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Virological and pathological investigation of *Canine parvovirus-2* (CPV-2) with the assessment of the genetic variability of field strains

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Abstract: *Canine parvovirus-2* (CPV-2), which is common worldwide, is a highly contagious pathogen in domestic dogs and some wild carnivore species. The agent causes a multisystemic infection in affected animals, especially acute hemorrhagic enteritis and fatal myocarditis in puppies. The study aimed to reveal the presence of virus in dogs with clinical symptoms of CPV-2 infection using virological, pathological, and immunohistochemical methods and make molecular characterization based on partial VP-2 gene of strains circulating in the region. As a result of PCR studies, 9 out of 16 rectal swab samples and 17 out of 38 blood samples were positive. A phylogenetic map was created by analyzing the partial VP2 gene sequences obtained from GenBank and the sequences obtained from this study. It was determined that 6 of the leukocyte samples obtained from the study were in CPV-2a, 5 of the leukocyte and 4 swab samples were in CPV-2b types. Amino acid differences were determined between the obtained sequences and the strains provided in GenBank. In the histopathological examination, lesions of varying degrees were found in all intestinal sections, while the most prominent lesions were seen in the ileocecal valve. Segmental or dense serosal hemorrhages were observed extending to the muscle layer and submucosa in the intestine. In the immunohistochemical examination, the most intense immune reaction was defined in the crypts. In order to control the infection, it is recommended to determine the circulating variants by conducting molecular epidemiological studies in different geographical regions, to support the research with different methods, and to develop the vaccines to be used according to the diversity of genetic variants.

Keywords: *Canine parvovirus-2*, molecular characterization, immunohistochemistry, histopathology, phylogenetic analysis

1. Introduction

Canine parvovirus-2 (CPV-2) which is quite frequent all around the world, is highly contagious and is characterized by acute, fibrinous, necrotic, or hemorrhagic enteritis [1, 2]. Even though the disease can be noticed in dogs of all ages, it has been reported that juvenile animals are more intensely affected by CPV-2. Sudden death as a result of the myocardial form characterized by nonsuppurative myocarditis is seen in newborn puppies [3,4]. The enteric form is frequently seen in mature animals and causes catarrhal, hemorrhagic, and fibrinous inflammation [2,5]. The agent is in the *Protoparvovirus* genus of the *Parvoviridae* family, the *Parvovirinae* subfamily. Although the agent is a nonenveloped DNA virus, new variants of the agent have emerged all over the world (2a/2b/2c). *Canine parvovirus* contains types with different antigenic properties, including CPV-1 (*Canine parvovirus-1*) and CPV-2. Most of the dogs with CPV-1 infection are asymptomatic. CPV-2 with important clinical findings was first described in 1978, and in 1980, CPV-2a, an antigenic variant of CPV-2, the CPV-2b variant of the virus was identified in 1984 [6,7]. In the 2000s, the CPV-2c variant of the virus was first described in Italy [2]. The genome is 5200 nucleotides long, single-stranded, and contains two open reading frames regions (ORFs). One of these encodes two nonstructural proteins (NS1 and NS2), while the other encodes three structural (VP1, VP2, VP3) proteins [8]. The VP2 gene is an important major antigenic capsid protein that determines the tissue selectivity (tropism) and host range of the virus [9]. There are many variants originating from CPV-2, and they are formed by the replacement of several amino acids in the VP2 protein [10]. According to the change in the amino acid in the capsid protein gene, it has been determined to be divided into antigenic types called CPV-2a (*Asparagine-Asn*) and CPV-2b (*Aspartic acid-Asp*) with the same pathogenesis [7,11]. Strains

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of CPV-2a and CPV-2b are interchangeable when two single-nucleotide polymorphisms (SNPs) changes occur in the viral capsid (SNP). It was determined that this change occurred depending on whether the amino acid at position 426 of the VP2 protein was *Asn* or *Asp* and the amino acid at position 555 was *Isoleucine* (*Ile*) or *Valine* (*Val*) [12]. CPV-2c variant first reported in Italy in 2000, arose as a result of the change in the 426th amino acid in the capsid protein (*Asp* → *Glu* (*Glutamic acid*)) [8,13]. CPV-2c, which is reported to be a more virulent antigenic variant, is circulating all over the world [13–17].

In this study, it was aimed to detect the presence of virus in blood, rectal swab samples, and different organ materials of dogs with symptoms of CPV-2 infection using molecular, pathological, and immunohistochemical methods in Balikesir, Türkiye and to make molecular characterization based on partial VP-2 gene of strains circulating in the region.

2. Materials and methods

2.1. Materials

In this study, 38 blood samples collected from private clinics, 16 rectal swabs and organ (heart, duodenum, jejunum, ileum, caecum, colon, and rectum) materials from 7 dead dogs were used. Tissue samples taken from dogs were brought to Balıkesir University Veterinary Faculty Pathology Department for necropsy. Samples were obtained from unvaccinated dogs with mixed breeds, aged between 2–6 months with gastroenteritis symptoms between December 2019 and November 2020. Permissions required for this study were obtained from Balikesir University Animal Experiments Local Ethics Committee (dated: 28/11/2019, numbered: 2020/4-19).

2.2. Virological methods

Blood samples taken into anticoagulant tubes were centrifuged at 1500 rpm for 10 min, and the buffy coats were transferred to an Eppendorf tube and stored at –20 °C until tested. Rectal swab samples were brought to the laboratory under a cold chain in sterile tubes containing 2 mL transport medium. After vortexing, the swab stick in the tube will be discarded and the swab samples will be centrifuged at 4500 rpm for 10 min. The supernatant was taken into stock tubes and stored at – 20 ˚C until testing.

