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The Investigation and Phylogenetic Analysis of Canine Parvovirus 2 Infection from Blood and Rectal Swab Samples from Dogs in Van Province, Turkey

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ABSTRACT In this study, it was aimed to determine the presence of CPV infection in the blood and rectal swaps of suspected dogs that they were handed to Yuzuncu Yıl University, Faculty of Veterinary, Department of Internal Disease using PCR techniques and molecular characterization of local viruses. 57 (75%) of the 76 rectal swabs used in the study and 40 (64%) of the 62 blood samples were determined to be positive by PCR obtained from dogs with clinical symptoms. Selected samples were positive with the virus carried the molecular characterization. The results of the sequence analysis were similar among themselves and they were found in CPV-2a and CPV-2b groups. It was determined that the sequences of virus strains showed 99-100% homology with other strains obtained from Genbank.

Keywords: *CPV, PCR, Molecular characterization, Phylogenetic analysis*

ÖZ

Türkiyede Van Yöresinde Bulunan Köpeklerden Sağlanan Kan ve Rektal Sıvap Örneklerinde Canine Parvovirus-2 Enfeksiyonunun Araştırılması ve Filogenetik Analizi

Bu çalışmada, Van ili Yüzüncü Yıl Üniversitesi Veteriner Fakültesi İç Hastalıkları Kliniğine getirilen Canine Parvovirus (CPV) Enfeksiyonu şüpheli köpeklerden alınan kan ve rektal sıvap örneklerinde PZR tekniği kullanılarak CPV enfeksiyonunun varlığının belirlenmesi ve yerel virusların moleküler karakterizasyonunun yapılması amaçlandı. Klinik olarak enfeksiyon belirtisi gösteren köpeklerden sağlanan 76 adet rektal sıvaptan 57 adedi (%75), 62 adet kan örneğinin ise 40 adedi (%64) PZR ile pozitif olarak belirlendi. Seçilen pozitif örnekler ile virusun moleküler karakterizasyonu gerçekleştirildi. Elde edilen sekans sonucu bilgileri değerlendirildiğinde örneklerin CPV-2a ve CPV-2b grublarında bulunduğu ve kendi aralarında benzerlik gösterdikleri belirlendi. Elde edilen virus suşu sekanslarının Genbankasından sağlanan diğer suşlarla %99-100 oranında homoloji gösterdiği belirlendi.

Anahtar Kelimeler: *CPV, PZR, Moleküler karakterizasyon, Filogenetik analiz*

INTRODUCTION

Parvovirus infection of dogs which is common all around the world is a disease characterized by hemorrhagic enteritis and myocarditis and caused by canine parvovirus type-2 (CPV-2) which is a non-enveloped, single stranded DNA virus It was distinguished from minute virus of canines (MVC) or CPV-1 that had been previously identified as parvovirus and had no antigenic relationship with CPV-2 (Buonavoglia et al. 2000). CPV-2 emerging on late 1970s (Kelly 1978; Appel et al. 1979) was identified to evolve into antigenic types being called as CPV-2a and CPV-2b and having the same pathogenesis (Parrish et al. 1985). Monoclonal antibodies are divided into CPV-2a (Asn) and CPV-2b (Asp) based on replacement of amino acid in Capsid protein gene (Dei Giudici et al. 2017). CPV-2c strain which was first identified in 2000 in Italy has been circulated as a mutant strain all over the world (Buonavoglia et al. 2001; Nakamura et al. 2004; Pérez et al. 2007; Ntafis et al. 2010). Antigenic variants are originated from CPV-2 and emerge with the variation of a few amino acids found in VP2 protein (Parrish et al. 1991; Martella et al. 2006).

Although symptoms of CPV infection vary in animals based on age, this infection causes watery, smelly, and sometimes bloody diarrhea, vomiting, exhaustion, depression, and anorexia in dogs with disease. Its myocardial form which is characterized by nonsuppurative myocarditis in newborn puppies becomes prominent and results in sudden deaths (Kelly 1978; Carmichael 1994). CPV-2 being substantially

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contagious among the dogs at all ages influences animals with prognosis of acute, fibrinous necrotic or hemorrhagic gastroenteritis and especially those without maternal antibody or vaccination (Carmichael 1994).

