ORIGINAL ARTICLE

Detection and Molecular Characterization of Calf Diarrhoea Bovine Coronaviruses Circulating in South Korea during 2004–2005

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Summary

Although the widespread occurrence of calf diarrhoea (CD) bovine coronavirus (BCoV) infections have been reported in most cattle producing countries, only the genetic differences in the BCoVs from American and Canadian isolates and/or strains have been identified and compared. Hence, it is unclear if the BCoVs circulating in the other countries have distinct genetic characteristics. The aim of this study was to determine the prevalence and genetic diversity of CD BCoVs based on the deduced amino acid (aa) sequences of the spike (S) and haemagglutinin/esterase (HE) proteins in South Korea. RT-PCR and nested PCR using the primer pairs specific to the nucleocapsid gene, BCoVs detected the BCoVs in 56 (15.6%) of 359 diarrhoeic faecal samples. Phylogenetic analysis of the entire S gene indicated that 10 Korean CD BCoV strains clustered with other Korean BCoV strains with different clinical forms but were different from the American and Canadian BCoV strains. Moreover, the phylogenetic data of the aa sequences of the HE gene revealed all the Korean CD strains to be distinct from the other Korean BCoV strains with different clinical forms. These results suggest that the Korean BCoVs cause endemic infections in diarrhoeic calves in Jeonnam province and have taken a different evolutionary pathway from the BCoVs in other countries. Moreover, the different BCoV strains are circulating in the different clinical forms in South Korea. These results also suggest that vaccines against the BCoVs can be developed with each Korean BCoV in different clinical forms.

Introduction

Coronaviruses (CoVs), belonging to the family *Coronaviridae*, are large, enveloped viruses 120–150 nm in diameter and possess a single-stranded, plus-sense RNA genome of approximately 26–30 kb in length (Lai and Holmes, 2001). Coronaviruses are now recognized as both veterinary and human pathogens that are associated with a wide range of economically important diseases in their respective hosts. CoVs have been separated into three distinct subgroups based on the serological cross-reactivity and genomic relatedness (Lai and Holmes, 2001).

The bovine coronavirus (BCoV) belongs to the second subgroup, which also contain a severe acute respiratory syndrome CoV, human respiratory CoV (HCoV-OC43), haemagglutinating encephalomyelitis CoV of swine, turkey enteric CoV and murine hepatitis CoVs (MHV) (Lai and Holmes, 2001; Snijder et al., 2003). The BCoV causes severe calf diarrhoea (CD) and is associated with winter dysentery (WD) in adult cattle and respiratory infections in feedlot cattle (Saif and Heckert, 1990; Clark, 1993; Cho et al., 2000; Lathrop et al., 2000). Clinically different forms of BCoV infections have been reported in most cattle producing countries including those in Europe, North America and East Asia (Saif and Heckert, 1990). It is believed that CD BCoV infections have caused enormous economic losses in the cattle industry of South Korea, such as WD in adult cattle (Jeong et al., 2005b).

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However, little is known about the precise epidemiology of CD BCoV infections in South Korea.

The BCoV contains five major structural proteins: the nucleocapsid (N), transmembrane (M), spike (S), haemagglutinin/esterase (HE) and small membrane (E) (Lai and Holmes, 2001). Although both the S and HE glycoproteins haemagglutinate erythrocytes by binding to *N*-acetyl-9-*O* acetyl neuraminic acid as a receptor determinant, the S glycoprotein requires fewer of these receptors on the surface of the erythrocytes for agglutination than the HE protein (Schultze et al., 1991a,b). Therefore, the S glycoprotein is believed to be the main haemagglutinin of BCoV and is responsible for the primary attachment of BCoV to other cell surface receptors (Schultze et al., 1991b). The variation in the host range as well as the tissue tropism of CoVs is largely due to variations in the S glycoprotein (Gallagher and Buchmeier, 2001).

