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A comprehensive study of canine parvoviruses (Carnivore protoparvovirus 1, Carnivore bocaparvovirus 1 and 2) from shelter dogs in Turkey

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Abstract: A total of 150 rectal swab samples were collected from diarrheic dogs from the Sivas Municipal Animal Shelter, Turkey in April 2018. While 127 faecal samples were gathered from adults, 23 samples were collected from puppies. Canine parvovirus type 2 (Carnivore protoparvovirus 1), Canine minute virus (Carnivore bocaparvovirus 1) and Canine bocavirus (Carnivore bocaparvovirus 2) were examined by PCR with three sets of novel primers. Some of the amplicons were subjected to molecular sequencing and molecular analysis. Three faecal (2.36%) samples were found to be positive for Carnivore protoparvovirus 1, five faecal samples (3.94%) were found to be positive for Carnivore bocaparvovirus 1 and three samples (2.36%) were found to be positive for Carnivore bocaparvovirus 2 among 127 adult dogs. On the other hand, 14/23 faecal samples (60.87%) were found to be positive for Carnivore protoparvovirus 1, 8/23 faecal samples (34.78%) were found to be positive for Carnivore bocaparvovirus 1, and 6/23 faecal samples (26.09%) were found to be positive for Carnivore bocaparvovirus 2 from the puppies. Moreover, we detected two distinct clades of Carnivore bocaparvovirus 1 and 2 according to the molecular analysis. To the best of our knowledge, this is the first report for the direct detection of Carnivore bocaparvovirus 1 and 2 in Turkey.

Keywords: dog parvovirus; molecular analysis; *NS1* gene; phylogeny; sequencing; *VP2* gene

The family *Parvoviridae* consists of two subfamilies: *Densovirinae*, which infects insects and *Parvovirinae*, which infects vertebrates.

According to new taxonomy records, the subfamily *Parvovirinae* consists of eight genera including *Amdoparvovirus*, *Aveparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Tetraparvovirus*, *Protoparvovirus* which includes the causative agent of classical canine parvoviral enteritis (Carnivore protoparvovirus 1) and *Bocaparvovirus* which includes Carnivore bocaparvovirus 1–6. In scientific literature, two *Bocaparvoviruses* have been isolated from dogs, which are Carnivore bocaparvovirus 1, formerly known as Canine minute virus, and Carnivore bocaparvovirus 2, formerly known as Canine bocavirus 1, thus far (Cotmore et al. 2014).

Since the International Committee on Taxonomy of Viruses (ICTV) changed the name of these viruses, we use the names Carnivore protoparvovirus 1 instead of Canine parvovirus 2, Carnivore bocaparvovirus 1 instead of Canine minute virus and Carnivore bocaparvovirus 2 instead of Canine bocavirus 1 in this paper according to the ICTV. Both of the Carnivore bocaparvoviruses (type 1 and 2) have shown to be causative agents of haemorrhagic enteritis of dogs (Macartney et al. 1988; Jarplid et al. 1996; Truyen et al. 1996; Bodewes et al.

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2014). A serological study indicated that Carnivore bocaparvovirus 1 has already been reported from Turkey, but without virological detection (Torun and Yilmaz 2005).

The aim of the study was the investigate the existence of parvoviruses in feacal samples of dogs at various ages in Turkey. To the best of our knowledge, the present report is the first report for the direct detection of Carnivore bocaparvovirus 1 and 2 in Turkey.

MATERIAL AND METHODS

Sampling

In this study, a total of 150 rectal swab specimens were collected from the Sivas Municipal Animal Shelter, Turkey. The sampled dogs had manifested gastro-intestinal problems, including 127 adult dogs (more than one-year-old) and 23 puppies that were approximately two to four months old. The collected samples were transported to the laboratory just after sampling and stored at –80 °C until being subjected to the DNA isolation.

The faecal samples were diluted to 1:10 with a 1 M phosphate buffered saline solution and centrifuged for 10 min at 1 000 *g* to remove the large cellular debris.

