

Sequence Analysis of VP2 Gene of the Parvoviruses Isolated from Fecal Samples of Domestic Cats in Tehran, Iran

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Abstract: Panleukopenia is a viral enteritis of high mortality rate in Feline population. It is caused by a small single-stranded DNA virus, belonging to the Family Parvoviridae, called Feline Panleukopenia Virus (FPV). Parvoviruses are capable of adapting to new hosts and can simply mutate like RNA viruses, resulting in the emergence of different strains with antigenic changes. Unlike the original Canine Parvovirus (CPV) type 2, the antigenic variants (CPV-2a, CPV-2b, CPV-2c) earned the ability to replicate in cat hosts either with clinical signs or without any symptoms. Cats may be able to shed Parvoviruses for a long time and can serve as an important reservoir. Co-infection with FPV and CPV-2 variants has been also reported. These characteristics make the cat an important host for Parvoviruses. Since no molecular study has reported on Parvoviruses infected cats in Iran, a decision was made to study these strains of Parvovirus. Twenty-eight fecal samples were collected from kittens under 6 months of age, both male and female and different breeds which had leukemia. PCR was performed on each sample and 26 samples tested positive for Parvoviruses. Randomly, the PCR products of eight samples were sequenced and their phylogenetic tree was generated. Analysis of the results revealed that all eight samples are FPV and it appears that FPV is more clonal than the CPV-2 variants in Iran. Amino acid residue 232 from the VP2 gene of FPVs has been changed from isoleucine to valine, which has only been reported in CPV-2 and its variants. It seems that some FPVs of Iran are changing to the CPV-2 variants. Hence, it is expected that in the future, the CPV-2 variants will be observed in cats.

Keywords: FPV, PCR, Genetic Analysis, VP2 Gene, Cats

Introduction

Feline panleukopenia is one of the most important viral diseases of high mortality rate in the feline population. It is caused by the Feline Panleukopenia Virus (FPV). The virus can be transmitted directly from a cat to another or from contaminated environment and objects. Major clinical signs of panleukopenia in cats are vomiting, diarrhea, fever, lethargy and inappetence and severe leukemia can be observed in complete blood count test.

According to the current taxonomy released, species of FPV belong to the Family Parvoviridae, subfamily Parvovirinae, Genus Protoparvovirus and Species Carnivore protoparvovirus 1 (ICTV, 2015).

Parvovirus is non-enveloped with an icosahedral symmetry of about 25 nm in diameter and its genome is

a single-stranded DNA with a size of 4.5-5.5 kb. Parvoviruses have two structural proteins namely VP1 and VP2. The amino acid residue changes the VP2 capsid protein, which affects the following: host range, virus strain and antigenic properties of the virus.

FPV has been known for about 100 years; however, CPV as a parvovirus that is able to infect dogs emerged in 1978 (Parrish, 1990). The original CPV-2 was rapidly replaced with a new antigenic type CPV-2a, CPV-2b in 1979-1984 (Parrish *et al.*, 1991) and finally, the newest strain of CPV-2c was detected in 2000 in Italy (Buonavoglia *et al.*, 2001). In contrast with the original CPV-2; CPV-2a/2b/2c earned the ability to replicate in a cat host and could also induce the clinical disease (Mochizuki *et al.*, 1993; Truyen *et al.*, 1996; Ikeda *et al.*, 2000; Battilani *et al.*, 2006a; Muz *et al.*, 2012;

Decaro *et al.*, 2008; 2011). CPV-2 variants (2a, 2b, 2c) are able to bind with feline Transferrin receptors and enter into the cells (Ikeda *et al.*, 2002; Hueffer and Parrish, 2003) Despite CPV, FPV is evolutionarily static and develops by random genetic drift (Decaro *et al.*, 2008) whereas the mutation rate of CPV is much higher. High mutation rate and the emergence of new strains of CPV are more similar to the characteristics of RNA viruses as compared to DNA viruses (Battilani *et al.*, 2006b). CPV-2 was replaced by CPV-2a and CPV-2b by changing 5-6 amino acid residues in the VP2 protein (Parrish, 1990; Parrish *et al.*, 1985).

