Genetic characteristics of canine bocaviruses in Korean dogs

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A B S T R A C T

To survey for canine bocavirus (CBoV) infection, 83 Korean dogs showing several clinical signs were collected in different provinces from January 2013 to July 2014. Using polymerase chain reaction (PCR) and in situ hybridization, CBoVs were detected in intestine and/or lung samples of 8 dogs (9.6%). To reveal the genetic characteristics of CBoVs, partial or complete regions of CBoVs were sequenced. In phylogenetic trees, 8 CBoVs fell into three clusters. The CBoV strains 13D226-1, 13D250, and 14Q216 were closely related to the CBoV HK831F strain, and the CBoV 14D142 strain was related to the CBoV HK882F strain. Lastly, CBoV 13D003, 13D095, 14D193, and 14Q209 strains were related to CBoV Dis-023, Dis-040, and Dis-046 strains. Interestingly, no canine pathogens were found in dogs in which four CBoVs (13D003, 13D0095, 14D142, and 14D193 strains) were detected and three of them (13D003, 13D095, and 14D193 strains) had a unique deletion (18 nucleotides) in the VP2 gene. Further, the open reading frame 4 (ORF4) region was absent in these 4CBoVs, but found in the other strains, which indicates that the absence of the ORF4 region rather than a unique deletion may have an influence on the pathogenesis of CBoV in dogs.

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1. Introduction

Parvoviruses in the family *Parvoviridae* are currently divided into two subfamilies, *Densovirinae* and *Parvovirinae*, members of which infect non-vertebrate and vertebrate hosts, respectively (Brown, 2010; Fauquet et al., 2005). The subfamily *Parvovirinae* has been classified into eight genera: *Amdoparvovirus, Aveparvovirus, Bocaparvovirus, Copiparvovirus, Dependoparvovirus, Erythroparvovirus, Protoparvovirus,* and *Tetraparvovirus* (Bodewes et al., 2014). The genus *bocavirus* within the subfamily *Parvovirinae* contains small, non-enveloped, autonomously replicating, single-stranded DNA viruses with an icosahedral capsid. Bocaviruses are unique among parvoviruses as they contain a third open reading frame (ORF) between the non-structural and structural-coding regions and the genome length of the viruses is approximately 5.4 kb. The genus was originally named according to the initial two members, bovine parvovirus (BPV) and minute virus of canines (MVC) (Binn et al., 1970; Mochizuki et al., 2002; Spahn et al., 1966; Storz et al., 1978).

The genus *Bocavirus* contains BPV, MVC (Fauquet et al., 2005), porcine bocaviruses (Cheng et al., 2010), gorilla bocavirus (GBoV) (Kapoor et al., 2010a,b), and four species of human bocaviruses (HBoV 1–4) (Allander et al., 2005; Arthur et al., 2009; Chieochansin et al., 2007; Kapoor et al., 2010a,b; Kapoor et al., 2009). Similar bocavirus-like sequences have also been detected in fecal samples of primates (Sharp et al., 2010). Four novel bocavirus species, California sea lion bocavirus 1 (CslBoV1) to CslBoV4, have been identified in the fecal flora of California sea lions (Li et al., 2011). More recently, a novel canine bocavirus (CBoV), phylogenetically distinct from MVC, was discovered in respiratory and intestinal samples from dogs (Kapoor et al., 2012).

At present, CBoV has been detected in fecal, nasal, urine, and blood samples of dogs with or without clinical signs. Recent studies reported that CBoV in dogs is genetically distinct from MVC and bocavirus genotypes found in other species because of a unique deletion in the VP2 gene that is substantially more prevalent in dogs with respiratory disease. Further, the ORF4 region, which was not found in other bocaviruses, was found in a recent study (Kapoor et al., 2012; Lau et al., 2012). However, a clear relationship between CBoV and respiratory or intestinal disease in dogs is yet to be established, and most of the clinical

and epidemiological features of CBoV infection are still unknown. Therefore, the present study was performed to investigate CBoV infection, and reveal any relationship between CBoV infection and pathological findings in dogs.

