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Genetic analysis of canine group 2 coronavirus in Korean dogs

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ARTICLE INFO

Article history:

Received 5 February 2009

Accepted 4 September 2009

Keywords:

CRCoV

BCoV

Phylogeny

Neighbor-joining

ABSTRACT

Three out of 109 mixed lung and tracheal tissue extracts originating from Korean dogs tested positive for infection with canine respiratory coronavirus (CRCoV) of *Coronaviridae* group 2. The three CRCoV-positive samples were found to be competent for viral propagation and isolation, using human rectal tumor cells (HRT-18), and the structure and nonstructure proteins encoded in the 3'-end of the CRCoV genome were sequenced. The small open reading frames situated between the spike and envelope genes of the three Korean CRCoV isolates were found to encode three nonstructural proteins (4.9 kDa, 2.7 kDa, and 12.8 kDa in size), as were the British (CRCoV G9142) and Italian (CRCoV 240-05) strains. Comparison of the deduced amino acid sequences of the spike protein in the CRCoV and bovine coronavirus (BCoV) strains revealed twenty sequence variations. The predicted spike protein of CRCoV contained 20 or 21 N-glycosylation sites, whereas that of BCoV contained 19 sites. Phylogenetic analysis of the spike gene from eight CRCoV and six BCoV strains, performed using the neighbor-joining approach, allowed us to classify into two clades (CRCoV and BCoV) and three Korean strains (CRCoV-K9, -K37, and -K39) related to the Japanese strain 06/075.

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1. Introduction

Canine respiratory coronavirus (CRCoV) belongs to coronavirus group 2 and is a causative agent in canine infectious respiratory disease (CIRD). Recently, this virus emerged in England as a novel pathogen in the respiratory tract of dogs suffering from severe respiratory disease (Erles et al., 2003). However, CRCoV infection is thought to cause only subclinical or asymptomatic conditions (Erles et al., 2003). CRCoV replication in the respiratory epithelium may damage the mucociliary system, and thereby lead to a more severe clinical course of infection

than other respiratory pathogens (Buonavoglia and Martella, 2007).

Antibodies against CRCoV are common in canine populations of the United Kingdom (36.2%), Republic of Ireland (30.3%), USA (54.7%), Japan (17.8%), and Italy (32.06%) (Kaneshima et al., 2006; Priestnall et al., 2006; Decaro et al., 2007). The CRCoV 4182 prototype strain was isolated from a dog in England that had been euthanized after severe respiratory disease (Erles et al., 2007). In 2002, CRCoV strain 02/005 was isolated from a nasal swab collected from a dog presenting with a cough and nasal discharge in the Chiba prefecture of Japan (Yachi and Mochizuki, 2006). CRCoV 240-05 was also isolated from the lungs of a dog in Italy that was co-infected with canine parvovirus type 2 (Lorusso et al., 2009).

The genomic organization of the CRCoV 4182 prototype strain reveals that its major structural and nonstructural proteins are genetically related to bovine coronavirus (BCoV), but that the genes encoding two or three small

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nonstructural proteins situated between the spike (S) and envelope (E) proteins differ from those of BCoV (Erles et al., 2007). This dissimilar region in BCoV has three different open reading frames (ORFs) and encodes three nonstructural proteins (4.9 kDa, 4.8 kDa, and 12.7 kDa in size), whereas the corresponding region in the CRCoV 4182 prototype strain encodes only two (8.8 kDa and 12.8 kDa) (Erles et al., 2007). Interestingly, British CRCoV strains T4182, T0715, T1030, and T1207 were found to encode two corresponding nonstructural proteins, but strain G9142 was shown to encode three putative nonstructural proteins (4.9 kDa, 2.7 kDa, and 12.7 kDa) (Erles et al., 2007). Sequence analysis of the viral RNA 3'-end of an Italian CRCoV, strain 240-05, also identified the genomic region between the spike and the envelope protein genes, as it did for strain G9142 (Lorusso et al., 2009).

