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Epidemiological investigation reveals genetic diversity and high co-infection rate of canine bocavirus strains circulating in Heilongjiang province, Northeast China

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ABSTRACT

To trace evolution of CBoV in Northeast China, 201 fecal samples from rectal swabs of diarrheic dogs collected from May 2014 to April 2015 were investigated using PCR targeting partial NS1 gene (440 bp). Furthermore, phylogenetic analysis of the identified CBoV strains was conducted using nucleotide sequences of the partial NS1 gene. The results indicated that 15 of 201 fecal samples (7.5%) were positive for CBoV; the partial NS1 genes of the 15 CBoV strains exhibited 83.1%–100% nucleotide identity, and 75.8%–100% amino acid identity; the entire VP2 gene of five selected CBoV strains exhibited 82.9%–96.8% nucleotide identity, and 90.4%–99.1% amino acid identity. The 15 CBoV strains exhibited high co-infection rates with CPV-2 (40%), CCoV (20%), and CaKV (26.67%). Phylogenetic analysis of the partial NS1 gene revealed that the 15 CBoV strains were divided into different subgroups of CBoV-2 when compared with CBoV-2 strains from South Korea, USA, Germany, and Hong Kong in China. Moreover, phylogenetic analysis of the VP2 gene indicated that five selected CBoV strains were divided into three different genetic groups of CBoV-2, involving in CBoV-2HK group, CBoV-2C group, and CBoV-2B group. The recombination analysis using the entire VP2 gene revealed three potential recombination events that occurred among five selected strains in our study. These data demonstrated that the CBoV strains circulating in Heilongjiang province, Northeast China showed genetic diversities, potential recombination events, and high co-infection rate. Further studies will be required to address the potential pathogenic role of these diverse CBoV strains.

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1. Introduction

Bocaviruses have a broad mammalian host range including humans, cows, pigs, gorillas, chimpanzees, Californian sea lions, dogs, cats, bats, and pine martens (Chen et al., 1986; Kapoor et al., 2010; Sharp et al., 2010; Li et al., 2011; Lau et al., 2012; Kapoor et al., 2012; van den Brand et al., 2012; Wu et al., 2012; Jiang et al., 2014). Bocavirus, a genus of the Parvovirinae subfamily (Parvoviridae family), is characterized by the presence of a third major open reading frame (ORF) named NP1. The genome of bocaviruses is single-stranded (ss) DNA and approximately 5 kb in length, coding for non-structural and capsid proteins. In the past 5 years, several novel animal bocaviruses from the pig, dog, and cat, have been discovered and characterized genetically (Lau et al., 2012; Kapoor et al., 2012; Jiang et al., 2014). Therefore, a wide ranging molecular epidemiological investigation is necessary to improve understanding of bocavirus evolution.

Recently, epidemics and apparent molecular evolution of human and porcine bocaviruses have been reported frequently in China (Liu et al., 2014; Wang et al., 2014; Zhang et al., 2014; Zhao et al., 2014; Lu et al., 2015; Luo et al., 2015), which has caused widespread concern. However, the genetic diversity of canine bocavirus (CBoV) strains circulating in China remains unclear. In the present study, the prevalence of CBoV was investigated in fecal samples from dog affected with diarrhea in Heilongjiang province, Northeast China. Furthermore, the genetic diversity and possible recombination events of the CBoV strains identified in China were analyzed. Our aim was to add basic information to increase the understanding of CBoV evolution.

2. Materials and methods

2.1. Sampling

In total, 201 rectal swab samples were collected from diarrhea-affected dogs from animal hospitals in three districts, Harbin, Daqing, and Mudanjiang, of Heilongjiang province in Northeast China

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from May 2014 to April 2015, using commercial virus sampling tubes (YOCON Biological Technology Co. Ltd. Beijing, China) with a volume of 3.5 mL. Of the 201 samples, 141 were collected from Harbin, 20 from Daqing, and 40 from Mudanjiang. The dogs were with or without a vaccination history, and of different breeds and both genders. All rectal swab samples were stored at -80°C , and they were also used for etiological investigations in our other studies (Geng et al., 2015; Li et al., 2016; Wang et al., 2016).

2.2. Primers for the NS1 gene of CBoV

Six nucleotide sequences of CBoV NS1 genes (Genbank accession no. JN648103, JQ692588, JQ692589, JQ692590, JQ692591, and KF771828) were obtained from the Genbank database of NCBI. The conserved regions of the NS1 gene of CBoV were analyzed using the Multiple Sequence Alignment program of the DNAMAN version 6.0 software (Lynnon BioSoft, Point-Claire, Quebec, Canada). A pair of specific primers for amplification of the NS1, NS1-F: 5' CTTCAGATGGTTCACA TTAAGAT 3' (1244 nt–1266 nt), NS1-R: 5' GGCAGYAGCTGAGAGTCTTT 3' (1664 nt–1683 nt), were designed on the basis of the conserved regions of the NS1 gene of CBoV. The amplification products (440 bp) representing the partial CBoV NS1 gene were sequenced for identification of CBoV infection and phylogenetic analysis. The position of the primers was calculated according to the complete genome of CBoV strain HK880N (GenBank accession no. JQ692588).

