Original Article

Co-circulation of canine chaphamaparvovirus and canine parvovirus 2 in dogs with diarrhea in Turkey

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Abstract

Chaphamaparvoviruses, a divergent group of parvoviruses (family *Parvoviridae*) infect both domestic and wild mammalian and avian species, including bats, chickens and pigs. They have recently been identified using metagenomic analysis from tissue and stool samples of healthy and sick animals. Here, dogs (60 healthy and 43 diarrheal) were investigated for the presence of canine chaphamaparvovirus, canine coronavirus (CCoV), canine adenovirus (CAV), canine distemper virus (CDV) and canine parvovirus 2 (CPV- 2) by rectal swabs using polymerase chain reaction (PCR). For chaphamaparvovirus, all the healthy dogs tested negative whereas three of the diarrheal dogs tested positive (3 /103; 2.9%). CPV-2 was also detected in diarrheal dogs (28/43; 65%) but no other viruses were found in the rectal samples of the sick dogs. The three chaphamaparvovirus-positive dogs were also positive for CPV-2. Phylogenetic analysis showed that the chaphamaparvoviruses were all related to American strains, forming a separate clade in the mammalian group. Amino acid sequence comparisons demonstrated that the Turkish strains had one substitution at 743 (Ser→Gly) and substitutions at 688 (Ser→Leu), 716 (Asp→His) and 743 (Cys→Ser/Gly) compared to the American strains. This study provides the first report of chaphamaparvovirus in Turkey. It also documents, for the first time, co-circulation of chaphamaparvovirus and CPV-2 in Turkey's dog population.

Keywords: Parvoviridae, chaphamaparvovirus, CPV-2, Molecular characterization, Turkey

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Introduction

The global dog population is estimated to be 900 million (Atitwa 2018). Many viral agents infect dogs, causing respiratory, gastrointestinal and nervous system diseases, including canine herpes virus (CHV), canine parainfluenza virus (CPIV), canine distemper virus (CDV), rabies virus, canine oral papillomavirus (CPV1), canine influenza (CIV), canine circovirus (canine CV), canine bocavirus (CBoV) and canine kobuvirus (CaKoV) (Aydin *et al.,* 2018; Caddy 2018; Thaiwong *et al.,* 2018). Canine diarrhea is one of the most significant diseases in dogs, whether caused by viruses, bacteria or parasites (Cave *et al.,* 2002). The main enteric viruses in dogs are canine adenovirus (CAV), canine corona virus (CCoV), mamastrovirus 5 (MAstV5) and canine parvovirus 2 (CPV-2) (De Benedictis *et al.,* 2011; Polat *et al.,* 2019; Timurkan *et al.,* 2018; Yesilbag *et al.,* 2004).

Parvoviruses are small, icosahedral symetric, non-enveloped, single-stranded linear DNA viruses approximately 5 kb in genome length. Structurally, the genome has two Open Reading Frames (ORF): a non-structural replicase gene (NS) expressing enzyme required for replication and a capsid (VP) gene expressing structural proteins (Calderon *et al.,* 2009; Miranda & Thomson 2016). The family *Parvoviridae* contains three subfamilies, *Parvovirinae*, *Densovirinae* and *Hamaparvovirinae* which infect vertebrate and invertebrate hosts. Chaphamaparvoviruses classified in *Hamaparvovirinae* infect vertebrate hosts. (Cotmore *et al.,* 2014). The subfamily *Parvovirinae* comprises 8 genus: *Dependoparvovirus*, *Copiparvovirus, Bocaparvovirus, Amdoparvovirus, Aveparvovirus, Protoparvovirus*, *Tetraparvovirus*, and *Erythroparvovirus*. More recently , two new genus in the subfamily Hamaparvovirinae were added: *Marinoparvovirus* and *Chaphamaparvoovirus*. *Marinoparvovirus* includes sesavirus, detected from rectal swabs of California sea lion offspring with malnutrition and pneumonia (Phan *et al.,* 2015).