Viruses circulating in geographically distinct areas or countries might have different antigenicity and pathogenicity. Like other RNA viruses, CoVs are believed to mutate at a high frequency because of the high error frequencies of the RNA polymerases (Steinhauer and Holland, 1986). Different BCoV strains or isolates are expected to show various degrees of substitutions particularly in the S1 subunit. In addition, mutations in the S1 sequences of MHV have been associated with altered antigenicity and pathogenicity (Fazakerley et al., 1992; Hingley et al., 1994).

Molecular analysis of the S and HE genes in the American and Canadian isolates and/or strains of BCoVs has been carried out and compared (Abraham et al., 1990; Boireau et al., 1990; Zhang et al., 1991, 1994 ; Rekik and Dea, 1994; Chouljenko et al., 1998; Gelinas et al., 2001; Hasoksuz et al., 2002). Recently, we reported that the Korean WD strains were genetically different from the American and Canadian BCoVs, which cluster on a separate major branch (Jeong et al., 2005a). However, the molecular characterization of the CD BCoVs circulating in South Korea has not been performed. The aim of this study was to obtain information on the prevalence and genetic diversity of CD BCoVs circulating in South Korea.

Materials and methods

Specimens

A total of 359 diarrhoeic faecal specimens from 343 Korean native beef (Hanwoo) calf herds were collected from Jeonnam province from 2004 to 2005 during the spring (121 samples/120 herds), summer (107 samples/98 herds), autumn (73 samples/69 herds) and winter (58 samples/56 herds). The ages of the sampled calves ranged from 2 to 90 days. The faecal samples were also examined for common bacterial enteric pathogens, as described elsewhere (Asakura et al., 1998; Park et al., 2006). For virology assays, faecal suspensions from each sample were prepared immediately by diluting the faeces in 0.01 M phosphate-buffered saline (PBS), pH 7.2, at a ratio of 1 : 10. The suspensions were vortexed for 30 s and centrifuged (1200 ***g*** for 20 min). The supernatants were collected and stored at −80°C for further testing.

RNA extraction

The RNA was extracted from a 200-μl starting volume of the centrifuged 10% faecal suspensions using the Trizol-LS (Gibco-BRL; Life Tech, Grand Island, NY) procedure. The recovered total RNA was suspended in 50 μl of RNase free water and stored at −80°C until needed.

RT-PCR and nested PCR

One-step RT-PCR and nested PCR assays primer pairs specific to BCoV nucleocapsid protein (Cho et al., 2001) were performed using the extracted RNA from the faecal samples. As a negative control, the RNA was extracted from the normal faeces of a mock-infected, colostrum-deprived calf. The amplification products were analysed by 1.5 or 2% agarose gel electrophoresis and visualized by UV irradiation of the ethidium bromide-stained samples.

BCoV isolation

Monolayers of human rectal tumour (HRT-18G) cell cultures grown in 6-well plates were used to isolate the BCoVs, as described previously (Benefield and Saif, 1990). The isolated BCoV were confirmed by direct immunofluorescent (IF) tests (Saif et al., 1988) and RT-PCR, as described above.

DNA sequencing

A one-step RT-PCR assay was performed using the primers specific to the S and HE genes of the Mebus strain, as described previously (Cho et al., 2001; Jeong et al., 2005a). The RT-PCR products were purified using a QIAEX II Gel Extraction kit (QIAGEN Inc., Valencia, CA) according to the manufacturer's instructions. DNA sequencing was performed using an automated DNA sequencer (ABI system 3700; Applied Biosystem Inc., Foster City, CA). The S and HE genes sequences of the BCoV isolates were compared with those of the other known BCoVs using the DNA Basic module (DNAsis MAX, Alameda, CA) (Table 1). The deduced amino acid (aa) sequences were then assembled and analysed on the Amino Acid Basic module (DNAsis MAX). A sequence similarity search was carried out for the BCoV S and HE

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Table 1. GenBank accession numbers of the reference strains of the bovine coronaviruses used in phylogenetic analysis