2.3. Sequencing of the NS1 gene

Genomic DNA was extracted from all 201 fecal samples using a commercial TIANamp Stool DNA Kit (Tiangen Biotech Co., LTD. Beijing, China) according to the manufacturer's instructions. The PCR amplification was carried out in a 50- μL reaction volume containing 0.1 μM of forward primer, 0.1 μM of reverse primer, 4 μL of genomic DNA, 25 μL of EmeraldAmp PCR Master Mix (2 \times Premix) (Takara Biotechnology (Dalian) Co. Ltd.), and an appropriate volume of ddH₂O. The mixtures were amplified by 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min, in an automated thermal cycler of the GeneAmp PCR System 9700 (Applied Biosystems). The amplification products were purified using an AxyPrep DNA Gel Extraction kit (A Corning Brand, Suzhou, China). The purified PCR products were directly subjected to sequencing using the Sanger sequencing method. Each sample was sequenced three times. All sequences were submitted to the NCBI Genbank (<http://www.ncbi.nlm.nih.gov>), and their accession numbers are as follows: KR998480–KR998494.

2.4. Phylogenetic analysis of the NS1 gene

For phylogenetic analysis of the NS1 gene, twelve NS1 genes of CBoV-2 and three NS1 genes of feline bocavirus (FeBoV) strains were retrieved from GenBank. To construct phylogenetic trees, multiple alignment of all target sequences was carried out using the ClustalX program version 1.83. Furthermore, the phylogenetic trees of all target sequences were generated from the ClustalX-generated alignments by MEGA6.06 software using the neighbor-joining method, respectively (Tamura et al., 2013). Neighbor-joining phylogenetic trees were built with the p-distance model, 1000 bootstrap replicates, and otherwise default parameters in MEGA 6.

2.5. Sequencing and phylogenetic analysis of the selected entire VP2 genes

A total of five strains HRB-F3, MDJ-26, DQ-beta8, MDJ-21, and HRB-C7 identified in our study, representing different genetic diversity in the phylogenetic tree of the NS1 gene, were chosen for sequencing of the entire VP2 genes. The primers specific for amplification of the entire VP2 gene, CBVvp2-F: 5' GCGGATACAAAGGGCAATCTCTG 3' and CBVvp2-R: 5' AAGTTTTCTTTTATGGCTTTATT 3' which resulted in an

1889-bp fragment covering the ORF of VP2 gene, were designed on the basis of the conserved regions of genome sequences of six CBoV strains (Genbank accession no. JN648103, JQ692588, JQ692589, JQ692590, JQ692591, KF771828, and KP281715). The PCR amplification was carried out in a 50- μL reaction volume containing 0.1 μM of forward primer, 0.1 μM of reverse primer, 4 μL of genomic DNA, 5 μL of 10 \times buffer, 0.5 U of ExTaq DNA polymerase (Takara, Dalian, China), and an appropriate volume of ddH₂O. The mixtures were amplified by 35 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, with a final extension at 72°C for 10 min, in an automated thermal cycler of the GeneAmp PCR System 9700 (Applied Biosystems). The purified products were directly subjected to sequencing by using Sanger sequencing method. Each sample was sequenced three times. Five entire VP2 sequences were submitted to the NCBI Genbank (<http://www.ncbi.nlm.nih.gov>), and their accession numbers are as follows: KT192699 for the strain MDJ-21, KT192700 for the strain MDJ-26, KT192701 for the strain DQ-beta8, KT192702 for the strain HRB-C7, and KT192703 for the strain HRB-F3.

To trace the genetic groups of the sequenced VP2 genes in our study, partial VP2 genes from different genetic groups according to the report described by Bodewes et al. (2014) were retrieved from GenBank. The phylogenetic tree of the partial VP2 genes was constructed from the ClustalX-generated alignments by MEGA6.06 software using the neighbor-joining method, respectively (Tamura et al., 2013). Neighbor-joining phylogenetic trees were built with the p-distance model, 1000 bootstrap replicates, and otherwise default parameters in MEGA 6.

2.6. Recombination analysis

The entire VP2 gene sequences of five CBoV strains, MDJ-26, DQ-beta8, MDJ-21, HRB-F3, and HRB-C7 identified in this study, were used for recombination analysis. The five VP2 genes of CPV-2 were re-aligned in the ClustalX (1.83) program. Detection of potential recombinant sequences, identification of potential parental sequences, and localization of possible recombination break points were determined using the Recombination Detection Program (RDP) (Martin and Rybicki, 2000), GENECONV (Padidam et al., 1999), BOOTSCAN (Martin et al., 2005), MaxChi (Smith, 1992), CHIMAERA (Posada and Crandall, 2001), and SISCAN (Gibbs et al., 2000) methods embedded in RDP4. Only potential recombination events detected by two or more of the programs coupled with phylogenetic evidence of recombination were considered significant with highest acceptable P-value cutoff of 0.05.

