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RESEARCH ARTICLE

Determination of Canine Parvovirus Variants in Puppies by Molecular and Phylogenetic Analysis

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Canine Parvovirus-2 (CPV-2) causes infectious viral disease characterized by severe diarrhea, vomiting, and high mortality, especially in young dogs. For routine laboratory diagnosis and molecular characterization of CPV-2, PCR analysis and phylogenetic analysis have been widely used methods in recent years. The aim of this study was to determine the molecular diagnosis and characterization of CPV-2 variants in puppies (0-6 months) with diarrhea symptoms and no vaccination against CPV-2 in the Central Anatolia region of Turkey. The phylogenetic analyses for VP2 gene were performed from the stools of the puppies (n = 50), which were found to be CPV-2 positive for the presence of nucleic acid by PCR, showing signs of gastroenteritis that were owned and cared for in the shelter environment. The results of the phylogenetic analysis, which included the study sequences of 4 each female and male puppies, indicated that both CPV-2a (4/8, 50%) and CPV-2b (4/8, 50%) variants were circulating in the study area. Molecular analyses also revealed that CPV-2 variants did not cause co-infection in these puppies. In conclusion, it can be stated that CPV-2a and CPV-2b variants continue to exist among the owned and stray dogs in the shelter in the Central Anatolia Region. Therefore, new vaccines should develop for successful fight against the infection and the vaccines should be prepared against CPV-2 virus antigenic types in different regions.

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INTRODUCTION

Canine parvovirus -2 (CPV-2) is one of the most important enteric viral agents with high mortality in carnivores. This highly contagious virus is frequently observed in shelters and pet shops where dogs live together (Miranda and Thompson, 2016). *Canine parvovirus* -2 is included in the *Parvoviridae* family, *Parvovirinae* subfamily *Protoparvovirus* genus. It is also classified in *Carnivore protoparvovirus 1* together with Feline panleukopenia virus (FPV) (Viscardi *et al.,* 2019). The CPV genome is 5,200 nucleotides long and contains two ORFs (open reading frame) (Qi *et al.,* 2020). The first ORF region encodes two non-structural proteins (NS1 and NS2), while the other ORF encodes three structural (VP1, VP2, and VP3) proteins (de Oliveira *et al.,* 2019). The VP2 gene is main capsid protein and main antigenic protein. It determines viral tissue tropism and host range (Liu *et al.,* 2021).

CPV-2 is spreading quite rapidly among dog populations worldwide. Although dogs of all ages are affected by this disease, it was reported that the younger dog population (6 weeks – 6 months) is more susceptible to CPV-2 (Miranda and Thompson, 2016). In 1970-1980, CPV-2 has adapted to dog populations as hosts. After the disease became widespread and worldwide, mutations in this virus created new antigenic variants called CPV-2a and CPV-2b. These antigenic variants were formed by amino acid substitution in the VP2 protein. Similarly, amino acid substitution 426. (Asp-426→Glu) in the VP2 capsid protein in the early 2000s revealed a new and more virulent antigenic variant (Buonavoglia *et al.,* 2001; de Oliveira *et al.,* 2019).

Recently, many studies were realized for the molecular detection and characterization of CPV-2 variants. Several studies demonstrated again the fact that puppies are at greater risk in terms of the harmful effects of CPV disease and reported novel variants of types CPV-2a and CPV-2b circulating in the canine population (de Oliveira *et al.,* 2019; Giraldo-Ramirez *et al.,* 2020; Hasib *et al.,* 2021; Singh *et al.,* 2021). Moreover, new mutations in the VP2 amino acid sequence (Y324I and T440A) have been reported due to antigenic drift in CPV-2 variants circulating worldwide (Zhou *et al.,* 2017).