In this study, we identified the sequence encoding the structural and nonstructural proteins in the 3'-end of the CRCoV genome, performed a molecular phylogenetic analysis, using the neighbor-joining approach, of the CRCoV spike gene isolated from mixed tissue samples (lung and trachea) of dogs from Korea, and conducted research to identify the number of the nonstructural proteins encoded by the region between the spike and envelope genes.

2. Materials and methods

2.1. Clinical samples

Respiratory tissue samples were collected from 109 dogs, 69 of which had been euthanatized and 40 of which had died from natural causes, from 13 local animal hospitals (four in Seoul, five in Kyunggi, two in Chungnam, one in Chungbuk, and one in Jeonnam province). Of the 109 samples collected, 69 were obtained from animals presenting with subclinical symptoms, 16 with complex skin disease, 15 with a combination of vomiting and

diarrhea, and 9 with symptoms of respiratory disease, such as cough and nasal discharge. The age distribution in the dog cohort was as follows: two to six months (16 animals), one year (29 animals), two years (30 animals), and three years and older (34 animals). Fifty-two of the dogs were female and fifty-seven were male. Lung and tracheal samples were collected and then placed in 30 ml of Hanks balanced salt solution (Invitrogen) and stored at -70°C until further use.

2.2. RNA and DNA preparation

For RNA and DNA extraction, approximately 25–40 mg of mixed lung and tracheal homogenate were used. Total RNA was extracted from each sample using the micro-column technique-based QIAamp Viral RNA Mini Kit (QIAGEN, Cat. No. 52906, USA). DNA was extracted from the same samples using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Cat. No. 56404, USA).

2.3. PCR and RT-PCR amplification

The canine parainfluenza virus (CPiV), canine herpesvirus (CHV), and canine adenovirus type 2 (CAV-2) were PCR-amplified as previously described (Erles et al., 2004). The canine adenovirus type 1 (CAV-1), canine distemper virus (CDV), and canine influenza virus (CIV) were also amplified using previously published methods (Hu et al., 2001; Frisk et al., 1999; Hoffmann et al., 2001). CRCoV was amplified using the primers described in Table 1. Samples from dogs determined by RT-PCR to be CRCoV-positive were then examined, using PCR analysis of appropriate enteric, fecal, lung or tracheal samples, for the presence of other pathogens, namely canine parvovirus (Ikeda et al., 2000; Kang et al., 2008), canine coronavirus (Pratelli, 2006), and *Bordetella bronchiseptica* (Hozbor et al., 1999). CRCoV cDNA was amplified using a one-step RT-PCR Kit (QIAGEN, Cat. No. 210212). The one-step RT-PCR program

Table 1
Sequence and position of oligonucleotide primers used in RT-PCR.

Primer	Sequence 5'–3'	Polarity ^a	Position ^b	Product size (bp)
NS21F	GCTAAATCCCGCTTAAGTT	+	1–20	1147
NS21R	CAATCTGAACGACTGTCACC	–	1147–1128	
HE1F	CATCACCGGCTAGACTTGAA	+	656–675	1258
HE1R	AGACTGCCTGGCATTGTTC	–	1913–1894	
HE2F	CTCTGCACAATCTACAGCTC	+	1478–1497	918
HE2R	GTCAACATCATTGATGGAAA	–	2395–2376	
S1F	GCTGCATGATGCTTAGACCA	+	2276–2295	1069
S1R	TTAATGGAGAAGGCACCGAC	–	3344–3325	
S2F	AACGGTTACACTGTTACGCC	+	3236–3255	1378
S2R	TCGATCTACGACTTCGTCTT	–	4613–4594	
S3F	TTCACGACAGCTGCAACCTA	+	4456–4475	1108
S3R	CTGAGCTTGGCTTCAAGAG	–	5563–5544	
S4F	GCAGCAGCAGGTGACCATT	+	5261–5280	1134
S4R	GTCGTCATGTAAGTITTAATTAC	–	6394–6371	
G3F	TATGTATGGCTTTAAITGG	+	6230–6239	1178
G3R	CTTCATCAGCAGTCCAAGTG	–	7407–7388	
M1F	AGACACTGTGTGGTATGTGG	+	7113–7132	1087
M1R	TTTGCTTGGGTTGAGCTCTT	–	8199–8180	
N1F	TAGTAGAGCGTCTCTGGAA	+	8087–8106	1600
N1R	TTCCAATTGGCCATAATTAA	–	9686–9667	

^a +, sense; –, antisense.

