

Molecular characterization of HE, M, and E genes of winter dysentery bovine coronavirus circulated in Korea during 2002–2003

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Abstract The different bovine coronavirus (BCoV) strains or isolates exhibited various degrees of substitutions, resulting in altered antigenicity and pathogenicity of the virus. In the previous our study, we demonstrated that the spike glycoprotein gene of Korean winter dysentery (WD) BCoV had a genetic property of both enteric (EBCV) and respiratory BCoV (RBCV) and were significantly distinct from the ancestral enteric strains. In the present study, therefore, we analyzed the other structure genes, the hemagglutinin/esterase (HE) protein, the transmembrane (M) protein and the small membrane (E) protein to characterize 10 WD BCoV circulated in Korea

Nucleotide sequence data reported is available in the GenBank database under the accession no. HE, M and E genes: KWD1; AH014866, KWD2; AH014867; KWD3; AH014868, KWD4; AH014869, KWD5; AH014870, KWD6; AH014871, KWD7; AH014872, KWD8; AH014873, KWD9; AH014874, and KWD10; AH014875.

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during 2002–2003 and compared the nucleotide and deduced amino acid sequences with the other known BCoV. Phylogenetic analysis indicated that the HE gene among BCoV could be divided into three groups. The first group included only RBCV, while the second group contained calf diarrhea BCoV, RBCV, WD and EBCV, respectively. The third group possessed only all Korean WD strains which were more homologous to each other and were sharply distinct from the other known BCoV, suggesting Korean WD strains had evolutionary distinct pathway. In contrast, the relative conservation of the M and E proteins of BCoV including Korean WD strains and the other coronaviruses suggested that structural constraints on these proteins are rigid, resulting in more limited evolution of these proteins. In addition, BCoV and human coronavirus HCoV-OC43 contained four potential O-glycosylation sites in the M gene. However, the M gene sequence of both BCoV and HCoV-OC43 might not contain a signal peptide, suggesting the M protein might be unlikely to be exposed to the O-glycosylation machinery in vivo.

Keywords Bovine coronavirus · E gene · HE gene · M gene · Phylogenetic analysis

Introduction

Bovine coronavirus (BCoV) causes severe diarrhea in new-born calves (CD) and winter dysentery (WD) in adult cattle, and is associated with respiratory tract infections in calves and feedlot cattle [1–5]. BCoV is a member of the family *Coronaviridae*, order *Nidovirales* [6]. The genome consists of a single molecule of positive-sense, non-segmented RNA, 30 kb in length, which

is transcribed into a nested set of several 3′-coterminal subgenomic mRNAs for the production of structural and non-structural proteins [7, 8]. The virion contains five major structural proteins; the nucleocapsid (N) protein, the transmembrane (M) protein, the small membrane (E) protein, the hemagglutinin/esterase (HE) protein and the spike (S) protein [9].

BCoV is a well characterized hemagglutinating coronavirus. As the name implies, HE has an acetyl esterase activity that cleaves acetyl groups from 9-*O*-acetylated neuraminic acid, thereby preventing or reversing the hemagglutination or hemadsorption induced by S or HE [10–14]. These properties suggest that HE may be involved in either virus entry or virus release from infected [10–14].

Since the molecular analysis of BCoV genes has been conducted and compared mainly among American and Canadian isolates and/or strains, it is unclear whether BCoV circulated in the other countries are distinctive in genetic characteristics [15–22]. Recently, we have shown that the WD strains circulated in Korea had a genetic property of both respiratory BCoV (RBCV) and enteric BCoV (EBCV) and were significantly distinct from the ancestral enteric strain [23]. These results prompted us to investigate whether the other structural genes of Korean WD strains have distinctive genetic property compared to those of the other known BCoV. We report here the complete nucleotide sequences of the HE, M, and E genes of WD BCoV circulated in Korea during 2002–2003 and their phylogenetic relatedness to other known BCoV.

Materials and methods

Viruses

Ten WD BCoV strains, KWD 1 to KWD 10, were originally isolated in the G clone of human rectal tumor cells (HRT-18G) from fecal samples of adult dairy or beef cows

with WD in South Korea and identified as a BCoV by immunoelectron microscopy and ELISA with BCoV-specific antisera and monoclonal antibodies, and RT-PCR specific for the part of BCoV N gene [24]. All KWD strains were tested at the fourth passages. Supernatant fluids from infected HRT-18G cells were collected and used for viral RNA extraction.