In Turkey, CVP-2 cases previously have been diagnosed in dogs. Further, the VP2 protein-coding gene characterization has been performed and CPV-2a, CPV-2b and CPV-2c variants reported (Timurkan and Oğuzoğlu 2015; Karapınar *et al.,* 2018; Polat *et al.,* 2019; Akkutay-Yoldar and Koc 2020).

The aim of this study was to determine the molecular characterization of CPV-2 nucleic acid fragments detected by conventional PCR method in the stool of the dogs in Central Anatolia (Konya Province) of Turkey.

MATERIALS AND METHODS

Samples and Viruses: In this study, the stool samples of 50 puppies (0-6 months old) which had diarrhea symptoms and no vaccination against CPV-2 in Selcuk University Faculty of Veterinary Medicine Department of Internal Medicine Clinic (n=10) and Konya Metropolitan Municipality Stray Animal Nursing Home and Rehabilitation Center (n=40) were used. The CPV-2 were determined as positive by PCR in the stool samples (Nandi *et al.,* 2010).

Viral DNA extraction and PCR analysis: Viral DNA was extracted from the stool samples (n=50) by the QIAcube robotic extraction method (Qiagen, Germany, Hilden) with the QIAamp Cador Pathogen Mini Kit (Indical Bioscience GmbH, Germany) according to the manufacturer's instructions. All DNA samples were kept at −20 °C until use. CPV-2 was investigated in the stool samples using the conventional PCR reported by Pereira *et al*. (2000) and Buonavoglia *et al*. (2001) methods. The PCR assay was performed using Taq DNA Polymerase (recombinant) (Thermo Scientific, Lithuania). The primer pairs used in these analyses are listed in Table 1. The positive control was obtained from the Department of Virology, Faculty of Veterinary Medicine, the University of Selcuk for the molecular assay.

PCR assay was carried out in a 50 µl reaction volume, containing 5µl of 10xPCR Buffer PCR master mix, 10 mM dNTP mix, 4µl of MgCI2 (25 mM), 0.25 µl of Taq DNA polymerase (5U/µl), equal concentration (0.5 µM) of each primer, 24.75 µl of nuclease-free water, and 5 µl of template DNA. Cyclic conditions for the assay included one cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were analyzed in 1.5% agarose gel electrophoresis and visualized under UV transilluminator Gel Doc™ (BIO-RAD).

Nucleotide sequencing and phylogenetic analysis: Sequence analysis was performed on all positive samples (n=50) mentioned above. The PCR assay was carried out following the methods of Buonavoglia *et al*. (2001) and Pereira *et al*. (2000). The PCR products were purified using the High Pure PCR Product Purification Kit (Roche, Germany, Mannheim) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Scientific, USA, Waltham) according to the manufacturer's instructions in the 3500 Series Genetic Analyser (Applied Biosystem, Japan, Tokyo). In addition, the fragment sequences of the corresponding regions for the CPV-2 isolates were derived from GenBank (http://www.ncbi.nlm.nih.gov) for comparative analysis. Multiple sequence alignments were implemented using the MUSCLE (Edgar, 2004) algorithm included in AliView software. The sequence data were submitted to the GenBank databases under the following accession numbers: MW685563 - MW685570.

A phylogenetic tree was constructed with sequences from both fecal samples (n = 8) and CPV-2 related sequences and two feline vaccine strain sequences stored in GenBank. The phylogenetic analyses were conducted maximum likelihood (ML) method and Tamura-Nei model with MEGAX software, and evaluated by bootstrapping using 1000 replicates. In addition, the AliView software was used for nucleotide and amino acid alignment comparisons.

Ethical approval: All procedures were approved by Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2019/39 on 25/04/2019).

RESULTS

PCR Analysis: The puppies were aged between 0 and 6 months; 50% (4/8) were female and 50% (4/8) were male. Among the breeds of these dogs were Kangal 50% (4/8) and Golden Retriever 12.5% (1/8), while 37.5% (3/8) of the dogs were crossbred (Table 2).

Table 1: Primers used in this study.