^b The oligonucleotide position is based on the sequence of CRCoV (EU999954).

consisted of 30 min at 42 °C, 15 min at 94 °C, 15 min at 94 °C, followed by 35 cycles of 30 s at 95 °C, 30 s at 48–52 °C, and 60 s at 72 °C, and with a final elongation step of 5 min at 72 °C. Amplification of specific genes from other viral strains was carried out according to previously published protocols (Erles et al., 2004; Hu et al., 2001; Frisk et al., 1999; Hoffmann et al., 2001; Ikeda et al., 2000; Pratelli, 2006; Kang et al., 2008; Hozbor et al., 1999). PCR products of the expected sizes were purified by electrophoresis on a 1% agarose gel, followed by extraction using a QIAquick Gel Extraction Kit (QIAGEN, Cat. No. 28706, USA). The extracted DNA fragments were subsequently subcloned using the pGEM-T Vector System II (Promega, Cat. No. A3610, USA).

2.4. Virus isolation

RT-PCR-positive samples were homogenized in RPMI supplemented with GIBCO™ Antibiotic–Antimycotic (100×). The homogenate was used to inoculate a 25 cm² tissue culture flask containing human rectal tumor (HRT-18) cells. Prior to HRT-18 cell inoculation, medium was removed and cells were washed twice with FCS-free medium supplemented with trypsin (5 µg/ml). The cells were then inoculated with homogenized sample and incubated at 37 °C for 60 min. The inoculum was replaced with fresh serum-free medium supplemented with trypsin (5 µg/ml), and harvested on post-infection day (PID) three or four.

2.5. Gene alignment and analysis

A 9.6-kb region encompassing the complete 3'-end of the viral genome of CRCoV strains was determined by RT-PCR amplification of CRCoV cDNA, cloning into the pGEM-T vector (Promega, Cat. No. A3610, USA), and sequencing with T7 and SP6 sequencing primers using an ABI Prism[®] 3730xi DNA Sequencer at the MacroGen Institute (MacroGen Co., Ltd.). The structural spike proteins of six BCoV strains (Accession Nos. AF058943, AF058942, AY935638, EF445634, DQ389639, and U00735) and five CRCoV strains (Accession Nos. DQ682406, EU999954, AY150272, AB242262, and AB370269) were obtained from the GenBank nucleotide database at the National Center for Biotechnology Information (NCBI). The accession numbers of the structural and nonstructural proteins encoded by the

complete 3'-end region of the viral genome of two CRCoVs, strain 4018 and 240-05, were also obtained from GenBank (NCBI). The spike gene is 4092 nucleotides long (encodes 1363 amino acids). The gene sequences were aligned using the CLUSTALX alignment program (Thompson et al., 1997), the TREE-PUZZLE 4.0.2 program (Strimmer and von Haeseler, 1996), and the BIOEDIT 7.053 program (Hall, 1999). N- and O-glycosylation sites and signal peptide cleavage sites were predicted using the NetNGlyc 1.0, NetOGlyc 3.1, and SignalP 3.0 analysis tools, respectively, from the Center for Biological Sequence Analysis (<http://www.cbs.dtu.dk/services/>).

2.6. Neighbor-joining

Neighbor-joining analysis (Saitou and Nei, 1987) was performed using the PHYLIP computer package, version 3.572c (Felsenstein, 1995), based on the formulae of Kimura (1980). The transition/transversion ratios for spike genes were estimated from the dataset using the TREE-PUZZLE 4.0.2 Program (Strimmer and von Haeseler, 1996) and were then used as inputs for the SEQBOOT, DNADIST, NEIGHBOUR, and CONSENS programs of the PHYLIP package. For all analyses, the MHV-JHM strain (Accession No: NC_006852) was specified as the outgroup. Graphic output was produced using TreeView 1.6.1 software (Page, 1996).