Metagenomic analysis has recently increased the number of identified chaphamaparvoviruses isolated from bat, bird and pig hosts (Palinski *et al.,* 2016; Reuter *et al.,* 2014). Since then, red – crowned crane parvovirus (RcPV) and Turkey parvovirus 1 (TP1) have been found from rectal swabs of both species (Reuter *et al.,* 2014; Wang *et al.,* 2019). Desmodus rotundus parvovirus 1 (DrPV- 1) was detected in vampire bat kidney tissue samples (*Desmodus rotundus*) in Brazil (Souza *et al.,* 2017). In China, porcine parvovirus 7 (PPV7) was obtained from rectal swab samples (Palinski *et al.,* 2016) while in the USA, a viral metagenomic analysis idenfitied a new parvovirus, chaphamaparvovirus, (previously named chapparvovirus; cachavirus) in a diarrhea outbreak in dogs (Fahsbender *et al.,* 2019). The other chaphamaparvovirus, peafowl parvovirus-1 (PePV1) and PePV2, were found in peafowls dying of diarrhea in a wildlife rescue center in China. PePV1 DNA was amplified by PCR from various tissues, including the liver, heart, and lungs and identified by immunohistochemical assay. The results indicated that PePV1 and PePV2 may be fatal pathogens in peafowl (Liu *et al.,* 2020).

Chaphamaparvoviruses have been identified over the past decade (Reuter *et al.,* 2014), with recent epidemiological studies revealing new chaphamaparvoviruses in various animal species. Moreover, chaphamaparvoviruses have recently been detected in humans (Fahsbender *et al.,* 2020), indicating that these viruses have a wider host distribution than is currently estimated.

In Turkey, the presence and distribution of chaphamaparvovirus remains unclear. According to our best information, neither serological nor virological studies have been conducted for chaphamaparvoviruses in humans or animals in Turkey. Accordingly, the present study aimed to investigate the presence and distribution of chaphamaparvovirus in Turkey by applying PCR to rectal swabs collected from both healthy and sick dogs from different georaphical regions.

Materials and Methods

Study region and samples: A total of 103 dogs (60 healthy and 43 diarrheal) were included in this study. Rectal swabs were collected from Mersin (the Mediterranean region), Sanliurfa (the South Anatolian region) and Erzurum province (theEastern Anatolia region) of Turkey for the detection of five viruses (CPV-2, CCoV, CDV, Chaphamaparvovirus, and CAV) that cause diarrhea (2018-2019). In addition, rectal samples were tested for chaphamaparvovirus and others viruses' presence in healthy animals. The 60 healthy dogs included 25 females and 35 males while 19 of the 43 diarrheal dogs were females and 24 were males. The age ranges of the healthy and diarrheal dogs were between 1 month and 9 months and 1.5 months and 8 months, respectively. No animals had a history of vaccination for CCoV, CAV, CPV-2, or CDV. Sixty of the dogs were mixed breeds while the other breeds were cocker (n=10), golden retriever (n=20), kangal (n=5), and German shepherd (n=8).

Hematological and Biochemical Analyses: Hematological and biochemical analyses were performed on whole blood and serum diagnostic samples taken from the dogs with diarrhea. The sera from all infected animals were analyzed for ALT, AST, BUN, cholesterol, total bilirubin and glucose using a biochemistry analyzer (Arkray Spotchem SP-4430 EZ, Japan). Whole blood samples of all the infected animals were analyzed for RBC, hemoglobin, PCV, WBC, neutrophils and lymphocytes using an automatic cell counter (Sysmex PocH-100iV Diff, Japan) (Table 1). Because whole blood and serum samples are routinely taken for the diagnosis and treatment of diarrhea in dogs, samples were not collected specifically for this study.