Primers	Sequence (5´→3´)	Amplicon size (bp)	Target gene	Reference
Hforc	CAGGTGATGAATTTGCTACA			
Hrevc	CATTTGGATAAACTGGTGGT	629		
555forc	CAGGAAGATATCCAGAAGGA			
555revc	GGTGCTAGTTGATATGTAATAAACA	583		(Buonavoglia *et al*., 2001)
Pbsb	CTTTAACCTTCCTGTAACAG			
Pbasb	CATAGTTAAATTGGTTATCTAC	427	VP2	(Pereira *et al*., 2000)

Table 2: Information on the samples in which CPV-2 was detected by PCR.

GenBank Accession Number	Sample	Age	Gender	Breed	Sample Place	CPV Type
MW685563	9	1-3 month	♂	Kangal	Clinical	2a
MW685564	10	1-3 month	♀	Kangal	Clinical	2a
MW685565	12	3-6 month	♂	Kangal	Clinical	2a
MW685569	64	0-1 month	♀	Crossbreed	Shelter	2a
MW685566	13	1-3 month	♀	G. Retriever	Clinical	2b
MW685567	19	1-3 month	♂	Kangal	Clinical	2b
MW685568	33	1-3 month	♀	Crossbreed	Shelter	2b
MW685570	100	1-3 month	♂	Crossbreed	Shelter	2b

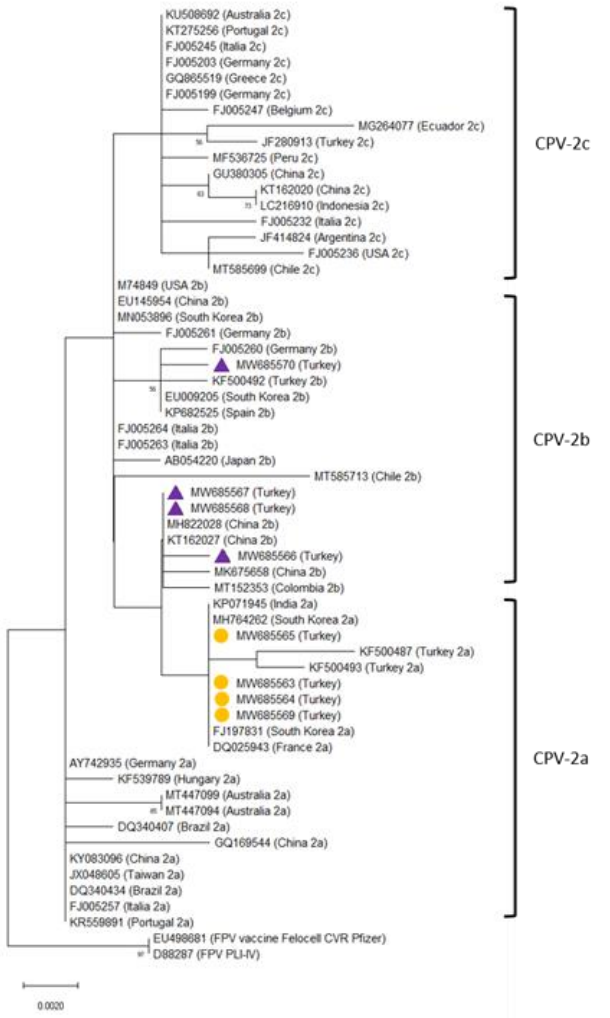

Fig. 1: Phylogenetic tree of VP2 gene nucleotide sequences of CPV-2 strains obtained from the GenBank database and Central Anatolia in Turkey. A phylogenetic tree including 60 partial sequences with 602 nucleotides was constructed by the MEGAX software using the ML method and Tamura-Nei model with a substitution model (TN93 + G), and a bootstrap value of 1,000 replicates. Branches marked with a triangle (▲) and circle (●) display the sequences obtained from the current study. The scale bar indicates 0.0020 nucleotide substitution per site.

