiley et al., 2002). Therefore, this study examined the prevalence and genetic diversity of unclassified BECs in diarrheic calves in South Korea.

2. Materials and methods

2.1. Specimens

Local veterinary clinicians sampled one or two diarrheic fecal samples from each farm and immediately submitted them to the College of Veterinary Medicine, Chonnam National University. Between January 2004 and December 2005, a total of 645 diarrheic fecal specimens were collected from 629 Korean native beef (Hanwoo) calf herds (aged 3–70 days) during the spring (407 samples/406 herds), summer (107 samples/98 herds), autumn (73 samples/69 herds) and winter (58 samples/56 herds). As the fecal samples had arrived, they were examined immediately for common enteric viral, bacterial and protozoan pathogens including unclassified BECs, groups A, B and C bovine rotaviruses (BRV A–C), bovine torovirus (BToV), bovine coronavirus (BCoV), BNoV, bovine viral diarrhea virus (BVDV), *Salmonella* spp., *Clostridium* spp., *Campylobacter* spp., shiga-toxin-producing *Escherichia coli*, *Coccidium* spp. and *Cryptosporidium* spp. (Asakura et al., 1998; Park et al., 2007a,b, 2008). The results of the BNoV, BToV and BCoV are reported elsewhere (Park et al., 2007a,b, 2008). Detailed data on the other enteric viruses will be reported elsewhere.

2.2. RNA extraction

For RNA extraction, fecal suspensions of each sample were prepared by diluting the feces 1:10 in

0.01 M phosphate-buffered saline, pH 7.2. The suspensions were then vortexed for 30 s and centrifuged ($1200 \times g$ for 20 min). The 200 μ l supernatant was used for RNA extraction using the Trizol-LS (Gibco-BRL, Life Tech, Grand Island, NY) procedure. The total RNA recovered was suspended in 50 μ l of RNase-free water and stored at -80°C until needed.

2.3. RT-PCR and nested PCR

The oligonucleotide primers as well as the RT-PCR and nested PCR conditions used to detect unclassified BEC, BToV, BCoV, BRV A-C, BNoV and BVDV are described elsewhere (Cho et al., 2001; Park et al., 2006, 2007a,b, 2008). As a negative control for all RT-PCR assays, RNA was extracted from the normal feces of colostrum-deprived calves that had been inoculated with 50 ml sterile PBS. In order to check for cross-contamination between RT-PCR and nested PCR, 5 μ l of the RT-PCR product with the RNA extracted from the normal mock-infected calves was used for all nested PCR assays. The amplification products were analyzed

by 1.5 or 2% agarose gel electrophoresis and visualized by irradiating the ethidium bromide stained samples with UV.

2.4. DNA sequencing

The RT-PCR products of a portion of the capsid gene (1692 bp), and the nested PCR products of a portion of the RdRp gene (194 bp) were selected from different test reactions and sequenced to confirm the specificity of the reaction as well as to obtain the genomic data for phylogenetic analysis. The RT-PCR products were purified using a GenClean II kit (BIO 101, Inc., La Jolla, CA) according to the manufacturer's instructions. DNA sequencing was carried out using an automated DNA sequencer (ABI system 3700, Applied Biosystem Inc., Foster City, CA).

2.5. Molecular analysis

Using the DNA Basic module (DNAsis MAX, Alameda, CA), the nucleotide (nt) and deduced amino

Table 1

Name and Genbank accession number of the reference unclassified bovine enteric caliciviruses (BEC) and other caliciviruses used in phylogenetic analysis