3. Results

3.1. Pathogen detection and CRCoV isolation

Of the 109 samples analyzed, samples from 40 dogs (36.7%) tested positive for one or more of the viral infections (CAV1, CAV2, or CDV), and samples from 69 dogs (63.3%) were negative for all viral infections tested. Three dogs (2.8%) were infected with CRCoV: one dog (K9) with CAV1 + CAV2 + CRCoV + CPV2a, one (K37) with CDV + CRCoV, and one (K39) with CDV + CRCoV + CPV2a. The clinical records of the three dogs infected with CRCoV showed that a two-month-old Pug (K9) had presented with nasal discharge and a mild cough, and a two-year-old Shih Tzu (K37) with a mild cough, loss of appetite, emaciation, and fever, and a three-year-old Maltese (K39) with a fever, vomiting, and diarrhea. These samples tested negative for infection with CHV, CPiV, CIV, CCV, and *Bordetella*

Table 2
Coding potential and putative transcription regulatory sequences of the 3'-end of the CRCoV genome.

Gene segment	Putative gene		Putative TRS ^a	
	Coding sequence	Length (aa)	Start nt position	TRS sequence
32 kDa	169–1005	278	156	CUAAC
HE	1017–2291	424	1002	CUAAC
S	2306–6397	1363	2300	CUAAC
4.9 kDa	6387–6521	44	6064	CAAAC
2.7 kDa	6557–6634	25		Not detected
12.8 kDa	6771–7100	109	6693	CUAAC
E	7087–7341	84	6958	CAAAC
M	7356–8048	230	7347	CAAAC
N	8058–9404	448	8045	CUAAC
I	8119–8742	207	8045	CUAAC

^a Transcription regulatory sequences.

Table 3

Nucleotide and amino acid sequence identity (%) between the nonstructural and structural proteins of CRCoV-K37 and other CRCoV strains and bovine coronavirus (ENT strain).

Strain	Country	Sequence acid identity (%) to CRCoV-K37									
		32 kDa	HE	S	4.9 kDa	2.7 kDa	12.8 kDa	E	M	N	I
K39	Korea	99.2 ^b	99.1	99.7	100	100	99.4	100	99.0	99.4	99.4
		98.6^c	99.1	99.3	100	100	98.2	100	99.1	99.8	98.1
K9	Korea	98.8	99.1	99.5	100	100	99.7	100	99.1	99.4	99.4
		97.8	98.6	99.0	100	100	99.1	100	99.1	99.3	98.1
240-05	Italy	98.3	97.8	98.9	97.8	96.2	98.5	99.6	97.4	97.9	97.8
		96.8	97.2	98.6	95.5	92.0	96.3	100	97.4	98.7	93.7
4182	UK	97.8	97.8	98.9	NP ^d	NP	98.2	100	97.7	97.9	97.3
		96.4	96.7	98.0	NP	NP	97.2	100	97.4	98.4	93.2
ENT ^a		97.6	98.1	96.4	NP	NP	98.2	99.6	98.8	97.8	97.8
		97.1	98.1	95.3	NP	NP	98.2	98.8	98.3	98.0	94.7

^a Bovine coronavirus ENT strain.

^b Nucleotide sequences homology is in non-bold.

^c Amino acid sequences homology is in bold.

^d NP, not present.

bronchiseptica. Isolation of CRCoV was successful for three samples (K9, K37, and K39) using HRT-18 cell lines.

