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Molecular Characterization and Phylogenetic Analysis of Canine Parvovirus 2 in Dogs, Mersin Province, Turkey

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Abstract: Canine parvovirus type 2 (CPV-2) is a highly contagious and lethal viral disease with acute gastroenteritis in young dogs. After it first appeared in USA in 1978, continuous evolution of virus genome has arisen new genetic and antigenic variants such as CPV-2a, CPV-2b, new CPV-2a, new CPV-2b and CPV-2c throughout the world. The aim of the present study was to detect CPV-2 genetic variants from blood samples of clinically ill dogs and to characterize CPV-2 genetic variants circulating in Mersin province (Turkey), by partial sequence analysis of VP2 gene. A total of 20 blood samples were collected from dogs with gastroenteritis from local veterinary clinics between April 2017 to September 2017. Out of 20, 7 samples were positive for CPV-2 genomic DNA using conventional PCR. Amino acid comparisons showed that Ile-324 mutation was detected in the VP2 of clinical CPV-2b samples, as differ previously studies Turkish genetic variants. A phylogenetic tree constructed based on VP2 gene sequences indicated that CPV-2b sequences from Mersin province were related to Indian genetic variants. Moreover, CPV-2b sequences from Mersin have clustered separately from other Turkish CPV-2b genetic variants previously reported. The results show that CPV-2b genetic variant is circulating among in dogs in Mersin province.

Key words: Canine parvovirus, dog, phylogenetic analysis, VP2 gene.

Türkiye'de Mersin İlinde Köpeklerde Canine Parvovirus 2'nin Moleküler Karakterizasyonu ve Filogenetik Analizi

Özet: Canine parvovirus 2 (CPV-2) genç köpeklerde akut kanlı gastroenteritis ile seyreden oldukça bulaşıcı ve ölümcül bir viral hastalıktır. 1978'de ABD'de ilk ortaya çıktıktan sonra, virus genomunun sürekli evrimi, tüm dünyada CPV-2a, CPV-2b, new CPV-2a, new CPV-2b ve CPV-2c gibi yeni genetik ve antijenik varyantların ortaya çıkmasına yol açmıştır. Bu çalışmanın amacı, Mersin (Türkiye) ilinde klinik olarak hasta köpeklerin kan örneklerinde CPV -2'yi tespit etmek ve dolaşımda olan CPV- 2 genetik varyatlarının karakterizasyonunu sağlamaktır. Bunun için 2017 Nisan ve 2017 Eylül ayları arasında, yerel kliniklerden 20 adet kan örneği gastroenteritisli köpeklerden toplandı. 20 örneğin 7'si PZR ile CPV- 2 yönünden pozitif olarak test edildi. Klinik CPV-2b örneklerinin VP2'sindeki amino asit karşılaştırmaları sonucunda daha önce çalışılan Türk soylarından farklı olarak Ile-324 mutasyonu tespit edildi. VP2 geni dizinlerinden köken alarak yapılan filogenetik ağaç, Mersin ilinden gelen CPV-2b dizilerinin Hindistan genetik varyantlarıyla ilişkili olduğunu göstermiştir. Aynı zamanda Mersin ilinden elde edilen CPV-2b dizinleri daha önce tespit edilen Türk genetik varyantlarından farklı bir dal şekillendirmiştir. Bu sonuçlar Mersin ilinde CPV-2b genetik varyantının köpekler arasında dolaştığını göstermiştir.

Anahtar kelimeler: Canine parvovirus, köpek, filogenetik analiz, VP2 gen.