Viruses	Strains	Source	Viruses	Strains	Source
BEC	Bo/MA16/04/Korea	DQ984640	BEC	Bo/MA474/05/Korea	EF528566
BEC	Bo/MA56/04/Korea	DQ984641	BEC	Bo/MA567-1/05/Korea	EF528567
BEC	Bo/SA68/04/Korea	DQ984642	BEC	Bo/MA567-2/05/Korea	EF528568
BEC	Bo/MA91/04/Korea	DQ984643	BEC	Bo/MA729/05/Korea	EF528569
BEC	Bo/MA95/04/Korea	DQ984644	BEC	Bo/NB/80/US	AY082891
BEC	Bo/MA99/04/Korea	DQ984645	BEC	Bo/CV23-OH/02/US	AY082890
BEC	Bo/MA120/04/Korea	DQ984646	BEC	Bo/CV504-OH/02/US	AY549168
BEC	Bo/MA129/04/Korea	DQ984647	BEC	Bo/CV519-OH/02/US	AY549169
BEC	Bo/MA137/04/Korea	DQ984648	BEC	Bo/CV526-OH/02/US	AY549170
BEC	Bo/MA164/04/Korea	DQ984649	BEC	Bo/CV531-OH/02/US	AY549171
BEC	Bo/HP167/04/Korea	DQ984650	BEC	Bo/CV548-OH/02/US	AY549172
BEC	Bo/MA176/04/Korea	DQ984651	BEC	Bo/CV562-OH/02/US	AY549173
BEC	Bo/SA181/04/Korea	DQ984652	BEC	Bo/Newbury1/76/UK	DQ013304
BEC	Bo/MA216/04/Korea	DQ984653	BEC	Bo/PenrithC39/00/UK	DQ228162
BEC	Bo/MA236/04/Korea	DQ984654	BEC	Bo/Penrith142/00/UK	DQ228160
BEC	Bo/MA360/04/Korea	DQ984655	BEC	Bo/Penrith143/00/UK	DQ228161
BEC	Bo/MA39/04/Korea	EF528558	BEC	Bo/Penrith150/00/UK	DQ228157
BEC	Bo/SA63/04/Korea	EF528559	BEC	Bo/Penrith151/00/UK	DQ228158
BEC	Bo/MA271/04/Korea	EF528560	BEC	Bo/Starcross93/00/UK	DQ228165
BEC	Bo/MA274/04/Korea	EF528561	BEC	Bo/Starcross117/00/UK	DQ228164
BEC	Bo/MA278/04/Korea	EF528562	Norovirus	Bo/Newbury2/76/UK	AF097917
BEC	Bo/MA298/04/Korea	EF528563	Sapovirus	Hu/Sapporo/82/JIP	U65427
BEC	Bo/MA362/04/Korea	EF528564	Lagovirus	Ra/RHDV/GH/88/GE	M67473
BEC	Bo/MA415/05/Korea	EF528565	Vesivirus	Fe/FCV/F9/58/US	M86379

Table 2

Amino acid and nucleotide identity of the partial polymerase and the full length capsid genes of the Korean unclassified bovine enteric caliciviruses compared with Bo/Newbury1/1976/UK and Bo/NB/1980/US

Isolate	Nucleotide (nt) and amino acid (aa) identity to							
	Bo/Newbury1/1976/UK				Bo/NB/1980/US			
	Polymerase		Capsid		Polymerase		Capsid	
	nt	aa	nt	aa	nt	aa	nt	aa
Bo/MA16/04/KOR	78.4	82.8		NA	87.1	93.8		NA
Bo/MA56/04/KOR	78.4	82.8		NA	87.1	93.8		NA
Bo/SA68/04/KOR	77.8	81.3		NA	85.6	92.2		NA
Bo/MA91/04/KOR	77.8	82.8		NA	86.6	93.8		NA
Bo/MA95/04/KOR	78.4	82.8		NA	87.1	93.8		NA
Bo/MA99/04/KOR	77.3	79.7		NA	85.1	90.6		NA
Bo/MA120/04/KOR	76.8	81.3		NA	86.6	92.2		NA
Bo/MA129/04/KOR	77.8	81.3		NA	85.6	92.2		NA
Bo/MA137/04/KOR	76.8	81.3		NA	86.6	92.2		NA
Bo/MA164/04/KOR	77.3	81.3		NA	86.1	92.2		NA
Bo/HP167/04/KOR	78.4	82.8		NA	87.6	93.8		NA
Bo/MA176/04/KOR	77.8	81.3		NA	86.1	92.2		NA
Bo/SA181/04/KOR	78.4	82.8		NA	87.1	93.8		NA
Bo/MA216/04/KOR	78.4	82.8		NA	86.1	93.8		NA
Bo/MA236/04/KOR	77.3	81.3		NA	86.1	92.2		NA
Bo/MA360/04/KOR	77.8	81.3		NA	85.6	92.2		NA
Bo/MA39/04/KO		NA	86.1	92.3		NA	87	92.9
Bo/SA63/04/KOR		NA	86.7	93.3		NA	87.6	93.8
Bo/MA271/04/KOR		NA	86.5	93.1		NA	87.5	93.6
Bo/MA274/04/KOR		NA	85.7	92.7		NA	86.9	93.1
Bo/MA278/04/KOR		NA	86.2	93.1		NA	87.4	93.6
Bo/MA298/04/KOR		NA	86.1	93.3		NA	87.3	93.8
Bo/MA362/04/KOR		NA	86.1	93.3		NA	87.2	93.6
Bo/MA415/04/KOR		NA	86.3	93.3		NA	87.3	93.8
Bo/MA474/05/KOR		NA	85.9	93.3		NA	87	93.8
Bo/MA567-1/05/KOR		NA	86.3	92.9		NA	87.4	93.4
Bo/MA567-2/05/KOR		NA	86.2	92.7		NA	87.3	93.3
Bo/MA729/05/KOR		NA	85.8	92.3		NA	86.8	92.9
Bo/Newbury1/76/UK		NA	NA	81.4		84.4	92.9	98.9
Bo/Penrith142/00/UK	94.3	96.6		NA	79.4	84.4		NA
Bo/Penrith143/00/UK	93.8	96.6		NA	78.9	84.4		NA
Bo/Penrith150/00/UK	92.8	95.3		NA	77.8	82.8		NA
Bo/Penrith151/00/UK	93.3	96.9		NA	78.4	84.4		NA
Bo/Starcross93/00/UK	80.9	84.4		NA	88.1	95.3		NA
Bo/Starcross117/00/UK	93.3	96.9		NA	78.4	84.4		NA
Bo/NB/80/US	81.4	84.4	92.9	98.9		NA		NA
Bo/CV23-OH/02/US	79.9	82.8		NA	85.6	92.2		NA
Bo/CV504-OH/02/US	NA		89.8	98	NA		92.3	98.5
Bo/CV519-OH/02/US	NA		86.9	93.8	NA		87.8	93.4
Bo/CV526-OH/02/US	NA		89.8	98	NA		92.2	98.5
Bo/CV531-OH/02/US	NA		89.9	98.4	NA		92	98.5
Bo/CV548-OH/02/US	NA		89.3	98.2	NA		91.4	98.7
Bo/CV562-OH/02/US	NA		89.6	98.4	NA		91.7	98.7