After centrifugation, the supernatants were submitted to a nucleic acid extraction procedure using a GF-1 Viral Nucleic Acid Extraction Kit (Vivantis, Selangor, Malaysia) according to the manufacturer's instructions. The eluted nucleic acids were stored at –80 °C until use.

Polymerase chain reaction (PCR)

The molecular detection of the partial *VP2* gene of Carnivore protoparvovirus 1 and Carnivore bocaparvovirus 1, and also the partial *NS1* gene of Carnivore bocaparvovirus 2 were conducted by our generic primer sets (Table 1). The polymerase chain reaction (PCR) mixture was prepared as a 50 µl final volume containing a 5 µl template, 5 µl of the 10 × PCR buffer, 10 mM of dNTP, 10 pmol/µl of each set of sense/antisense primers, and 5 IU of Taq DNA polymerase (Vivantis, Selangor, Malaysia). The conditions of the PCR were adjusted accordingly to 95 °C for 2 min for the pre-denaturation for 40 cycles, 94 °C for 45 sec of denaturation, 51 °C (Carnivore bocaparvovirus 1), 55 °C (Carnivore bocaparvovirus 2) or 52 °C (Carnivore protoparvovirus 1) for 45 s of annealing, 72 °C for 1 min for the extension and lastly 10 min at 72 °C for final extension. The PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide.

Sequencing and phylogenetic analysis

The corresponding amplicons were directly submitted to the sequencing. The amplicons were purified with a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA, USA). All of the sequenced products were used to obtain the

Table 1. Oligonucleotide primers for the detection and sequencing of the partial *VP2* and *NS1* genes of Carnivore protoparvovirus 1, Carnivore bocaparvovirus 1 and 2, respectively

Primer ID	Sequence (5'-3')	Target gene	Amplicon size
CPV2-3140F	CCTGACAGAGCAACTCCGTTT	*VP2* gene of Carnivore protoparvovirus 1	640 bp
CPV2-3779R	TGTGAACCATCTGAAGCAAGGT		
CPV2-3778F	GCTGAGGTTGGTTATAGTGCR		642 bp
CPV2-4419R	TGGATTCCAAGTATGAGAKGCT		
CPV2-3824Fn	CACAAGGGCCATTTAAAACACC		591 bp
CPV2-4414Rn	TCCAAGTATGAGAGGCTCTTAG		
CMV4064F	TGTGGGTGGGTCAATAATGA	*VP2* gene of Carnivore bocaparvovirus 1	500 bp
CMV4563R	TTGTTTGTTCCGTCTTGCAC		
CBoV999F	CCTGACAGAGCAACTCCGTTT	*NS1* gene of Carnivore bocaparvovirus 2	400 bp
CBoV1398R	TGTGAACCATCTGAAGCAAGGT		

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phylogenetic data. The partial sequences were compared with other sequence data which are provided online by the National Center for Biotechnology Information (NCBI). The sequence alignment and phylogenetic analysis based on the partial nucleotide sequences of 642 and 541 bp *VP2* gene (Carnivore protoparvovirus 1), 500 bp *VP2* gene (Carnivore bocaparvovirus 1), and 400 bp *NS1* gene (Carnivore bocaparvovirus 2) were constructed with the use of Geneious Prime Software v2019.2.3.

RESULTS AND DISCUSSION

Five faecal samples (3.94%) were found to be positive for the 500 bp partial *VP2* gene of Carnivore bocaparvovirus 1. While, three samples (2.36%) were found to be positive for the 400 bp partial *NS1* gene of Carnivore bocaparvovirus 2 and three samples (2.36%) were found to be positive for the 640 bp partial *VP2* gene of Carnivore protoparvovirus 1 within 127 adult dogs. On the other hand, eight faecal samples (34.78%) were found to be positive for the 500 bp partial *VP2* gene of Carnivore bocaparvovirus 1, six faecal samples (26.09%) were found to be positive for the 400 bp partial *NS1* gene of Carnivore bocaparvovirus 2 and fourteen faecal samples (60.87%) were found to be positive for the 640 bp partial *VP2* gene of Carnivore protoparvovirus 1 from the puppies. The overall PCR results are illustrated below (Table 2). Ten samples, collected from nine puppies and an adult, were found to be positive for a mixed infection. The summarised graphical representation of the infections is illustrated below (Figure 1). The detection confirmation was conducted by multiple alignments of the sequenced amplicons together with the canine parvovirus sequences gathered from GenBank.