Introduction

Canine parvovirus 2 (CPV-2) is small, non-enveloped, single-stranted DNA virus, belonging to genus *Parvovirus*, the family *Parvoviridea* with genome 0f 5, 2 Kb in lenght. CPV-2 has two open reading frames (ORFs). One of them encodes non-structural proteins (NS1, NS2) and the other encodes structural proteins (VP1, VP2). Amino acid changes in the VP2 protein, main viral protein of capsid, alter the antigenic properties of the virus in determining the viral host distribution and tissue tropism [6, 13]. After the emergence of the CPV-2 in 1978, two new genetic variants (CPV-2a and CPV-2b) were characterized in 1979 and 1984, respectively. Monoclonal antibodies can distinguish as CPV-2a (426Asn) and CPV-2b (426Asp) by amino acid substitution in the capsid protein gene. In 2000, CPV-2c (Asp-426 to Glu) is a new genetic variant was detected in Italy [6, 11]. These three genetic variants occur in dogs throughout the world; CPV-2a was found in Nigeria, South Africa, Turkey, Greece and other European countries [14]. CPV-2b was reported from USA, Turkey and China [6, 23]. Morever,

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CPV-2c has been reported in Italy, Spain, Germany, the United Kingdom, the United States, Australia, Portugal, Vietnam and some other European countries [1,5,7,10,12,13,19].

CPV-2 is a highly contagious disease that causes acute hemorrhagic enteritis and myocarditis in dogs with high fatality rate ranges from %24 to %43 [26, 27]. The virus is usually transmitted through direct contact from dog to dog or acquired indirectly environment contaminated with feces [27]. Although CPV-2 is common in all dogs, puppies between 6 weeks and 6 months are more susceptible to disease. The incubation period of disease is between 3 and 7 days and main characteristics of the disease are depression, loss of appetite, fever, vomiting, hemorrhagic diarrhea and dehydration. Vaccination is main method of protection from disease. Commercial vaccines are available for CPVs genetic variants worldwide and are able to protect against 4 genetic variants; CPV, CPV - 2a, CPV-2b and CPV- 2c respectively [13]. CPV vaccines (Vanguard, Pfizer; Nobivac, Intervet; Parvodog, Merial; Quantum, Schering) used for dogs include 2a and 2b genetic variants in Turkey. However, antigenic changes occurring in the VP2 protein of CPV reduce efficacy of vaccine in dogs [23]. Mutations such as 87, 101 297, 300,305, 323, 324, 426, 440, 555 occurring of VP2 protein of CPV result in new genetic variant and pathogenic features [21, 25]. Carmichael et al.,[3] reported that CPV -2 variants were detected higher titres than original virus in faeces of naturally infected dogs.

The aim of the present study was to detect CPV from blood samples of dogs and to characterize CPV genetic variants circulating in Mersin province (Turkey), by partial sequence analysis of VP2 gene.

Materials and Methods

1. Sample collection

Blood samples (n=20) were obtained from local veterinary clinics in Mersin province between April and September 2017. Anticoagulated blood samples were centrifuged at 3000 rpm for 7 minutes and the buffy coat was collected and stored at -20°C until use. Individual information such as age, gender, breed, clinical symptoms and vaccination history about the animals involved the study is given in Table 1.

Table 1. Year, sample type, Age , gender, vaccination status, clinical sings of dogs infected and non - infected with CPV - 2b (F: Famale, M: Male, NV: Non - vaccinated).

Year	Sample type	Age(month)	Sex	Vaccination status	Clinical symptoms	Type	Breed
2017	Blood 1	1,5ay	M	NV	Depression, vomiting	-	Mix
2017	Blood 2	2ay	F	NV	Diarrhea	CPV -2b	Mix
2017	Blood 3	2ay	F	NV	Anorexia,anemia depression	-	golden retriever
2017	Blood 4	3ay	M	NV	Anorexia, vomiting	-	german shepherd
2017	Blood 5	1,5	F	NV	diarrhea	CPV -2b	german shepherd
2017	Blood 6	5ay	M	NV	Anorexia, Diarrhea	-	Mix
2017	Blood 7	2ay	M	NV	Diarrhea	-	Mix
2017	Blood 8	3ay	M	NV	Diarrhea	-	rottweiler
2017	Blood 9	1,5	F	NV	Diarrhea	-	pointer
2017	Blood 10	3ay	F	NV	Anorexia, vomiting	-	pointer
2017	Blood 11	2ay	F	NV	Anorexia, vomiting	CPV -2b	Mix
2017	Blood 12	1,5	M	NV	Depression, vomiting	CPV -2b	Mix
2017	Blood 13	2ay	M	NV	Diarrhea	CPV -2b	golden retriever
2017	Blood 14	1,5	M	NV	Diarrhea	-	golden retriever
2017	Blood 15	2ay	F	NV	Vomiting, diarrhea	-	Mix
2017	Blood 16	2ay	M	NV	Anorexia, depression	CPV -2b	Mix
2017	Blood 17	3ay	M	NV	Diarrhea	-	Mix
2017	Blood 18	1,5ay	M	NV	Vomiting	CPV -2b	golden retriever
2017	Blood 19	2ay	F	NV	Vomiting	-	rottweiler
2017	Blood 20	5ay	M	NV	Anorexia, Diarrhea	-	rottweiler