Extraction of viral RNA

RNA was extracted based on the acid guanidinium–phenol–chloroform RNA extraction method as described by Cho et al. [25, 26]. Briefly, 500 µl Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and 50 µl 20 M sodium acetate (pH 4.0) were added to individual tubes containing 200 µl of each of the supernatant fluids from infected HRT-18G cells. After mixing, 500 µl of water-saturated phenol (pH 4.5) and 100 µl of chloroform and isoamylalcohol (49:1) were added, vortexed briefly and placed on the ice for 15 min. The mixture was centrifuged at 20,800 g for 20 min at 4°C and the supernatant was transferred into a new tube. To remove non-specific inhibitors of the PCR reaction present in the extracted samples, the supernatant was purified using the RNaid kit according to the manufacturer's instructions (BIO 101, Inc., LaJolla, CA). The extracted RNA was resuspended with 30 µl DEPC treated water. As a negative form of control, RNA was extracted from mock-infected HRT-18G cells.

Preparation of oligonucleotide primers

The oligonucleotide primers used in the RT-PCR were designed from the published sequence of the HE, M, and E genes of the Mebus strain (GenBank accession No. U00735). The primer sequences and predicted product sizes are shown in Table 1.

Table 1 The oligonucleotide primers designed from the HE, M, and E genes of the BCoV Mebus strain (GenBank accession No. U00735) used for DNA sequencing

Gene name	Primer name ^a	Sequence	Location	Product size
HE	HEAF	5′-CAG TGA AGA AGA CTA AAC TCA GT-3′	32 kDa putative non-structural protein 821–HE 698	741 bp
	HEAR	5′-TAA ATA ACA CCA GTG TCT TTA TT-3′		
	HEBF	5′-TGA CGA GTA TAT CGT ACC ACT T-3′	HE gene 591–1275	684 bp
	HEBR	5′-CTA AGC ATC ATG CAG CCT AGT ACC-3′		
M	MF	5′-CCA CCA GTT CTT GAT GTG GA-3′	E gene 226–N gene 59	817 bp
	MR	5′-CCA GAA CGA TTT CCA AAG GA-3′		
E	EF	5′-CGK AGA CAG GAG TTA AAT GTT T′-3′	12.7 kDa protein 301–M gene 265	280 bp
	ER	5′-TTT GGA TTA ACT AAA CGT CA-3′		

^aF: upstream primer. ^bR: downstream primer.

RT-PCR

A one-step RT-PCR assay was performed as described by Cho et al. [25, 26]. Briefly, the tube containing 15 μ l of the RNA sample and 1 μ l of DMSO was incubated at 70°C for 10 min and then quenched on ice. Subsequently, 44 μ l of the RT-PCR mixture was added. The RT-PCR mixture consisted of 5 μ l of 10 \times buffer [100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM MgCl₂, 0.01% gelatin), 5 μ l of MgCl₂ (25 mM), 1 μ l of 10 mM dNTPs, 1 μ l of the upstream primer (50 pmol), 1 μ l of the downstream primer (50 pmol), 0.5 μ l of RNasin (Promega Corporation), and 0.5 μ l of AMV RT (Promega Corporation), 0.5 μ l of Taq polymerase (Promega Corporation) (5 U/ μ l). The mixture was incubated for 60 min at 42°C, preheated for 5 min at 94°C, and subjected to 35 cycles of PCR. The PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

DNA sequencing

The RT-PCR products were purified using a GenClean II kit (BIO 101, Inc., LaJolla, CA) according to the manufacturer's instructions. The DNA sequencing was done using an automated DNA sequencer (ABI system 3700, Applied Biosystem Inc., Foster City, CA).