The aim of the study was to perform PCR detection of parvovirus circulating in dogs in the region of Van and its molecular characterization.

MATERIALS and METHODS

Samples

The samples used in the study were obtained from unvaccinated dogs which were brought to Yüzüncü Yıl University Veterinary Faculty Department of Internal Diseases for treatment. Animals used as sample generally had clinical findings such as fever, anorexia, depression, gastrointestinal problems, vomiting, and diarrhea. Even though breeds of dogs were not certain, mixed ages of breeds varied between 2 and 6 months. While blood samples were taken into 10 mL of tubes with anticoagulant from 62 dogs, rectal swab samples were taken into sterile tubes containing 2ml transport medium-DMEM (1% antibiotic and 2% fetal calf serum) from 76 dogs. Blood samples taken into tubes with anticoagulant were centrifuged in a cooling centrifuge at +4°C, at 8000xg for 10 minutes. Following centrifugation, leucocyte layer in the middle was transferred into sterile tubes containing 2 ml PBS with the help of a Pasteur pipette and was centrifuged again in the cooling centrifuge at +4°C, at 1000 rpm for 10 minutes, and it was taken into 2.5 ml of stock tubes by adding 10% DMSO after this process was repeated 2 times in total. After vortexing rectal swab samples taken into laboratory using the cold chain, the swab stick in the tube was removed and swab samples were centrifuged in the cooling centrifuge at +4°C at 6000xg for 10 minutes. Supernatant was transferred into 2.5 ml of stock tubes and kept in the deep freezers at -20°C.

PCR

Viral nucleic acid was isolated from blood and swab samples using viral DNA and RNA purification kit (GeneJET, Thermo scientific, Lithuania) for extraction of viral DNA. The set of 555 for-CAGGAAGATATCCAGAAGGA and 555 rev- GGTGCTAGTTGATATGTAATAAACA primers, which were used in the study by Buonavoglia et al. was used for nucleic acid amplification (Buonavoglia et al. 2001). The amplified PCR products were separated on a 2% agarose gel containing ethidium bromide and the results were obtained under ultraviolet (UV) light by transilluminator.

Nucleotide sequences and Phylogenetic analysis

Sequence analysis was performed for PCR products that were detected to be as strong as being appropriate for sequence analysis among samples identified as positive after tests. The PCR products were purified using a commercial kit (Gel and PCR purification kit, GenMark, Taiwan) and then the samples were confirmed as a loading agarose gel subjected directly to sequencing in CEQ 8000 Genetic Analyzer (Beckman Coulter, USA) using the DyeTermination Cycle Sequencing Kit (DTCS, Beckmann Coulter, USA). Nucleotide sequences were compared with the corresponding reference Canine parvovirus isolates by using BLAST software in NCBI database. Phylogenetic analysis (Neighbor-Joining) with bootstrap analysis (1.000 replicates) and the Kimura 2-parameter correction was conducted by using the MEGA packaged software v5.0 (1).

RESULTS

The animals used as sample in the study generally had clinical findings such as fever, anorexia, depression, gastrointestinal problems, vomiting, and diarrhea. The pair of primer used in PCR tests applied for investigating the presence of CPV infection was chosen as specific to a wide gene site coding capsid protein of the virus and includes a locus consisting of at least 6-7 amino acids that are considerably important variables providing biological diversity of the virus. Table 1 shows the distribution of PCR positive samples by gender and age. It is showed that ethidium bromide stained agarose gel electrophoresis image of the PCR products in Figure 1.