Sample processing, DNA extraction, and PCR: Rectal swabs from the healthy and diarrheal dogs were moved to appropriate storage containers. Each rectal swab was homogenized with PBS (Phosphate Buffer Saline) and centrifuged at 3,000 rpm for 10 minutes. The supernatant taken into the sterile tube was stored at -80 ºC until analysis. 200 µl supernatant was used for nucleic acid extraction. For the extraction of nucleic

acids, High Pure Viral Nucleic Acid kit (Roche Diagnostic, Mannheim, Germany) was used. The obtained nucleic acids were stored at -80 ºC. Complementary DNA (cDNA) strands were generated using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystem, CA, USA) according to the manufacturer's instructions. For the detection of chaphamaparvovirus, a nested PCR protocol was used as described elsewhere (Fahsbender *et al.*, 2019) while PCR and RT-PCR protocols were used to detect the viruses (CAV, CCoV, CPV-2 and CDV) causing diarrhea and possible co-infections in dogs (Castilho *et al.*, 2007; Timurkan *et al.*, 2018; Timurkan and Oguzoglu, 2015; Yesilbag *et al.*, 2004) (Table 2). After electrophoresis in 1% agarose gel, PCR products were visualized in a ChemiDoc XRS+ imaging system (Bio-Rad Laboratories, Munich, Germany).

Sequence Analysis: Samples identified as positive by PCR were sent to a commercial firm for sequence analysis (Medsantek, Istanbul, Turkey, http://www.medsantek.com). The PCR products were sequenced bi-directionally in an ABI PRISM 3500xL Dx Genetic Analyzer (Thermo Fisher Scientific, Hennigsdorf, Germany). The raw data from the analysis was examined for nucleotide and amino acid sequences using BLASTn and BLASTp from the National Center Biotechnology Information (NCBI) service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1990). The data was then analyzed with CLUSTAL W program for nucleotide and amino acid similarities and differences (Hall 1999). MEGA 6 was used to create the maximum likelihood tree using the obtained sequences and other sequences in the GenBank (Tamura *et al.*, 2013).

Table 1 Hematological and sera biochemical results of dogs with CPV-2 and CPV-2 + chaphamaparvovirus.

Parameter	CPV-2 infected dogs (n=28) Mean ± SD	CPV-2 + chaphamaparvovirus infected dogs (n=3) Mean ± SD	Reference range*
RBCs $\times 10^6$/µL	4.1±3.20	4.0±2.0	4.95–7.87
Hemoglobin g/dl	10±0.23	9.8±13.25	11.9–18.9
PCV %	32±1.22	32±0.12	35–57
WBCs $\times 10^3$/µL	3.8±2.54	3.6±2.15	5.0–14.1
Neutrophil $\times 10^3$/il	2.5±0.41	2.4±3.15	2.9–12.0
Lymphocyte $\times 10^3$/il	0.9±0.21	0.9±0.33	1-4.8
ALT u/l	114.3±3.44	116.3±0.41	10-109
AST u/l	15.0±1.92	14.8±0.36	13–15
BUN mg/dl	33.52±7.53	34.10±3.04	8–28
Cholesterol mg/dl	167.12±15.4	173.12±10.2	135–278
Total bilirubin mg/dl	0.61±1.24	0.63±0.32	0–0.3
Glucose mg/dl	73±0.14	72±21.2	76–119

*International reference range (Tvedten, H., et al., 2004; Merck Manual,2020)

Table 2 Information on the primers used for virus detection in the present study (*annealing tempure changes with primer).