3.2. Characterization of the genome

The gene segments and the predicted molecular weights of the encoded proteins of the CRCoV Korean isolates are as follows: NS (nt 169–1005; 32 kDa), HE (nt 1017–2291; 47.7 kDa), S (nt 2306–6397; 151.1 kDa), 4.9 kDa (nt 6387–6521; 4.9 kDa), 2.7 kDa (nt 6557–6634; 2.7 kDa), 12.8 kDa (nt 6771–7100; 12.8 kDa), E (nt 7087–7341; 9.2 kDa), M (nt 7356–8048; 26.4 kDa), N (nt 8058–9404; 49.3 kDa), and I (nt 8119–8742; 23.3 kDa) (Table 2). The predicted positions of transcription regulatory sequences (TRSs) for each ORF encoding protein were located from several to hundreds of nucleotides upstream of the AUG protein initiation codon (Table 2). The TRS sequence occurs in two forms, the CUAAC type (upstream of genes encoding NS, HE, S, 12.8 kDa, N, and I proteins) and the CCAAAC type (upstream of genes encoding 4.9 kDa, 2.7 kDa, E, and M proteins) (Table 2). Nucleotide and amino acid sequence identity for the 4.9 kDa and 2.7 kDa proteins and for the protein encoded by the E gene were 100% among the three CRCoV Korean isolates (Table 3). The E protein was highly conserved in five CRCoV strains (K9, K37, K39, 240-05, and 4182), with perfect identity at the amino acid level (Table 3). The HE protein of three Korean isolates was found to contain nine potential glycosylation sites upon analysis with the NetNglyc 1.0 server, while CRCoV strain 240-05 was found to have eight potential N-glycosylation sites (Lorusso et al., 2009). A potential signal peptide of three Korean CRCoV HE proteins was identified at amino acids 1–18, with a potential cleavage site between amino acids 18 and 19, using the SignalP 3.0 server. The M protein was found to contain just one potential N-glycosylation site in all of the CRCoV strains tested, while the CRCoV strain 240-05 contained five potential O-glycosylation sites and the remaining CRCoV strains contained four potential O-glycosylation sites.

The 9.6-kb sequences encompassing the complete 3'-end of the viral genome of the three Korean CRCoV strains K9, K37, and K39 are available under accession numbers GQ918141, GQ918142, and GQ918143, respectively.

3.3. Analysis of the spike gene

The S protein was found to be 1363 amino acids long, and the TRS was predicted to be located six nucleotides upstream of the AUG codon. The nucleotide and deduced amino acid sequences of the S gene of strain K37 were compared with those of seven CRCoV strains (K39, K9, 240-05, 4182, T101, 02/005, and 06/075) and six BCoV strains (LUS-94, LY-138, KWD2, 339/06, KCD8, and Mebus). The percentage of amino acid identity between the S protein of strain K37 and of each of the seven strains of CRCoV was as follows: K39 (99.3%), K9 (99.0%), 240-05 (98.6%), 4182 (98.0%), T101 (97.9%), 02/005 (98.8%), and 06/075 (98.7%). The percentage of amino acid identity between the S protein of strain K37 and that of the six BCOVs was: LUS-94 (95.8%), LY-138 (96.3%), KWD2 (95.5%), 339/06 (95.3%), KCD8 (95.4%), and Mebus (95.4%). A potential N-terminal signal peptide of the CRCoV S protein was identified at amino acids 1–17 using SignalP-HMM, and at amino acids 1–14 using SignalP-NN. Amino acid analysis of the S protein revealed 20 different sequence variants between the CRCoV and BCoV strains (Table 4). In addition, the amino acids encoded by the CRCoV spike gene were found to contain 21 (CRCoV-4182, -240/05, -K9, and -K37) or 20 (CRCoV-K39) N-glycosylation sites, whereas those of BCoV contained only 19 (data not shown).

3.4. Genetic analysis of the 4.9 kDa protein and envelope protein

The 4.9 kDa, 2.7 kDa, 12.8 kDa, and E protein of three CRCoV Korean isolates were 44, 25, 109, and 84 amino acids long, respectively. The TRS of the 4.9 kDa, 12.8 kDa,

Table 4
Comparison of the amino acid sequences of spike proteins in CRCoV and BCoV strains.