Clinical forms	Strains	S gene	HE gene
Calf diarrhoea	BCQ9	U06091	
	BCQ20	U06092	
	BCQ571	U06093	U06093
	BCQ701		AF230527
	BCQ1523	AH010062	AH010062
	BCQ2070	U06090	
	BCQ3708		AF230528
	KCD1	DQ389632	DQ389642
	KCD2	DQ389633	DQ389643
	KCD3	DQ389634	DQ389644
	KCD4	DQ389635	DQ389645
	KCD5	DQ389636	DQ389646
	KCD6	DQ389637	DQ389647
	KCD7	DQ389638	DQ389648
	KCD8	DQ389639	DQ389649
	KCD9	DQ389640	DQ389650
	KCD10	DQ389641	DQ389651
Respiratory infection	BCQ3994	AF339836	AF339836
	LSU	AF058943	AF058943
	OK	AF058944	AF058944
	LUN	AF391542	AF391542
	G95	M80844	
	BCO43277		AH010241
	BCO44175		AH010063
Enteritis	LY-138	AF058942	AF058942
	F15	D00731	
	Quebec	AF220295	
	ENT	AF391541	AF391541
Winter dysentery	BCQ7373	AH010061	AH010061
	BCQ2590		AH010256
	KWD1	AY935637	DQ016118
	KWD2	AY935638	DQ016119
	KWD3	AY935639	DQ016120
	KWD4	AY935640	DQ016121
	KWD5	AY935641	DQ016122
	KWD6	AY935642	DQ016123
	KWD7	AY935643	DQ016124
	KWD8	AY935644	DQ016125
	KWD9	AY935645	DQ016126
	KWD10	AY935646	DQ016127
	KWD11	DQ389660	DQ994162
	KWD12	DQ389659	DQ994163
	KWD13	DQ389658	DQ994164
	KWD14	DQ389657	DQ994165
	KWD15	DQ389656	DQ994166
	KWD16	DQ389655	DQ994167
	KWD17	DQ389654	DQ994168
	KWD18	DQ389653	DQ994169
	KWD19	DQ389652	DQ994170
Avirulent	Vaccine	M64668	
	Mebus	U00735	U00735
	L9	M64667	M76372

protein genes using the LALIGN Query programme of the GENESTREAM network server at Institut de Génétque Humaine, Montpellier, France (http://www.eng.uiowa.edu/~tscheetz/sequence-analysis/examples/LALIGN/lalign-guess.html). Phylogenetic and bootstrap (1000 replicates) analyses based on the nucleotide and aa alignments were constructed using the neighbour-joining method as well as the unweighted-pair group method using the average linkages of Molecular Evolutionary Genetics Analysis (MEGA version 3.1 Arizona State University, Tempe, USA) with the pair-wise distance (Kumar et al., 2004).

Results

Detection of BCoV in the faecal samples of diarrhoeic calves

Using a one-step RT-PCR assay, 14 (3.9%) of 359 diarrhoeic faecal samples were positive for BCoV. Nested PCR assay detected 56 positive faecal samples (15.6%) from 55 herds (16.0%). Of these 56 BCoV-positive faecal specimens, 17 faecal samples (4.7%) from 16 calf herds (4.7%) tested positive for the BCoV alone, while the remaining 39 BCoV-positive faecal samples (10.9%) from the 39 BCoV-positive herds (11.4%) also tested positive for other enteric pathogens.

BCoV was detected in the faecal samples obtained from four seasons but was most prevalent in summer: 11 (3.1%) of 121 faecal samples (11/120 herds) in spring, 41 (11.4%) of 107 faecal samples (40/98 herds) in summer, one (0.3%) of 73 faecal samples (1/69 herds) in autumn and three (0.8%) of 58 faecal samples (3/56 herds) in winter tested positive for the BCoV by either RT-PCR or nested PCR (data not shown).