2.7. Screening for canine enteric pathogens

Fecal samples that tested positive for CBoV were also screened for canine parvovirus type 2 (CPV-2), canine coronavirus (CCoV), canine astrovirus (CaAstV), canine norovirus (CNoV), canine kobuvirus (CaKV), and group A-rotavirus (RV-A) by either PCR or RT-PCR and sequencing, according to the protocols previously described (Buonavoglia et al., 2000; Gómez et al., 2011; Cattoli, 2012; Mesquita and Nascimento, 2012; Di Martino et al., 2013; Costa et al., 2014).

3. Results

3.1. PCR detection of CBoV

A total of 201 samples were investigated by PCR targeting 440 bp of the partial NS1 gene of CBoV. The characteristics of the CBoV positive dogs in different locations in Heilongjiang province of Northeast China are shown in Table 1. Of the 201 fecal samples, 7.5% (15/201) were positive for CBoV; the positive rate for CBoV in Harbin, Daqing, and Mudanjiang was 2.8% (4/141), 15% (3/20), and 20% (8/40), respectively (Table 2). Of the 15 CBoV positive samples, 40% (6/15) were positive for CPV-2; the positive rate for CPV-2 in Harbin and Mudanjiang was 75%

Table 1
Characteristics of the CBoV positive dogs in different location in northeast China.

Strain	Accession no.	Collection date	Location	Breed	Gender	Age	Vaccined	Other enteric viruses (accession no.)
MDJ-12	KR998480	Feb-2015	Mudanjiang	GM	F	3 M	Yes	—
MDJ-13	KR998481	Oct-2014	Mudanjiang	LR	F	2.5 M	NA	CaKV (KT210391)
MDJ-15	KR998482	Nov-2014	Mudanjiang	RC	F	1.5 M	Yes	CPV-2a (KT074261)
MDJ-17	KR998483	Dec-2014	Mudanjiang	CO	F	1.5 M	NA	CCoV (KT192645)
MDJ-21	KR998484	Dec-2014	Mudanjiang	RC	M	3 M	No	CPV-2a (KT074264)
MDJ-23	KR998485	Apr-2015	Mudanjiang	TM	M	2.5 M	NA	—
MDJ-26	KR998486	Apr-2015	Mudanjiang	PD	F	4 M	Yes	CCoV (KT192648)
MDJ-27	KR998487	Apr-2015	Mudanjiang	NA	F	NA	NA	CPV-2a (KT074266), CaKV (KT210392)
HRB-a1	KR998488	Sep-2014	Harbin	NA	F	3.5 M	Yes	CCoV (KT192656), CPV-2a (KT074276), CaKV (KT210394)
HRB-b2	KR998490	Oct-2014	Harbin	GM	NA	3 M	NA	CPV-2b (KT074323)
HRB-C7	KR998493	Oct-2014	Harbin	NA	M	2 M	NA	CPV-2a (KT074288)
HRB-F3	KR998494	Nov-2014	Harbin	JS	F	3.5 M	NA	—
DQ-alpha9	KR998489	Feb-2015	Daqing	CH	M	1.5 M	Yes	CaKV (KT210401)
DQ-beta7	KR998491	Feb-2015	Daqing	NA	M	1 M	No	—
DQ-beta8	KR998492	Jan-2015	Daqing	PD	M	1.5 M	Yes	—

Note. For breed, GM = Golden Malinois, LR = Labrador Retriever, RC = Rough Collie, CO = Caucasian Owtcharka, TM = Tibetan Mastiff, PD = Poodle, CH = Chihuahua, and JS = Japanese Spitz; For gender, F = female, and M = male; for age, M = month. For vaccine, the animals were vaccinated for CPV-2, canine distemper virus, canine parainfluenza virus, canine adenovirus type 1, and canine adenovirus type 2.

(3/4), and 37.3% (3/8), respectively. In addition, 20% (3/15) were positive for CCoV: the positive rate for CCoV in Harbin and Mudanjiang was 25% (1/4), and 25% (2/8), respectively; 26.67% were positive for CaKV: the positive rate for CaKV in Harbin, Daqing, and Mudanjiang all was 25% (1/4); 6.7% (1/15) were positive for CPV-2 and CaKV; 6.7% (1/15) were positive for CPV-2, CCoV, and CaKV (Table 2).

3.2. Analysis of sequence data and phylogenetics of CBoV strains

Sequence analysis revealed that the partial NS1 genes of the 15 CBoV strains exhibited 83.1%–100% nucleotide identity, and 75.8%–100% amino acid identity; the entire VP2 genes of the five selected CBoV strains (the strain HRB-b2 not available) showed 82.9%–96.8% nucleotide identity, and 90.4%–99.1% amino acid identity. For partial NS1 genes, the four CBoV strains from Harbin showed 84.8%–97.2% nucleotide identity and 76.5%–98.5% amino acid identity, the three CBoV strains from Daqing showed 83.1%–100% nucleotide identity and 91.7%–100% amino acid identity, and the eight CBoV strains from Mudanjiang showed 94.2%–100% nucleotide identity and 91.7%–100% amino acid identity (Table 2).