Virus	Clinical forms	Strain, clone or isolate	Country	Accession No.	Host	Amino acid at residue	40	101	103	118	235	407	440	492	528	540	582	695	744	828	887	933	1069	1256	1257	1289	
CRCoV	Respiratory infection	K9^a	Korea	EU983105	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	V	F	D	A	L	M	H	
		K37	Korea	EU983106	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		K39	Korea	EU983107	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		4182	UK	DQ682406	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		240-05	Italy	EU999954	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		T101	UK	AY150272	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		02/005	Japan	AB242262	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		06/075	Japan	AB370269	Dog	V	D	V	V	V	H	M	I	N	N	L	K	S	S	S	V	F	D	A	L	M	H
		LSU-94	Respiratory			AF058943	Cow	T	N	I	K	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q
		LY-138	Enteritis			AF058942	Cow	T	N	I	M	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q
KWD2	Winter dysentery			AY935638	Cow	T	N	I	M	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q		
339/06	Summer dysentery			EF445634	Cow	T	N	I	M	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q		
KCD8	Calf diarrhea			DQ389639	Cow	T	N	I	M	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q		
Meibus	Avirulent			U00735	Cow	I	N	I	M	N	L	V	D	A	T	Q	A	V	A	V	E	S	S	V	Q		

^a The three CRCoV strains isolated from dogs in Korea are in bold.

and E protein is predicted to be located at 323, 78, and 129 nucleotides upstream of the AUG codon, respectively, whereas we were unable to predict the position of the TRS of the 2.7 kDa protein. The three genes positioned between the BCoV spike and envelope genes encode the 4.9 kDa, 4.8 kDa, and 12.8 kDa proteins, which are conserved in the BCoV-179, -LY138, and -Meibus strains, while the corresponding gene region in CRCoV-4182 encodes only two proteins, which are 8.8 kDa and 12.8 kDa large. Interestingly, three Korean strains encode protein species of 4.9 kDa, 2.7 kDa, and 12.8 kDa, as do CRCoV strain G9142 and 240-05. Analysis of the amino acid sequences encoded by the CRCoV and BCoV envelope genes showed one sequence variation (data not shown). The nucleotide sequence identity between the 4.9 kDa, 2.7 kDa, and E gene of CRCoV strain K37 and 240-05 was 97.8%, 96.2%, 98.5%, and 99.6%, respectively, while identity of the corresponding deduced amino acid sequences was 95.5%, 92%, 96.3%, and 100%, respectively (Table 3).

3.5. Phylogenetic tree analysis

The transition/transversion parameter estimated from the dataset of the Spike genes of CRCoV and BCoV was 1.40, and 14 strain sequences consisting of 4092 nucleotides in total contained 2574 (62.9%) constant sites. The topology of the spike genes indicated that all of the CRCoV and BCoV strains belonged to one of two clades: CRCoV or BCoV (Fig. 1). Using the neighbor-joining approach and murine hepatitis virus as an outgroup, the first branch was found to have a strong bootstrap value on the node between the CRCoV and BCoV strains. Eight CRCoV strains from dogs in England (CRCoV-4182 and -T101), Japan (CRCoV-02/005 and -06/075), Korea (CRCoV-K9, -K37 and -K39), and Italy (CRCoV 240-05) revealed that the three Korean strains were related to the Japanese strain 06/075 and that the Italian strain 240-05 was related to the Japanese strain 02/005 (Fig. 1).

4. Discussion

The etiological agents thought to be involved in CIRD are complex, and generally include CRCoV, CAV-1 and -2, CPiV, CHV, CIV, reoviruses, and *Bordetella bronchiseptica* (Erles et al., 2004; Buonavoglia and Martella, 2007). A longitudinal study of viruses associated with endemic CIRD at a rehoming center in the United Kingdom reported the presence of CPiV, CHV, and CRCoV, whereas CAV2 and CDV were absent from the population (Erles et al., 2004).

