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Short Paper

Phylogenetic analysis of canine parvovirus 2 subtypes from diarrheic dogs in Iran

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Abstract

Background: Canine parvovirus type 2 (CPV-2) causes gastroenteritis and leukopenia in dogs worldwide. They are three subtypes of CPV-2 including CPV-2a, CPV-2b, and CPV-2c. The distribution status of CPV-2 subtypes has been shown differences in many countries. **Aims:** The aim of the present study was detection and phylogenetic analysis of different subtypes of CPV-2 circulating in two provinces of Iran, Tehran and Alborz. **Methods:** CPV-2 was detected using 555 primer pairs in collected samples. Phylogenetic analysis of CPV-2 subtypes was done using sequencing of the partial length of *VP2* gene. **Results:** Twenty-eight CPV-2 were detected using 555 primer pair. The sequences of isolates were deposited in the GenBank database. Phylogenetic analysis revealed that all CPV-2c subtype isolates had very high sequence identity to China and Zambia that form a distinct cluster. **Conclusion:** In conclusion, this study revealed the emergence of all CPV-2 variants in dogs in Iran. Thus, the continual monitoring of CPV-2 in domestic dogs should be further conducted on a large scale to determine the predominant variants and their distributions in the country and to follow the dynamics of CPV-2 in the Middle East region of Asia.

Key words: Canine parvovirus type 2, Detection, Dog, Iran, Phylogenetic analysis

Introduction

Canine parvovirus type 2 (CPV-2) is a small non-enveloped virus with a single strand DNA genome containing approximately 5000 nucleotides. The virus capsid symmetry is icosahedral and assembled from 54 copies of VP2 and 6 copies of VP1 (Zhou *et al*., 2017). Although CPV-2 is a DNA virus, its nucleotide substitution rate (10^{-4} per site per year) is similar to RNA viruses (Voorhees *et al*., 2020).

CPV-2 was discovered in 1978, and from 1978 to 1979 spread worldwide. CPV-2 may be derivate from feline panleukopenia virus (FPV). The original CPV-2 differs from FPV in six amino acids (aa) residues in VP2 protein. These changes may have contributed to the gain of affinity for canine transferrin receptor (Tfr) observed during the shift from FPV to CPV-2 and accelerate easy transfer of CPV-2 between dogs (Decaro and Buonavoglia, 2012; Zhou *et al*., 2017). The mutated aa of VP2 protein include 80, 93, 103, 323, 564, and 568 residues. CPV modifies more rapidly than FPV (Decaro and Buonavoglia, 2012; Kapiya *et al*., 2019). After a few years, two new antigenic variants, CPV-2a and CPV-2b, appeared and they completely replaced the original CPV-2. CPV-2a and CPV-2b differ from the original CPV-2 in five to six aa of VP2 protein including 87, 101, 297, 300, 305, and 426 residues. The third variant, CPV-2c, was reported in Italy in 2000. The difference between the three subtypes is related to aa 426 possessing N in the original CPV-2 and CPV-2a, D in CPV-2b and CPV-2c, and E in the residue of VP2 protein (Zhou *et al*., 2017; Kapiya *et al*., 2019). Now three antigenic variants of CPV-2 including 2a, 2b, and 2c are circulating in many countries (Decaro *et al*., 2007; Battilani *et al*., 2019). At present, original CPV-2 is not circulating in dog population but it exists in most vaccines (Day *et al*., 2016; Zhou *et al*., 2016). Subsequently, CPV-2c was shown to affect adult and vaccinated dogs and even cats (Decaro and Buonavoglia, 2012; Charoenkul *et al*., 2019). The global distribution of CPV-2 subtypes indicates that CPV-2a is the predominant subtype in Asia, while CPV-2c is predominant in South America and Europe (Zhou *et al*., 2017). Previous studies indicated that CPV-2b is the predominant subtype in Iran and the US (Firoozjaii *et al*., 2011; Miranda and Thompson, 2016; Zhou *et al*., 2017; Nikbakht *et al*., 2018).