2.2.1. Viral DNA extraction, polymerase chain reaction (PCR), sequence analysis

Extraction of viral DNA from blood and swab samples; was performed in suitability with the kit's procedure using a commercial viral DNA isolation kit (Viral RNA+DNA Preparation Kit, Jena Bioscience, Germany). PCR method and CPV VP-2 gene region-specific primers were performed using H for 5'-CAGGTGATGAATTTGCTACA-3'; H rev5'-CATTTGGATAAACTGGTGGT-3' (629 bp) [13]. PCR conditions were implemented as after initial denaturation 94 ˚C for 10 min, 40 cycles at 95 ˚C for 50 s, 58 °C for 60 s, 72 °C for 60 s and a final extension for 7 min at 72 °C. The obtained PCR products were visualized on a 1.5% agarose gel. Sequence analysis of positive PCR products was performed. The data obtained after the sequence analysis were aligned to the Clustral W algorithm using the BioEdit (7.0.5) program [18]. Canine parvovirus nucleotide sequences were compared with different reference sequences in the NCBI database using BLAST software and phylogenetic analysis studies were completed using the MEGA v11.0 program. The neighbor-joining method was used in the analysis and the bootstrap value was calculated as 1000 repetitions [19].

2.3. Pathological methods

Different tissue samples from 7 dogs with suspected CPV were used to evaluate pathological findings. Tissue samples taken from duodenum, jejunum, ileum, caecum, colon, and rectum were fixed in formalin buffered (10%) for at least 24 h. The tissues were trimmed, embedded in paraffin, and 5-mm sections were stained with hematoxylin and eosin (HE). In order to detect the presence of parvovirus virus antigen in all intestinal parts of each case, immunostaining was performed with mouse anticanine/feline parvovirus (CPV1-2A1 clone, BIO-RAD, USA) antibody using the Avidin-Biotin-Immunoperoxidase complex method. Sections of 4 µm thick from paraffin blocks were taken on a poly-L-lysine coated slide. After the sections were deparaffinized, they were washed with PBS (Phosphatized Buffer Solution) and kept in 3% hydrogen peroxide solution for 15 min to block the endogenous peroxide activity. Then, the tissues washed using PBS were kept in a pH 6.0 citrate buffer solution in the microwave for 14 min to expose the antigenic receptors and allowed to cool. After the cooled sections were washed with PBS, they were incubated with Large Volume Ultra V Block (TA-125-UB, Thermo, USA) for 30 min at room temperature to prevent nonspecific staining (30 min). At that time, without washing the sections, they were incubated with mouse anticanine/feline parvovirus (1/200, CPV1-2A1 clone, BIO-RAD, USA) antibody for 1 h at 37 °C. At the end of this period, sections washed 3 times with PBS at 5-min intervals were treated with Biotinylated Goat Anti-Poyvalent (TP-125-BN, Thermo, USA) secondary antibody for 30 min. Afterward, the sections were washed at PBS 3 times at 5-min intervals and incubated with Streptavidin Peroxidase (TP-125-HR, Thermo, USA) for 30 min. At the end of the period, the sections were washed with PBS 3 times at 5-min intervals and 3.3-diaminobenzidine tetrahydrochloride-H2O2 (DAB) solution was applied to the sections for 30 s. At the end of the time, the reaction was stopped with distilled water. As a final step, the sections were stained in Mayer

Hematoxylin for 45 s, followed by 70%, 96%, 100%, and 100% alcohol, respectively, followed by 10 min in xylol and examined under a light microscope.

3.Results

3.1. Virological findings

As a result of PCR studies, 9 out of 16 rectal swab samples and 17 out of 38 blood samples were positive. Fifteen of the samples (11 leukocytes, 4 rectal swabs) determined as positive were subjected to sequence analysis. GenBank accession numbers of the samples used in phylogenetic analysis are given in Table 1.

It was determined that the sequences obtained as a result of this study and the partial VP-2 gene sequences obtained from GenBank were similar at different rates (Figure 1). When amino acid similarity was investigated, it was determined that there were differences between the strains obtained as a result of the study and the strains compared from the gene bank. It was determined that 6 of the leukocyte samples obtained from the study were in CPV-2a, 5 and all swab samples were in CPV-2b types (Table 1). Amino acid differences (297, 300, 305, 316, 341, 375, 426, 440) appeared between the strains obtained as a result of sequence analysis and the reference sequences obtained from GenBank (Table 2). The place of our strains in the *Parvovirinae* subfamily and the similarity with CPV-1 are shown in Figure 2.

