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Genetic characteristics of canine bocaviruses in Korean dogs

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ABSTRACT

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.

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

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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).