Virus name	Gene name	Primer sequence (5′→3′)	Product (bp)	PCR conditions	References
Canine coronavirus	M	CCoV1–TCCAGATATGTAATGTTCGG CCoV2-TCTGTTGAGTAATCACCAGCT	409	95 ˚C for 4 min; 40 cycles 95 ˚C for 30 sec, X*˚ C for 45 sec, 72 ˚C for 1 min; final extension at 72 ˚C for 7 min	Yesilbag *et al.*, 2004
Canine distemper	N	F- 5′-ACTGCTCCTGATACTGC-3′ R- 5′-TTCAACACCAAC(T/C)CCC-3′	480		Castilho *et al.*, 2007
Canine parvovirus-2	VP2	Hfor - CAGGTG ATGAATTTGCTACA Hrev - CATTTGGATAAACTGGTGGT	610		Timurkan and Oguzoglu, 2015
Canine Chaphamaparvo virus	NS1	CPV-625F- CAACTAGCCGAATGCAGGGA CPV- 948R - CGATAACATCCCCGGACTGG	323		Fahsbender *et al.*, 2019
		CPV18-687FN-AGCTCAGTTTGGCCCAGATC CPV- 911RN - AGAGGGATCGCTGGATCTGT	224		
Canine Adenovirus	E3	HA1- CGCGCTGAACATTACTACCTTGTC HA2-CCTAGAGCACTTCGTGTCCGCTT	For CAV2 1030 For CAV1 508		Timurkan *et al.*, 2018

Results

Rectal swab samples from 103 dogs (60 healthy and 43 diarrheal) were investigated for chaphamaparvovirus presence using nested PCR. Chaphamaparvovirus was only detected in 3 unhealthy dog samples. These dogs were aged 3, 5 and 7 months. One was female and the others were male.

The rectal samples were also tested using PCR and RT-PCR to identify four other viruses (CCoV, CAV, CPV-2, and CDV) and possible co-infections causing diarrhea. The PCR analysis demostrated that 28 of the

43 diarrheal dogs were positive for CPV-2. However, these 43 dogs were negative for CCoV, CDV and CAV (Table 3). Moreover, the healthy dogs were negative for the remaining viruses. The negative samples were investigated no further for etiological factors causing diarrhea. The PCR analysis also showed that 3 dogs were positive for both chaphamaparvovirus and CPV-2.

The sequences of the PCR amplicons of the NS1 partial gene region of chaphamaparvovirus were compared phylogenetically with the American strains. This demonstrated that the Turkish strains (accession numbers; MT076059, MT076060 and MT076061) were located in the same branch as the American strains (accession numbers MK448316 and MK893826) within the mammalian group (Figure 2). The nucleotide sequences of the three chaphamaparvovirus positive samples were 99.5-100% identical with each other and 98.6% identical with the American strains.

Partial amino acid sequences of the NS1 protein were investigated to detect differences between the Turkish and American strains. As shown in Figure 1, the amino acid sequence comparison indicated that the TR-Erz- chaphamaparvovirus strain had a substitution at 743 (Ser) whereas the TR-Mers-chaphamaparvovirus strain and the TR-Urf-chaphamaparvovirus had one at 743 (Gly). There were also substitutions at 688 (Ser→Leu), 716 (Asp→His) and 743 (Cys→Ser/Gly) compared to the American strains (Figure 1) (Reference gene number, MK448316, using amino acid comparisons).

Hematological and biochemical analyses were performed on whole blood and serum diagnostic samples from the diarrheal dogs. Firstly, both the dogs with CPV-2 and CPV-2 + chaphamaparvovirus had reduced RBC, HB and PCV values compared to the reference values. This may explain the anemia detected in these patients. Secondly, dogs with CPV-2 and CPV-2 + chaphamaparvovirus also had significantly lower WBC, netrophil and lymphocyte values than the reference values. Thirdly, dogs with CPV-2 and CPV-2 + chaphamaparvovirus had greater ALT and total bilirubin levels than the reference values. Finally, the animals with diarrhea were hypoglycemic, although their AST and cholesterol values were within the reference range (Merck Manual 2020; Tvedten *et al.*, 2004) (Table 1).

Table 3 Result of viruses screened in this study and samples collected from provinces of Turkey (n= number).