3.2. Pathological findings

In the necropsies of dogs with suspected parvovirus, severe fibrino hemorrhagic enteritis was observed in almost all animals. The intestinal wall was macroscopically excessively thickened and oedematous. It was observed that Peyer's patches became well known in the serosa and mucosa in a way that could be easily noticed. Segmental and dense serosal hemorrhages were observed extending to the muscle layer and submucosa in the intestine. It was observed that all intestinal sections from the duodenum to the rectum were excessively swollen due to oedema and bleeding. It was noted that especially the ileum and cecum parts were dark red and Peyer's patches became visible from the serosa. The intestinal lumen had a dark red, bloody watery content (Figure 3). Central collapsed bleeding areas were observed especially in the ileocecal valve mucosa. The periphery of the bleeding foci was covered with dull white-yellow fibrin. Mesenteric lymph nodes were enlarged and pale. In the histopathological examination, lesions of varying degrees were found in all intestinal sections, while the most prominent lesions were seen in the ileocecal valve. It was observed that the uppermost part of the mucosa in this region was covered with a layer consisting of desquamated epithelial cells, erythrocytes, and fibrin. The villi under this layer were massed and the epithelial parts were shed (Figure 4a). In addition, it was determined that the upper 1/3 of the villi were necrotic in 3 cases. While intense mononuclear cell infiltration was noted in the lamina propria, it was seen to be accompanied by neutrophil leukocytes in some cases (Figure 4b). In 7 of all cases, the crypt lumens were filled with necrotic epithelial cells. The central part of Peyer's patches was filled with reticulum cells. In the

Table 1. GeneBank accession numbers and sample type of CPV-2 strains obtained in the study.

No	Strain Name	Accession No	Genotype	Sample
1	TR/BAL/CPV-2a/4/L/2020	MZ197813	2a	Leukocyte
2	TR/BAL/CPV-2a/9/L/2020	MZ197814	2a	Leukocyte
3	TR/BAL/CPV-2a/14/L/2020	MZ197815	2a	Leukocyte
4	TR/BAL/CPV-2a/28/L/2020	MZ197816	2a	Leukocyte
5	TR/BAL/CPV-2a/2/L/2020	MZ197817	2a	Leukocyte
6	TR/BAL/CPV-2a/6/L/2020	MZ197818	2a	Leukocyte
7	TR/BAL/CPV-2b/19/S/2020	MZ197819	2b	Swab
8	TR/BAL/CPV-2b/16/L/2020	MZ197820	2b	Leukocyte
9	TR/BAL/CPV-2b/22/S/2020	MZ197821	2b	Swab
10	TR/BAL/CPV-2b/25/S/2020	MZ197822	2b	Swab
11	TR/BAL/CPV-2b/20/L/2020	MZ197823	2b	Leukocyte
12	TR/BAL/CPV-2b/30/L/2020	MZ197824	2b	Leukocyte
13	TR/BAL/CPV-2b/29/L/2020	MZ197825	2b	Leukocyte
14	TR/BAL/CPV-2b/37/S/2020	MZ197826	2b	Swab
15	TR/BAL/CPV-2b/32/L/2020	MZ197827	2b	Leukocyte