Table 1. Distribution of PCR-positive samples age and gender

Sample (n)	Gender	Age (months)				
		0-2	2-6	6-12	12 and >	Total (%)
Rectal Swab (57)	Female	5	8	2	2	17 (29.8)
	Male	8	21	9	2	40 (70.2)
Blood (40)	Female	3	6	2	1	12 (30)
	Male	6	14	8	-	28 (70)

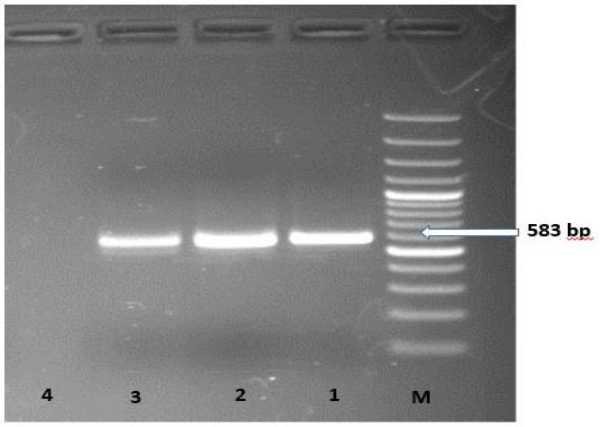

Figure 1. Ethidium bromide stained agarose gel electrophoresis image of the PCR products. [M: Marker (100 bp DNA ladder) 1,2,3: spesific product for CPV, 4: negative control (Distiled water)]

In the study, the sequence analysis was carried out by choosing samples that were determined to be strongly positive via PCR. As results of the sequence analysis were evaluated, these samples were too much similar and 4 of them were used for phylogenetic analysis. These samples were determined as Gene bank accession numbers of these samples were V1 (rectal swab)-MG545538, V2 (leucocyte)-MG545539, V3 (rectal swab)- MG545540, V4 (leucocyte)-MG545537, respectively. Figure 2 shows phylogenetic map prepared via comparison of sequences from gene locus coding capsid protein obtained from the study with each other and with reference sequences obtained from Gene Bank. While V4 (leucocyte)- MG545537 sample was determined to be CPV-2a as a result of phylogenetic analysis of samples, V1 (rectal swab)-MG545538, V2 (leucocyte)-MG545539, V3 (rectal swab) - MG545540 samples were found to be CPV-2b. Samples found in CPV-2b group shared similarity with each other.

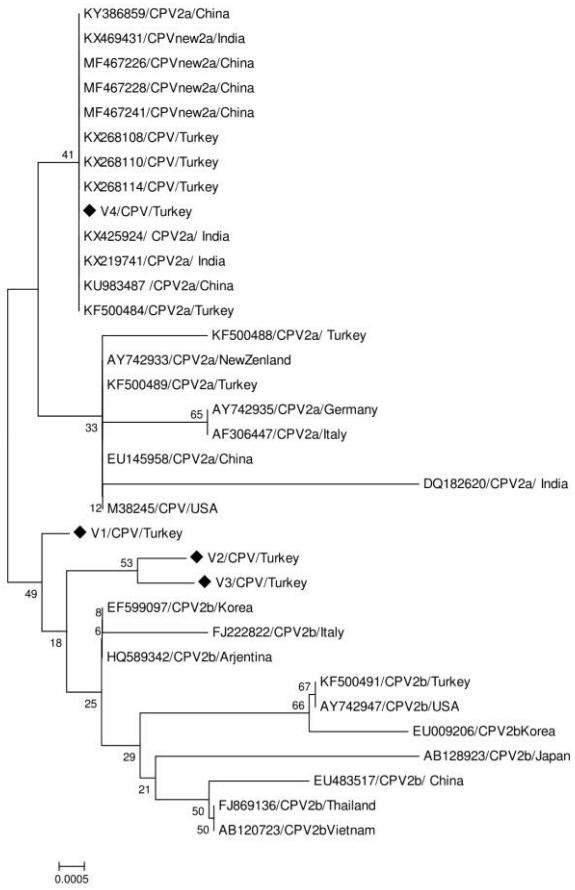

Figure 2. Phylogenetic tree of sequences from this study and reference sequences

DISCUSSION

While CPV infection commonly seen among dogs in the world and in Turkey causes severe enteritis in adult animals, it results in death causing myocarditis especially in puppies. Infection can be controlled by choosing appropriate methods of control and vaccines all over the world.