NA, genomic sequence for region not available.

acid (aa) sequences of the capsid gene and a portion of the RdRp gene were compared with those selected from other known caliciviruses (Table 1). The variation in the nt sequences within the capsid gene was analyzed using the sliding-window genetic diversity plot (SimPlot, version 3.5.1; <http://sray.med.som.jhmi.edu/RaySoft>).

Phylogenetic and bootstrap (1000 replicates) analyses based on the nt sequences of the capsid gene (1650 bp without primer sequence) and a portion of the RdRp gene (154 bp without primer sequence) was carried out using the neighbor-joining method and the unweighted-pair group method of Molecular Evolutionary Genetics analysis (MEGA version 3.1) with a pairwise distance (Kumar et al., 2004). A sequence similarity search was performed for the unclassified BEC capsid and RdRp genes using the LALIGN Query program of the GENESTREAM network server at Institut de Génétique Humaine, Montpellier, FRANCE (<http://www.eng.uiowa.edu/~tscheetz/sequence-analysis/examples/LALIGN/lalign-guess.html>).

3. Results and discussion

A total of 645 diarrheic fecal samples from 629 calf herds were screened by RT-PCR and nested PCR. Nine positive fecal samples from 9 herds were detected using the 1-step RT-PCR assay, which targeted a 594 bp fragment of the RdRp region in the unclassified BECs. Fifty-nine fecal samples (9.2%) from 57 herds (9.3%) were found positive by the nested PCR assay, which targeted a 194 bp fragment of the RdRp region. Of the 59 unclassified BECs-positive fecal specimens, 19 fecal samples (2.9%) from 18 herds (2.8%) tested positive to this virus alone. The other 40 fecal samples (6.2%) from the 39 herds (6.2%) also tested positive to other enteric pathogens including BRV A-C, BCoV, BNoV, BVDV, BToV and *E. coli*. In addition, 402 fecal specimens from the 401 herds that tested negative for unclassified BECs tested positive for other enteric pathogens. No enteric pathogens were detected in the 184 fecal samples from 171 herds. The reported fecal prevalence of the BECs in calf diarrhea ranged from 8.4% in the UK (Oliver et al., 2006) to 28.0% in the US (Smiley et al., 2003). These results suggest that BEC infections are endemic in diarrheic calves in South

Korea in a similar manner to the prevalence of BEC infections in the UK (Oliver et al., 2006).