2. Materials and methods

2.1. Clinical samples

To investigate the prevalence of CBoV in Korean dogs, intestine, lung, brain, kidney, liver, lymph node, spleen, and heart samples from 83 dogs which were dead with several clinical signs were collected by Animal and Plant Quarantine agency (QIA) from January 2013 to July 2014. Kidney and liver samples were mixed and ground in phosphate buffered-saline solution (PBS) at a concentration of ~1 g/mL. Lymph node and spleen samples were mixed and ground in PBS at a concentration of ~1 g/mL. The other samples, intestine, lung, brain, and heart, were ground separately in PBS at a concentration of ~1 g/mL. All ground samples were stored at −80 °C until use.

2.2. Polymerase chain reaction (PCR)

All sample suspensions were centrifuged at 8000 rpm for 10 min to remove large debris. Total DNA/RNA was extracted directly from samples using the Viral DNA/RNA Extraction Kit (iNtRON, Seongnam, Korea), according to the manufacturer's directions. Intestine and/or lung samples were screened for CBoV using polymerase chain reaction (PCR) by Maxime PCR premix kit (iNtRON, Seongnam, Korea). We performed PCR using previously reported bocavirus screening primers (Lau et al., 2012). Oligonucleotide primers were designed by multiple alignments of the nucleotide sequences in NS1 regions of known bocaviruses including HBoV, BPV, and MVC. The forward primer was 5′-GCCAGCACNGGNAARACMAA-3′ and reverse primer was 5′-CATNAGNCAYTCYTCCCACCA-3′. The PCR conditions were 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 50 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min in an automated thermal cycler (Applied Biosystems). The size of the expected amplified DNA was 141 bp. For the lung or intestine samples in CBoV positive dogs, CBoV was examined from other organ samples including brain, kidney, liver, lymph node, spleen, and heart using the same protocol.

2.3. Histological examination

After necropsy, all parenchymal organs were fixed in 10% neutral-buffered formalin and embedded in paraffin wax, sectioned at 4 μm thickness, stained with hematoxylin and eosin, and processed by routine histopathological methods.

2.4. In situ hybridization

For the generation of a CBoV-specific in situ probe, PCR labeling with the PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH, Mannheim, Germany) was performed, according to the manufacturer directions. Briefly, the specific region (nucleotides 765–1509) of the NS1 gene in 13D003 strain was amplified by PCR with NS1 forward (5′ TTCAGTCTCAGCTCGAGATTAACG 3′) and NS1 reverse (5′ TGAAAGATCAAGAGCTGTATAG 3′) primers. This PCR product was directly generated and labeled, and subsequently used as hybridization probes. With the obtained probes, lung and intestine tissues of the infected dogs were investigated for the presence of CBoV nucleic acid. To detect bocavirus DNA, additional paraffin embedded tissues were sectioned at 4 μm thickness and in situ hybridized using the fully automated system (NexES IHC instrument; Ventana Medical Systems, Inc., Tucson, AZ, USA) and DAB detection system (Ventana Medical Systems Inc.). The tissue sections were deparaffinized (standard xylene and industrial methylated spirits) and fixed in 4% paraformaldehyde for 10 min. Tissues were then permeabilized by incubation in 1 mg/mL pepsin in 0.1 mol/L HCl for 20 min at 37 °C followed by denaturation at 70 °C for 10 min before hybridization with DIG-labeled riboprobe at 65 °C for 6 h. Washes after hybridization were performed using 0.1 × SSC at 75 °C twice for 6 min followed by PBS at room temperature for 5 min. After hybridization, slides were incubated with QDs 605-conjugated anti-DIG antibody (Invitrogen, Carlsbad, CA) for 30 min and rinsed thereafter twice in PBS for 5 min. All tissue sections were coverslipped using 90% (v/v) glycerol/PBS mounting solution. The DAB detection system was applied and then the sections were incubated at 37 °C. Sections were counterstained with hematoxylin and post-counterstained with bluing reagent.

2.5. Complete and partial sequencing of CBoVs

The genetic diversity of CBoV strains was investigated by amplifying and sequencing of the partial capsid protein sequencing using primers (forward primer 5′-GGAGGAGGTGGAGGACAT-3′ and reverse primer 5′-CGTCCGTCAGGTCAGATT-3′) targeted to a 526 bp region of theVP1/VP2 genes (Lau et al., 2012). The amplified DNA fragment was purified using an Agarose Gel DNA Extraction Kit (INtRON, Korea) and subcloned into the pGEM-T vector (Promega, Madison, WI, USA), according to the manufacturer's instructions. Automated nucleotide sequencing was performed on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). All nucleotide positions were confirmed by three or more independent sequencing runs in both directions. The nucleotide and putative amino acid sequence alignments were created using BioEdit (Ibis Biosciences, Carlsbad, CA, USA).