There are a few studies investigating Canine Parvovirus in Turkey. In the study conducted by Özkul et al. in 2002, parvovirus was identified in a dog displaying enteritis finding for the first time in Turkey using polymerase chain reaction (PCR). The obtained PCR products were subjected to RFLP analysis by fractioning them with restriction endonuclease enzymes. RFLP analysis performed using the enzymes Apa LI and EcoRV detected homology at least in VP2 coding sites of DNAs identified in feces and prepared as positive control from live attenuated parvovirus vaccine (Özkul et al. 2002).

In the study conducted by the sample of 60 dogs with hemorrhagic enteritis, antigenic characterization of canine parvovirus type 2 (CPV2) was evaluated by reactivity occurring in four monoclonal antibodies (mAbs) against CPV-2 using hemagglutination inhibition (HI) test, and CPV-2 was identified in 21 of samples (35%). Typology study was conducted in 16 of them; while CPV-2a was identified for 9, CPV-2b was identified for 7 (Yılmaz et al. 2005). Another study by Torun et al. investigated the presence of Canine Parvovirus Type-1 in dogs in Turkey. The presence of antibody in serum samples obtained from dogs displaying symptoms of gastrointestinal and respiratory system infection was surveyed via immunofluorescence (IF) test and CPV-1 antibodies were identified in 18 (18%) dogs of 100. These results revealed CPV-1 as serological in Bursa region where samples were collected in Turkey (Torun et al. 2005). In the study conducted by Timurkan and Oğuzoğlu in 2014, the presence of virus was investigated in rectal swab and blood samples of 65 dogs in the city of Ankara between April 2009 and February 2010. 25 of the samples (38.4%) were determined to be positive and sequence analysis was carried out. It was determined that 17 of the analyzed samples were CPV-2a (68%) and 8 were CPV-2b (32%) (Timurkan and Oğuzoğlu 2015). In a study using 20 dogs as sample in the city of Mersin, 7 PCR positive samples were determined to be CPV-2b (Dinçer 2017). In the present study, 57 (75%) out of 76 rectal swabs displaying clinical infection symptoms and 40 (64%) out of 62 blood samples were found to be positive. The distribution of samples' PCR results was determined by age and gender. According to these results, the ratio of positivity determined in male animals from all age groups for both leucocyte and rectal swab samples was higher compared to female animals. While 17 (29.8%) of 57 positive rectal swab samples were determined in females, 40 (70.2%) were identified in male animals. While 12 (30%) of 40 leucocyte samples that were found to be positive via PCR were determined in female animals, 28 (70%) were determined in male animals. Aktaş et al., stated in their study that incidence rate of infection was likely higher in male dogs than female dogs (Aktaş et al. 2011). As indicated by Houston et al. in 1996, this can be explained as the fact that male dogs are at higher risk for exposure to infectious agents since they tend to stroll around more than female dogs (Houston et al. 1996).

Comparison between age groups in the present study revealed that rate of incidence of CPV enteritis was higher in dogs in the age group of 2-6 months which is in parallel to findings of previous researchers (Houston et al. 1996; Homem et al. 1999; Castro 2007).

All of the materials used in the present study were tested via PCR and the product could not be obtained from some of different materials belonging to an animal. This was associated with the fact that viral load could vary based on organs and time of infection.

When considering the results of studies conducted in Turkey, it was determined that the ratio of CPV-2a circulating in Turkey was higher. When other CPV strains of samples that were found in GeneBank and in limited number were compared, these were observed to show similarity at various rates. The sample V4- MG545537 was found to be similar with CPV-2a strains from Turkey, China, and India. This sequence of virus strain was determined 99-100% homology between other strains from GeneBank. Also V1 (rectal swab)-MG545538, V2 (leucocyte)-MG545539, V3 (rectal swab) - MG545540 samples were determined to be CPV-2b as a result of phylogenetic analysis. These sequences was determined close to with CPV-2b strains from Korea, Italy, Argentina.

Control and fighting programs have been applied to prevent CPV infection that has an important place among pet animal infections in the world. Vaccination is applied more in western provinces since having pets is common so that infection has been prevented considerably. The present study is considered to be important for further studies and control programs to be developed because it contains current data about presence of the infection in

the region of Van and has molecular characterization data of circulating field strains of CPV. It was also concluded to apply routine vaccination as well as taking more effective precautions for follow-up of the infection in dogs.

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