2. Template DNA Preparation and PCR

Nucleic acids were extracted from 200µL blood samples using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannhaeim, Germany) following the manufacturer's recommendations. Purified DNAs were stored at -20°C until PCR. The PCR targeting partial VP2 gene (629bp) was performed as described elsewhere [2]. Amplified PCR products were visualized under ultraviolet light via ethidium bromide staining after electrophoresis using in %1 agarose gel in TAE (Figure 1). Consequently, 100µL amplicon from each sample was purified using a commercial kit (GeneJET, Thermo Scientific, Lithuania) according to manufacturer's instructions. Purified DNA was used for DNA sequencing and subsequent phylogenetic analysis.

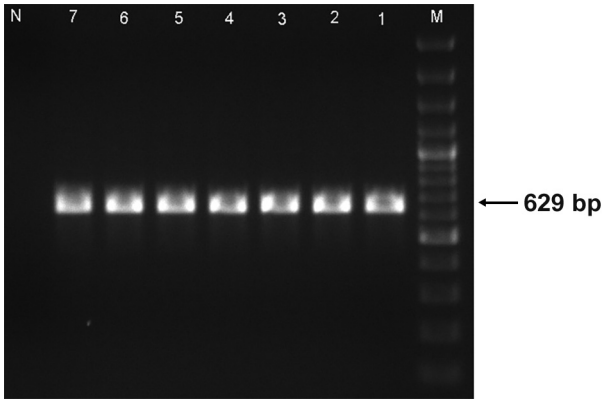

Figure 1. Agarose gel image of the PCR products (629 bp) of Canine parvovirus DNA using by Hfor and Hrev primers. Lane M: 100bp DNA ladder (Thermoscientific, USA); Lane: 1 - 7 positive samples; Lane 8: Negative control.

3. DNA sequencing and phylogenetic analysis

Sequencing was performed in ABI PRISM 310 Genetic Analyzer (Applied Biosystem, CA) using same primers that were used in the PCR. The obtained DNA sequences were compared to other CPV sequences available from GenBank Database (http://www.ncbi.nlm.nih.gov). Alignment and pairwise comparisons were carried out in CLC Main Workbench v7.7 (CLCBio, Aarhus, Denmark). Phylogenetic tree was drawn in MEGA software v.6.06 using neighbor-joining (NJ) method and bootstrap values were calculated with 1,000 replicates [19].

Results and Discussion

Twenty dogs with clinically defined gastroenteritis (especially vomiting and diarrhea) signs were tested with conventional PCR assay for the presence of CPV-2. Seven (35%) of the dogs were CPV-2 positive. Samples from negative dogs were not re-investigated for the other etiological causes of gantroenteritis. CPV- 2 positive dogs were between 1, 5 and 5 months old. Four of the positive dogs were male and three were female. According to the information received from the pet clinics, no vaccination was administrated to dogs. Breeds of dogs involved the study were recorded as Rottweiler, Golden Retriever, Pointer, German Shepherd and mix breed (Table 1).