Using the DNA Basic module (DNAsis MAX, Alameda, CA), nucleotide sequences of our BCoV isolates were first compared for the HE sequence of BCoV as follows: the CD strains including Mebus (GenBank accession No. AAA66393), L9 (GenBank accession No. AAB20434), BCQ571 (GenBank accession No. AAA92991), BCQ1523 (GenBank accession No. AAG40600), BCQ3708 (GenBank accession No. AAG40593), and BCQ701 (GenBank accession No. AAG40592), the WD strains including BCQ7373 (GenBank accession No. AAG40594) and BCQ2590 (GenBank accession No. AAA92989), the RBCV strains including BCQ3994 (GenBank accession No. AAK14397), LSU (GenBank accession No. AAF25508), OK (GenBank accession No. AAF25518), LUN (GenBank accession No. AAL57307), BC043277 (GenBank accession No. AAG60544) and BC044175 (AAG40622), EBCV strains including LY-138 (GenBank accession No. AAF25498) and ENT (GenBank accession No. AAK83355). The M gene sequence compared were as follows: Mebus (GenBank accession No. AAK29779), LY-138 (GenBank accession No. AAF25504), ENT (GenBank accession No. AAK83361), LSU (GenBank accession No. AAF25514), OK (GenBank accession No. AAF25524) and LUN (GenBank accession No. AAL57312). The E gene sequence compared were Mebus (GenBank accession No. AAA42914), BCQ571 (GenBank accession No.

AAK01079), BCQ1523 (GenBank accession No. AAG40605), BCQ7373 (GenBank accession No. AAG40599), LY-138 (GenBank accession No. AAF25503), ENT (GenBank accession No. AAK83360), BCQ3994 (GenBank accession No. AAG40609), LSU (GenBank accession No. AAF25513), OK (GenBank accession No. AAF25523) and LUN (GenBank accession No. AAL57311). The deduced amino acid (aa) sequences were then assembled and analyzed on the Amino Acid Basic module (DNAsis MAX, Alameda, CA). A sequence similarity search was performed for the BCoV HE, M and E protein using the LALIGN Query program of the GENESTREAM network server at Institute de Génétique Humaine. Phylogenetic analyses were conducted using PhyolDraw program at the Graphics Application Laboratory, Pusan National University.

Results

Molecular analysis of HE gene

The entire HE gene of the 10 KWD strains contained an ORF of 1272 nucleotides, encoding a predicted protein of 424 aa residues and having a molecular weight of approximately 47.6 kDa, respectively. Among all strains analyzed, a total of 24 polymorphic nucleotides were identified in the HE gene of BCoV, compared with the Mebus strain (data not shown). These polymorphisms led to 10 aa changes at 10 distinct sites which seemed to be distributed randomly. There were no frameshift, deletion, or insertion, and nonsense mutations, compared with the Mebus strain. In addition, all KWD strains shared several conserved features with all BCoV; a hydrophobic putative signal sequence of 18 amino acids at the N-terminus; the 9 potential N-linked glycosylation sites; the 14 cysteine residues; the putative active site for neuraminidase activity, FGDS, at the N-terminus; an extremely hydrophobic region of 26 amino acids near the C-terminus which may serve as a potential membrane-anchoring domain; a stretch of 10 hydrophilic amino acids at the C-terminus which may be the intravirion domain.

Based on the total number of aa substitutions, a phylogenetic tree of the entire HE gene sequences was constructed using the Clustal method (DNASIS) with CD, RBCV, EBCV and WD strains or isolates (Fig. 1a). The alignment indicated that the HE genes among BCoV could be divided into three groups. The first group includes only RBCV, while the second group contains CD, RBCV, WD, and EBCV, respectively. The third group possesses only all Korean WD strains which were more homologous to each other and were sharply distinct from the other known