The phylogenetic analysis based on the NS1 genes revealed that the 15 CBoV strains from China were divided into four different groups: GI (strains HRB-F3 and MDJ-17), GII (MDJ-26, DQ-alpha9, DQ-beta8, MDJ-12, and MDJ-13), GIII (MDJ-15, MDJ-27, MDJ-21, and MDJ-27), GIV (HRB-C7, HRB-a1, and DQ-beta7) and one separate branch (HRB-b2) in the phylogenetic tree (Fig. 1). The phylogenetic analysis of the selected VP2 genes revealed that the strains MDJ-26 and DQ-beta8 were clustered with the Hong Kong strains; the strains MDJ-21 and HRB-F3 were clustered with the CBoV-2C reference strains; the strain HRB-C7 was clustered with the CBoV-2B reference strain (Fig. 2).

3.3. Recombination analysis

A total of three potential recombinant events were found among the strains MDJ-26, DQ-beta8, MDJ-21, HRB-F3, and HRB-C7 on the basis of

the entire VP2 gene. The Fig. 3 revealed the recombination event that occurred between DQ-beta8 strain (accession no. KT192701) and HRB-C7 strain (accession no. KT192702), which led to the recombinant HRB-F3 strain (accession no. KT192703). The Fig. 4 revealed the recombination event that occurred between DQ-beta8 strain (accession no. KT192701) and HRB-C7 strain (accession no. KT192702), which led to the recombinant MDJ-21 strain (accession no. KT192699). The Fig. 5 revealed the recombination event that occurred between DQ-beta8 strain (accession no. KT192701) and MDJ-21 strain (accession no. KT192699), which led to the recombinant MDJ-26 strain (accession no. KT192700).

4. Discussion

In the past few years, novel bocaviruses identified in animals and humans has attracted worldwide attention. In the present study, investigation and phylogenetic analysis of CBoV was conducted in dogs in Heilongjiang province, Northeast China. Among all fecal samples investigated, the positive rate for CBoV was 7.5%, which is higher than previously reported (4.6%) by Lau et al. (2012). Among the three areas investigated, the positive rate for CBoV in the Mudanjiang area reached 20%, higher than the rates in Harbin (2.8%) and Daqing (15%). These data suggest that CBoV has a high prevalence in Northeast China, and that there are differences among regions. In our study, the CBoV positive fecal samples were also screened for the leading causes of canine viral enteritis, including canine parvovirus-2 (CPV-2), canine coronavirus (CCoV), canine astrovirus (CaAstV), canine norovirus (CNoV), canine kobuvirus (CaKV), and canine group A-rotavirus (CRV-A). Co-infection with CPV-2, CCoV, and CCoV was identified in the 15 CBoV positive samples; co-infection of CBoV with CPV-2 reached 40%, co-infection of CBoV with CaKV reached 26.67%, and co-infection of CBoV with CCoV reached 20%. Bodewes et al. (2014) reported a novel CBoV strain associated with severe enteritis in a litter of puppies. In our study, the high co-infection rate of CBoV with other enteric viruses suggests that CBoV, as a potential enteric pathogen, may be associated with viral diarrhea in dogs in Northeast China.

Table 2
Analysis of the CBoV positive samples, identity of partial NS1 genes, and other enteric viruses in CBoV positive samples.

Location	Numbers of sample	Positive rate for CBoV	Percent identity		Other enteric viruses in the CBoV positive samples				
			Nucleotide level	Amino acids level	CPV-2	CCoV	CaKV	CPV-2 + CaKV	CPV-2 + CCoV + CaKV
Harbin	141	2.8% (4/141)	84.8%–97.2%	76.5%–98.5%	75% (3/4)	25% (1/4)	25% (1/4)	—	25% (1/4)
Daqing	20	15% (3/20)	83.1–100%	91.7%–100%	—	—	25% (1/4)	—	—
Mudanjiang	40	20% (8/40)	94.2%–100%	91.7%–100%	37.3% (3/8)	25% (2/8)	25% (2/8)	12.5% (1/8)	—
Total	201	7.5% (15/201)	83.1%–100%	75.8%–100%	40% (6/15)	20% (3/15)	26.67% (4/15)	6.7% (1/15)	6.7% (1/15)