CD BCoV isolation in HRT-18G cells

Of the 14 BCoV-positive faecal samples detected by RT-PCR or nested PCR, CD BCoV was isolated from 10 faecal samples from 10 herds. Of these isolates, seven (KCD1–7) were isolated from the faecal specimens sampled in spring and three (KCD 8–10) in summer. After the second or third passage, CPE which is characterized by enlarged, rounded and densely granular cells in clusters was observed in the cultures inoculated with each of the faecal samples at post-inoculation days 2–3. There were no differences in the CPEs observed between isolates. CPE was not observed in the mock-infected HRT-18G cells. The direct IF test detected the BCoV-specific cytoplasmic fluorescence in the HRT-18G cells inoculated with each of these samples at the third or fourth passage. The number of fluorescing cells increased with increasing number of serial passages. A specific band was detected after amplification

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with all 10 isolates using a RT-PCR assay targeting a 730 bp fragment of the N gene of BCoV. These 10 isolates were designated as the KCD1–10 strains respectively.

Molecular analysis of S and HE genes of Korean CD BCoV strains

All the S and HE genes of the 10 KCD strains had an open reading frame (ORF) of 4092 and 1272 nucleotides respectively. These nucleotide sequences encoded the predicted proteins containing 1363 and 424 aa residues respectively. The S protein consisted of the S1 and S2 segments at the cleavage site of aa 768, which were approximately 86 and 65 kDa respectively. A total of 123 polymorphic nucleotides were identified in the S gene of the BCoV strains compared with the prototype Mebus strain (data not shown). These polymorphisms led to 51 aa changes at the 48 distinct sites. A nucleotide substitution of the HE gene of the BCoVs, which was not detected in the prototype Mebus strain, was detected in 10 sites, leading to 10 aa changes at different sites. No frameshift, deletion, insertion or nonsense mutations were observed in the nucleotide sequences of the S and HE genes in the strains isolated in this study compared with those of the prototype Mebus strain.

A comparison of the deduced amino acids of the S and HE genes between our strains and the other known BCoVs revealed that the two most similar sequences were those of the KCD2 and WD KWD13 strains (99.56%) for the S gene, and the KCD2 and KCD4 and CD BCQ701 strains (100.0%) for the HE gene respectively. In addition, the most distant were those of the KCD9 and WD BCQ7373 strains (96.48%) for the S gene, and the KCD1, KCD5, KCD7 and KCD9 strains and WD KWD18 (97.41%) for the HE gene. All the virulent KCD strains tended to be distant from the ancestral enteric strain, Mebus (below 97.51%).

The N-terminal region of the S1 subunit (aa 1–330; Laude et al., 1995), which was shown to function as a receptor-binding domain in the mouse hepatitis virus, had a total of 21 aa changes compared with the Mebus strain. A unique aa substitution at aa 149 within this region was observed in all the Korean strains including the 10 KCD strains and 19 KWD strains isolated from the adult diarrhoeic cattle (Fig. 1). The S1A and the S1B immunoreactive domains identified within the amino acids 351–403, and the amino acids 517–621, had three aa changes, respectively, compared with the Mebus strain (data not shown).