For the determination of the complete nucleotide sequence of CBoV, DNA extracted directly from the samples was used as the template and amplified by degenerate primers designed from multiple alignments of the genomes of CBoV, MVC, HBoV, BPV, and PBoV. Primer sequences are available upon request. The complete or near complete sequence of the CBoVs has been deposited in GenBank under accession numbers KP281713-KP281720.

2.6. Phylogenetic analysis

The partial VP1/VP2 sequences and complete genomic sequence of CBoV were compared to those of reference bocavirus strains in GenBank by using BLAST. The phylogenetic tree of the Korean CBoV with bocavirus reference strains and other representative bocaviruses based on the nucleotide and amino acid alignments was constructed using the neighbor-joining method and Molecular Evolutionary Genetics Analysis (MEGA version 4.0). Bootstrap analysis was carried out using 1000 replications, and the phylogenetic tree was visualized using Treeview (Tamura et al., 2007), and MVC was specified as an out-group.

2.7. Screening of other pathogens

All samples were also screened for canine parvovirus (CPV), canine distemper virus (CDV), canine adenovirus (CAdV), canine influenza virus (CIV), canine herpesvirus (CHV), canine parainfluenza virus (CPIV), and canine coronavirus (CCV) using a commercial detection kit (iNtRON Biotechnology, Korea), as described previously (Woo et al., 2010; Yoon et al., 2009).

3. Results

3.1. Detection of CBoV and other pathogens in dogs

Of 83 Korean dogs, CBoV was detected in the intestine and/or lung samples of eight dogs (9.6%) using PCR. We detected CBoV in dogs of different ages between 5 days and 3 months (Table 1). We also detected CBoV in the other organ samples of eight CBoV-positive dogs including in the intestine, kidney and liver mixture, and lymph node and spleen mixture of all CBoV-positive dogs, and CBoV was lowest in the brain and observed in just 2/8CBoV-positive dogs. The strong positive bands from PCR for CBoV were similar in the kidney and liver mixture and lymph node and spleen mixture samples compared with the other organ samples (data not shown). Eight CBoV-positive samples were also tested for other pathogens, e.g., CHV, CIV, CPIV, CDV, CPV, CCV, and CAdV. Of the concurrent infections of CBoV with other pathogens, mixed infections with 2 pathogens (CBoV + CPV, $n = 1$) and 3 pathogens (CBoV + CPV + CCV, $n = 1$; CBoV + CAdV + CCV, $n = 1$; CBoV + CPV + CHV, $n = 1$) were identified by PCR. No pathogens except for CBoV were detected in 4 dogs (13D003, 13D095, 14D142 and 14D193; Table 1). In bacteriologic and parasitological examination, any bacteria or parasites were not detected in the eight dogs in which CBoV was found. Of eight CBoV-positive dogs, 4 dogs (13D226-1, 14D142, 14D193 and 14Q206) showed clinical signs of respiratory dysfunction, such as nasal discharge or cough, and 7 dogs except 14Q216, had lesions in the lung (Table 1). In two cases of infant CBoV-positive dogs (13D003 and 14D142), the other infant dogs from the same mothers of 13D003 and 14D142 were also dead (Table 1).

