Research Article

Molecular Characterization and Evolutionary Analysis of Canine Parvo Viruses in Dogs

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Abstract | Canine parvoviruses (CPV) are posing significant threats to the dog population, around the globe. To ascertain the current situation and genetics of CPVs in Pakistan, a total of fifty samples from sick dogs suspected for parvovirus infection were collected from different geographic areas of the country. Samples were screened for positivity and verified for CPV using PCR. From a 30% positive samples for hemagglutination, nineteen (38.4%) samples were positive in PCR. Sequence analysis of the selected positive samples revealed that both CPV-2 and new CPV-2a strains are circulating in the domestic dog poputlation in Pakistan. This pioneer research on the molecular characterization of CPVs in dogs would establish foundations to carry out effective control strategies in future.

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Introduction

Canine parvovirus (CPV) is one of the deadliest and most contagious viruses in dogs. Based on the surface genes, CPVs can be divided into two types: CPV-1 and CPV-2. CPV-1 strains are unable to cause significant infection in dogs, and are thus named as minute canine virus. On the other hands, CPV-2 strains produce the most severe form of the disease primarily in domesticated dogs and wild canids. Three variants of CPV type 2 (2a, 2b, and 2c) have been identified. Variant type 2c was first identified in Italy and has a different pattern of antigenicity as compared to variant 2a and 2b which are nearly similar to parental CPV-2 (Jacobs et al., 1980; Buonavoglia et al., 2001).

Several evolutionary events have been suggested that dictate the emergence of two important viruses of dogs and cats. Point mutations in (93 Lys → Asn and 323 Asp → Asn) VP2 gene of feline parvoviruses (FPV) may result in the emergence of CPV. Other substitutions at different regions in the VP2 gene of CPV-2 can also lead to emergence of new strains of CPV-2a (87 Met → Leu, 300 Ala → Gly, 305 Asp → Tyr), CPV-2b (426 Asn → Asp, 555 Ile → Val) and CPV-2c (426 Asp → Glu) (Decaro et al., 2006; Stucker et al., 2012).

Currently, the predominant strains reported in Paki-

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stan are CPV-2 and CPV-2b (Towakal et al., 2010). Owing to high disease prevalence and significant impact on canine population, we aim to identify the currently prevailing strain of CPV in Pakistan as well as to identify any molecular differences in prevailing CPV strains by sequence analysis of the partial VP2 gene region of the Pakistani strains. These strains were also compared at molecular level with vaccine strains commonly used to prevent CPV infections in Pakistan.

Materials and Methods

Sample collection

A total of 50 samples of all parvovirus suspect cases were collected via rectal swabs from sick dogs from different geographical areas of Pakistan, showing signs of diarrhea. In addition, information such as date of sampling, age, sex, breed, housing location, and clinical history were also recorded. Each sample was properly labeled. A total of 0.5 mL of phosphate buffer saline was added to each fecal sample for transportation purposes. Fecal swabs were cut and transferred to Eppendorf tubes containing 0.5 mL PBS, and were centrifuged at 4000 g for 15 minutes and supernatants were collected individually. Processed samples were stored at -20^0 C until further processing.

Screening, confirmation and sequencing of positive samples

Haemagglutination test was used to screen all collected samples by assessing agglutinating activity as described by Desario et al. (2005). Positive samples were selected for polymerase chain reaction for confirmation purposes. DNA of selected samples was extracted using the FavroPrep Viral Nucleic Acid Extraction Kit Favrogen (Cat # FAVNK001-1) method according to protocol provided by the manufacturer. Both the quality and quantity of extracted viral DNA were analyzed by NanoDrop 2000 (Thermo Scientific Company, USA). PCR was performed using previously reported primers for detection of CPV (Buonavoglia et al., 2001), named as H-par-for (5'- CAGGTGATGAATTTGCTACA -3') targeting position 3556-3575 & H-par-Rev (5'- CATTTGGATAAACTGGTGGT -3') targeting the 4185 – 4166 region of VP2 gene of CPV-2 and giving rising a production of 610 bps. For both primer pairs, the temperature profile included an initial denaturing at 95 C for 5 minutes, 35 cycles of denaturing at 94 C for 30 seconds, annealing at 51.3 C for 1 minute, extension at 72 °C for 45 seconds, and a final extension at 72 C for 10 minutes. Annealing temperatures were the same in both primers. The PCR product was confirmed by running the PCR product on 1.2% agarose gel. Electrophoresis was performed at 110 V for 30 minutes. The amplified PCR product was further purified using GeneJet Gel Extraction Kit (cat K0691) using instructions provided by the manufacturer. Purified samples were sent to a commercial laboratory for DNA sequencing using Sanger's chemistry. The sequences obtained were examined using BLAST (Basic Local Alignment Search Tool). Sequences were compared with other sequences already obtained from the GenBank database (http://www.ncbi.nlm.nih.gov) and were carefully aligned by BioEdit software (version 7.2.5.0) using the ClustalW method (Thompson et al., 1994). The MEGA software program, version 6.0, was used to construct a phylogenetic tree using the neighbor joining method, with bootstrap values calculated with 1,000 replicates (Figure 1).