Two new findings emerged from our study. First, CRCoV infection alone was not found in the lung and tracheal tissues of sampled dogs; and second, complex CRCoV infection occurred together with CAV-1 or -2, CDV, or CPV2a infection, whereas infection with CHV, CPiV, CIV, canine coronavirus (CCV), or *Bordetella bronchiseptica* was absent in all six CRCoV-positive dogs. Furthermore, among the three CRCoV-positive dogs reported here, two (the K9 and K37 isolate) displayed mild respiratory clinical symptoms, and one (the K39 isolate) presented with a combination of vomiting and diarrhea. However, two dogs (the K9 and K39 isolates) were infected with CPV type 2a

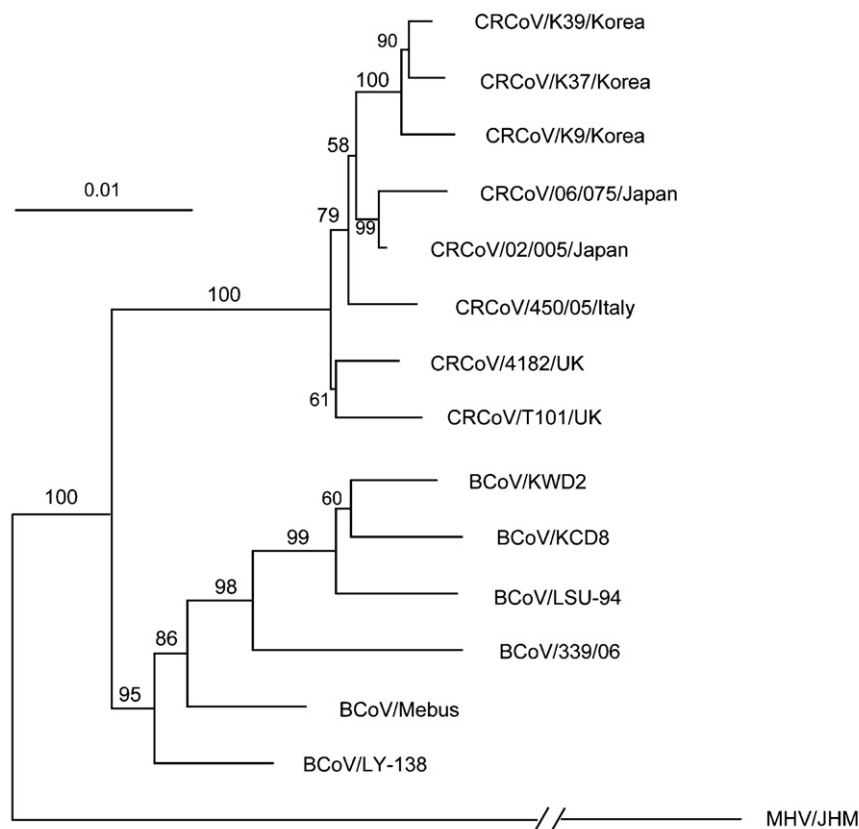


Fig. 1. Phylogenetic tree of CRCoV and BCoV strains. The phylogeny was based on spike gene sequences from eight CRCoVs, six BCoVs, and an outgroup from one MHV spike gene nucleotide sequence, using the neighbor-joining method. Sequences for dogs of English (CRCoV-T101 and -4182), Italian (CRCoV-240/05), Japanese (CRCoV-02/005 and -06/075), and Korean origin (CRCoV-K9, -K37 and -K39) were obtained from the GenBank nucleotide database at the National Center for Biotechnology Information (NCBI). Numbers at nodes indicate the neighbor-joining bootstrap value ($\geq 50\%$).

according to CPV diagnosis, and not with CPV type 2a variant.

Erles et al. (2007) previously reported that infection with CRCoV alone caused subclinical or mild respiratory symptoms, while CRCoV infection in conjunction with other pathogens resulted in severe respiratory disease and possible exacerbation of disease phenotypes caused by other pathogenic agents (Erles et al., 2003). Further, they stated that additional epidemiological studies are required to determine the exact biological and immunological roles of single or combined CRCoV infection.

H3N8 equine influenza virus was described as a novel viral pathogen causing CIRDC in different dog populations of the United Kingdom and Florida, USA (Crawford et al., 2005; Daly et al., 2005), and H3N2 canine influenza virus has since been described as a novel candidate pathogen for CIRDC in Korea (Song et al., 2008). However, the absence of CIV in three CRCoV-positive canine samples, as well as in the remaining 106 CRCoV-negative dogs reported here, suggests that H3N2 may not be related to CIRDC in Korea.