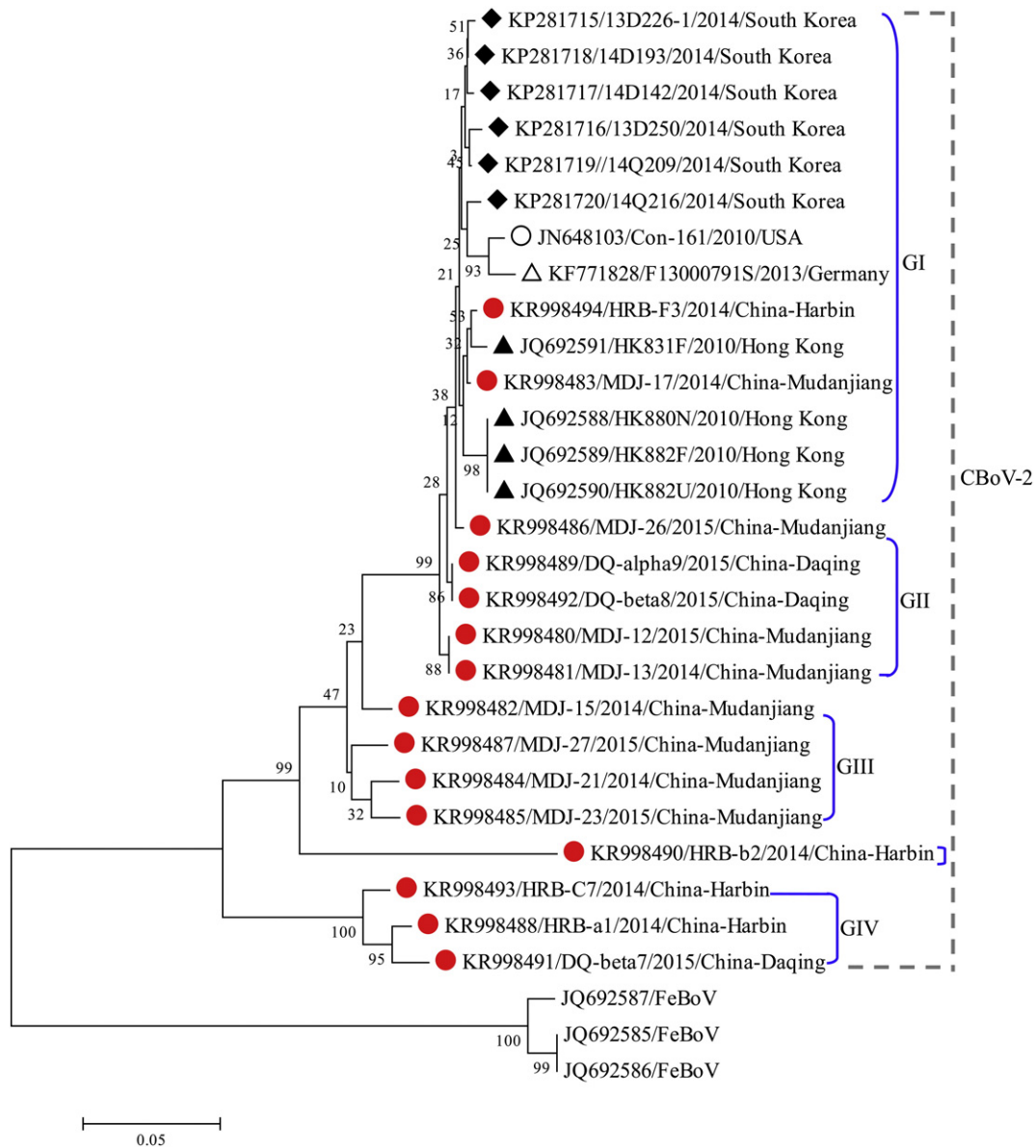


Fig. 1. Phylogenetic analysis of CBoV strains among China, Hong Kong, USA, South Korea, and Germany based on the NS1 gene. *Note.* ● The strains identified in our study; ▲ Hong Kong of China; ○ USA; ◆ South Korea; △ Germany.

Recently, novel bocavirus strains have been reported in dogs and cats (Lau et al., 2012; Bodewes et al., 2014). In our study, although the 15 CBoV strains were identified in Heilongjiang province of northeast China, the identified CBoV strains exhibited differences at the nucleotide and deduced amino acid levels. In the phylogenetic tree based on the partial NS1 genes, the 15 CBoV strains identified from northeast China formed four different groups and one separate branch. These data demonstrated that the CBoV strains from the same area showed genetic diversity. Based on reference strains from CBoV-2, CBoV-3, and CBoV-1, the 15 CBoV strains identified in China belong to CBoV-2. The strains HRB-F3 and MDJ-17 showed a close relationship with BCov strains from Hong Kong of China, Germany, USA, and South Korea, forming the GI group. However, the other 13 CBoV strains, forming three separate groups (GII, GIII, and GIV), and one separate branch (HRB-b2), differ genetically from the strains HRB-F3, MDJ-17, and 10 reference strains of CBoV-2, especially the GIV group. However, the NS1 genes of CBoV-2B and CBoV-2C were not available, and therefore the genotypes of the 15 CBoV strains could not be defined precisely. Moreover, phylogenetic analysis based on the VP2 gene revealed that

the selected five CBoV strains were divided into different genetic groups, involving in CBoV-2HK group (the strains MDJ-26 and DQ-beta8), CBoV-2C group (the strains MDJ-21 and HRB-F3), and CBoV-2B group (the strain HRB-C7). These data demonstrated that the CBoV strains circulating in Northeast China show differential genetic evolution.