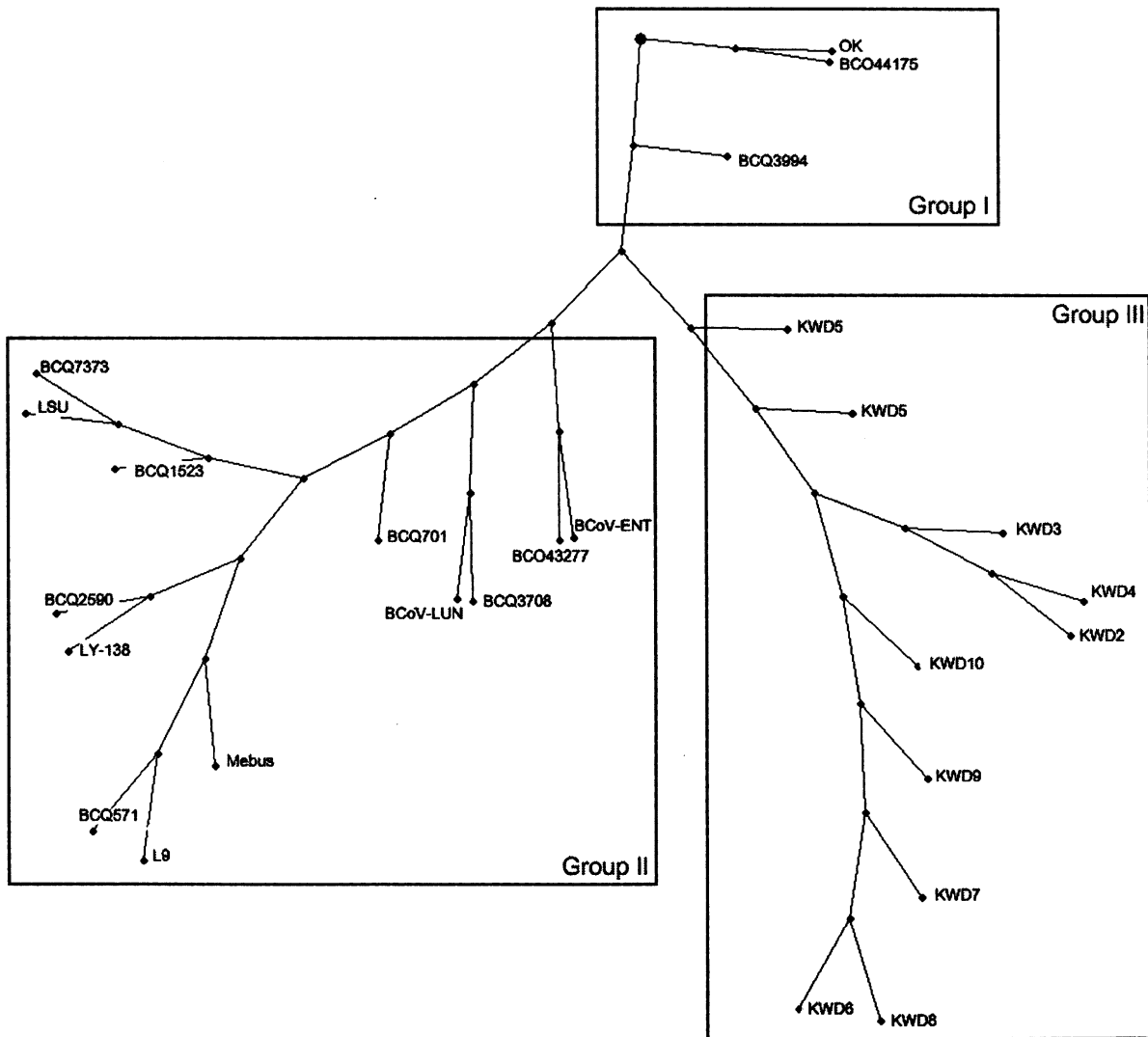


Fig. 1 The phylogenetic tree of the HE, M and E genes of respiratory (RBCV), enteric (EBCV), calf diarrhea (CD), winter dysentery (WD), and avirulent strains or isolates was made using PhyloDraw at the Graphics Application Laboratory, Pusan National University. KWD1-

KWD10: Korean WD strains; BCQ7373 and BCQ2590: WD strains; Mebus, BCQ571, BCQ1523, BCQ3708 and BCQ701: CD strains; BCQ3994, LSU, OK, BCoV-LUN, BCO43277 and BCO44175: RBCV strains; LY-138 and BCoV-ENT: EBCV strains; L9: avirulent strain

BCoV (Fig. 2). The two most distant sequences were those of KDW3 strain and BCQ2590 WD BCoV strain (96.69%) in paired comparisons. The virulent Korean WD isolates tended also to evolutionally distant from L9 and Mebus strains which were considered to be rather avirulent.

All KWD strains had unique aa substitution at aa 173 in comparison to all other known BCoV strains (Fig. 2). This substitution (A → V) did not alter the charge but increased slight hydrophobicity. RBCV- and EBCV-specific aa sites were reported in the HE gene of BCoV, respectively [17, 21]. RBCV-specific substitution (aa 66; D → G) was also detected in all 10 KWD strains (Fig. 2). In addition, both EBCV- and virulent-specific substitutions at aa 5 (L → P) and 367 (S → P) were conserved in all BCoV including 10 KWD strains but not in the avirulent Mebus and L9 CD strains (Fig. 2).