3.2. In situ hybridization and pathological findings

The nucleic acid of CBoV was detected in six of 8 dogs (13D003, 13D095, 13D250, 14D142, 14D193 and 14Q216) by in situ hybridization (Fig. 1). In situ hybridization analysis of the lung tissues of 13D003, 14D142, and14D193 and intestine tissues of 13D095, 13D250, and 14Q216 revealed CBoV nucleic acid positive cells. In pathological examination, edema in tracheal submucosa and interstitial pneumonia were revealed in 13D003. Alveolar septa and glomerulus degeneration were revealed in 13D095. The variant 13D226-1 showed alveolar wall thickening, pulmonary hyperemia, thrombosis, and cryptitis. The variant 13D250 showed pulmonary hyperemia, bronchiolitis, villous atrophy, and squamous villous epithelial cell. The variant 14D142 also showed pulmonary hyperemia and thrombosis, and 14D193 showed fibrinous and serous exudates in the alveolar space. Interstitial pneumonia and severe bronchitis were shown in 14Q209 and hemorrhagic enteritis was shown in 14Q216 (Fig. 2).
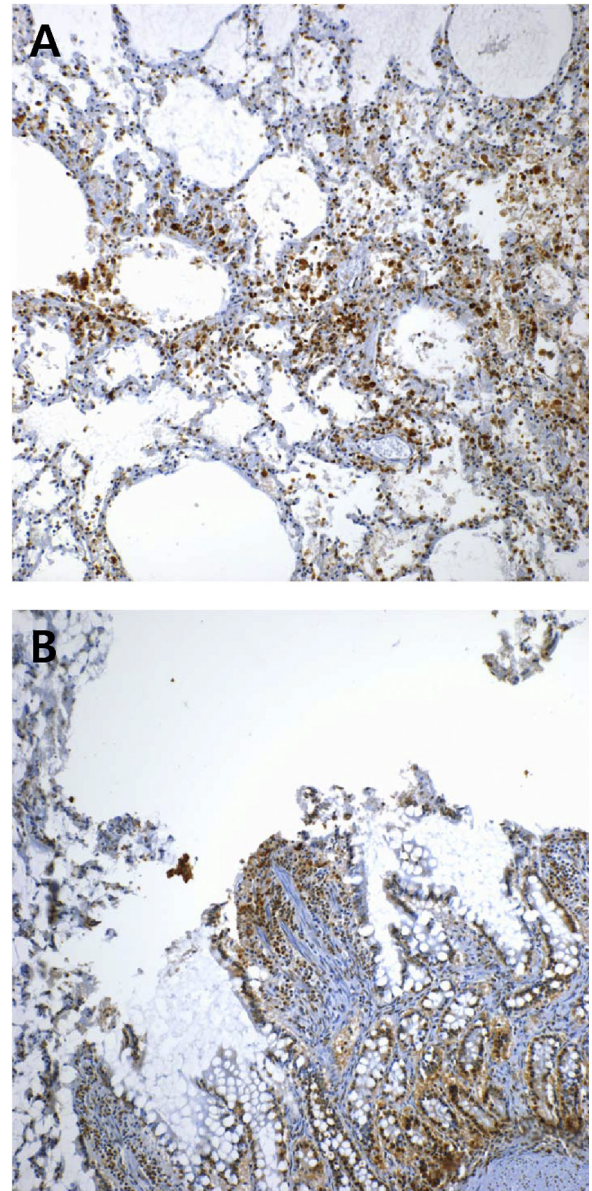

Fig. 1. The nucleic acid of CBoV (dark brown) was observed in lung tissue of 14D193 (A: ×200) and intestine tissue of 14Q216 (B: ×200) in situ hybridization.

3.3. Genome analysis and phylogenetic relationship

Partial VP1/VP2 regions of eight representative CBoVs were sequenced and aligned with other CBoVs obtained from GenBank, using BioEdit. In phylogenetic trees, eight CBoVs fell into three

Table 1
Detection of canine bocavirus and other viral pathogens in canine intestine and lung samples.

ID	Age	PCR		In situ hybridization		Clinical signs and histological findings	Mixed infections
		L	I	L	I		
13D003	10 days	+	+	+	−	Loss of appetite, Death of dogs from same mother dog, Interstitial pneumonitis	−
13D095	3 months	+	+	−	+	Lethargy, Alveolar septa degeneration, Glomerulus degeneration	−
13D226-1	2 months	−	+	−	−	Nasal discharge, Diarrhea, Cryptitis, Pulmonary hyperemia	CPV, CCV
13D250	2 months	−	+	−	+	Diarrhea, Vomiting, Intestinal villous atrophy, Cryptitis, Pulmonary hyperemia	CCV, CAdV
14D142	5 days	+	+	+	−	Nasal discharge, Death of dogs from same mother dog, Pulmonary hyperemia	−
14D193	2 months	+	+	+	−	Nasal discharge, Pulmonary fibrosis	−
14Q209	3 months	−	+	−	−	Nasal discharge, Cough, Bronchiolitis	CPV, CHV
14Q216	2 months	−	+	−	+	Vomiting, Diarrhea, Enteritis	CPV