Phylogenetic Analysis: Fifty fecal samples previously detected to be CPV-2 positive were tested by CPV-2 specific PCR for sequence analysis. Sequences in the PCR products were analyzed using the Jalview2 program and eight sequences were used in phylogenetic analyses. The phylogenetic analysis revealed variant CPV-2a and CPV-2b (Fig. 1).

Identifying changes in the VP2 gene: The sequence analysis disclosed amino acid changes between these sample sequences and reference sequences from GenBank at residues 423, 426, 440, and 562 (Fig. 2). Moreover, these amino acid changes revealed the characterization of CPV-2 variants in fecal samples relative to 426 residues of the amino acid sequence (Table 3).

The phylogeny was inferred from the VP2 protein-coding regions of CVP-2 revealing 602 nucleotides (Fig. 1). The bootstrap support values (>50%) were relatively high in the sequence distribution in the tree. The phylogenetic tree revealed that four of the Turkish variants fall into two separate groups within the reference CPV-2b sequences and the other Turkish variants within the reference CPV-2a sequences.

Data from sequence analysis demonstrated some changes in residues 423 (Pro→Ser), 426 (Asn→Asp), 440 (Thr→Ala), and 562 (Val→Glu) compared to the reference amino acid sequences (Fig. 2).

DISCUSSION

In this study, when the puppies with clinical signs of gastroenteritis were evaluated in terms of CPV-2 subtypes in 0-6 months old puppies that were not vaccinated against CPV and found in both owned and homeless animal shelters, the subtypes causing CPV-2 in Konya were CPV-2a (4, 50%) and CPV-2b (4, 50%) were determined in equal proportions. Many recent studies have revealed that different CPV-2 genotypes are common in distinct regions of the world (Decaro *et al.*, 2013; Miranda and Thompson 2016; Akkutay-Yoldar and Koc 2020). Although CPV-2a is common in Europe (Ntafis *et al.*, 2010; Filipov *et al.*, 2011; Decaro *et al.*, 2013; Filipov *et al.*, 2016) and China (Yi *et al.*, 2016), CPV -2b was more common in the USA (Hong *et al.*, 2007) and Brazil (Pinto *et al.*, 2012). However, novel variants of the CPV-2a and CPV-2b types circulating globally in the canine population continue to be reported (de Oliveira *et al.*, 2019; Akkutay-Yoldar and Koc 2020; Giraldo-Ramirez *et al.*, 2020).

The studies between 2000 and 2010 showed that the dominant variant circulating in Turkey is CPV-2a (Yilmaz *et al.*, 2005; Timurkan and Oğuzoğlu 2015). However, in later studies conducted (Karapınar *et al.*, 2018; Akkutay-Yoldar and Koc 2020), it was reported that the active variant was CPV-2b. Also, after detection of the CPV-2c variant in blood samples from cats (Muz *et al.*, 2012), the presence of CPV-2c was reported for the first time in the dog population of the Southeastern Anatolia Region of Turkey (Polat *et al.*, 2019). As a result of the current study, CPV-2a and CPV-2b were determined equally. This result was similar to the results reported in Turkey so far and revealed that both CPV-2a and CPV-2b, which are subtypes of CPV circulating in Turkey, continue to be seen as effective.

Phylogenetic trees are a frequently used current method for analysing and genotyping viruses with a phylogenetic reference (Abbass *et al.*, 2021). Thus, important data are obtained about the development dynamics of field isolates. Moreover, these data are very important for developing protection and control programs against a possible pandemic threat. According to the phylogenetic tree, it was determined that both genotypes of CPV-2 were present in our field strains (Fig. 1). The phylogenetic clustering that separated these 2 genotypes suggested that the CPV-2 isolates circulating in the field may belong to different genetic groups that evolved from a common ancestor as a heterogeneous group. The phylogenetic analysis revealed that the Turkish CPV-2a strains (MW685563, MW685564, MW685565, and MW685569) clustered together with Turkey, South Korea, India, and France isolates. Moreover, Turkish CPV-2b variants fall into two separate groups within the reference CPV-2b sequences. According to the phylogenetic