One hundred fifty (127 adults and 23 puppies) diarrheic dogs were examined for canine parvoviruses from the city shelter by using three sets of novel primers. Nearly ten times higher levels of positiv-

Table 2. Overall PCR results for the detection of Carnivore protoparvovirus 1, Carnivore bocaparvovirus 1 and 2 both from adults and puppies

	Total samples	Carnivore protoparvovirus 1 (Canine parvovirus 2)	Carnivore bocaparvovirus 1 (Canine minute virus)	Carnivore bocaparvovirus 2 (Canine bocavirus 1)
Adults	127	2.36% (3/127)	3.94% (5/127)	2.36% (3/127)
Puppies	23	60.87% (14/23)	34.78% (8/23)	26.09% (6/23)

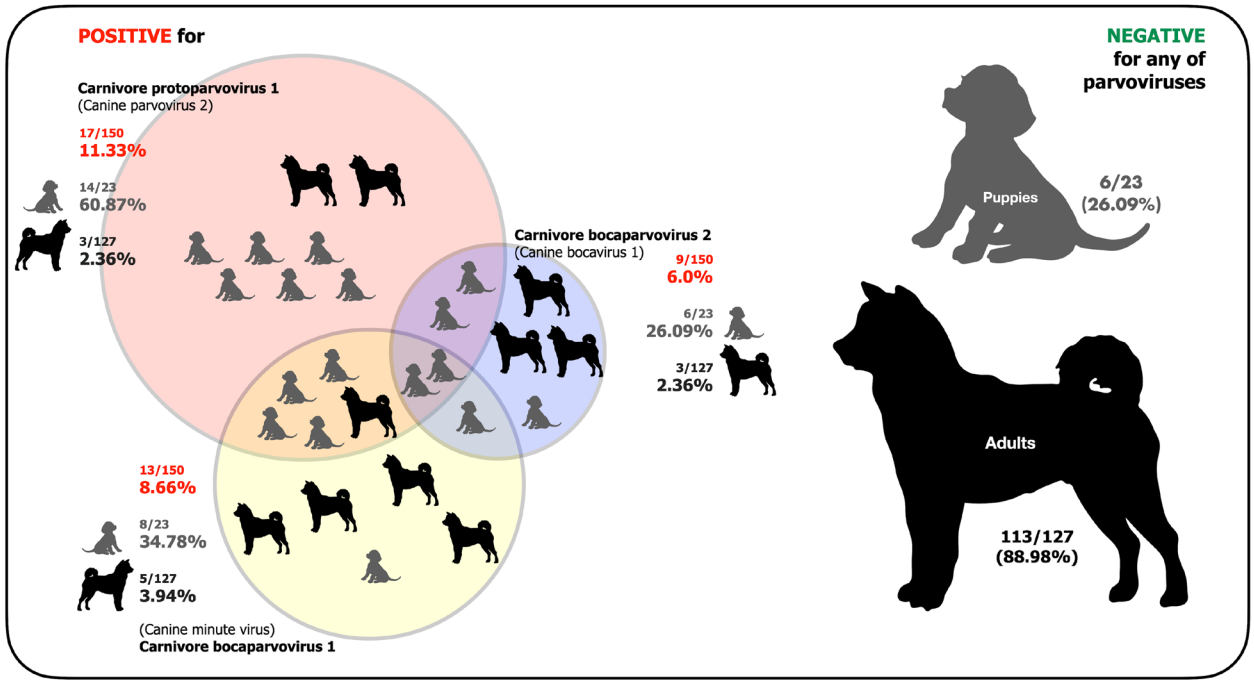

Figure 1 . Detailed diagram of the positive samples and the point of mixed infections

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ity were found in the puppies than the adults for both Carnivore bocaparvovirus 1 and 2. On the other hand, about 25 times higher levels of positivity were found in the puppies than the adults for Carnivore protoparvovirus 1. The presence of Carnivore protoparvovirus 1 has been reported from Turkey already (Ozkul et al. 2002; Sahna et al. 2008; Timurkan and Oguzoglu 2015). On the other hand, serological evidence of Carnivore bocaparvovirus 1 had also been reported by Torun and Yilmaz (2005) in Turkey. The seroprevalence was reported as 18% from 100 diarrheic dogs at that time. However, there is no record about Carnivore bocaparvovirus 2 by either conducting serological or virological methods in Turkey.