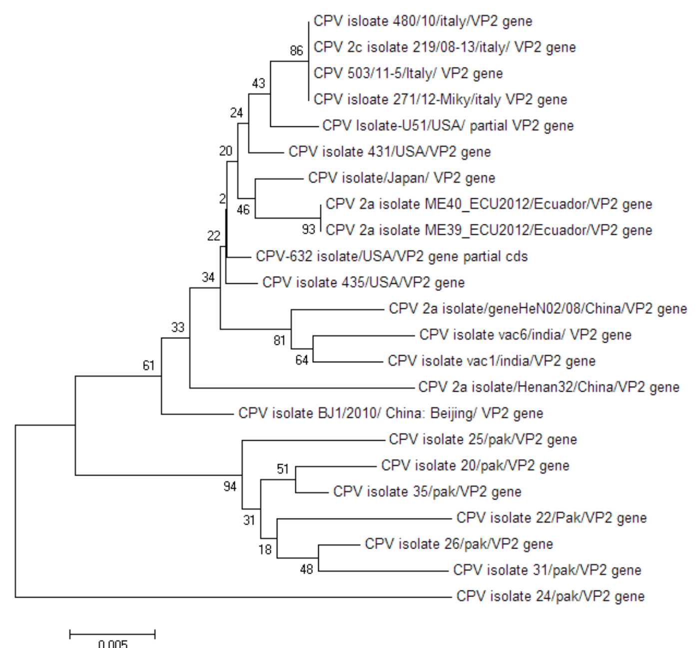

Figure 1: *Phylogenetic tree of partial VP2 nucleotide sequences of canine parvovirus strains obtained from the GenBank database and Pakistani CPV strains. The following sequences were added in the GenBank database: CPV isolate 435/USA (AY742953.1), CPV isolate 431/USA(Y742951.1), CPV2aisolate/Henan32/China(KM924289.1),CPV2aisolate/geneHeN02/08/China(EU441280.1), CPV isolate BJ1/2010/ China: Beijing/ VP2 gene, CPV 2a isolate ME40_ECU2012/Ecuador(KF149983.1), CPV 2a isolate ME39_ECU2012/Ecuador(KF149982.1), CPV isolate/Japan (AB128923.1), CPV isolate vac6/india (JN625224.1),CPV isolate vac1/india (JN625219.1), CPV isloate 271/12-Miky/italy (JX305965.1), CPV 503/11-5/Italy(JX305964.1), CPV isloate 480/10/Italy(JX305957.1), CPV 2c isolate 219/08-13 (FJ005250.1), CPV isolate-U51/USA(AY742942.1), and CPV-632 isolate/USA(AY742940.1).*

Table 1: *Age, breed, sex, and immunization status of dogs selected for sequencing.*

Sample ID	Age(mo)	Breed	Sex	Location	Vaccination	Genotype
CPV isolate 20/pak	6.0	Labrador	M	Lahore	No	2a
CPV isolate 22/pak	3.0	Pitbull	M	Quetta	No	2a
CPV isolate 24/pak	3.5	GSD*	M	Gujranwala	Incomplete	2
CPV isolate 25/pak	5.0	GSD	M	Lahore	incomplete	2a
CPV isolate 26/pak	2.5	GSD	M	Islamabad	No	2a
CPV isolate 30/pak	3.0	GSD	F	RYK	Incomplete	2a
CPV isolate 31/pak	2.5	Labrador	F	Narowal	No	2a

*: *German Shepherd Dog*

Table 2: *Variable nucleotides in the VP2 gene partial sequences of the canine parvovirus strains found in dogs of Pakistan*

Nt. position© aa residue	3675 aa297	3685 aa300	3699 aa305	3753 aa323	3757 aa324	3953 aa389	3954 aa390	4104 aa440	4176 aa464	4177
Reference CPV (M38245.1)	T	C	G	A	A	C	A	A	T	A
Primodog Vacc	T	C	G	A	T	C	A	A	T	A
CPV-Isolate 20/pak	G	G	T	A	T	C	A	G	A	A
CPV-Isolate 22/pak	G	G	T	A	T	C	A	G	A*	T*
CPV-Isolate 24/pak	T	C	G	G	A	T	G	A	T	A
CPV-Isolate 25/pak	G	G	T	A	T	C	A	G	T	A
CPV-Isolate 26/pak	G	G	T	A	T	C	A	G	A	A
CPV-Isolate 31/pak	G	G	T	A	T	C	A	G	A	A
CPV-Isolate 35/pak	G	G	T	A	T	C	A	G	A	A
aa. change	TCT GCT S→A	GCT GGT A→G	GAT TAT D→Y	GAC AAC D→N	TAT ATT Y→I	ACC ACT T→T	GCA ACA A→T	ACA GCA T→A	TAT AAT Y→N	TAT ATT Y→⇉*

©: *nucleotide positions are referred to CPV complete sequence obtained from GenBank under accession no. M38245.1*
*: *indicates that in CPV isolate 22, T and A swaps their positions as A and T at 4176 and 4177 respectively and a very new amino acid is coded i.e. isoleucine*

Results and Discussion

The present study was designed to assess the genetics of CPVs based on the partial gene sequence, variation and phylogenetic analysis of VP2 gene. Initially, a total of 50 samples were processed for screening. Only 30% (15/50) samples showed hemagglutination positivity which ranged from 1:4 to 1:256. Positive samples, based on HA titre, were subjected to PCR and target-specific amplicons were detected in 38% (19/50) of the samples.