It has been reported that inter-genotype and intragenotype recombination are present among bocaviruses (Fu et al., 2011, Jiang et al., 2014). In this study, three inter-species recombination events were identified in VP2 genes of the five selected CBoV strains which were divided into different genetic groups. Our findings are in line with a recent report that showed the presence of recombination among different regions of VP2 genes by analysis of porcine bocavirus strains in USA (Jiang et al., 2014). In our study, two potential recombinant strains HRB-F3 and MDJ-21, belonging to the CBoV-2C group, were generated by recombination of the strain DQ-beta8 (CBoV-2HK group) and the strain HRB-C7 (CBoV-2B); the potential recombinant strain MDJ-26, belonging to the CBoV-2HK group, was generated by recombination of the strain DQ-beta8 (CBoV-2HK group) and the strain MDJ-21 (CBoV-2C). The

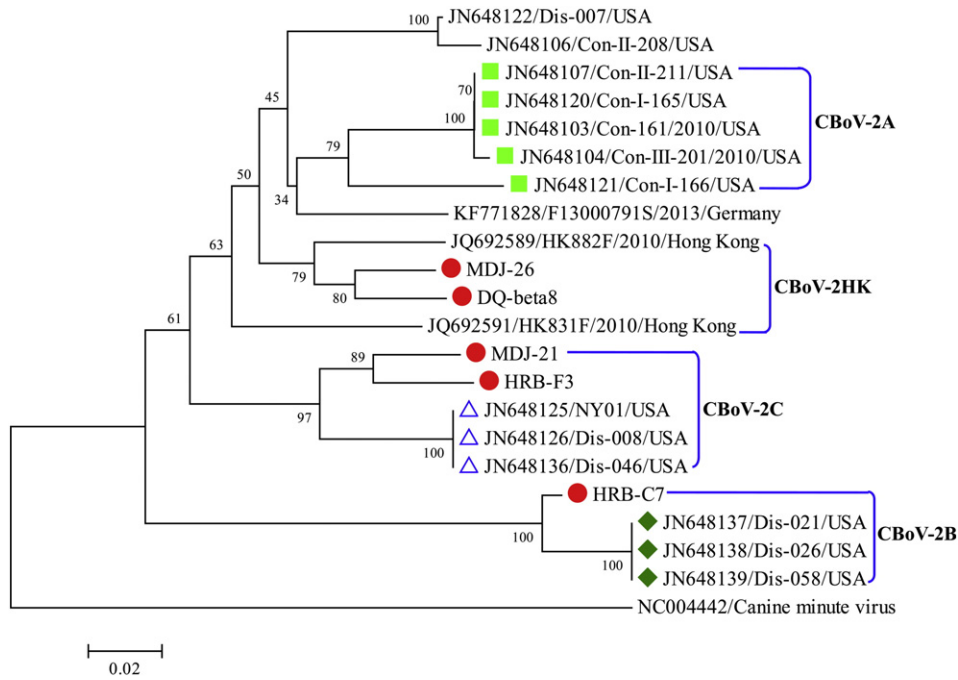


Fig. 2. Phylogenetic analysis of CBoV strains among China, Hong Kong, USA, South Korea, and Germany based on the parial VP2 gene. Note: ■ CBoV-2A group; ● The strains identified in our study; ▲ CBoV-2C group; ◆ CBoV-2B group.

limited data suggests that the recombination events among different subgroups of the CBoV-2 strains can occur frequently. This is further supported by the extensive investigation of molecular epidemiology of CBoV strains in different districts or countries. In addition, for better monitoring recombination events, it is suggested that the co-infection

of the different CBoV subgroup strains should be investigated in China in future study.

In conclusion, investigation of a limited number of fecal samples revealed a high prevalence of CBoV in dogs affected with diarrhea in Heilongjiang province of Northeast China, and the positive rate