The amino acid residue 300(Gly) that was a dog specific amino acid changed to Asp in the CPV-2a and CPV-2b isolated from cats. This mutation led to a loss of canine host specificity in these variants (Ikeda *et al.*, 2000). There are reports about isolating CPV variants from domestic and wild cats in Asia and other continents around the world (Ikeda *et al.*, 2000; Steinel *et al.*, 2000). FPV and CPV are more than 98% similar in the nucleotide and amino acid sequence and slight changes in amino acids of VP2 capsid protein makes a difference between the FPV and CPV-2 (6 amino acid: 80, 93, 103, 323, 564, 568) (Chang *et al.*, 1992; Steinel *et al.*, 2000).

There are reports about the presence of two or more strains of parvovirus, simultaneously in one cat as a host (Battilani *et al.*, 2006a; 2013; 2011) that make cats a suitable host for evolution and genetic recombination of parvoviruses. Based on the authors' knowledge, no molecular study has been performed on parvoviruses which infect cats in Iran, Tehran and therefore, it was considered as the objective of this study.

Materials and Methods

Sampling

Twenty-eight fecal samples were collected with sterile swabs from cats and under 6 months of age, both male and female and different breeds that referred to the Veterinary Hospital of the University of Tehran. Leukopenia was observed in their CBC (complete blood cell count) of all patients. The cats presented clinical signs of Panleukopenia including vomiting, diarrhea, depression and anorexia; except one cat that had no gastroenteric signs while others had only one of the signs, vomiting or diarrhea.

Collected samples were stored in 2 ml microtubes at a temperature of -20°C.

DNA Preparation

Samples were centrifuged (5000 rpm, 5 min) until the stool was deposited, the supernatant was pipetted out and filtered and used for DNA extraction (MBST DNA Isolation Kit, Iran), according to the manufacturer's instruction. Sterile distilled water was used as negative control; CPV and FPV live attenuated vaccines

(Fortdodge, USA) were used as positive control. The extracted DNA was stored at -20°C until PCR.

PCR

Primers M₁: 5'-AGC TGT CGA CGA AAA CGG ATG GGT GGA AAT-3' and M₁₁: 5'-ACT GGT GGT ACA TTA TTT AAT GCA G-3' were chosen from the VP2 gene (1201bp) segment that was designed by Steinel *et al.* (2000). This selected segment contains most amino acid residues that differentiate between CPV-2 variants and FPV.

To prepare a master mix, 12.5 µl from ready to use Mastermix (Sina Clon, Iran), 8.5 µl distilled water, 1 µl from each primers and 2 µl from the extracted DNA were mixed in final volume of 25 µl. The prepared mixture was transferred to an Eppendorf (Germany) Thermocycler. Thermal cycling contains: Initial denaturation at 94°C for 5 min, Denaturation at 94°C for 2 min, Annealing at 60°C for 2 min, Elongation at 72°C for 4 min (Repeated final three steps for 5 times), Denaturation at 94°C for 1 min, Annealing at 60°C for 1 min, Elongation at 72°C for 2 min (Repeated final three steps for 30 times), Final elongation was at 72°C for 10 min.

PCR products were electrophoresed in 1.2% agarose gel at 90 V and for 75 min, then the gel was stained in ethidium bromide for 15 min and visualized with a UV-light transilluminator. Expected band size was 1201 bp.

Sequencing, Sequence Analysis and Phylogenetic Tree

Eight PCR products were randomly selected and sequenced with an ABI sequencer after purification by Takapouzist Company. Sequences were trimmed and aligned (with BLAST in NCBI) to the reference FPV and CPV-2 strain sequences and other FPV sequences were reported from other countries. Analysis and generation of phylogenetic tree were performed with CLC sequence viewer v.6 software which is a user friendly software for analyzing sequence data and phylogenetic tree generation. Phylogenetic trees were generated using a neighbor-joining algorithm with bootstrap value of 1000.

Result

PCR and Sequencing

In this study, 26 of 28 samples were positive in PCR and showed 1201 bp size bands Fig. 1 and 8 sequenced samples were all diagnosed with FPV according to the different amino acid residues.

In the sequences of samples no. 2, 17 and 20, some disorderliness was observed, as two or three types of nucleotide's peak in its chromatogram (not only at the beginning but also in the middle parts of chromatogram) which may indicate co-infection with two or three similar viruses Fig. 2.

Sequence Analysis

Considering 1% difference between nucleotide sequences, there was no contamination. There was no clone but different strains existed.

The remarkable characteristic among sequenced samples was the mutation in amino acid residue 232 from Valine to Isoleucine, CPV-2 strains specific amino acid in this residue. This may indicate a comprehensive mutation among FPVs circulating in Tehran Table 1.