Molecular analysis of M and E genes

The entire M and E genes of the 10 KWD strains contained ORFs of 690 and 252 nucleotides, respectively. These nucleotide sequences of M and E genes encoded a predicted protein of 230 and 84 aa residues, respectively. In comparison with the Mebus strain, a total of 15 and 8 polymorphic nucleotides were identified in the M and E genes of all KWD strains, respectively. These polymorphisms led to 6 and 5 aa changes which seemed to be distributed randomly. N-linked glycosylation was detected in one site of M gene but not in the E gene.

The M protein has previously been shown to be O-glycosylated in MHV, HCV-OC43 and BCoV [27–29]. HCV-OC43 has six potential O-glycosylation sites which are well conserved in all BCoV including Korean WD

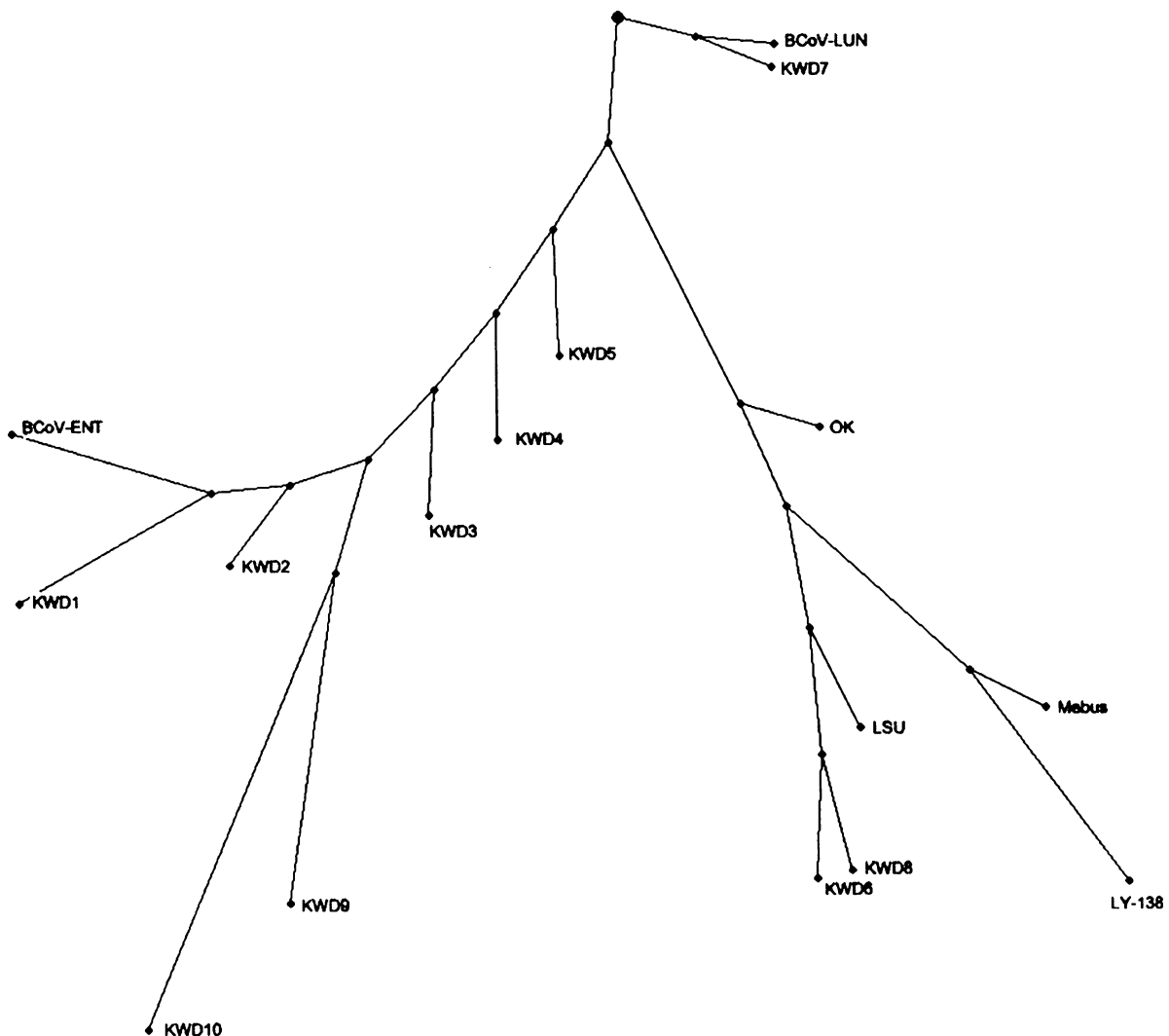


Fig. 1 (Continued)

strains [28]. By using the NetOGlyc 3.1 Server program at Technical University of Denmark, interestingly, only four potential O-linked glycosylation sites (aa 2, 3, 5 and 6) were detected in the aminoterminal domain of M gene of BCoV and HCV-OC43.

By drawing comparisons of deduced aa between our strains and the other known BCoV, KWD8 and Mebus showed the two most distant (96.42%) in the M gene sequences, while KWD10 and LY-138 (97.82%) in the E gene. However, phylogenetic analysis of aa sequences of M and E genes revealed no characteristic pattern between our strains and the other known BCoV; all KWD strains were sparse between the other known BCoV in the phylogenetic tree (Fig. 1b, c).

Discussion

The different BCoV strains or isolates exhibited various degrees of substitutions. Therefore, mutations of BCoV

genes have been associated with altered antigenicity and pathogenicity of the virus [30, 31]. Furthermore, genetic differences between RBCV and EBCV and virulent and avirulent strains were identified especially within the S gene [17, 18]. We have also identified genetic differences between RBCV, CD, and WD strains [19]. To identify a phylogenetic characterization among the other known BCoV and Korean WD strains, we compared the nucleotide and amino acid sequences of HE, M and E genes. The phylogenetic data of the HE protein indicated that Korean WD strains were more homologous to each other and were sharply distinct from the other known BCoV, suggesting Korean WD strains had evolutionary distinct pathway. This is also supported by our previous results in which S protein of all Korean WD strains had distinct genetic property in comparison with that of the other BCoV [23]. Besides, the S protein of all Korean WD strains revealed a genetic property of both RBCV and EBCV. However, HE protein of these strains did not showed a genetic property of both