	Regions of Turkey (province)		
	Mersin	**Sanlıurfa**	**Erzurum**
Diarrhea (dogs)	n=15	n=11	n=17
Canine Parvovirus-2	Positive	Positive	Positive
	(n=10; 66,6%)	(n=8; 72,7%)	(n=10; 58,8%)
Canine Chaphamaparvovirus	Positive	Positive	Positive
	(n=1; 6,6%)	(n=1; 9%)	(n=1; 5,8%)
Canine Coronavirus	Negative	Negative	Negative
Canine Distemper virus	Negative	Negative	Negative
Canine Adenovirus	Negative	Negative	Negative

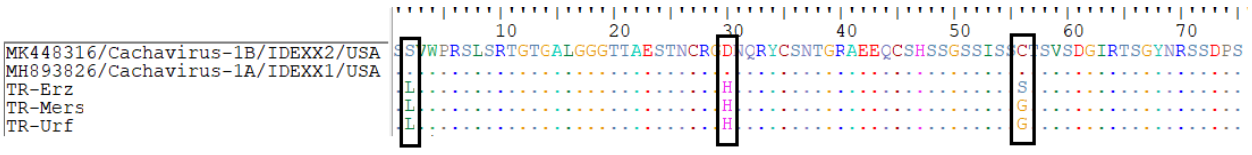

Figure 1 Amino acid changes in NS1 partial protein of chaphamaparvovirus.

Discussion

The global importance of emerging and re-emerging infections has increased recently due to their potential effects on human and animal health. Infections may be caused by new (emerging) viruses (Zhou *et al.*, 2020) or mutations of circulating viruses (re-emerging) (Polat *et al.*, 2019; Timurkan and Aydin, 2019). Thus, regular epidemiological investigation and identification of viruses are essential to determine replication mechanisms, host ranges and geographical distributions. Until recently, information about whether or not chaphamaparvoviruses infect vertebrate hosts was uncertain. However, several recent studies have revealed infections of vertebrate hosts. One study demostrated that MKPV causes kidney disease in laboratory mice (Roediger *et al.*, 2018) while the detection of DrPV-1 DNA in the kidneys of vampire bats caught in Brazilian forest areas indicates this virus may adapt to kidney niche of vampire bats (Souza *et al.*, 2017). Overall, these studies suggest that chaphamaparvoviruses can be pathogenic for various animal species. Specifically, some mammal chaphamaparvoviruses may be nephrotropic and

cause kidney disease in animals exposed to stress (Lee *et al.*, 2020).

A previous study reported chaphamaparvovirus in 4% of dogs with diarrhea, although there was no information about their ages. While this indicates that chaphamaparvovirus causes infections in dogs, the low viral load suggests that chaphamaparvovirus may result from ingesting contaminated foods rather than infection of intestinal tissue (Fahsbender *et al.*, 2019). Thus, it is necessary to investigate whether chaphamaparvovirus can be morbigenous for dogs. There are no previous reports of chaphamaparvovirus presence in Turkey. In the present study, rectal swabs of both healthy and diahrreal dogs were collected from three different georaphical regions, including Mersin (the Mediterranean region), Sanliurfa (the South Anatolian region) and Erzurum province (the Eastern Anatolia region) of Turkey. These were screened for the partial-length NS1 gene of chaphamaparvovirus by nested PCR and were also tested for the presence of four other viruses (CCov, CDV, CAV, and CPV-2). Unsurprisingly CPV-2 was detected in the majority of dogs with diarrhea (28/43; 65%). Previous studies suggest that CPV-2 is widely dispersed in Turkey's dog population (Dincer 2017; Karapınar *et al.*, 2018; Timurkan and Oguzoglu, 2015). Morever, the CPV-2c genotype has recently been detected in dogs with gastrointestinal symptoms (Polat *et al.*, 2019). In the present study, chaphamaparvovirus was also detected in CPV-2 positive dogs across a wide range, including Mersin (1/15), Erzurum (1/17) and SanlıUrfa (1/11). All chaphamaparvovirus-positive dogs were under one year old. Enteric viruses that cause diarrhea are known to infect susceptible animals together. For example, rotavirus and CPV-2 have been detected in dogs with gastroenteritis in Mexico (Ortega *et al.*, 2017) while co-infections with turkey astrovirus (TAstV) and turkey coronavirus (TCoV) cause more severe morbidity and higher mortality than single infections (Yu *et al.*, 2000). In our study, the co-detection of CPV-2 and chaphamaparvovirus in three dogs with diarrhea indicates that these viruses may circulate together and increase the severity of the disease as in the aforementioned viruses.