Seasonally, BEC infections were less prevalent in diarrheic fecal samples of calves obtained in spring than in the other seasons; 30 (7.4%) out of 407 fecal samples [13 (6.6%) out of 198 samples in 2004 and 17 (8.1%) out of 209 samples in 2005] tested positive in spring; 14 (13.1%) out of 107 fecal samples [6 (11.5%) out of 52 samples in 2004 and 8 (14.5%) out of 55 samples in 2005] tested positive in summer; 8 (11.0%) out of 73 fecal samples [4 (10.5%) out of 38 samples in 2004 and 4 (11.4%) out of 35 samples in 2005] tested positive in autumn; and 8 (13.8%) out of 58 fecal samples [4 (12.9%) out of 31 samples in 2004 and 3 (11.1%) out of 27 samples in 2005] tested positive in winter. To our knowledge, there is no report showing a clear seasonal distribution of BEC infections in calves. Therefore, more epidemiological studies throughout the world will be needed to properly understand the seasonal pattern of

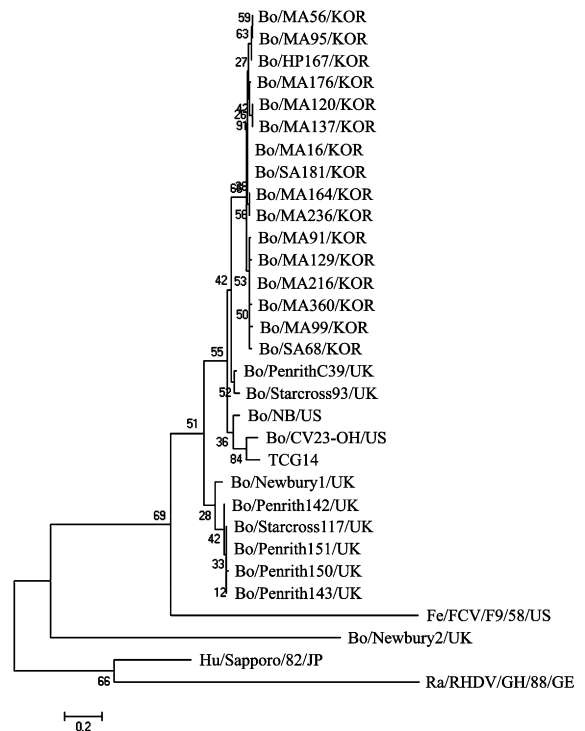


Fig. 1. The phylogenetic trees of the nucleotide sequences of the partial RdRp gene of caliciviruses were constructed using the neighbor-joining method of Molecular Evolutionary Genetics Analysis (Kumar et al., 2004). The names of the viruses used are listed in Table 1.

BEC infections and to establish BEC surveillance programs to prevent BEC infections.

Molecular analysis was performed by sequencing 194 nt of the nt 5698 to nt 6454 region of the RdRp gene from 16 BECs, which were sequenced from the 59 positive samples for the RdRp region by nested PCR. The paired comparisons of these viruses revealed the nt and aa sequences of the Korean viruses RdRp gene to be more homologous to each other (96.4–100% nt and 95.3–100% aa) and more closely related to the NB-like viruses (80.9–88.1% nt and 84.5–98.4% aa) than to the NA1-like viruses (75.8–78.4% nt and 79.7–82.8% aa) (Table 2). Phylogenetic analysis of the nt sequences of the partial RdRp gene from the unclassified BECs also revealed the 16 Korean RdRp gene to cluster together and closely in a branch with the NB-like strains, particularly with the PenrithC39 and Starcross93 strains (Fig. 1). The NA1-like viruses including the NA1, Penrith142, Starcross117, Penrith151, Penrith143 and Penrith150 strains clustered on a separate major branch (Fig. 1). These results suggest that the Korean BECs are closely related to NB-like but

distantly related to the NA1-like viruses. A previous study reported that unclassified BECs can be divided into two potential genotypes, NA1-like and NB-like, according to phylogenetic analysis of the RdRp gene (Oliver et al., 2006). It was reported that both genotypes are circulating in the UK but the US has only NB-like viruses (Oliver et al., 2006). The genetic similarity of the Korean BECs to NB-like viruses from the US can be explained by the fact that from the 1960s, South Korea imports live cows mainly from countries in North American and Oceania, not from European countries. However, the possibility of NA1-like virus infections in South Korea, even with a very low infection status, cannot be excluded. Therefore, continuous monitoring and intensive study of the BECs with a larger number of bovine fecal samples will be needed.