To understand better the reasons for this difference, a phylogenetic analysis of the CPV-2 subtypes was done using sequencing of partial length of *VP2* gene.

Materials and Methods

Samples

A total of 47 rectal samples were collected from dogs with clinical signs such as vomiting and diarrhea using sterile swabs. Rectal swabs were kept in 2 ml sterile phosphate buffered saline (PBS). The samples were transported to the laboratory under cool condition and stored at -70°C until use. The samples were collected from Tehran and Alborz provinces in Iran, during 2019 and 2020.

DNA extraction and PCR amplification

CPV-2 DNA was extracted using DynaBio™ (viral nucleic acid extraction mini kit, Takapozist Co., Iran) according to the manufacturer's instructions. CPV-2 was detected using 555 primer pairs from the samples. The primer pairs 555 forward (5´-CAG GAA GAT ATC CAG AAG GA-3´) and reverse (5´-GGT GCT AGT TGA TAT GTA ATA AAC A-3´) amplify a 583 bp fragment of capsid protein including the codon for residue 426 (Buonavoglia *et al*., 2001). PCR was conducted using a Taq DNA polymerase (Yektatajhiz, Iran), in the ESCO thermal cycler. The following thermal condition was used: initial denaturation step at 94°C for 10 min followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s. A final extension step was performed at 72°C for 10 min.

Sequencing

The PCR products obtained with 555 primer pairs were gel purified and sequenced using Sanger sequencing. Nucleotide sequences were edited and analyzed using BioEdit software, version 7.2.5. After editing the sequences, 483 bp fragments were used in the phylogenetic analysis. Phylogenetic analysis was conducted in the MEGA X using the maximum likelihood method based on the Kimmura 2-parameter model (Fagbohun and Omobowale, 2018). A total of 28 nucleotide sequences were included in the dataset. Also, 25 reference sequences were obtained from GenBank of which 10 were from Asia and 15 were from other parts of the world, including Africa, North, and South of America, Europe, and Australia. Selection of reference sequences was based on similarity to CPV-2 sequences of Iran. For this purpose, analysis of sequences by basic local alignment search tool (BLAST), which is available in GenBank was done (Fig. 1). Furthermore, some aspects such as genotype and geographical origin were considered in the reference sequences collection. In addition, two feline parvovirus sequences were used as outgroups. The phylogenetic tree reliability was estimated with bootstrap method 1000 replicates.

Results

PCR detection

CPV-2 DNA was detected in 28 (59.5%) of 47 fecal samples by PCR using primer pairs 555. Figure 1 shows the PCR products of some of the isolates. As shown in Table 1, All 28 samples that had clinical signs and were positive by PCR were collected from cases that were not vaccinated against CPV-2 except two.

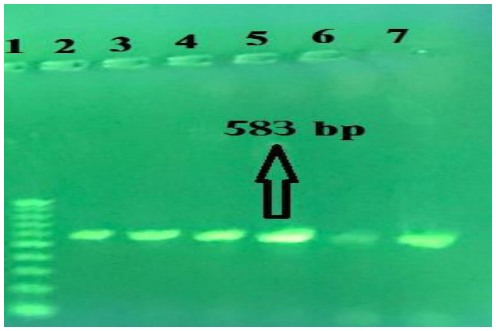

Fig. 1: PCR products of CPV-2, 583 bp fragments of capsid protein. Lane 1: DNA molecular weight marker (100-1000 bp), Lane 2: Positive control, and Lanes 3-7: Positive isolates