L: lung tissues; I: intestine tissues; CHV: canine herpes virus; CPV: canine parvovirus; CAdV: canine adenovirus; CCV: canine coronavirus.
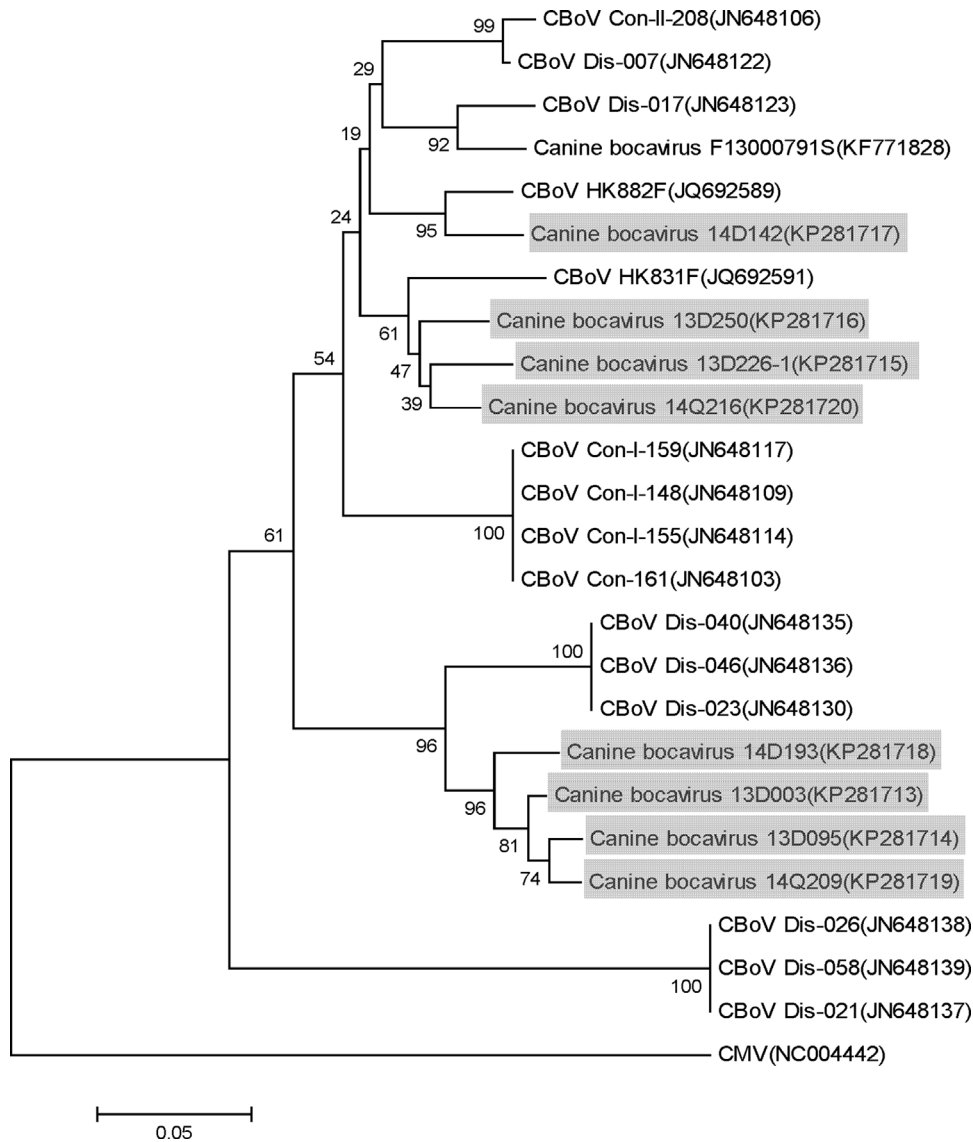
Fig. 2. Phylogenetic relationship between the VP2 gene nucleotide sequences from different canine bocavirus strains. GenBank accession numbers are in parentheses. The tree was constructed by the neighbor-joining tree with 1000 bootstrap replications.
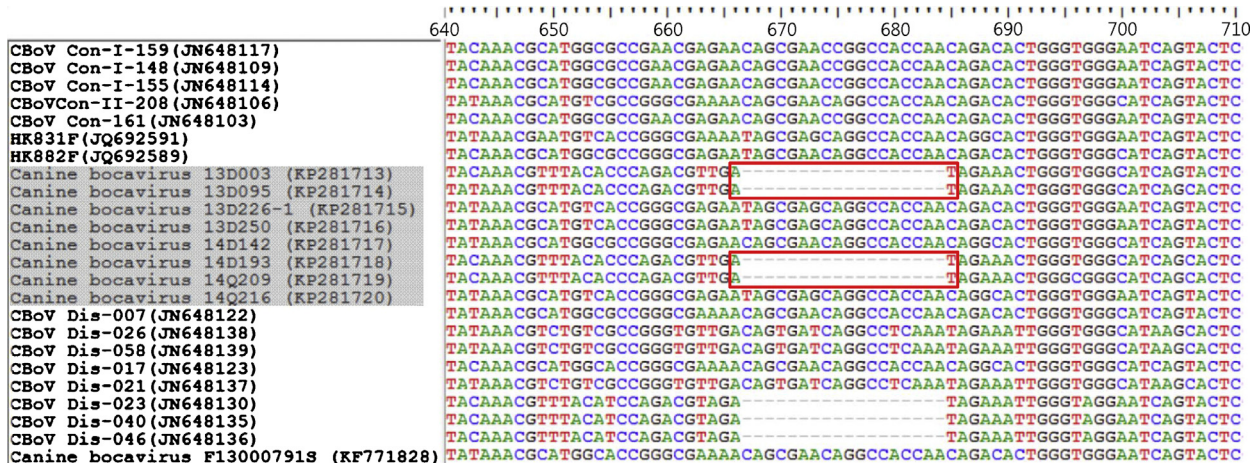CBoV, canine bocavirus; CMV, Canine minute virus; CPV.