The classical type canine parvovirus (Carnivore protoparvovirus 1) is a well-known canine pathogen. There are numerous research studies concerned with Carnivore protoparvovirus 1 worldwide including Turkey (Yesilbag et al. 2007; Sahna et al. 2008; Touihri et al. 2009; Mohan Raj et al. 2010; Ntafis et al. 2010; Yi et al. 2016). In these reports, the positivity of puppies varies from 35% to 90%. In this study, we detected 640 bp partial *VP2* gene amplicons from 2.36% (3/127) of the adults and 60.87% (14/23) of the puppies.

Fourteen PCR products from the Carnivore protoparvovirus 1 positive samples were sequenced and the data were submitted to the GenBank (accession codes: MK503182-5 and MN171410-19). Multiple alignments of 455 previous strains, which were gathered from the GenBank, as well as our strains were examined. All of the new strains reported here are genotype 2b, according to the 426th aa of the VP2 protein. Four aa changes were observed on our partial sequences in comparison with the 469 total strains. These are the 267th position "TTT/Phe to TAT/Tyr", the 324th position "TAT/Tyr to ATT/Ile", the 440th position "ACA/Thr to GCA/Ala", and also the 426th "GAT/Asp" position which is a marker for the genotype distinction. On the other hand, 25 nucleotide silent mutations were observed which does not affect the aa substitution. Moreover, one of the silent mutations "ACT/Thr to ACC/Thr" is unique for our strains at the 445th aa position (nt: 1335) (Figure 2).

Experimental and/or field surveys indicate that Carnivore bocaparvovirus 1 causes pneumonitis and/or enteritis and varies from mild to severe in puppies while adults look healthy (Ohshima et al. 2010). The seropositivity of Carnivore bocaparvovirus 1 varies from 5% to 70% worldwide (Carmichael et al. 1994; Pratelli et al. 1999; Torun and Yilmaz 2005; Sahna et al. 2008; Touihri et al. 2009; Timurkan and Oguzoglu 2015). However, there is only one molecular epidemiological study of Carnivore bocaparvovirus 1, other than seroepidemiological studies, which detected the viral genome in 1.2% (4/346) of dogs among enteric and/or respiratory diseased domestic dogs in Japan (Mochizuki et al. 2002). In this research, we detected the 500 bp partial *VP2* gene amplicons of Carnivore bocaparvovirus 1 from 3.94% (5/127) of the adults and 34.78% (8/23) of the puppies. The results indicate that the prevalence of Carnivore bocaparvovirus 1 was detected higher that it is supposed to in puppies.