Table 1: Detailed descriptions of CPV-2 characterized in this study

Breed	Age (month)	Vaccination status	CPV-2 subtype (sequencing)
Shih Tzu	5	-	CPV2a/Iran/2019/19
German Shepherd	6	-	CPV2b/Iran/2019/21
Mixed	4	-	CPV2a/Iran/2020/01
Mixed Terrier	3	-	CPV2b/Iran/2020/22
Chihuahua	9	+	CPV2a/Iran/2019/11
Mixed Terrier	2	-	CPV2c/Iran/2019/27
German Shepherd	3	-	CPV2c/Iran/2020/05
German Shepherd	3	-	CPV2c/Iran/2020/07
Mixed	3	-	CPV2a/Iran/2019/18
Mixed Terrier	2	-	CPV2a/Iran/2019/20
Rottweiler	3	-	CPV2a/Iran/2020/02
German Shepherd	2	-	CPV2a/Iran/2019/13
Pomeranian	3	+	CPV2b/Iran/2020/23
Shih Tzu	4	-	CPV2a/Iran/2019/12
German Shepherd	2	-	CPV2a/Iran/2020/03
Mixed Terrier	2	-	CPV2a/Iran/2019/16
German Shepherd	4	-	CPV2a/Iran/2020/04
German Shepherd	2	-	CPV2c/Iran/2020/26
Pomeranian	3	-	CPV2c/Iran/2020/06
Pomeranian	9	-	CPV2c/Iran/2020/28
Mixed	7	-	CPV2b/Iran/2020/24
Mixed Terrier	6	-	CPV2b/Iran/2020/25
German Shepherd	10	-	CPV2c/Iran/2020/08
Dobermann	2	-	CPV2a/Iran/2019/15
Dobermann	2	-	CPV2a/Iran/2019/14
German Shepherd	3	-	CPV2a/Iran/2019/17
German Shepherd	6	-	CPV2c/Iran/2020/09
German Shepherd	6	-	CPV2c/Iran/2020/10

Sequencing and phylogenetic analysis

According to the amino acid sequence at 426 residue, 14, 5, and 9 isolates belong to CPV-2a (50%), CPV-2b (17.8%), and CPV-2c (32.1%), respectively (Fig. 2). Sequence alignment analysis showed three non-silent mutations at some isolates that residue 440 (Thr to Ala) were seen in 16 isolates. Another non silent mutations were at residue 553 (Asn to Ile) and 556 (Asp to Gly) at isolate CPV2a/Iran/2019/15 and, 568 (Gly to Val) at isolates CPV2a/Iran/2019/16 and CPV2a/Iran/2019/17.

The sequences were deposit in the GenBank data base (accession number was MW444897-MW444916, MN906317-MN906321, and MN990465-MN990469).