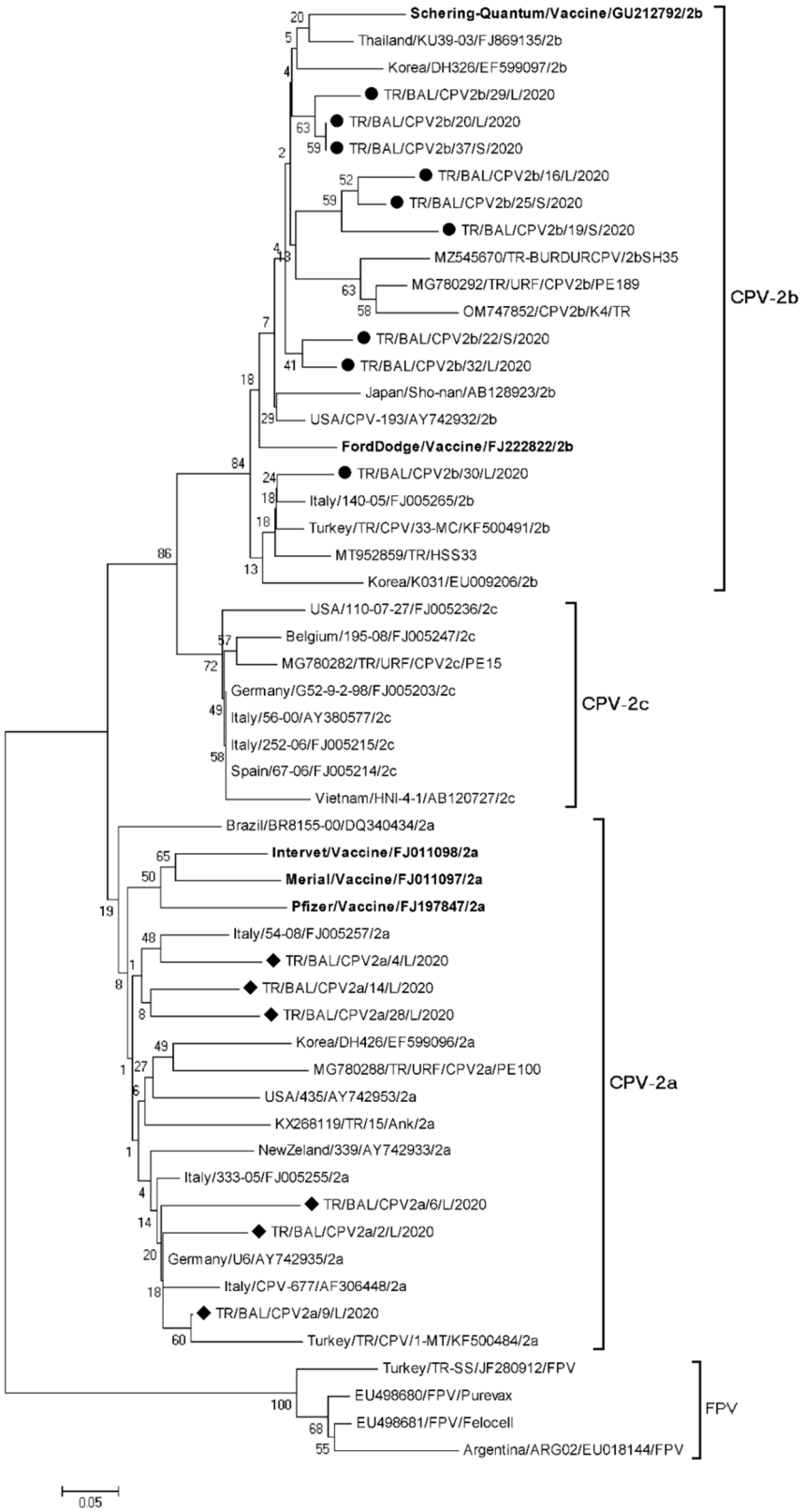

Figure 1. Phylogenetic analysis of the canine parvovirus VP-2 gene.

Table 2. Amino acid changes of CPV-2 strains obtained in the study.

Accession Number	Amino acid position number							
	297	**300**	**305**	**316**	**341**	**375**	**426**	**440**
M38245/CPV2a	S	A	D	V	P	N	N	T
Italy/333-05/FJ005255/2a	A	G	Y	V	P	D	N	T
Intervet/Vaccine/FJ011098/2a	A	G	Y	I	P	D	N	T
Merial/Vaccine/FJ011097/2a	A	G	Y	I	P	D	N	T
Pfizer/Vaccine/FJ197847/2a	A	G	Y	V	P	D	N	T
TR/BAL/CPV2a/9/L/2020 (MZ197814)	A	G	Y	V	P	D	N	T
TR/BAL/CPV2a/4/L/2020 (MZ197813)	A	G	H	I	P	D	N	T
TR/BAL/CPV2a/14/L/2020 (MZ197815)	A	G	Y	I	P	D	N	T
TR/BAL/CPV2a/6/L/2020 (MZ197818)	A	G	Y	V	P	D	N	T
TR/BAL/CPV2a/28/L/2020 (MZ197816)	A	G	Y	M	P	D	N	T
TR/BAL/CPV2a/2/L/2020 (MZ197817)	A	G	Y	V	P	D	N	T
KX268119/TR/15/Ank/2a	A	G	Y	I	P	E	N	T
TR/CPV/1-MT/KF500484/2a	A	G	Y	V	P	D	N	A
MG780288/TR/URF/CPV2a/PE100	A	G	Y	I	P	D	N	T
Korea/DH326/EF599097/2b	A	G	Y	V	S	D	D	A
FordDodge/Vaccine/FJ222822/2b	A	G	Y	V	S	D	D	T
Scher-Quan/Vaccine/GU212792/2b	A	G	Y	V	S	E	D	A
TR/BAL/CPV2b/19/S/2020 (MZ197819)	A	G	Y	V	S	D	D	A
TR/BAL/CPV2b/16/L/2020 (MZ197820)	A	G	Y	V	A	D	D	A
TR/BAL/CPV2b/22/S/2020 (MZ197821)	A	G	Y	V	S	D	D	A
TR/BAL/CPV2b/25/S/2020 (MZ197822)	A	G	Y	V	A	D	D	A
TR/BAL/CPV2b/20/L/2020 (MZ197823)	A	G	Y	V	S	D	D	A
TR/BAL/CPV2b/30/L/2020 (MZ197824)	A	G	Y	V	S	D	D	A
TR/BAL/CPV2b/29/L/2020 (MZ197825)	A	G	Y	V	T	D	D	A
TR/BAL/CPV2b/37/S/2020 (MZ197826)	A	G	Y	V	S	D	D	A
TR/BAL/CPV2b/32/L/2020 (MZ197827)	A	G	Y	V	P	D	D	A
MG780292/TR/URF/CPV2b/PE189	A	G	Y	V	T	D	D	A
OM747852/CPV2b/K4/TR	A	G	Y	V	T	D	D	A
MT952859/TR/HSS33	A	G	Y	V	S	D	D	A
MZ545670/TR-BURDURCPV/2bSH35	A	G	Y	V	S	D	D	A
TR/CPV/33-MC/KF500491/2b	A	G	Y	V	S	D	D	A
Belgium/195-08/FJ005247/2c	A	G	Y	V	P	D	E	T
MG780282/TR/URF/CPV2c/PE15	A	G	Y	V	P	D	E	T

immunohistochemical examination, the most intense immune reaction was detected in the crypt epithelium. The intracytoplasmic immunoreaction was observed in macrophages infiltrating this region, crypt epithelial cells, and the cytoplasm of some enterocytes (Figure 4c, Figure 4d). The intracytoplasmic immunoreaction is observed in cardiomyocytes (Figure 4e, Figure 4f).