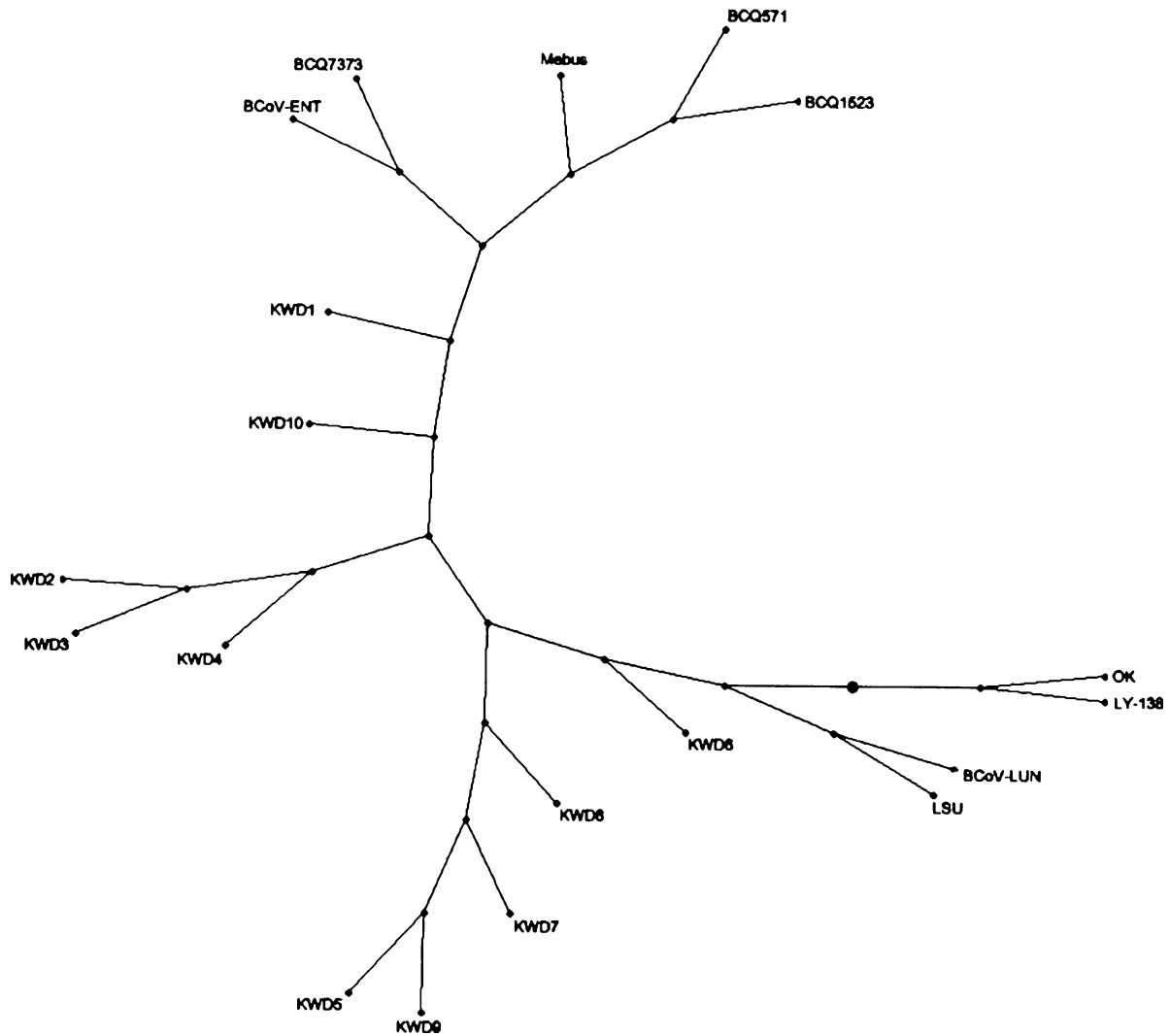


Fig. 1 (Continued)

RBCV and EBCV. Since HE gene is known to be derived by a recombination between an HE mRNA of influenza C virus and the genomic RNA of an ancestral coronavirus and is not absolutely necessary for virus infection [31], these may result in lower evolutionary pressure to HE gene than S gene of BCoV. In addition, the relative conservation of the M and E proteins of all coronaviruses including BCoV suggests that structural constraints on these proteins are rigid, resulting in more limited evolution of these proteins.

Coronaviruses are thought to mutate at a high frequency like most RNA viruses because of the high error frequencies of RNA polymerases [32]. The most striking example of the biologic importance of coronavirus mutants is the emergence of porcine respiratory coronavirus from transmissible gastroenteritis virus [33, 34]. BCoV causes variable clinical signs; calf diarrhea, WD in adult cattle and respiratory infection in feedlot cattle [1–5]. Therefore, bovine coronavirologists tried to find genetic

characterization of BCoV responsible for their clinical signs. Chouljenko et al. [17] reported that RBCV- and EBCV-specific aa substitutions in the S, HE, M and N genes, respectively, can be predictive. Likewise Mabs-specific strains for these regions may also be distinguished among respiratory, enteric, and vaccine BCoV strains [17]. In our recent results, however, these substitutions in the S gene of BCoV were also detected in all Korean WD strains, suggesting these aa substitutions does not appear to be a potential marker of respiratory or enteric tropisms [23]. In the present study, the aa substitutions at aa 5 (L → P) and aa 367 (S → P), and aa 66 (D → G) in HE gene of all Korean WD strains were identical with that believed to be specific for enteric and respiratory tropisms, respectively. Therefore, this result can support our statement that they may not be RBCV- and EBCV-specific aa substitutions. In contrast, putative virulent-specific sites are all conserved in Korean WD strains with those of the other virulent strains