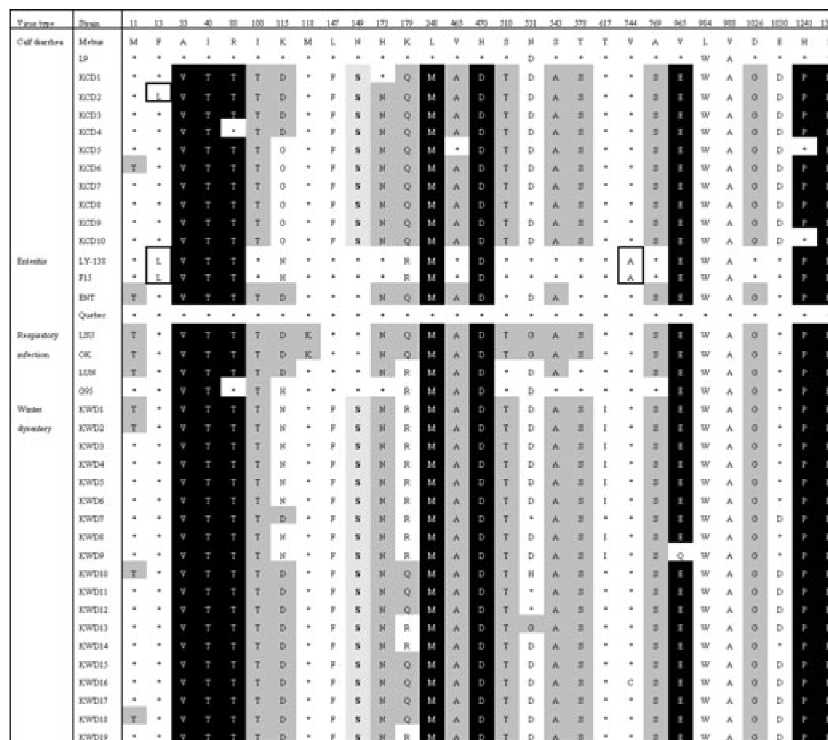

Fig. 1. Comparison of the predicted aa sequences of the BCoV S protein in the different strains. The light-grey box contains the aa sites known for being RBCV-specific (Chouljenko et al., 1998), the dark boxes are virulent-specific (Zhang et al., 1991), the clear boxes are EBCV-specific (Chouljenko et al., 1998) and the slant-lined box is Korean BCoV strains specific.

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The putative virulent-specific aa sites (at aa 33, 40, 89, 248, 470, 965, 1241 and 1341) in the S gene were compared between avirulent strains (Mebus and L9) and all Korean CD BCoVs. All the virulent-specific aa sites were well conserved in all the KCD strains except for KCD4 at aa 88, KCD5 and KCD10 at aa 1241 (Fig. 1). The putative respiratory bovine coronavirus (RBCV)-specific aa substitutions in all Korean BCoV and RBCV strains were compared. These aa substitutions were similar or identical in all Korean BCoV strains (Fig. 1). In addition, enteric bovine coronavirus (EBCV)-specific aa substitutions at aa 13 and 744 in all the Korean BCoVs and other known BCoVs were compared. None of these aa substitutions were observed in the CD BCoVs and WD BCoVs. Moreover, none of these substitutions were observed in the EBCV strains, ENT and Quebec (Fig. 1).

In all the known BCoVs, the S protein has a cleavage site located at the sequence 763–768 (KRRSRR). The KRRSRR sequences were conserved in all KCD strains. The aa sequences of the S2 subunit of the present KCD strains were comparatively well conserved compared with the S1 subunit of the Mebus strain. The only remarkable finding in the S2 subunit was found in the first hydrophobic region. All known virulent BCoVs including the BRCV LSU and OK strains, EBCV LY and F15 strains, all Korean strains including KWD1–8, 10–19 and KCD1–10 strains except for KWD9 had a markedly higher hydrophilicity due to an aa substitution at aa 965 (V $\rightarrow$ E) than the avirulent strains, L9 and Mebus strains. All the KCD strains had several conserved features of the HE gene with all the BCoVs, i.e. a hydrophobic putative signal sequence of 18 amino acids at the N-terminus of HE, eight potential N-linked glycosylation sites of HE, a putative active site for neuraminate-*O*-acetylesterase activity, FGDS, and at the N-terminus of HE.