Phylogenetic Tree

In this study, sequenced samples formed a separate clade from the reference FPV strains and most of the strains were reported from other countries except the strain reported from India and that reported from Egypt. They seem to be from a common ancestor. In the phylogenetic tree, strains from Iran are similar to those from developing countries like India and Egypt and different from developed countries Fig. 3.

Table 1. Amino acid residue table of the sequenced samples compared with reference strains

	FPV ¹	CPV-2 ²	CPV-2a ³	CPV-2b ⁴	CPV-2c ⁵	2	4	7	15	17	19	20	22
80	Lys	Arg	Arg	Arg	Arg	Lys	-	Lys	Lys	-	Lys	Lys	-
87	Met	Met	Leu	Leu	Leu	Met	Met	Met	Met	Met	Met	Met	Met
91	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
93	Lys	Asn	Asn	Asn	Asn	Lys	Lys	Lys	Lys	Lys	Lys	Lys	Lys
98	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu
103	Val	Ala	Ala	Ala	Ala	Val	Val	Val	Val	Val	Val	Val	Val
110	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
232	Val	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
282	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn
297	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
300	Ala	Ala	Gly	Gly	Gly	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
305	Asp	Asp	Tyr	Tyr	Tyr	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
323	Asp	Asn	Asn	Asn	Asn	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
340	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
373	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
375	Asp	Asn	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp
415	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile	Ile
426	Asn	Asn	Asn	Asp	Glu	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn
440	Thr	Thr	Thr	Thr	Thr	Thr	-	Thr	-	-	-	Thr	-

¹FPV_M38246, ²CPV2_38245, ³CPV2a_M24003, CPV2a_AF306445, ⁴CPV2b_M74849, CPV2b_AF306450, ⁵CPV2c_AY380577

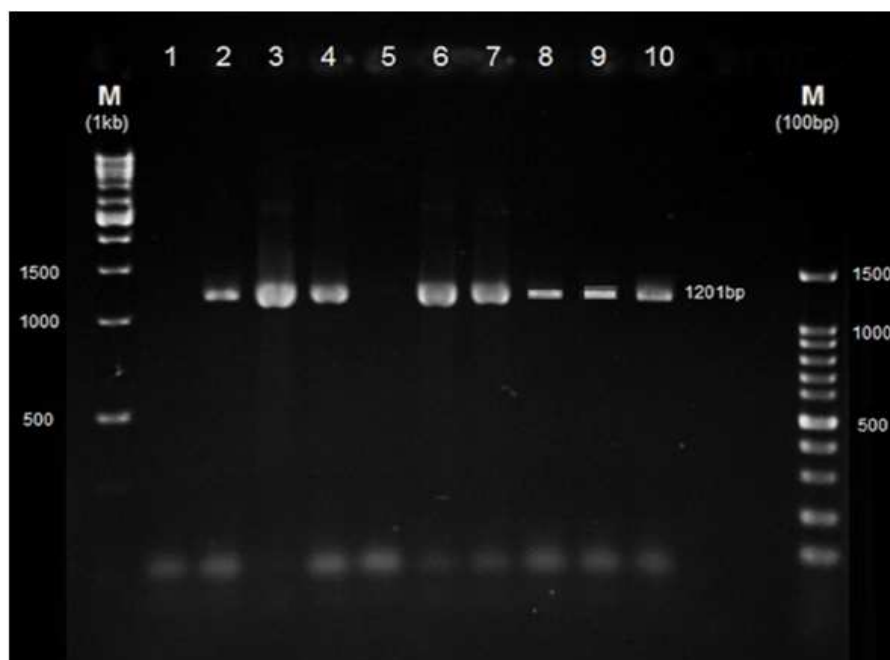


Fig. 1. PCR results of some samples. M: DNA Ladder (1kb, 100bp), 1: Negative control, 10: Positive control