Fig. 3. An 18 nucleotide deletion (red box) found previously in CBoV Dis-023, Dis-040, and Dis-046 strains, located at the variable exposed loop of VP1/VP2 (Kapoor et al., 2012), was present in the 13D003, 13D095, 14D193, and 14Q209 strains.. The genome size shown in this figure is from 640 to 710 nucleotide of CBoV VP1/VP2.

clusters. The CBoV strains 13D250, 13D226-1, and 14Q216 were closely related to the CBoV HK831F strain and the CBoV 14D142 strain was related to the CBoV HK882F strain. The CBoV 13D003, 13D095, 14D193, and 14Q209 strains were related to CBoV Dis-023, Dis-040 and Dis-046 strains (Fig. 2). In addition, the six amino acid (aa) deletion found previously in the CBoV Dis-023, Dis-040 and Dis-046 strains, located at the variable exposed loop of VP1/VP2 (Kapoor et al., 2012), was present in the CBoV 13D003, 13D095, 14D193, and 14Q209 strains (Fig. 3).

The complete genome of the CBoV 13D003 strain was 5348 nucleotides (GenBank accession no. KP281713) and the near-complete genome sequences of CBoV strains 13D095, 13D226-1, 13D250, 14D142, 14D193, 14Q209, and 14Q216 were 5156–5177 nucleotides (GenBank accession no. KP281714–KP281720). The non-coding region of the 13D003 strain on left-hand side (LHS) terminus, located at the 5′ end of positive-sense ssDNA genomes was 313 nucleotides. The right-hand side (RHS) non-coding region of the 13D003 strain, found at the 3′ end of positive-sense ssDNA genomes was 199 nucleotides. The ORF1, ORF2, and ORF3 of the eight strains encode 649–794 aa non-structural (NS) protein, 706-724 aa overlapping the VP1/VP2 capsid protein and 196 aa NP1 protein, respectively. The ORF4 region reported in a previous study (Lau et al., 2012) was found in the genome of CBoV 13D226-1, 13D250, 14Q209, and 14Q216 strains, and encodes 145 aa. The eight CBoV strains showed the genome organization of various forms depending on the length of NS1 protein, the length of VP1 protein, or the presence of the ORF4 region (Fig. 4).

Eight CBoV strains showed nucleotide similarities between 91.2–97.9% and fell into three clusters based on the phylogenetic analysis of the partial VP1/VP2 region (Fig. 2). Further, the genome sequences of the eight strains shared 87.6–94.9% nucleotide identities with those of other known CBoV strains.