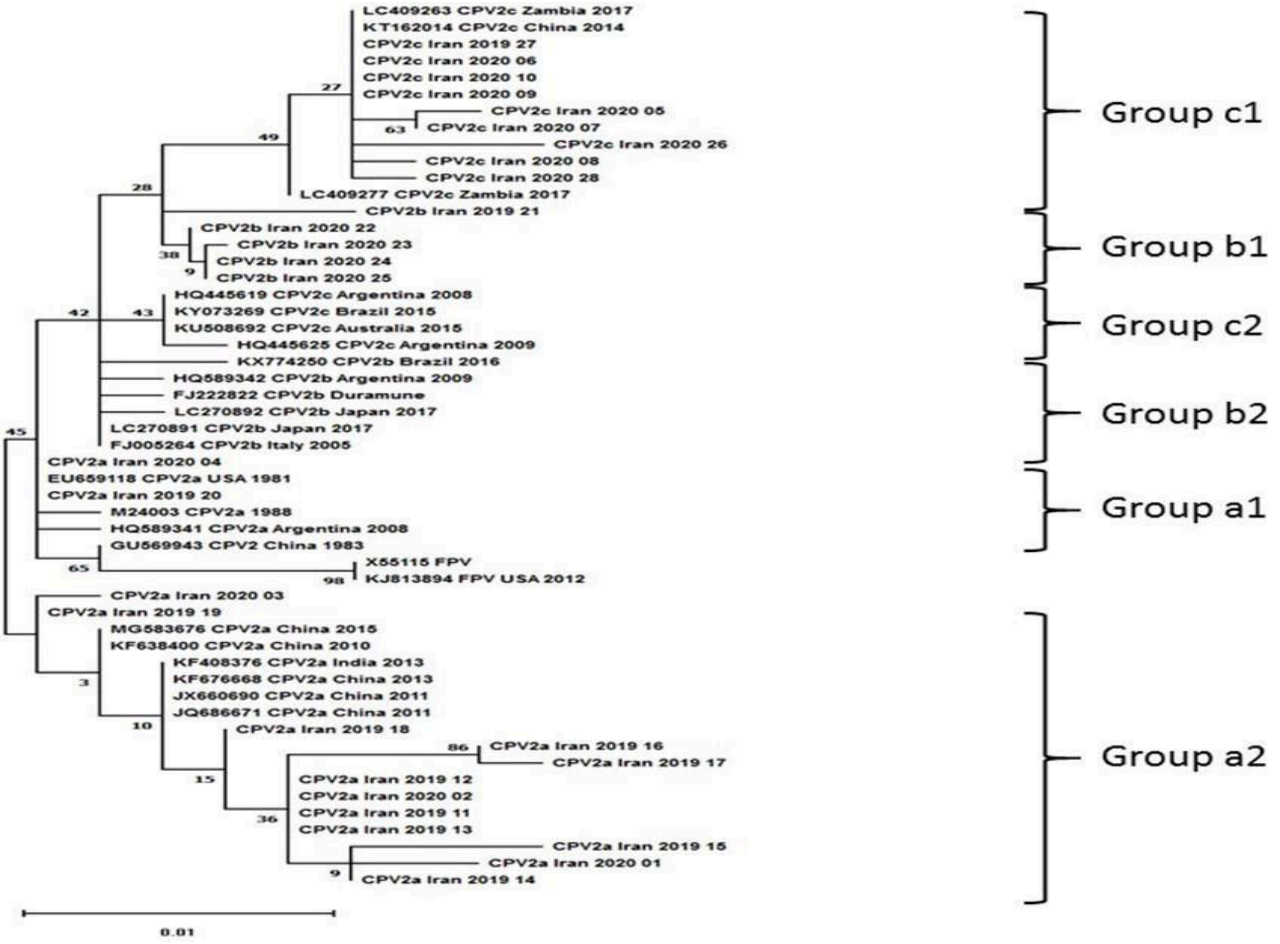

Fig. 2: Molecular phylogenetic analysis of *VP2* gene by maximum likelihood method. The evolutionary history was inferred using the Maximum Likelihood method and Kimura 2-parameter model. The tree with the highest log likelihood (-1030.46) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 53 nucleotide sequences. Codon positions were 1st+2nd+3rd+Noncoding. There were a total of 490 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

Discussion

CPV-2 is one of the most important infectious diseases of dogs that causes highly contagious gastroenteritis and leukopenia with high mortality in dogs (Figueiredo *et al*., 2016). Currently, there are three different CPV-2 subtypes, including CPV-2a, CPV-2b, and CPV-2c, that cause disease in dogs worldwide but there is no report of disease by original CPV-2 (Mosallanejad *et al*., 2014; Zhou *et al*., 2017; Novosel *et al*., 2019). The results of the current study indicate a high prevalence of CPV-2 (59.5%) among dogs that is presented to veterinary clinics with gastroenteritis symptoms. We found that all three CPV-2 subtypes are cocirculating in Iran, CPV-2a was the predominant subtype (50%), followed by CPV-2c (32.1%), and CPV-2b (17.8%). While previous studies in Iran indicated that CPV-2b was the predominant subtype in Iran (Firoozjaii *et al*., 2011; Nikbakht *et al*., 2018) but, the studies in Asia showed that CPV-2a was the predominant subtype (Zhao *et al*., 2013; Yi *et al*., 2016; Zhou *et al*., 2017). The prevalence of CPV-2c not only has been reported from some countries in Asia, including India, Vietnam, China, and Taiwan (Charoenkul *et al*., 2019), but also it is the dominant subtype found in Europe and America except for the US (Hong *et al*., 2007; Calderon *et al*., 2011; Zhou *et al*., 2017). This indicates that ecology can influence the geographic distribution of CPV-2 subtypes, and currently CPV-2a instead of CPV-2b, becomes the predominant subtype in Iran. Until 2019, the CPV-2b and CPV-2a subtypes were present in Iran and there were a few reports of CPV-2c (Saei *et al*., 2017; Nikbakht *et al*., 2018). There is no report about identification of original CPV-2 from diarrheic dogs in Iran.