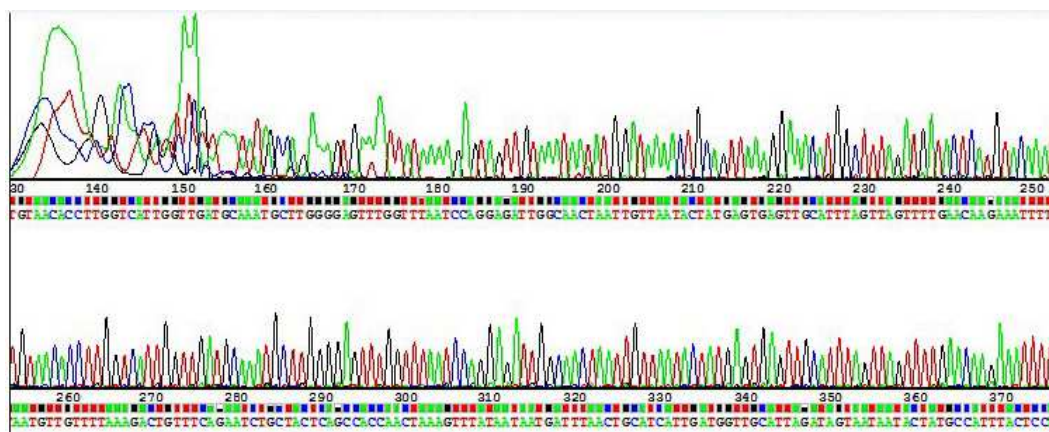


Fig. 2. Chromatogram of one of the samples with suspected Co-infection

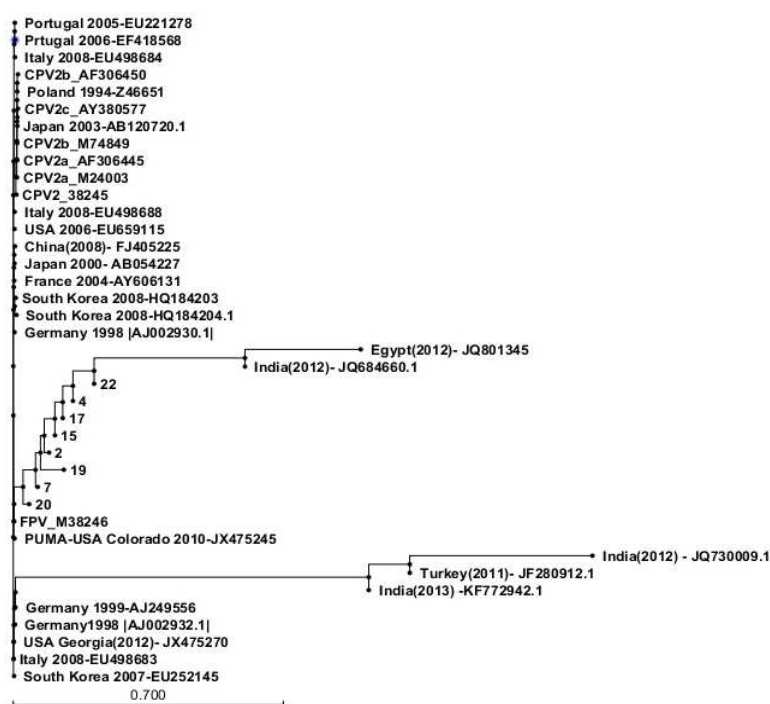


Fig. 3. Phylogenetic tree of the sequenced samples, reference and some countries. Japan - AB054227, China- FJ405225, South Korea -HQ184204, USA Georgia- JX475270, Italy -EU498683, South Korea -HQ184203, Portugal -EF418568, Portugal -EU221278, Italy -EU498684, South Korea -EU252145, USA Colorado -JX475245, Italy -EU498688, France -AY606131, Poland -Z46651, USA -EU659115, Japan -AB120720, Germany -AJ002930, Germany -AJ002932, Germany -AJ249556, India -KF772942, Turkey- JF280912, India- JQ684660, India - JQ730009, Egypt- JQ801345

Discussion

Panleukopenia is a highly contagious viral disease with variable strains which infect cats on a daily basis, all over the world. Many studies have been performed on this virus and many different results were obtained. There is no information about the parvoviruses that infect cats in Iran, to show which strain is dominant in this country and the probable genetic changes of the virus. In this study, PCR was conducted on the fecal

samples that were collected from cats which were suspected to have panleukopenia and 8 PCR products were randomly sequenced. All the sequenced samples seem to be FPV and three were suspected to be co-infected. Mutation was observed in amino acid residue 232 from valine to isoleucine and it was considerable.

There are many reports on the isolation of parvovirus strains from cats with clinical signs and even apparently healthy ones but only CPV-2 variants isolated from healthy cats (Mochizuki *et al.*, 1993; Clegg *et al.*, 2012).