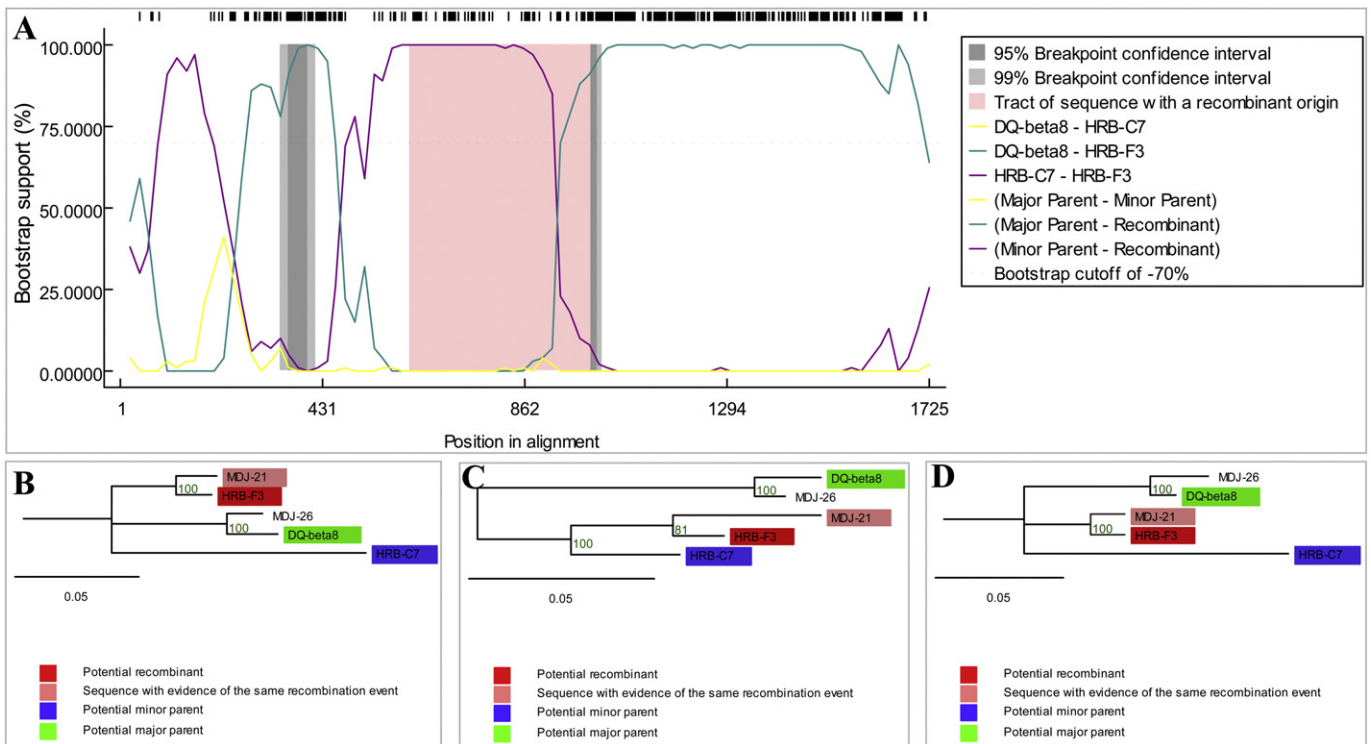


Fig. 3. Identification of the recombination event between the minor parent strain HRB-C7 (blue) and the major parent strain DQ-beta8 (green), leading to the recombinant HRB-F3 strain (red). A. Bootscan evidence for the recombination origin on the basis of pairwise distance, modelled with a window size 200, step size 20 and 100 bootstrap replicates; B, C, D. Neighbor-joining tree (1000 replicates, Kimura two-parameter distance) constructed using the regions derived from minor parent strain (1–615, 1005–1725) (B), the recombination region (616–1004) (C), and non-recombinant region (D).