Phylogenetic analysis of S and HE proteins of CD BCoV strains

The alignment of the deduced amino acids of the entire S gene sequences indicated that the BCoVs could be divided into three groups (Fig. 2). The first group consisted mainly of all the Korean BCoV strains including CD, WD and other BCoV strains with virulent BRCV BCQ3994

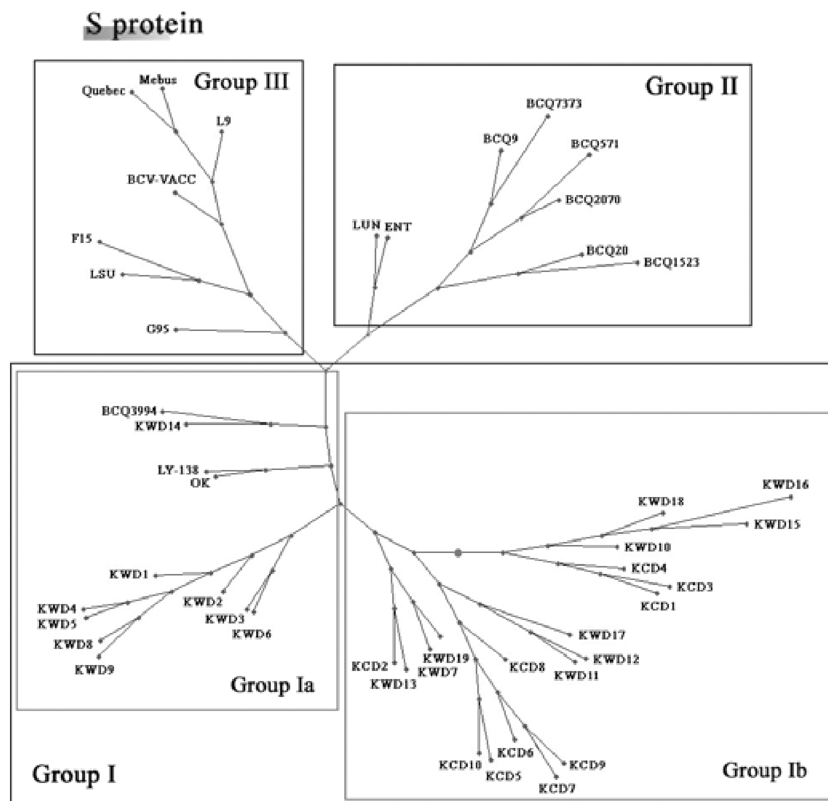

Fig. 2. The phylogenetic tree of the S gene indicates that the BCoVs could be divided into three groups. The first group could be further divided into two subgroups. The first group contains most of the Korean BCoV strains. The second group included only Canadian strains. The third group clustered with the American RBCV LSU and G95 strains, the French EBCV F15 strain, the Canadian EBCV Quebec strain, as well as the avirulent VACC, L9 and Mebus strains.

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and OK strains, and the virulent EBCV LY-138 strain. Moreover, the first group could be further divided into two subgroups, in which the first subgroup included mainly the Korean WD strains with BRCV BCQ3994 and OK strains, EBCV LY-138 strain and KWD14 strain, which was isolated from an adult diarrhoeic cow in summer. The second subgroup consisted mainly of all Korean CD BCoV strains and Korean BCoV strains isolated from adult diarrhoeic cattle in the warmer seasons as well as two Korean WD strains (KWD7 and KWD10). The second group included only Canadian strains; the Canadian CD strains (BCQ9, -20, -571, -1523 and -2070), WD BCQ7373 strain, EBCV ENT strain and RBCV LUN strain. The third group clustered with the American RBCV LSU and G95 strains, the French EBCV F15 strain, the Canadian EBCV Quebec strain, as well as the avirulent VACC, L9 and Mebus strains.