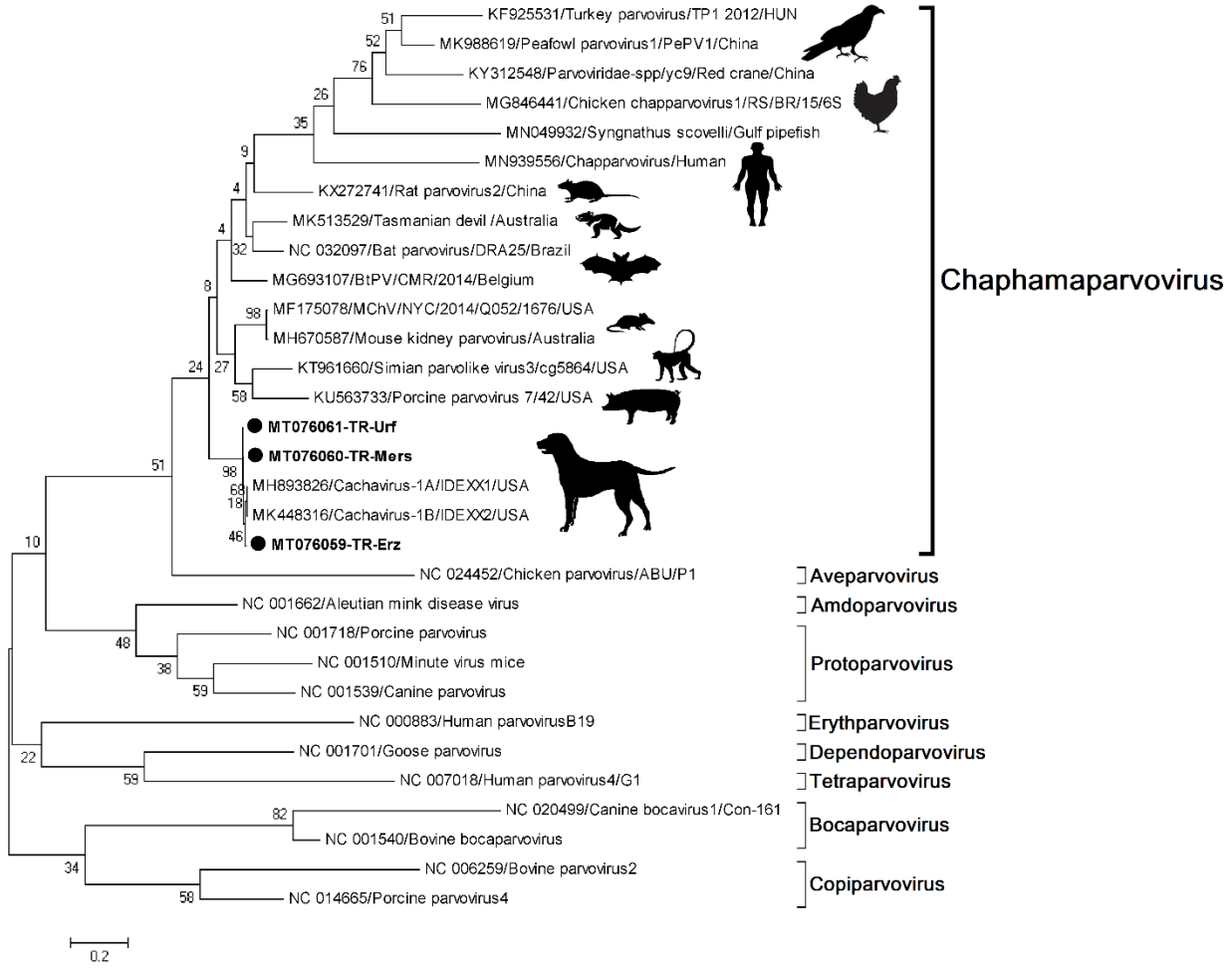

Figure 2 Phylogenetic tree based on the partial NS1 sequence (225 nucleotides) of chaphamaparvoviruses. The phylogeny was performed with maximum likelihood analysis based on 1000 replicates by MEGA 6 software. In this study, chaphamaparvovirus sequences are indicated with a '*circle*'.

Recent studies have demonstrated that chaphamaparvoviruses infect different organs, such as the kidneys, heart and lungs (Liu *et al.*, 2020; Roediger *et al.*, 2018). Thus, it is unsurprising that chaphamaparvovirus is detected in organs (especially kidney tissue) other than the intestines. Although all the chaphamaparvoviruses detected in our study were in dogs with diarrhea, recent studies show that chaphamaparvoviruses are found in different organs. Thus, this should be investigated in future

experimental studies of chaphamaparvoviruses. The presence of chaphamaparvovirus in three major regions in Turkey shows the need for large-scale screening followed by detailed epidemiological observation. Since our study was epidemiological, we did not gather information about pathogenesis in these dogs. Nevertheless, we demonstrated that all the detected chaphamaparvovirus strains were found in dogs with diarrhea; hence, they may be associated with gastrointestinal pathogenesis. No significant biochemical or hematological differences were found between CPV-2 positive and CPV-2 + chaphamaparvovirus animals. The absence of a significant difference in serum and whole blood values between these two groups is not sufficient to evaluate possible infection caused by chaphamaparvovirus in dogs. Future studies of more animals and exclusively on dogs with diarrhea caused by chaphamaparvovirus can help to elucidate clinical presentaion in dogs.