Phylogenetic analysis revealed that CPV-2c subtypes are clustered in two distinct groups. All CPV-2c of Iran are located in group c1 and they were accompanied with some sequences from Zambia and China (accession No. LC409263, and KT162014, respectively). This indicates that CPV-2c subtypes from Iran showed high similarity to CPV-2c in Africa and East of Asia. The reason for this fact is not distinguished, but it can be related to

commercial trade practices between these countries, such as import of keeping equipment of dogs, like dog food. There are some sequences from South of America, Australia located in group c2. This indicates that they are two different clusters of CPV-2c subtypes that are spreading in Iran. CPV-2b subtypes are clustered in two distinct groups too. Group c2 is located between b1 and b2. All CPV-2b subtypes of Iran that are located in group b1 showed very high identity. The unique cluster also indicates that CPV-2b circulating in Iran are different from other parts of the world. But, in group b2 there are some sequences from South America, Europe, and East Asia. CPV-2a subtypes are clustered in two distinct groups too, but they show more divergence in comparison with CPV-2b. The CPV2-a sequences are located in group a1, including some sequences from America, Brazil, Argentina, China, and Iran and they are the oldest CPV-2. Interestingly, most CPV-2a subtypes from Iran (group a2) are located with some sequences from India and China. These indicate that CPV-2a subtypes have the most diversity and CPV-2a subtypes in Iran are from two ancestors Euro-America and Asia. According to topology of phylogenetic tree, CPV2c/Iran/2020/26, CPV2a/Iran/2019/15, and CPV2a/Iran/2019/17 are the youngest isolates, and CPV2a/Iran/2020/04, and CPV2a/Iran/2019/19 are the oldest isolates (Fig. 2).

Vaccination of dogs is the best method to protect them from infection with CPV-2 (Day *et al*., 2016; Ford *et al*., 2017) but, maternal antibodies can cause failure of vaccine to raise immune system and increase antibodies against CPV-2 (Chastant and Mila, 2019). The results of the current study indicate that two vaccinated dogs were affected by CPV disease (CPV2a/Iran/2019/11 and CPV2b/Iran/2020/23). One of the dogs was nine-month-old when infected with CPV-2. According to the history, this case received two doses of vaccine; the latest one was 20 days before the infection. In order to accomplish maternal antibodies in dogs older than 16 weeks, there should not be any interference with the vaccine. So, a single dose of CPV-2 vaccine has the potential to induce an immune response (Day *et al*., 2016; Decaro *et al*., 2020). One reason for the infection of the mentioned case could be attributed to vaccine-related immunization failure such as improper vaccination schedules; the other can be related to host immunization failure. Some cases without protective immune response after vaccination in age older than 16 weeks can be considered as genetically immune non-responder (Day *et al*., 2016; Decaro *et al*., 2020). Finally, mutation at residue T440A is also responsible for the vaccination failure. The isolates with 440A may become the novel CPV-2 sub-variant (Zhou *et al*., 2017) that was presented in 16 CPV-2 isolates in the current study.

In conclusion, this study revealed the emergence of all CPV-2 variants in dogs in Iran. Thus, the continual monitoring of CPV-2 in domestic dogs should be further conducted on a large scale to determine the predominant variants and their distributions in the country, and also to detect the dynamics of CPV-2 in the Middle East region of Asia. The whole genome sequencing of all CPV-2 variants is imperative to recognize all mutations that can cause vaccine failure.

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Conflict of interest

The authors declare that they have no conflict of interest.

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