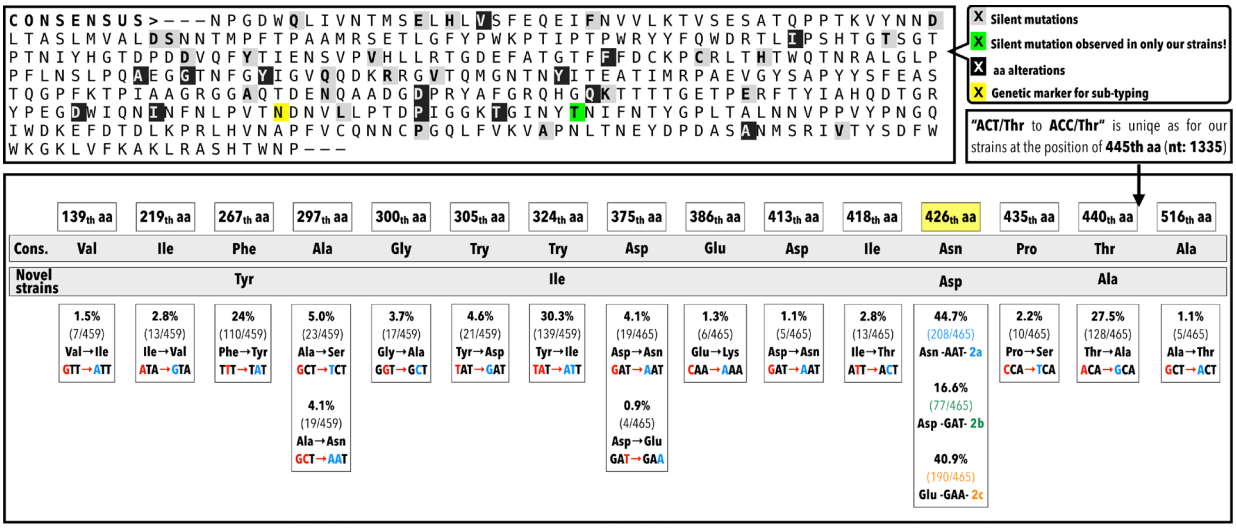

Figure 2. Sequences of the Carnivore protoparvovirus 1 strains based on the partial VP2 protein showing mutations and silent mutations – only mutations found in more than 5 of the total 469 strains are illustrated

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Carnivore bocaparvovirus 1 and 2 have also been reported in both respiratory and enteric diseased puppies (Hashimoto et al. 2001; Jang et al. 2003; Kapoor et al. 2012; Lau et al. 2012; Bodewes et al. 2014). However, the epidemiological data about Carnivore bocaparvovirus 1 and 2 is limited in the literature. On the other hand, some research indicates that the positivity of Carnivore bocaparvovirus 2 varies, such as 4.1% in China (Lau et al. 2012), 9.6% in South Korea (Choi et al. 2015), and 22.78% in the USA (Kapoor et al. 2012). In this study, we detected 400 bp partial *NS1* gene amplicons in 2.36% (3/127) of the adults and in 26.09% (6/23) of the puppies.

According to the molecular analysis based on the 500 bp partial sequences of the *VP2* gene of Carnivore bocaparvovirus 1, two distance lineages were observed (Figure 3). All the Turkish isolates were constituted at lineage 2 together with three Chinese strains (MH540355.1–MH540357.1). The nucleotide identity of the lineage 1 isolates varies from 97.0% to 99.6% and the lineage 2 isolates varies from 97.60% to 100%. The nucleotide identity between lineage 1 and 2 was 94.4% to 97.0%. We also detected two unique mutations ("GCC/Ala to ACC/Thr") on MK503188 and ("AGC/Ser to AAC/Asn") on MK503186, MK503187, and MK503188 (Figure 4). As a result, we discovered Carnivore bocaparvovirus 1 isolates, which are genetically distinct from previously known isolates of Carnivore bocaparvovirus 1. Further investigations will be needed to study Carnivore bocaparvovirus 1 infections in dogs, since we must consider that Carnivore bocaparvovirus 1 is more widely distributed than previously recognised.

The consensus (1 000 replicates) neighbour joining phylogenetic tree of the Carnivore bocaparvovirus 2 strains also indicated that two distinct lineages were substituted (Figure 5) based on the 400 bp partial *NS1* gene. All the Turkish isolates and a South Korean isolate (KF771828.1) were situated in the same lineage. The nucleotide identity of lineage 1 isolates varied from 97.5% to 100% and lineage 2 isolates varied from 98.50% to 99.0%. The nucleotide identity between lineage 1 and 2 was 95.25% to 97.0%. We also detected a unique