Conventional PCR was carried out using Hfor and Hrev primers. Sequence analysis of PCR amplicons were performed with same primer sets. The obtained sequence results were compared to gene bank reference sequences (Figure 2). Phylogenetic (NJ) analysis revealed that all CPV-2 detected in the study were found to be CPV-2b. Other genetic variants (CPV-2a and CPV-2c) were not identified in the study. In order to investigate the variations occurring on VP2 gene, reference strain (GenBank Accession # M38245) was compared to amino acid residues obtained from clinical samples (Table 2).

Table 2. Amino acid changes in VP2 partial gene of CPV - 2b type

Aa no.	297	300	305	324	336	375	426	440
M38245	S	A	D	Y	A	N	N	T
P5	A	G	Y	I	G	D	D	A
P12	A	G	Y	I	G	D	D	A
P2	A	G	Y	I	G	D	D	A
P13	A	G	Y	I	G	D	D	A
P18	A	G	Y	I	G	D	D	A
P11	A	G	Y	I	G	D	D	A
P16	A	G	Y	I	G	D	D	A
Aa change	TCT→GCT S→A*	GCT→GGT A→G*	GAT→TAT D→Y*	TAT→ATT Y→I*	GTA→GTG V→V	AAT→ GAT N→D*	ATT→GAT N→D*	ACA→GCA T→A*

Amino acid substitutions are indicated by asterisks (*).

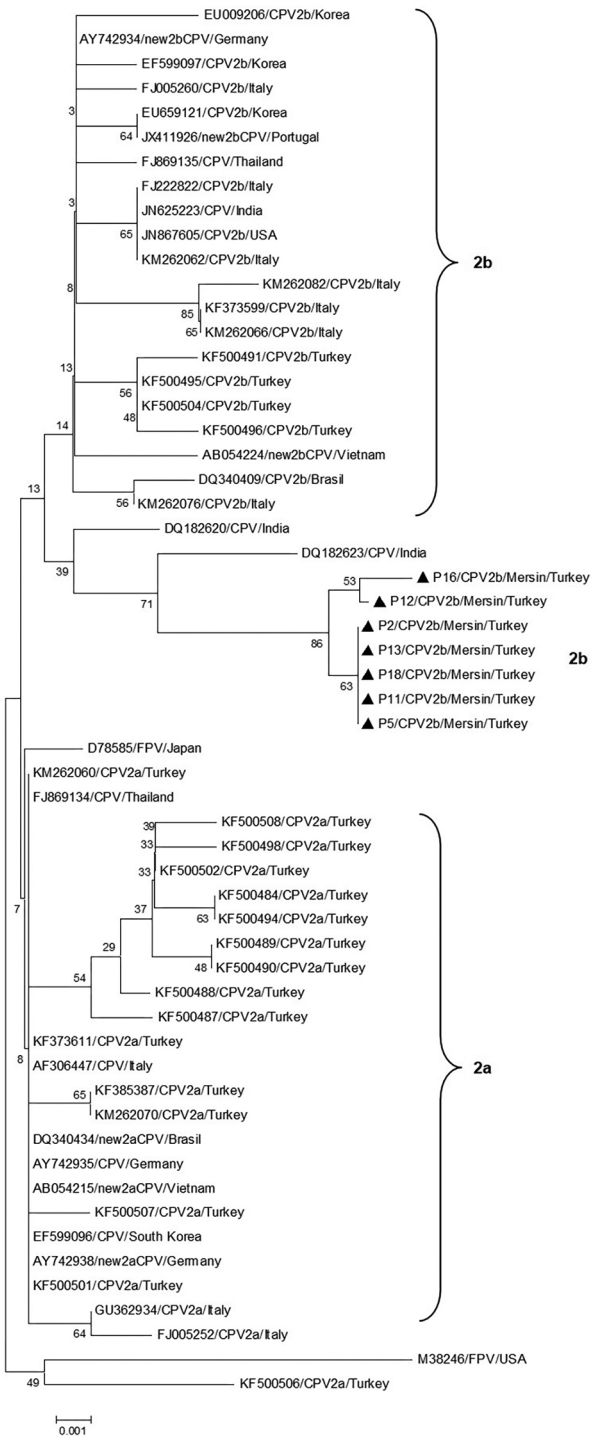

Figure 2. Neighbor joining tree (Mega 6) was constructed using canine parvovirus partial VP2 gene sequences under study (consensus sequences are indicated *filled triangle* in this study) and reference sequences.