The RT-PCR assay with the primer pair specific to the whole capsid gene (1692 nt of the nt 5035 to nt 6726) carried out using the 59 fecal samples testing positive to the RdRp gene by nested PCR. Among the positive amplicons, 12 amplicons that had been amplified strongly by RT-PCR were selected and

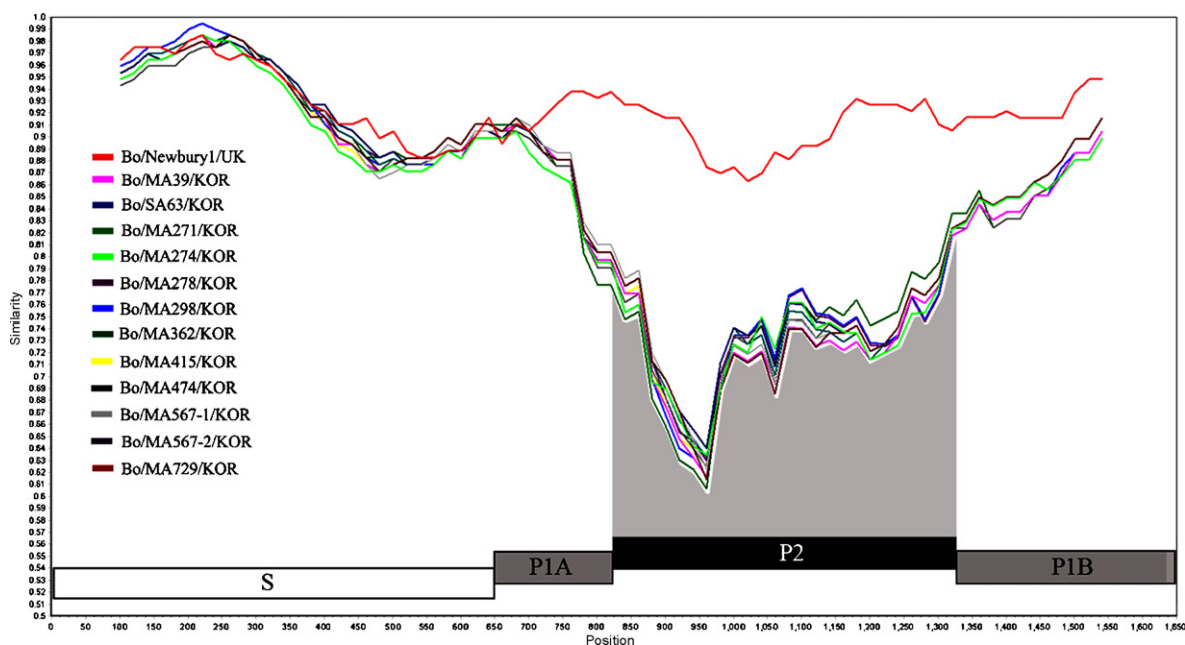


Fig. 2. The nucleotide identity plot of the Newbury1 and all Korean unclassified bovine calicivirus strains was generated in comparison with the Nebraska strain using Simplot with a 200-nucleotide window moving along in 20 nucleotide steps. The capsid gene was divided into the S (white box), P1A and P1B (gray box) and P2 (black box) domains (Chen et al., 2004).

the most hypervariable region was observed in the P2 region (Fig. 2). In addition, an analysis of the nt and aa sequences of the Korean BEC capsid as a whole, S and P regions were more homologous to each other (Fig. 2).

Unlike the phylogenetic data of RdRp region, the phylogenetic tree of the nt sequences of the whole capsid gene did not show any characteristic clustering to the NB-like or NA1-like viruses. Moreover, all Korean BECs clustered on a separate branch. The NB-like and NA1-like viruses were on a different branch (Fig. 3). In addition, the Korean BECs in the phylogenetic analysis of the RdRp and capsid genes clustered together, and the other known BECs clustered on a separate branch. In contrast to genotyping by the RdRp gene, it was reported that there are no capsid genotypes (Oliver et al., 2006). Therefore, this data can support the above suggestion (Oliver et al., 2006) and suggests that the Korean BECs may have a distinct evolutionary pathway from the NB- and NA1-like BECs.

In summary, unclassified BEC infections are endemic in diarrheic calves in South Korea. The infecting strains are genetically closer to NB-like viruses but may have a distinct evolutionary pathway from the NB- and NA1-like BECs.

Acknowledgments

This study was supported by the funds from the National Veterinary Research and Quarantine Service (NVRQS), Ministry of Agriculture and Forestry, and the Regional Technology Innovation Program (RTI05-01-01) of the Ministry of Commerce, Industry and Energy (MOCIE), Republic of Korea. The authors acknowledge a graduate fellowship provided by the Korean Ministry of Education and Human Resources Development through the Brain Korea 21 project.

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