4. Discussion

Viruses in the *Parvoviridae* family have a wide host spectrum and the *Parvovirinae* subfamily, which infects vertebrates includes 10 genera (*Amdoparvovirus, Artiparvovirus, Aveparvovirus, Bocaparvovirus, Dependoparvovirus, Erythroparvovirus, Loriparvovirus, Protoparvovirus, Tetraparvovirus* and *Copiparvovirus*) [20]. The place of our

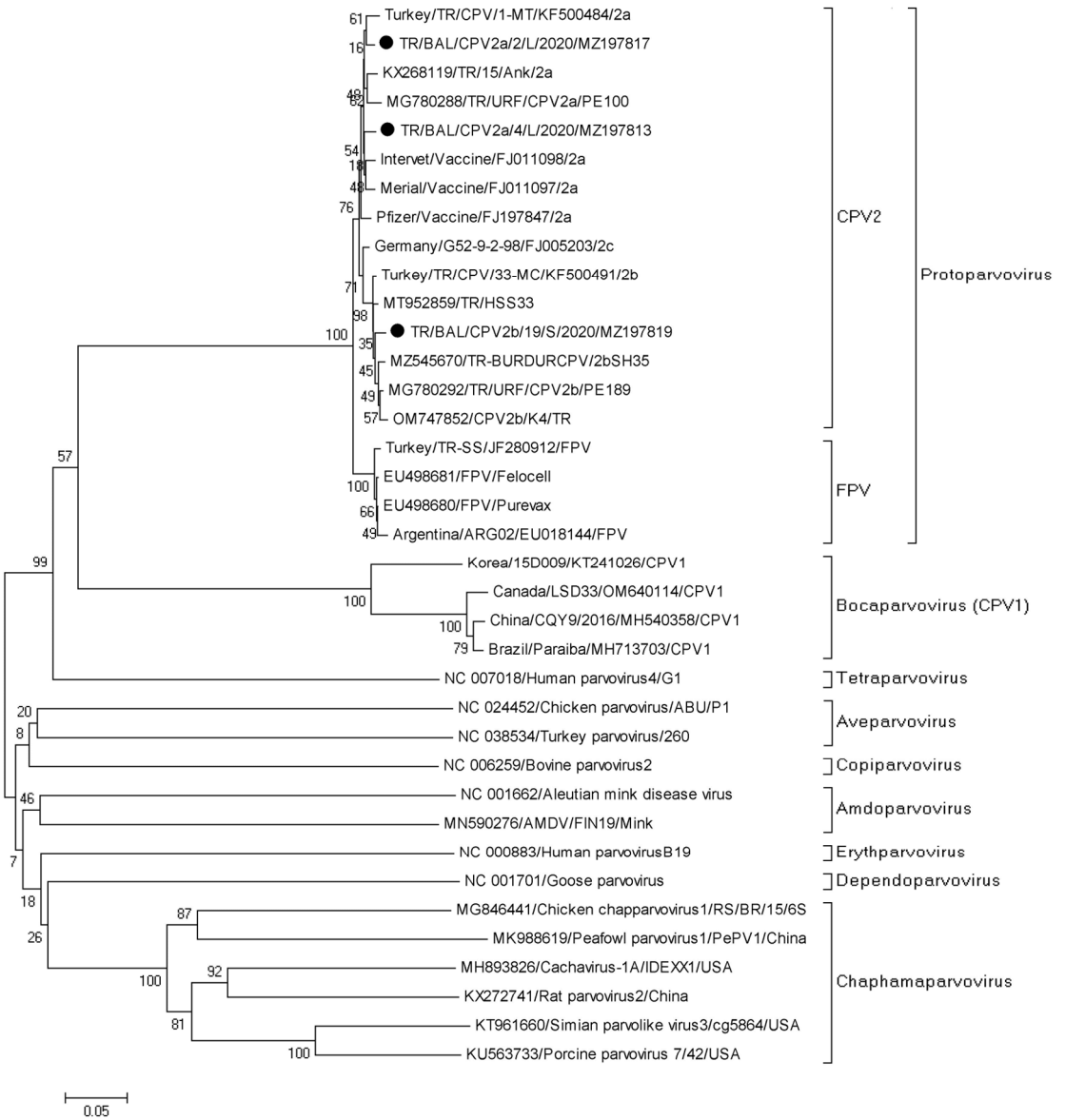

Figure 2. The place of our strains in the *Parvovirinae* subfamily. Strains from our study are marked with black circles.

strains determined as CPV-2 in *Parvovirinae* subfamily *Protoparvovirus* genus and similarity with other genera were revealed in the phylogenetic tree (Figure 2).

CPV-1 (Canine Minute Virus-CMV), which was first identified in 1970, progresses with resorption, abortion, embryonic and fetal deaths in pregnant dogs, and enteric and respiratory symptoms in neonates, and occurs subclinically in most infections. It was reported that CPV-2, which is completely different from the first identified virus, emerged in 1978 as a result of the mutation of the *Feline Panleukopenia Virus* (FPV), to which it is closely related [21,22]. CPV-2 infection, which is common all over the world, causes severe enteritis in adult animals and myocarditis and it can result in death in puppies [23]. Studies have reported that age, gender, secondary infections, lack of vaccination, immunosuppression, individual and regional factors play a role in the prognosis of infection [24,25]. Different antigenic variants of CPV-2 are common to varying degrees in different countries. According to epidemiological studies conducted all over the world, the distribution of CPV-2 variants varies by geographic region, and accordingly, the dominant CPV-2 variant in circulation may also vary. CPV-2a has been reported in the USA, Japan, Taiwan, Australia, Belgium,