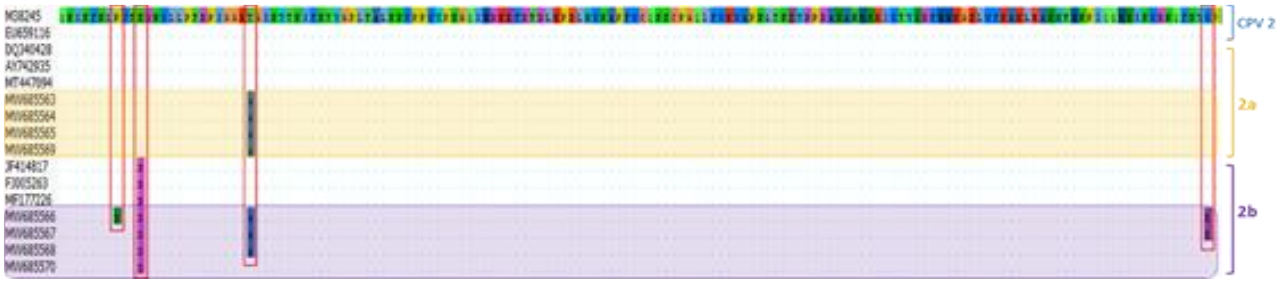

Fig. 2: Multiple alignments to compare the amino acid changes that occur in the VP2 gene of CPV in between 8 representative strains and the sequences in the GenBank database. Dots indicate amino acids identical to the reference CPV sequence. Red boxes demonstrate amino acid changes. The sequences obtained in this study were marked with orange and purple. 4053 C/T, 4062 A/G, 4104 A/G, 4471 T/A) *Reference: M38245: CPV-2 whole genome (Parrish, 1991).

Table 3: Comparison of working nucleotide sequences with reference sequences

GenBank Accession number	3586	3617	3675	3685	3699	3713	4038	4062	4104	4471	4496
M38245*	T	T	T	C	G	A	A	A	A	T	A
EU659116*	T	T	T	C	G	A	A	A	A	T	A
MW685563/2a	T	T	*G*	*G*	*T*	A	A	A	*G*	T	*G*
MW685564/2a	T	T	*G*	*G*	*T*	A	A	A	*G*	T	*G*
MW685565/2a	T	T	*G*	*G*	*T*	A	A	A	*G*	T	*G*
MW685566/2b	*A*	*C*	*G*	*G*	*T*	A	A	*G*	*G*	*A*	*G*
MW685567/2b	T	T	*G*	*G*	*T*	A	A	*G*	*G*	*A*	*G*
MW685568/2b	T	T	*G*	*G*	*T*	A	A	*G*	*G*	T	*G*
MW685569/2a	T	T	*G*	*G*	*T*	A	A	A	*G*	T	*G*
MW685570/2b	T	T	*G*	*G*	*T*	*G*	A	*G*	A	T	*G*

*CPV-2 whole genome references: M38245: CPV-2 (Parrish, 1991), EU659116: (Hoelzer *et al.*, 2008).

tree, while the MW685566, MW685567, and MW685568 strains were more similar to China and Colombia isolates, the MW685570 strain was closely related to Turkey, Germany, South Korea, and Spain isolates (Fig. 1). This result was similar to what has been reported until now from Turkey and revealed that both CPV-2a and CPV-2b, which are subtypes of CPV circulating in Turkey, continue to be seen as effective.