Phylogeneticly, the aa sequence of the HE gene also showed distinct patterns. The alignment of the deduced aa sequences of the HE genes indicated that the BCoVs could be divided into four groups (Fig. 3). The first group consisted mainly of Korean CD strains (KCD1–7 and -9) with the Canadian CD BCQ701 strain. The second group clustered with the Canadian CD strains (BCQ571, -1523, -2590), the Canadian WD strain (BCQ7373), the American EBCV strain (LY-138), the American RBCV strain (LSU) and avirulent strains (Mebus and L9). The third group consisted of the Korean CD strains (KCD8 and 10), RBCV strains (OK, BCQ3994, BCO14175, BCO43277, LUN), EBCV strain (ENT), and the Canadian CD strain (BCQ3708). The fourth group consisted of only the Korean BCoV strains isolated from the faecal samples of adult diarrhoeic cattle, regardless of the season. This group could be further divided into two subgroups. The first subgroup consisted mainly of the Korean WD BCoV strains isolated in winter along with KWD13, KWD16 and KWD 18 strain isolated in autumn and summer, while the second subgroup consisted of Korean BCoV strains isolated from adult diarrhoeic cattle in the warmer seasons.

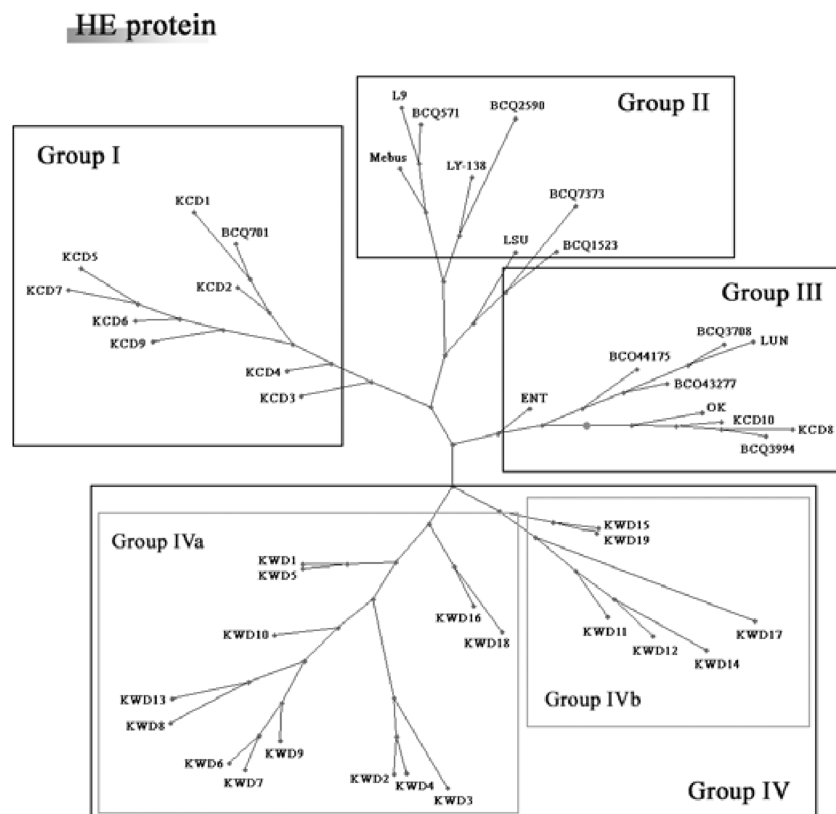

Fig. 3. The phylogenetic tree of the HE protein showed that the BCoVs could be divided into four groups. The first group mainly contained the Korean CD strains (KCD1–7 and -9) and the Canadian CD BCQ701 strain. The second group clustered with the Canadian CD strains (BCQ571, -1523, -2590), the Canadian WD strain (BCQ7373), the American EBCV strain (LY-138), the American RBCV strain (LSU) and avirulent strains (Mebus and L9). The third group consisted of the Korean CD strains (KCD8 and 10), RBCV strains (OK, BCQ3994, BCO14175, BCO43277, LUN), EBCV strain (ENT), and the Canadian CD strain (BCQ3708). The fourth group contained only the Korean BCoV strains isolated from the faecal samples of adult diarrhoeic cattle.