The nucleotide sequence of the 3'-terminal portion of CRCoV strain 4182 and 240-05 was determined, as were the open reading frame sequences of the gene encoding the 32 kDa protein to the N gene (Erles et al., 2007; Lorusso et al., 2009). Three proteins (4.9 kDa, 4.8 kDa, and 12.8 kDa) were found to be encoded by the region between the spike

and envelope genes, while the corresponding gene region in CRCoV-4182 encodes only two proteins (8.8 kDa and 12.8 kDa) (Erles et al., 2007). It was recently reported that CRCoV strain 240-05 isolated from Italy encodes proteins of 4.9 kDa, 2.7 kDa, and 12.8 kDa (Lorusso et al., 2009). The 4.9–12.8 kDa proteins encoded by three Korean strains were consistent with those of CRCoV strain G9142 and 240-05. The CRCoV-4182 strain isolated from the UK is likely to have undergone genetic mutation. Therefore, it is likely that the proteins encoded by the region between the spike and envelope genes in the CRCoV genome are three nonstructural proteins that are 4.9 kDa, 2.7 kDa, and 12.8 kDa. Coronavirus genes are preceded at the 5'-end by TRSs that commonly include the consensus sequence CYAAAC (Hofmann et al., 1993; Zuniga et al., 2004). In the genetic characterization of the bubaline coronavirus (BuCoV), the predicted nucleotide sequence of TRS encoding the 12.8 kDa protein were shown to be different for BCoV and CRCoV, which were CCAAAC and CUAAAC, respectively.

Structural predictions of the protein encoded by the spike gene from the CRCoV strains identified in this study showed 21 or 20 N-glycosylation sites, while the spike protein of the six BCoV strains (-LUS/94, -LY/138, -KWD2, -339/06, -KCD8, and -Mebus) analyzed showed 19. Four CRCoV strains (-4182, -240/05, -K9, and K37) had 21

N-glycosylation sites (at amino acid positions 59, 133, 198, 359, 437, 444, 492, 649, 676, 696, 714, 739, 788, 895, 937, 1194, 1224, 1234, 1253, 1267, and 1288), while CRCoV-K39 had 20 N-glycosylation sites, lacking the one at amino acid position 437. The N-glycosylation sites of the four CRCoV strains and the six BCoV strains differed at two sites (amino acid position 444 and 492).

The predicted C-terminal membrane-anchoring domain of bovine enteric coronavirus spike protein was previously reported to contain eight cysteine residues (Boireau et al., 1990), in agreement with the results presented here, at amino acid positions 1329–1331, 1334, 1338, 1342, 1445, and 1446.

Comparison of spike gene topology indicated that all CRCoV and BCoV strains belonged to two clades (CRCoV and BCoV). The first branch, divided using murine hepatitis virus as an outgroup, produced a strong bootstrap value by the neighbor-joining approach at the node between the CRCoV and BCoV strains. The phylogenetic tree of spike genes from four CRCoV strains (-T101, -4182, -02/005, and -06/075) also showed two branch points (in dogs of English and Japanese origin) (Mochizuki et al., 2008). Until now, few CRCoV isolates from the United Kingdom (2), Japan (2), Italy (1), and Korea (3) have been reported. Eight CRCoVs were predicted to occur in distinct geographical regions such as Asia and Europe. However, three Korean strains related to Japanese strain 06/075 and Italian strain 240-05 related to Japanese strain 02/005. For a more precise analysis of the geographical clades in the phylogenetic tree of the CRCoV strains, more CRCoV strains should be isolated from a variety of countries and studied.

In summary, small nonstructural proteins encoded by the region between the spike and envelope genes of three CRCoV strains isolated from Korean canine samples were found to contain three ORFs, which encode proteins of 4.9 kDa, 2.7 kDa, and 12.8 kDa. The neighbor-joining approach was used to infer CRCoV and BCoV phylogenies, which comprise two clades (CRCoV and BCoV) and three Korean strains that are related to Japanese strain 06/075.

Acknowledgments

We are grateful to Ms. Sung-Eun Lee and Mr. Jung-Sam Kim for technical assistance.

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