3.4. Genetic diversity of CBoV

Calculation of pair-wise distances using the NS1, NP1, and VP1/VP2 regions of 12CBoV variants resulted in up to 20.7%, 4.1%, and 12.1% (mean diversities of 8.3%, 1.9%, and 8.5%) nucleotides and 20.6%, 2.6%, and 10.5% (mean diversities of 8.6%, 1.3%, and 6.2%) protein sequences divergences, respectively (GenBank acession nobers KP281713-KP281720, JQ692589, JQ692591, JN648103, and KF771828). The sequences of the ORF4 region in 6CBoV strains showed that they possessed up to 3.7% (mean diversity of 2.7%) nucleotide and 4.2% (mean diversity of 3.1%) protein differences from each other (GenBank accession numbers KP281715, KP281716, KP281719, KP281720, JQ692589, and JQ692591). These results of these genetic diversities from the CBoV strains indicate that NS1 and VP1/VP2 regions are highly variable while NP1 and ORF4 regions are relatively conserved.

4. Discussion

The present study examined the viral nucleic acids of CBoV in the lungs and intestines of dogs with clinical signs relating to problems in these organ systems. We detected CBoV in approximately 9.6% of Korean dogs that died of some severe disease and this rate was higher than the positive rate (6.3%) of CBoV in Hong Kong (Lau et al., 2012). The infection rate of CBoV in Korean dogs can be higher or lower than that observed in the present study, because we examined a limited number of organs and samples from dogs referred to Animal and Plant Quarantine agency (QIA) and subclinical infection can still exist. All dogs infected with CBoV were less than three months old (Table 1). The age of dogs infected with CBoV indicates that younger dogs may be more susceptible to CBoV than adult dogs. Commonly, young animals suffer from disease by bocavirus, whereas in adults subclinical infection is common (Kapoor et al., 2012).

All CBoV-positive dogs showed respiratory system, gastrointestinal tract, or abortion problems in clinical and histopathological results, which are consistent with previous studies of CBoV infection (Bodewes et al., 2014; Kapoor et al., 2012; Lau et al., 2012). Further, CBoV was detected in 4 dogs (strains 13D003, 13D095, 14D142, and 14D193) that did not possess any other pathogens related to disease or death (Table 1). In situ hybridization studies of
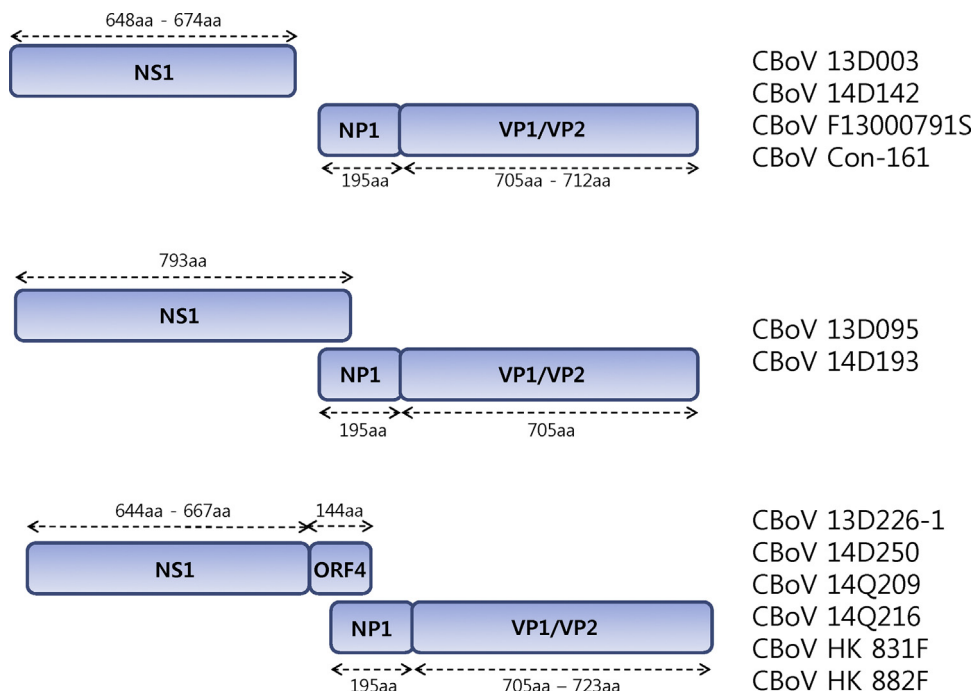

Fig. 4. Comparative genome organization of canine bocaviruses isolated from Korea and other canine bocaviruses.

the lungs and intestines from 13D003, 13D095, 14D142, and 14D193 strains revealed positive findings for CBoV nucleic acids in alveolar macrophages and intestinal lymphocytes (Fig. 1). These results indicate that CBoV may have pathogenicity in young puppies by a single infection.