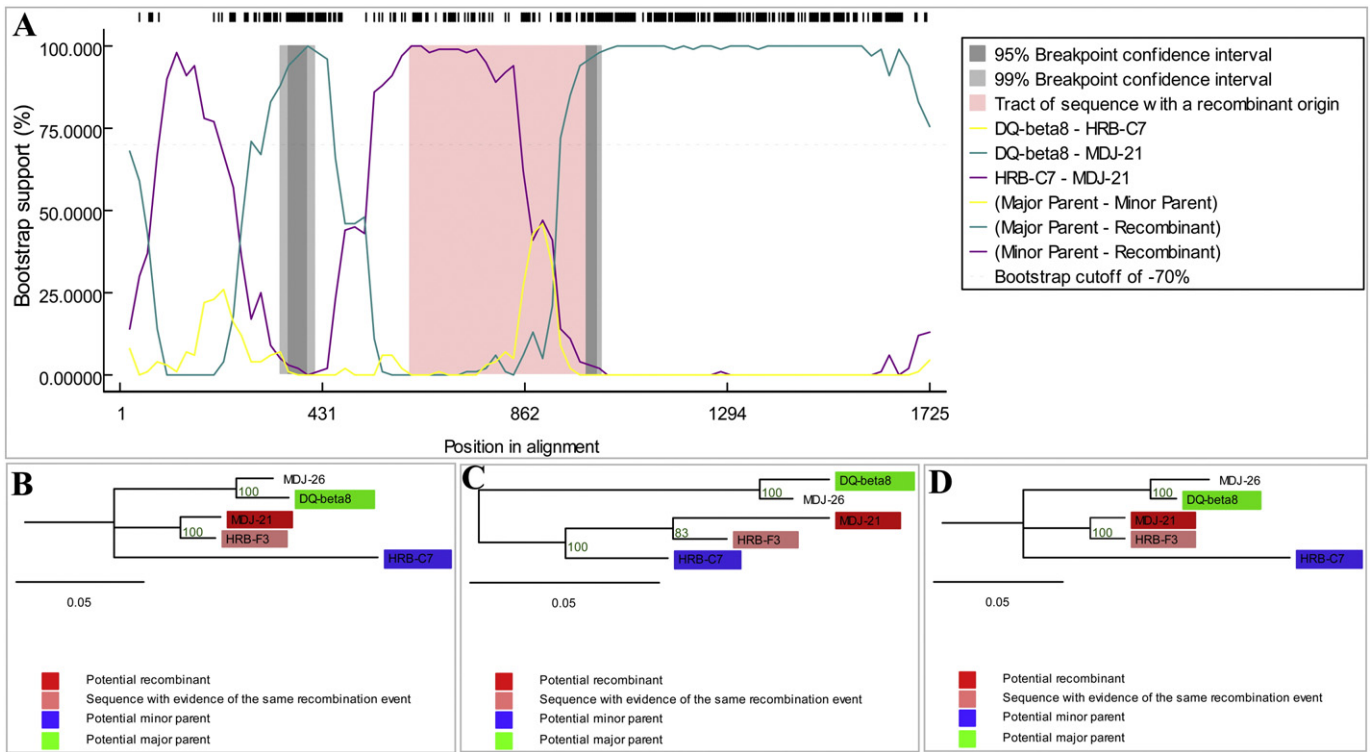


Fig. 4. Identification of the recombination event between the minor parent strain HRB-C7 (blue) and the major parent strain DQ-beta8 (green), leading to the recombinant MDJ-21 strain (red). A. Bootscan evidence for the recombination origin on the basis of pairwise distance, modelled with a window size 200, step size 20 and 100 bootstrap replicates; B, C, D. Neighbor-joining tree (1000 replicates, Kimura two-parameter distance) constructed using the regions derived from minor parent strain (1–615, 944–1725) (B), the recombination region (616–993) (C), and non-recombinant region (D).

for CBoV varied among the different geographical areas. The high co-infection of CBoV strains with CPV-2, CCoV, and CaKV were found in different proportions. The CBoV strains identified from China showed

genetic diversity and potential recombination events. In future, the extensive epidemiologic surveillance and comprehensive characterization of the CBoV strains circulating in China should be done to clarify the

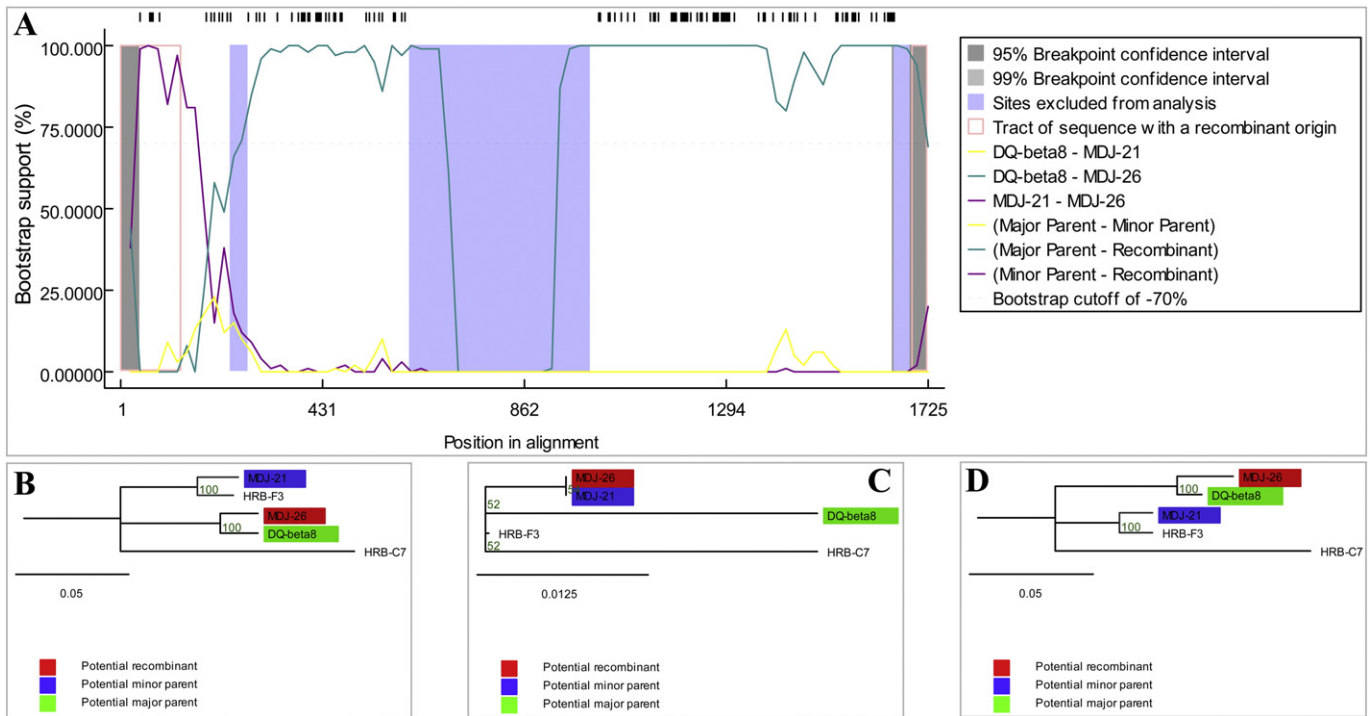


Fig. 5. Identification of the recombination event between the minor parent strain MDJ-21 (blue) and the major parent strain DQ-beta8 (green), leading to the recombinant MDJ-26 strain (red). A. Bootscan evidence for the recombination origin on the basis of pairwise distance, modelled with a window size 200, step size 20 and 100 bootstrap replicates; B, C, D. Neighbor-joining tree (1000 replicates, Kimura two-parameter distance) constructed using the recombination region (133–1688) (B), the regions derived from minor parent strain (1–132, 1689–1725) (C), and non-recombinant region (D).

genetic diversity of CBoVs and the association of CBoV with enteric diseases in dogs.

Conflict of interest statement

The authors have no conflicts of interest.

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