Clinical disease	Strain or isolation	HE gene											M gene					E gene						
		2	5	49	66	78	173	200	289	311	367	392	4	13	38	39	106	221	7	8	11	12	53	
Calf diarrhea	Mebus	F	L	N	D	I	A	Y	V	Y	S	L	V	W	I	I	R	M	V	F	T	V	G	
	L9	*	*	T	*	*	*	*	*	*	*	*												
	BCQ571	*	*	*	*	*	*	*	*	*	*	I							*	*	*	*	V	
	BCQ1523	*	P	T	G	*	*	*	*	*	*	P	I							*	*	*	*	V
	BCQ3708	*	P	T	G	*	*	*	*	*	*	P	I											
BCQ701	*	*	T	G	*	*	*	*	*	*	P	I												
Enteritis	LY-138	*	P	*	*	*	*	*	*	*	P	*	*	*	*	*	*	*	*	*	*	*	V	
	ENT	*	P	T	G	*	*	*	*	*	P	I	*	*	V	*	*	L	*	*	*	*	V	
Respiratory infection	BCQ3994	*	P	T	G	*	*	*	*	*	P	I							*	*	*	*	*	
	LSU	*	P	T	*	*	*	*	*	*	P	I	*	*	V	*	*	*	*	*	*	*	V	
	OK	*	P	T	G	*	*	*	*	*	P	I	*	*	V	*	*	*	*	*	*	*	V	
	LUN	*	P	T	G	*	*	*	*	*	P	I	*	*	V	*	*	L	*	*	*	*	V	
	BCO43277	*	P	T	G	*	*	*	*	*	P	I												
	BCO44175	*	P	T	G	*	*	*	*	*	P	I												
Winter dysentery	BCQ7373	*	P	T	*	*	*	*	*	*	P	I							*	*	*	*	V	
	BCQ2590	*	P	*	*	*	*	*	*	*	P	*												
	KWD1	*	P	T	G	*	V	*	*	*	P	I	G	*	V	*	*	L	*	*	*	*	V	
	KWD2	*	P	T	G	*	V	H	I	N	P	I	*	*	V	*	*	L	*	*	*	*	V	
	KWD3	I	P	T	G	*	V	H	I	N	P	I	*	*	V	*	*	L	*	*	*	*	V	
	KWD4	*	P	T	G	*	V	H	I	N	P	I	*	*	V	*	*	L	*	*	*	*	V	
	KWD5	*	P	T	G	*	V	*	*	*	P	I	*	*	V	*	*	L	S	*	*	*	V	
	KWD6	S	P	T	G	*	V	*	*	*	P	I	*	*	V	*	*	M	*	*	*	*	V	
	KWD7	S	P	T	G	*	V	*	*	*	P	I	*	*	V	*	*	L	*	*	A	*	V	
	KWD8	S	P	T	G	L	V	*	*	*	P	I	*	*	V	*	*	M	*	C	*	A	V	
KWD9	S	P	T	G	*	V	*	*	*	P	I	*	*	V	*	G	L	S	*	*	*	V		
KWD10	C	P	T	G	*	V	*	*	*	P	I	*	R	V	L	*	L	*	*	*	*	V		

Fig. 2 Comparison of the predicted amino acid (aa) sequences of the BCoV HE, M and E proteins specified by different strains. Amino acids that are different in at least one strain are shown. Light-gray box

contains aa sites known for being RBCV-specific [17], dark boxes are both virulent- and EBCV-specific [17], and clear box contains Korean strains-specific aa changes

but not in the avirulent Mebus and L9 strains, implying Korean WD strains are virulent [17, 21].

Glycosylation of the aminoterminal domain of M gene has been known to be O-linked for MHV, HCV-OC43 and BCoV, and N-linked for IBV and TGEV [27–29, 35, 36]. Like those of TCV, HCV-OC43 and MHV, one potential N-glycosylation site was also detected in the predicted all BCoV including Korean WD strains; it is located near the N-terminal, presumably exposed portion of the molecule [27, 29, 35]. In contrast, six potential O-glycosylation sites have been detected in M gene of HCV-OC43 and BCoV [28]. However, only four aa sites among them could be defined as potential O-glycosylation sites by the analysis of NetOGlyc 3.1 Sever program at Technical University of Denmark. Moreover, this program indicated that M gene sequence of both BCoV and HCV-OC43 might not contain a signal peptide. Therefore, proteins without signal

peptides are unlikely to be exposed to the O-glycosylation machinery and thus may not be glycosylated in vivo even though they contain potential motifs. Further study will be needed on in vivo O-glycosylation machinery in MHV, HCV-OC43 and BCoV.

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