Amino acid analysis revealed that all positive specimens were had a substitution N426D, which is characteristic of CPV-2b variant (A→G transversion). Nucleotide variation at position 948 resulted in A→G (synonymous mutation) codon change from GTA→ GTG, they both encode the same amino acid (V→V). In this study, substitutions were detected in S297A (nt3675), A300G (nt3685), D305Y (nt3699), Y324I (nt3757), N375D (nt3912) and T440A (nt4104) (Table 2). The residue T440A, which is known to be main antigenic site of the virus, locates at the three-fold spike on VP2 protein [6]. Synonymous and non-synonymous mutations have been described previous studies [6, 11, 17, 24, 26]. However, mutation in residue Y324I was the first reported in Turkey genetic variants (Table 2). The Y324I mutation has been reported in CPV genetic variants in China, India, Korea and Japan [10, 16, 18, 22]. It was reported that residue 324 shows strong positive selection all carnivore parvovirus. [8, 9]. It is adjacent to residue 323, which is responsible for host range like previously reported [6, 13, 23] .and residue 93, which determines the host distribution with binding canine transferrin receptor (TrR) [16]. As a result, residue 324 mutation may have a role on CPV host range together with residue 323 and 93. Although the function of the residue 324 is not fully known, Lin et al.,[9] reported that Ile324 mutation resulted in viral shedding up to 63 days in dogs. These mutations can lead to changes in the VP2 protein of CPV and cause new genetic variants. Moreover, the emergence of new genetic variants reduces the efficacy of vaccines used in the field [15].

Phylogenetic tree indicated that all CPV-2b genetic variants in Mersin province were close-related and allocated in a separate branch (Figure 2). Comparison of CPV-2b VP2 sequences obtained from clinical samples were 99% identical to each other and CPV-2b sequences detected in this study were associated with 98% identical to Indian sequences.

CPV-2 is highly infectious and deadly viral disease of dogs seen in many countries around the world. Study performed in Greece showed that all three CPV-2 genetic variants (CPV-2a, 2b and 2c) are circulating in dogs [11]. In one of the most recent investigation Dei Giudici et al., [6] performed in dogs and cats, all variants CPV- 2a/2b/2c have been shown to be in circulation by conventional PCR. Another study carried out in Lithuania re-

vealed CPV-2a and 2b genetic variants are present in dogs [26]. Moreover, CPV-2a has been reported in rectal swaps obtained from dogs in India [17]. In Turkey, there is limited number of studies on CPV genetic variants in dogs [21, 22, 23]. Timurkan et al., [20] carried out the first molecular characterization of CPV-2a and CPV-2b genetic variants in Ankara province. Results of the study showed presence of CPV-2b genetic variant in dogs in Mersin province. All of these investigations show that the CPV-2a and CPV-2b are predominant strains in dog population in Turkey and CPV-2c variant has not yet been identified in Turkey so far. Further molecular and epidemiological studies are needed to determine on CPV infections and to characterize circulating types in dogs.

In conclusion, the presence of amino acid mutations suggests that the virus has evolved continuously. Although parvovirus is a DNA virus, it has high genetic substitution rate, which causes emerging new genetic variants as well as RNA viruses [5]. Differences between field and vaccine strains are the most important reason for some immune failures [17]. Periodic epidemiological survey and molecular studies can be identified new CPV-2 genetic variants and mutations. In addition, comparison of strains obtained from different geographical areas can be provided the development of used vaccines and CPV 2 infections can be effectively controlled in the country.

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