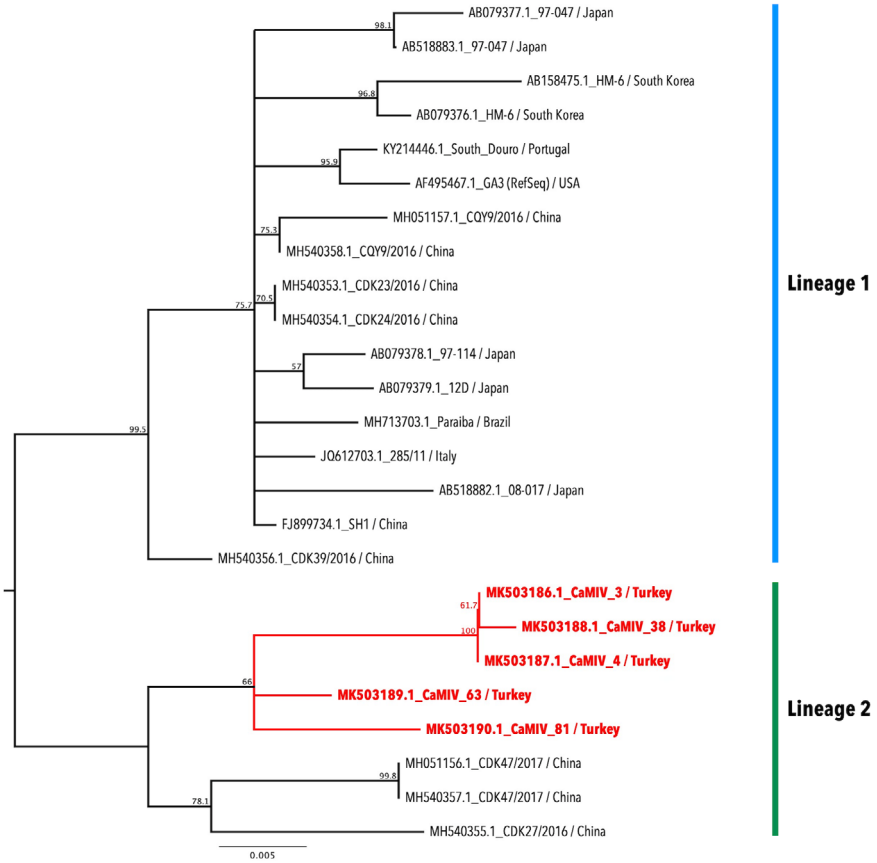

Figure 3. A cladogram representing the consensus (1 000 replicates) neighbour joining phylogenetic tree of the Carnivore bocaparvovirus 1 strains based on the 500 bp partial *VP2* gene

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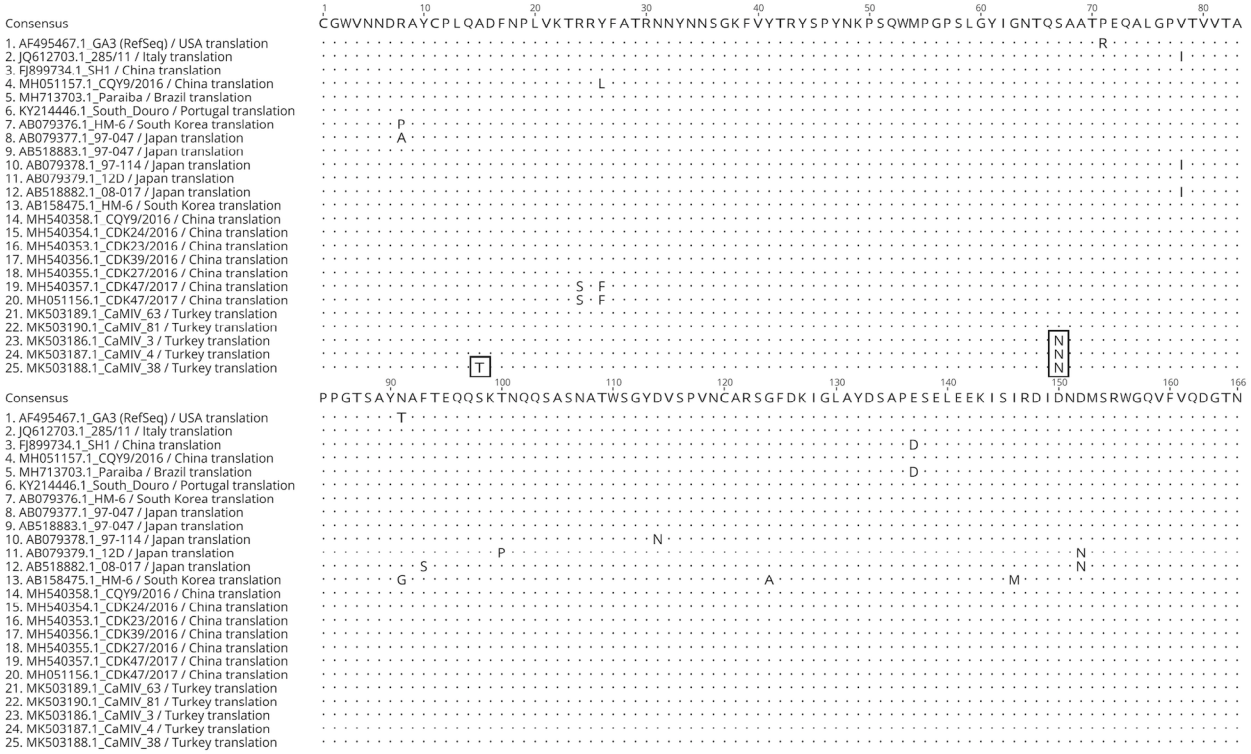