The CBoV strains from 13D003, 13D095, and 14D193 had a unique deletion (18 nucleotides) in the VP2 gene that appeared to fall into separate clusters distinct from the other detected strains. In histological findings, respiratory system problems such as interstitial pneumonitis, pulmonary hyperemia, or alveolar septa degeneration were observed in lung tissues of 13D003, 13D095, and 14D193 (Table 1). These results correspond with an earlier study that found CBoV was associated with respiratory disease (Kapoor et al., 2012) and support the notion that the unique deletion may have an influence on the pathogenesis of CBoV in dogs. However, the case of 14D142 also showed respiratory system problems, such as nasal discharge and pulmonary hyperemia, even though the CBoV detected in lung tissues of 14D142 did not have a unique deletion. This result is not consistent with the hypothesis that a relationship exists between a unique deletion and respiratory disease (Kapoor et al., 2012). In addition, the F13000791S strain, which does not have a unique deletion, was reported as a pathogen for enteritis (Bodewes et al., 2014).

The genome organization of the CBoV strains assessed in the present study was varied in their forms (Fig. 4). Each protein of bocavirus strains is known to possess varying lengths depending on the host species (Lau et al., 2012); however, bocaviruses from canine had different genome organizations and protein lengths in the same species. Recent studies have reported that CBoV genomes posses a second exon encoding the C-terminal region of NS1 and conserved RNA-splicing signals near the end of NS1, which may generate a longer NS1 (Lau et al., 2012). The predicted NS1 protein of CBoV 13D095 and 14D193 strains was relatively long, encoding 793 aa, compared with 648–674 aa in the other CBoV strains examined in the present study. This longer length supports the possibility that the putative second exon and conserved RNA-splicing signals may generate a longer NS1. In addition, the presence of the ORF4 region, immediately downstream of ORF1, was found. In CBoV strains, the ORF4 region was reported previously (Lau et al., 2012). The ORF4 region was found in 13D226-1, 13D250, 14Q209, and 14Q216 strains, but was not found in 13D003, 13D095, 14D142, and 14D193 strains, which are expected pathogens that can cause disease by a single infection. Further, CBoVs were detected in lung tissues of 13D003, 13D095, 14D142, and 14D193 dogs and all these dogs showed clinical respiratory system symptoms and histologically. Therefore, the absence of the ORF4 region can be one of the possible factors that influence the pathogenesis of CBoV. The ORF4 region encodes a 144 aa protein, which overlaps with the predicted second exon of NS1, which indicates that the ORF4 region might also be generated by a second exon and conserved RNA-splicing signals near the end of NS1. However, the ORF4 region may be translated discontinuously from the NS1 region by, for example, alternative splicing or specific cleavage by a protease, unlike the cases that have a continuous and long NS1 region. The ORF4 region made this way may affect the function of NS1 proximally, and it may influence the pathogenicity of CBoV. An earlier study suggested that typical bocavirus NS1 protein might be functional during virus infection, such as transactivation (Chen et al., 2010). Until recently, the unique deletion in the VP2 gene of CBoV was the only possible factor that had been proposed to determine pathogenicity (Kapoor et al., 2012). However, this study indicates that the absence of the ORF4 region rather than the unique deletion may have an influence on the pathogenesis of CBoV in dog. Further studies, such as animal inoculation using CBoV-positive samples

or CBoV isolate, are necessary to determine which factors, unique deletion, the ORF4 region, or both, actually influence the pathogenesis of CBoV.

In conclusion, CBoV can be a pathogen that causes diseases such as pneumonia or enteritis by a single infection in young puppies, and the pathogenesis of CBoV might be influenced by the absence of the ORF4 region rather than the presence of a unique deletion. Further studies that examine animal inoculations are essential for the prevention, diagnosis, and treatment of CBoV, which will lead to a better understanding of the distribution, diversity, and pathogenesis of canine bocavirus in domestic dogs and wild mammals in the Canidae family.

Conflict of interests

The authors declare that they have no conflict of interests.

Acknowledgements

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