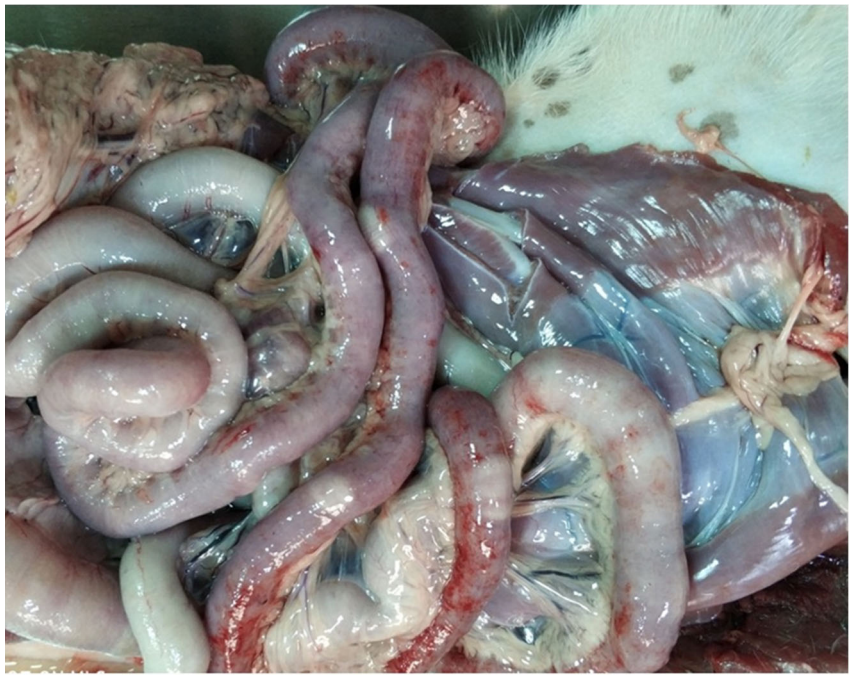

Figure 3. Thickened bowel wall, prominent Peyer's patches discernible from the serosa, and heavy serosal hemorrhages.

Denmark, and France [26,27]. Both variants of CPV-2 have been circulating worldwide for over 40 years and have been described in Europe, Asia, Africa, Oceania, and America [28].

In our country, there are many studies on the epidemiology and genotyping of infection. In the studies conducted, 2a [17,29–32] and 2b [17,29,31–36] variants have been reported to be widely circulated (Table 3). CPV-2c causes more severe disease in adult dogs than CVP-2a/2b variants [37]. In the study, 6 of the samples found to have CPV-2 were in CPV-2a and 9 of them were in CPV-2b. Despite the limited presence of CPV-2c in Türkiye previously, the results of our study support that CPV-2a and CPV-2b continue to circulate as dominant variants in dog populations.

According to the phylogenetic tree revealed as a result of sequence analysis, it was determined that both genotypes of CPV-2 were found in the field strains obtained from our study. CPV-2a strains from the study TR/BAL/CPV-2a/14/L/2020 and TR/BAL/CPV-2a/28/L/2020 formed a separate branch, while TR/BAL/CPV-2a/4/L/ It was determined to be similar to the 2020 strain and the Italy strain. TR/BAL/CPV-2a/2/L/2020 and TR/BAL/CPV-2a/6/L/2020 strains are closely related to each other and to Italy, and Germany strains. The TR/BAL/CPV-2a/9/L/2020 strain was similar to the strain previously obtained in Türkiye (Ankara). Among the CPV-2b strains obtained from the study, TR/BAL/CPV-2b/29/L/2020, TR/BAL/CPV-2b/37/S/2020, TR/BAL/CPV-2b/20/L/2020 separately were found to be similar to Korean and Thai strains when forming a branch. TR/BAL/CPV-2b/22/S/2020, and TR/BAL/CPV-2b/32/L/2020 strains also formed a separate branch. TR/BAL/CPV-2b/16/L/2020, TR/BAL/CPV-2b/19/S/2020, and TR/BAL/CPV-2b/25/S/2020 strains formed a separate branch and more strains in Türkiye. It was determined that the strains were similar to strains in different regions (Burdur, Şanlıurfa, Elazığ). These results are similar to the results reported from Türkiye so far. The data obtained as a result of the study show that both CPV-2a and CPV-2b subtypes are circulating in the region.

Amino acid differences (297, 300, 305, 316, 341, 375, 426, 440) appeared between the strains obtained by our study and the reference sequences obtained from GenBank (Table 2). In recent studies, it has been determined that the changes in the 297th, 300th, 305th, 426th, and 440th amino acid residues of the VP2 protein are among the amino acid changes that are important for antigenicity [38,39]. The 297th aa *Ser→Ala* (*Serine* 297 *Alanine*) mutation in the VP2 protein of the virus has been detected in both CPV-2a and CPV-2b variants. The amino acid change determined in the CPV-2 strains obtained in our study was also reported in previous studies [13,40,41] and it was determined that it affected an antigenic residue close to epitope B on the shoulder region of the capsid. The amino acid exchange *Ala→Gly* (*Alanine* 300 *Glycine*) is localized in a minor