Figure 4. Multiple alignment of the 500 bp partial *VP2* gene sequences of the Carnivore bocaparvovirus 1 strains indicating two point mutations on MK503186, MK503187 and MK503188

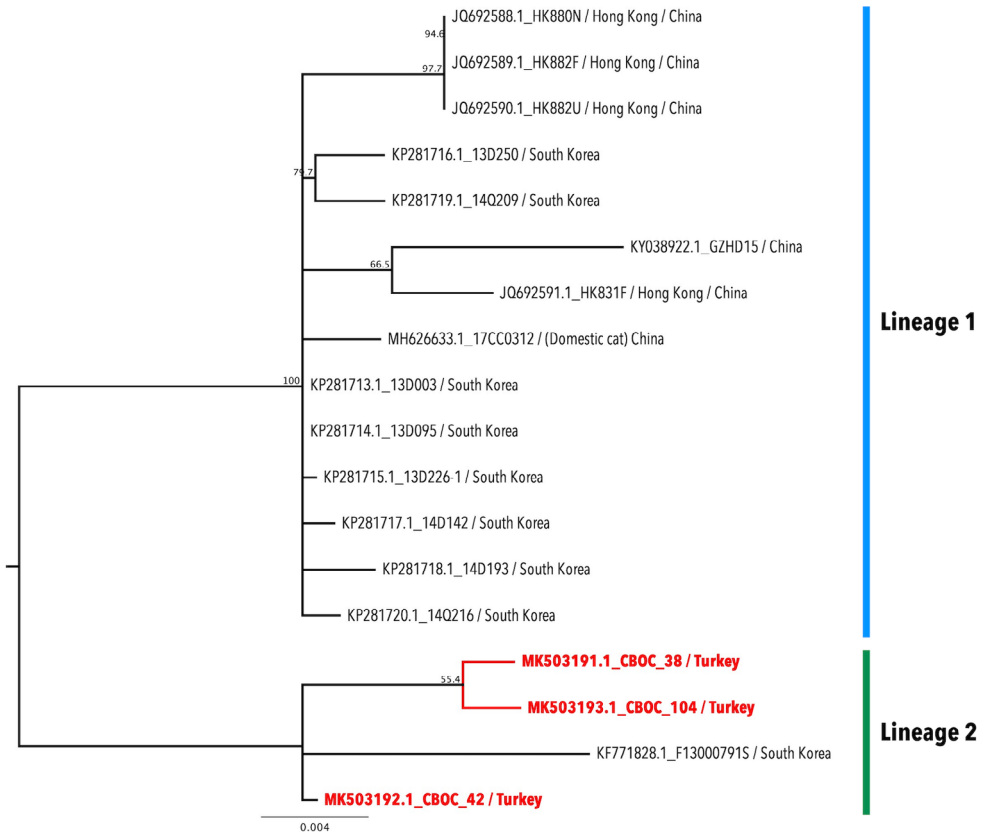

Figure 5. A cladogram representing the consensus (1 000 replicates) neighbour joining phylogenetic tree of the Carnivore bocaparvovirus 2 strains based on the 400 bp partial *NS1* gene