The emergence of CPV-2 (2a, 2b, 2c) is associated with the ability to proliferate in cat hosts and since then, there are many reports on the isolation of these variants from clinically ill cats (Ikeda *et al.*, 2000; Decaro *et al.*, 2011; Mochizuki *et al.*, 1996; Decaro *et al.*, 2010). Mutations that led to the emergence of new variants mostly occurred on the VP2 protein genome. Despite being single stranded DNA viruses, the nucleotide substitution of parvoviruses is similar to that of RNA viruses (Shackelton *et al.*, 2005).

The sequencing of PCR products is one of the most reliable methods of distinguishing between virus strains. Specific amino acid residues in the capsid protein of the virus are used to identify the strain which are different from the FPV and CPV-2 variants (Table 1). Many studies have been performed on these residues and many changes have been detected. Some of these residues include 80, 93, 103, 232, 300, 305, 323 and 426 distinguished FPV from CPV-2 variants. Residues 92, 103 and 323 have effects on the dog host range (Truyen *et al.*, 1995). Residue 426 not only differs between FPV and CPV-2 but differs between the CPV-2 variants (2a, 2b, 2c), hence they can be discriminated through that feature (Buonavoglia *et al.*, 2001; Martella *et al.*, 2006). Therefore, residue 426 is an important key to detect the strain of the parvoviruses.

In this study, according to the PCR results, 26 samples from the 28 tested positive for the presence of the parvovirus and all 8 sequenced samples were FPV, considering the FPV specific amino acid residues except residue 232 that changed from Valine (Val) to Isoleucine (Ile).

The Isoleucine in residue 232 was only seen in CPV-2 and its variants. Based on the authors' knowledge, this mutation has not been reported before in FPV. This mutation constantly occurred in all 8 samples that were sequenced and it probably indicates that the FPVs of this study are changing to CPV.

In addition to the ability of parvoviruses to mutate, it seems that the presence of multiple variants in a host can also lead to recombination and creation of new strains. The co-infection of CPV variants was reported for the first time in a cat in 2006 (Battilani *et al.*, 2006b) and afterwards, the co-existence of 8 new variants of CPV-2c in one sample and CPV-2a, FPV and the intermediate strain of CPV-FPV simultaneously in a cat host were reported (Battilani *et al.*, 2013; 2011). In this study, the nucleotide sequence of samples 2, 17 and 20 were obscure and two or more nucleotides were detected in some point of the sequence, probably because of co-infection and the presence of two or more strains of parvoviruses in these samples. To ensure that, these samples have to be cloned and sequenced in future studies.

According to the results of this study, it seems that FPV is the dominant strain among kittens suffering from panleukopenia in Tehran. However, the possibility of the presence of CPV-2 strains cannot be ruled out,

considering the report of isolating CPV-2 strains from cats in the neighboring country "Turkey" (Muz *et al.*, 2012).

In the phylogenetic tree, Iran and one of the strains reported from India and a strain reported from Egypt were placed in the same clade with strains of this study. They were separated from other strains reported from other countries. In developed countries compared to developing countries like Iran, India and Egypt probably due to antigenic drift caused by widespread vaccination and subsequent selective pressure and high hygiene standards in these countries, as well as more transportation and communication between developed countries, strains are more similar and different from the developing countries.

Conclusion

According to the findings of this study, it is speculated that FPV is the dominant strain among kittens with clinical sign of panleukopenia in Tehran. However, considering the mutation of amino acid residue 232 in all sequenced samples, it is possible that the FPV strains of Tehran are being converted to the CPV-2 strains.

Samples 2, 17 and 20 were suspected of co-infection. To confirm that, they have to be cloned and more studies are needed to evaluate the strains circulating among cats in Iran. However, due to the changes in amino acid residue 232 to the CPV type (Isoleucine) observed in all sequenced samples and the possibility of presence of co-infection which can lead to emerging CPV from FPV, it is expected that CPV variants will be observed in cats in the future. Finally, due to the presence of CPV-2 variants in cats in Turkey, there is also the possibility that these strains are already present in Iran.

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Author's Contributions

Negin Jahanbin: Contribute to clinical part, study, sampling and diagnostic procedures, writing the manuscript and molecular studies.

Shahram Jamshidi: Contribute to clinical part, study, sampling and diagnostic procedures. Supervisor of the project.

Omid Madadgar: Contribute to molecular studies and sequencing.

Ethics

Authors should address any ethical issues that may arise after the publication of this manuscript.

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