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Discussion

This study is the first large-scale epidemiological examination to determine the prevalence of CD BCoVs in diarrhoeic calves in Jeonnam province of South Korea. Based on the present RT-PCR and nested PCR assays for detecting the BCoV in faecal samples from diarrhoeic calves, the CD BCoVs were detected in 15.6% faecal samples (16.0% calf herds), which is consistent with the results showing the BCoVs in 8–69% of diarrhoeic claves (Clark, 1993; Lathrop et al., 2000). These results suggest that CD BCoV infections are endemic in diarrhoeic calves in Jeonnam province of South Korea.

Interestingly, the Phylogenetic data for the aa sequence of the S gene showed that the CD BCoV strains isolated in summer and spring clustered with the Korean BCoV strains isolated from adult diarrhoeic cattle in the warmer seasons. In contrast, the Korean WD BCoV strains isolated in the winter were Phylogenetically different. Considering the results of the Phylogenetic analysis of the S gene, the Korean CD BCoV strains in the warmer seasons had genetic similarities to the Korean BCoV strains isolated from adult diarrhoeic cattle in the warmer seasons. In addition, all the Korean BCoV strains regardless of their different clinical forms (CD, WD and adult cattle diarrhoea forms in the warmer seasons) were Phylogenetically different from the Canadian and the American BCoVs, which clustered on a separate major branch. This suggests that all the Korean BCoV strains resulted from a different evolutionary pathway from those of the other countries. Moreover, the Phylogenetic data of the aa sequences of the HE gene revealed all the Korean CD strains to be different from the previous known Korean WD strains, and the BCoV strains isolated from adult diarrhoeic cattle in the warmer seasons. This indicates that different BCoV strains circulate in the different clinical forms in South Korea. In addition, the CD BCoV strains isolated in this study tended to be distant from the ancestral enteric strain, Mebus, which further supports the hypothesis that genetic differences may be more related to the time of the appearance of an outbreak (Hasoksuz et al., 2002; Jeong et al., 2005a).

Amino acid substitutions in a putative receptor-binding domain in the N-terminal region of the S gene can alter the tropism of the coronavirus (Laude et al., 1995). In this study, 21 aa substitutions in the Korean CD strains were detected, which were not found in the Mebus strain, suggesting that it may induce either a change in the receptor binding ability during viral invasion or pathogenicity to cattle. Virulent BCoVs are known to have putative virulent-specific sites at 10 aa sites in the S gene through comparison of avirulent and vaccine strains (Zhang et al., 1991). The virulent-specific aa sites of all Korean CD strains were compared with other known BCoVs including virulent and avirulent strains. These virulent-specific sites were well conserved in all the Korean CD strains, suggesting that all the Korean CD strains are virulent. However, the Korean CD BCoV strains, KCD4, KCD5 and KCD10, had aa substitutions at aa 88 and 1241. Therefore, further experimental inoculation studies will be needed to determine if these aa substitutions change the viral pathogenicity in calves.

The hypervariable region spanning aa 452–593 of the S1 subunit contains the S1B immunoreactive epitope, which is the target for virus neutralizing Mabs, and showed various antigenicity (Cavanagh, 1995). It was reported that four RBCV-specific aa substitutions at aa 510, 531, 543 and 578 can be predictive, and that this region can be used to distinguish between enteric and vaccine BCoV strains (Chouljenko et al., 1998). In this study, these aa substitutions were similar or identical in all Korean BCoV strains. These results are consistent with our previous results showing that there are no RBCV-specific aa substitutions (Hasoksuz et al., 2002; Jeong et al., 2005a). In addition, aa 13 and 744 of the S1 subunit have been reported for EBCV-specific sites, which were observed in EBCV strains F15 and LY (Chouljenko et al., 1998). However, these substitutions were similar or identical to RBCV, CD and WD BCoVs, even in the same EBCV strains (ENT and Quebec), suggesting that there are no EBCV-specific sites in the S gene (Hasoksuz et al., 2002; Jeong et al., 2005a).

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