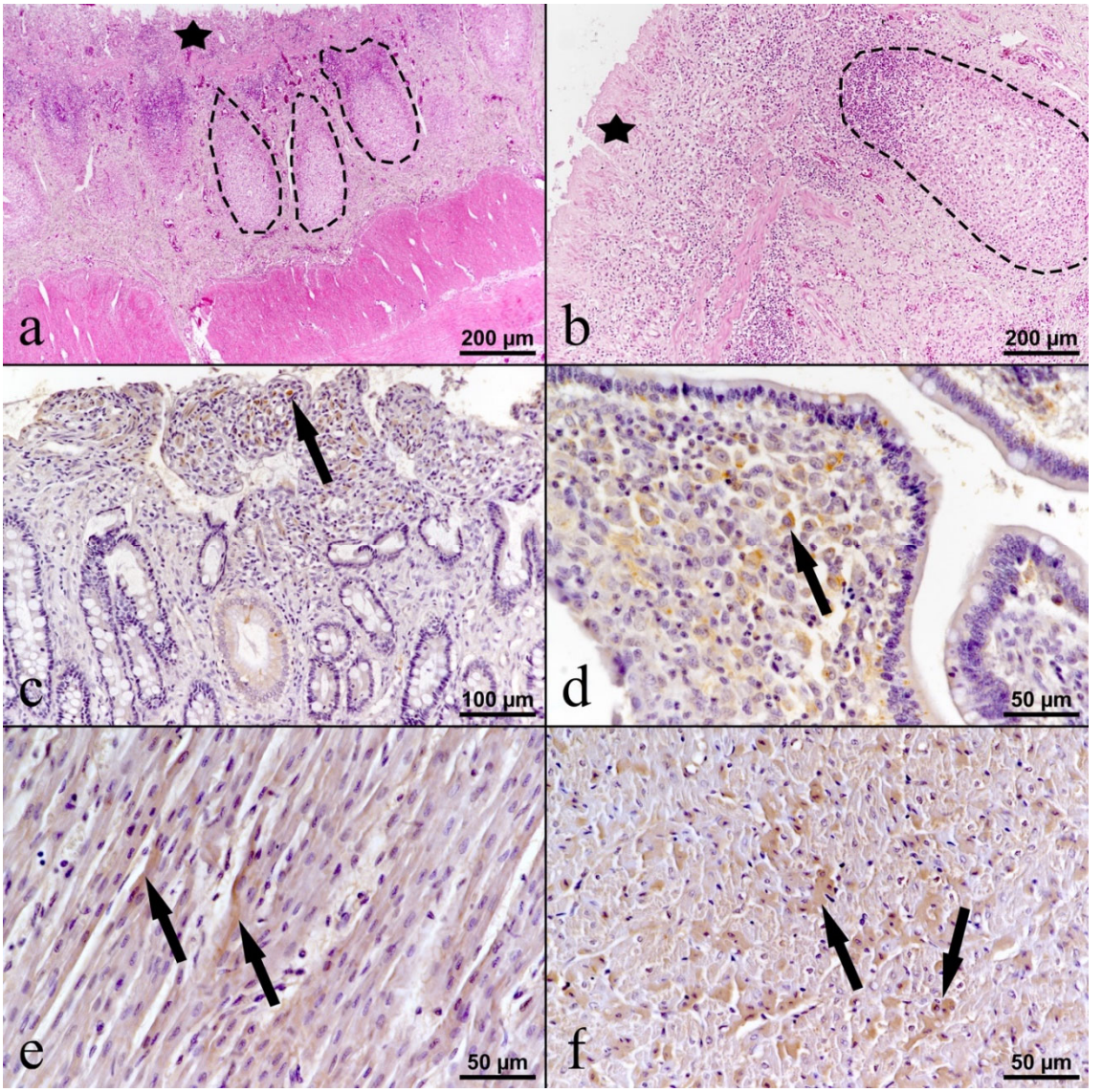

Figure 4a. Necrosis and atrophy in villi (star) and lymphoid depletion in Peyer's patches (dotted areas). HE. **b.** Atrophy and mononuclear cell infiltrations in villi (star) and lymphocytolysis (dotted area). HE. **c.** Intracytoplasmic immunohistochemical positive reaction in the epithelial cell (arrow). IHC. **d.** Intracytoplasmic immunohistochemical reaction in the macrophages (arrow). IHC. **e.** Intracytoplasmic immunohistochemical reaction in the cardiomyocyte (arrows). IHC. **f.** Intracytoplasmic immunohistochemical reaction in the cardiomyocyte (arrows). IHC.

antigenic region and occurs between strains CPV-2a and CPV-2b. The 316th aa changed as *Ile* in the reference strain *Val* CPV-2a vaccine strains and MZ197813, and MZ197815 strains obtained from our study. This result is similar to previous studies in our country (Ankara- KX268119/TR/15/Ank/2a, Şanlıurfa- MG780288/TR/URF/CPV2a/PE100). In addition, MZ197816 obtained from our study was transformed into *Methionine* (*Met*) very different from the others. While the 341st amino acid was *Proline* (*Pro*) in CPV-2a and CPV-2c strains, it changed to *Ser* in CPV-2b vaccine strains and MZ197819, MZ197821, MZ197823, MZ197824, MZ197826 strains obtained from our study. This change was also detected in previous studies in our country (Ankara- MT952859/TR/HSS33, Burdur-MZ545670/TR-BURDURCPV/2bSH35). The same amino acid was changed to *Ala* in MZ197820, MZ197822 strains, and *Threonine* (*Thr*) in strains detected in previous studies in Türkiye (Şanlıurfa, Elazığ) and MZ197825 strain obtained from our study. These changes in amino acid 341 are thought to be effective in the emergence of different

Table 3. CPV-2 genotypes and provinces previously determined in Türkiye.