In the family parvoviridae, the NS1 protein plays a major role in various processes, including viral cytotoxicity and pathogenicity in host cells. Analysis of the NS1 protein in CPV-2 isolates identified several amino acid changes although information about the functions of amino acids on the NS1 protein is limited (Mira *et al.,* 2020). The detected changes may be essential in virus replication and host distribution. We detected amino acid changes in the NS1 protein of chaphamaparvovirus in specimens obtained from dogs in Turkey. These amino acid changes reflected differences between our Turkey strains and the American strains, which indicates that NS1 protein mutations may occur frequently. Thus, more studies are needed to determine the effects of these amino acid changes on chaphamaparvovirus replication and pathogenesis. Chaphamaparvovirus has been very recently reported in human plasma samples in Brazil (Fahsbender *et al.,* 2020). The present study showed that phylogenetically comparisons of partial NS1 gene sequences indicated an identitiy of 44% between all isolated chaphamaparvovirus (American and Turkish strains) and human chaphamaparvovirus. That is, chaphamaparvoviruses are probably species specific while chaphamaparvoviruses and human chaphamaparvovirus have large genetic distances.

In conclusion, this study provides the first report of chaphamaparvovirus from the dog population in Turkey and also assesses co-circulation of CPV-2 and chaphamaparvovirus for the first time in dogs showing clinical signs of diarrhea. Despite its DNA structure, chaphamaparvovirus may be evolving through amino acid mutations in the NS1 protein gene. Further epidemiological studies are needed to determine the real incidence of the chaphamaparvovirus in dogs in Turkey. Moreover, molecular and serological studies using more samples will help to better understand chaphamaparvovirus's prevalance and kidney cell tropism, identify specific antibodies and determine its replication mechanisms. Finally, new data will help describe chaphamaparvovirus-dog interaction.

Compliance with ethical standards

Author's contributions: All authors contributed equally to this work.

Conflict of interest: The authors declare no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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