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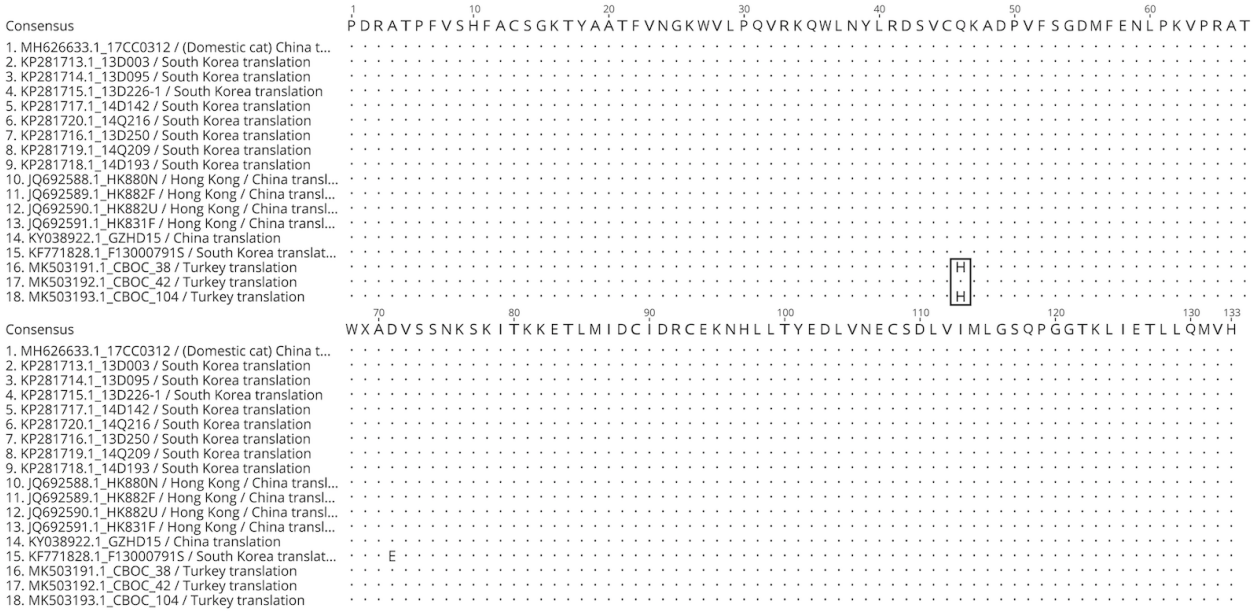

Figure 6. Multiple alignment of the 400 bp partial *NS1* gene sequences of the Carnivore bocaparvovirus 2 strains indicating a point mutation on MK503191 and MK503193

mutation ("CAG/Glu to CAC/His") on MK503191 and MK503193 (Figure 6). As a result, we propose that the Turkish Carnivore bocaparvovirus 2 isolates, which are genetically distinct from previously known isolates, are just the same as Turkish Carnivore bocaparvovirus 1 strains.

It is plausible that we had a considerable drawback in our study. Despite that some of the causal agents that subjected to in this study are intermittently detected in clinically healthy animals, i.e., bocaparvoviruses (Manteufel and Truyen 2008; Martella et al. 2018), our study focused on diarrheic dogs only. Thus, we conjecture that this study implies the need for more comprehensive studies in the subfamily *Parvovirinae* in clinically healthy and diarrheic dogs.

In conclusion, we report that this is the first virological detection of these two viruses (Carnivore bocaparvovirus 1 and 2) in shelter dogs in Turkey. It is also the only study that examined 3 carnivore parvoviruses in dogs with diarrhoea. We also believe that this study will especially contribute to expanding of the scientific knowledge on Carnivore bocaparvovirus 1 and 2.

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Conflict of interest

The authors declare no conflict of interest.

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