Accesion Number	City	Genotype (CPV-2)	Genbank Date	References
KF500505	Ankara	a,b	23.05.2016	[29]
KX268119	Ankara	a,b,c	11.09.2017	Unpublished
MG545540	Van	a,b	06.03.2018	[30]
MG780292	Şanlıurfa	a,b,c	22.09.2018	[17]
MK248878	Ankara	b	24.06.2019	[33]
MZ545670	Burdur	b	18.08.2021	[35]
MT952859	Aydın	-	22.09.2021	Unpublished
MW685570	Konya	a,b	21.06.2022	[31]
OM747859	Elazığ	b	12.07.2022	[34]
MZ197827	Balıkesir	a,b	17.09.2022	This study
MZ391101	Aydın	a,b	04.10.2022	Unpublished

variants of CPV-2b. This amino acid change could not be detected in our strains. Mutations in the 426th amino acid of the VP2 protein cause important differences in the antigenic structure of the virus, and CPV-2a, CPV-2b and CPV-2c (Asn (N) in FPV, CPV2 and CPV-2a), *Asn* in CPV-2b *Aps* and *Glu* in CPV-2c [26]. The 426th amino acid in CPV-2b strains in this study (MZ197819, MZ197820, MZ197821, MZ197822, MZ197823, MZ197824, MZ197825, MZ197826, MZ197827) was determined as *Asp*. The 440th aa *Thr* →*Ala* (*Thr* 440 *Ala*) change, which has been reported [28,39] in many CPV2a/2b/2c variants and has a role in the antigenicity of the virus, was also detected in our CPV-2b strains.

When molecular characterization studies conducted in our country and all over the world are evaluated, it is seen that CPV-2 strains circulating in the field show similarity at different rates with *Feline Panleukopenia virus* (FPLV) strains obtained from cats [42,45]. Infections caused by each of the different variants of the virus progress with similar clinical findings in carnivores and often coinfect the host [46]. Studies have shown that the canine and feline host ranges of CPV and FPV are predominantly formed by amino acid residues in the viral capsid. It has been reported that mutations in CPV-2 that affect the ability to infect and reproduce in cats are related to amino acid changes 87, 300 and 305 in the VP2 gene [47]. In the study, while *Asp* in the 305th amino acid reference strain was *Tyr* in all other compared strains and vaccine strains, it changed as *Histidine* (*His*) in MZ197813, unlike all strains. This suggests that the strain may be of feline origin. In this context, it should not be forgotten that cats and dogs can be carriers and sources of infection for each other.

The enteric form of CPV-2 infection is common and is characterized by catarrhal, hemorrhagic, and fibrinous enteritis [48–51]. In all cases, macroscopic findings were evident, subserosal hemorrhage or congestion was detected in the affected segments of the intestine, and the intestinal lumen was empty, and watery undigested contents were present. In this study, it was determined that the intestinal wall was excessively thickened, the ileum and caecum parts were dark red and Peyer's patches became visible from the serosa in the necropsies of the dogs. Segmental or dense serosal hemorrhages extending to the submucosa in the intestine were observed. The intestinal lumen had a dark red, bloody watery content and it was determined that the hemorrhage foci were covered with dull white-yellow fibrin.

In studies using immunohistochemical techniques, it has been reported that viral antigens are seen especially in epithelial, endothelial, and lymphoid cells with high mitotic activity [52,53]. Pathological lesions defined as villous atrophy in the small intestines, necrosis of epithelial cells in the region extending from the villi to the crypts, necrotic cells in the crypt lumens, and dense mononuclear cell infiltration located in the lamina propria are common in cases [48,50–52]. In the current study, significant dense mononuclear cell infiltration in the lamina propria was noted in all cases while neutrophil leukocytes were found to accompany them in 7 cases.

One of the important pathological lesions observed in parvovirus-infected dogs is the depletion of Peyer's patches [54]. This finding indicates the initial replication of the virus in lymphoid tissues and then spread to the cryptic epithelium. In general, it was observed that this region where the middle part of Peyer's patches was emptied was filled with reticulum cells. In the examinations, it was determined that the crypt lumens were filled with necrotic epithelial cells in most of the cases. These findings are consistent with findings from previous studies [53,54].

Immunohistochemical examinations have a principal place in the detection of infection. In the immunohistochemical examinations, intracytoplasmic immunoreaction was remarked in macrophages and crypt epithelial cells infiltrating the crypts. The immunohistochemistry result correlates with [5]. Since pathological findings may not develop in all cases, immunohistochemical methods may be recommended in addition to virological methods for definitive diagnosis.

The positive immunoreaction seen in cardiomyocytes in 2 of the dead puppies (30 days old and 45 days old) included in the study samples revealed that myocarditis occurred in these puppies. Additionally, it was determined that the virus obtained from these puppies was the CPV-2b variant. Studies have reported that despite effective vaccination in many countries, the circulation of the virus in dog populations cannot be prevented [27,28]. However, since the vaccination information of the mothers of the puppies was not available in this study, the connection between the variants identified in the puppies and the vaccine cannot be evaluated.

5. Conclusion

Control programs are implemented all over the world against CPV-2 infection, which is considered one of the most important viral agents for dogs. Selection of appropriate vaccines is important in the control of infection. Although the causative agent is a DNA virus, the presence of amino acid mutations has been demonstrated in all studies conducted to date and in our study. These results show that the virus is constantly evolving and genetic variants are emerging. Many studies conducted all over the world have revealed that the current vaccines used do not provide adequate protection against different CPV variants. The reason for this is the immune deficiencies that occur with the differences between the field and vaccine strains [55,56]. In addition, when the findings determined in the dead puppies were evaluated, it may be recommended that mothers should be vaccinated before pregnancy, and that they should be vaccinated 3 doses together with at least 2 booster vaccines and circulating variants should be included in the vaccine.

In order to control CPV-2 infection, it is recommended to detect virus variants circulating in different regions and develop vaccines containing these variants.

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Conflict of interest

There is no conflict of interest between the authors.

Ethical statement

Ethics committee permissions for this study were obtained from Balikesir University Animal Experiments Local Ethics Committee, and the study was carried out within the scope of the permission of this committee, dated 28/11/2018 and numbered 2019/12-3.

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