Nucleotide substitutions within the viral genome can result in the emergence of variants and developmental diversity of antigenic characters across host range tropisms. When the nucleotide sequences of the reference genomes and the samples of the current study were evaluated, it was determined that there were changes in certain regions between all the reference genomes and the samples of this study (Table 3). 3675 (T-G), 3685 (C-G), 3699 (G-T), 4062(A-G), and 4104 (A-G) (Fig. 2). The changes in nucleotides were found to be similar to the nucleotide changes reported in a study conducted in Turkey (Timurkan and Oğuzoğlu 2015). Also in the study reported by Wang *et al*. (2016), when the nucleotide sequences of the new CPV-2a and new CPV-2b isolates and the samples of the current study were evaluated, it was determined that the changes in the 4062. nucleotide (426.aa) were the same and gave the same results in the differentiation of CPV-2a and CPV-2b.

Certain amino acid changes on VP2 play an important role in typing (Steinel *et al.*, 2001; Decaro and Buonavoglia, 2012). In particular, the emergence of remarkable differences in antigenic structure at amino acid 426 and allows for distinction between CPV-2a, CPV-2b, and CPV-2c. 426. Aa is ASN (N) in FPV, CPV-2 and CPV-2a, ASP (D) in CPV-2b, and GLU (E) in CPV-2c (Steinel *et al.*, 2001; Decaro and Buonavoglia, 2012). When the data obtained in the current study and the nucleotide changes of the selected reference sequences are examined (Table 3), in the MV685563, MV685564 MV685565, and MV685569 amino acid sequence, which was determined as CPV -2a in the phylogenetic tree, 426.aa were determined as ASN (N), while MV685566, MV685567, MV685568 and MV685570 4 426.aa were found to be ASP (D), thus confirming the typing results determined by the phylogenetic tree (Table 3).

The puppies (0-6 months old) sampled in this study were mostly in the 1-3-month age group (Table 2). Studies of canine parvovirus infection have reported that age has a significant effect on the disease, with a higher incidence in puppies under 6 months old that are not given colostrum (Aktas *et al.*, 2011; Sakulwira *et al.*, 2003). It recently has been emphasized again that puppies are at serious risk for the harmful effects of CPV disease (Singh *et al.*, 2021). The study results revealed the detrimental effects of CPV disease in puppies and supported the previous results reported by Singh *et al.* (2021). Moreover, the presence of both types of CPV was detected in owned puppies in this study (Table 2), which is consistent with has been reported before study in Turkey (Aktas *et al.*, 2011). Some reports suggest that female or male dogs are at higher risk for CPV infection (Sakulwira *et al.*, 2003; Aktas *et al.*, 2011). In the present work, it used a sample size that limits determining the true association between gender and CPV status. Therefore, we could not evaluate the relationships between CPV infection and gender. In addition, colostrum should be given to newborns and the vaccination schedule should be carefully followed to protect or fight against this disease in the dog population globally (Hasan *et al.*, 2016; Hasib *et al.*, 2021).

In conclusion, despite vaccination applications against CPV-2 infections, which are considered to be one of the most important viral agents for all dogs, it is observed very intensely today. It is thought that this situation may develop since the subtypes of CPV-2 infections are not fully known locally and that

vaccinations are not performed for these subtypes. In addition, reported studies at different times both in Turkey and in the world reveal that CPV is in constant change and therefore current vaccination strategies are insufficient. It was concluded that the most important step in the fight against this infection is to make the correct and rapid diagnosis together with the correct vaccinations and to ensure that the newborns receive colostrum in a sufficient time. Hence, regional phylogenetic trees covering wider regions should be constructed and regular typing studies and appropriate vaccination studies should be carried out periodically. Besides, it is thought that data about the circulation of the virus in natural life and among animals can be provided if samples can be made from wild dogs.

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Authors contribution: Motivation/Concept: ID, AS. Design: ID, OA, MEO. Control/Supervision: AS, OA. Data Collection and / or Processing: ID. Analysis and / or Interpretation: ID, MEO. Writing the Article: ID Critical Review: ID, AS, OA.

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