According to data collected from pet owners it was suggestive that infection rate was higher in non-vaccinated, young, male dogs of German Shepherd breed. The maximum numbers of positive cases were recorded from the Lahore region (9/16). It may be due to any temperature fluctuation or mishandling during transportation of sample from other regions of Pakistan.

The successful PCR was carried out on partial VP2 gene of the virus that was present in the stool samples of dogs, with already reported primers and the purified product was sequenced. From 19 CPV-2 positive samples, seven were selected randomly based on their HA titers, for sequencing and all were identified as CPV-2a except for one (CPV-isolate 24/pak) which was CPV-2. It was noticeable that neither CPV-2b nor CPV-2c strains were identified. Table 1 shows the age, vaccine status, sex, and breed information of the animals selected for gene sequencing. Table 2 shows the targeted segment of the VP2 protein and the eight variable positions that were detected when compared to reference sequences.

As shown in Table 2, some substitutions were observed in all of our CPV-2a strains, specifically, 297 (Ser → Ala), 300 (Ala → Gly), 305 (Asp → Tyr), and 323 (Asp → TAsn). A mutation in residue 440 (Thr → Ala) was detected in six samples (Table 2). It was

noted that at position 3954 our reference CPV strain had nucleotide A coding for the amino acid threonine (ACA) but our CPV-isolate 24 possessed nucleotide G coding for amino acid alanine (GCA), it was surprising to note this as up till this point every nucleotide of this sample was showing 100% similarity with reference CPV-2 strain while other samples were already showing mutations leading to categorize them as CPV-2a variants. Another new mutation was seen in sample ID CPV-isolate 22, that at position 4176 & 4177, both the nucleotides T & A swap their positions as A & T respectively, and a new amino acid was coded, i.e. isoleucine (ATT) instead of tyrosine (TAT). Further, two samples (CPV isolate 24 & 25) resembled the reference strain and the remaining four samples showed mutations at position 4176 forming asparagine (AAT) instead of tyrosine (TAT). Likewise, the same mutation from tyrosine to isoleucine occurred at AA 324 leading to mark the strain as new CPV-2a. Amino acid mutations of the VP2 gene sequences analyzed in this study as shown in Table 3.

Table 3: *Mutation seen in amino acids at different positions*

Isolate	Amino acid at position					
	297	324	370	420	426	440
CPV-2 (M38245)	Ser	Tyr	Gln	Phe	Asn	Thr
Partial-length VP2						
CPV-2 (n = 1)	Ser	Tyr	Gln	Phe	Asn	Thr
CPV-2a (n = 6)	Ala	Ile	Gln	Phe	Asn	Ala
Primodog	Ser	Tyr	Gln	Phe	Asn	Thr

The phylogenetic tree (Figure 1) was constructed using partial VP2 nucleotide sequences of canine parvovirus strains obtained from the GenBank database and obtained local CPV strains. The Pakistani CPV-2 and CPV-2a strains were placed in different branches. The phylogenetic analysis of strains showed that all the samples collected from different regions of Pakistan fall into one clade and the branching pattern is shared with the neighboring countries, especially China. This information reveals that our virus is genomically similar to our neighboring countries India and China. The reason behind this may be that we share the borders closely with them. The tree also tells us that our clade is far from USA and Italy, which may indicate that the variant may vary from the virus of these countries. It was also noted that our CPV-2 strain branched independently from other Pakistani as well as reference strains.

There have been few studies on CPV infections in dogs in Pakistan and the molecular characterization has not been done so far. In the past few years, only one study had been conducted for variant detection in Pakistan by using specific primers for strain CPV-2 and 2b, revealing occurrence of both strains in Pakistan (Towakal et al., 2010).

In this study, partial-length VP2 gene sequencing of CPV was done in order to check the prevalence of CPV and study the genetic evolution of CPV strains prevailing in the sequences obtained were also compared with strain present in vaccine (Primadog®). In the current study, variable amino acids were identified at similar positions in different samples.

VP2 partial gene analysis indicated an asparagine at position 426 of six out of seven samples, thus identifying them as CPV-2a. Further, a nucleotide substitution was observed at position 970–972 of the VP2 gene, forming the amini acid isoleucine (ATT) from tyrosine (TAT) at position 324. The sequence analysis of selected samples showed that the most common variant in Pakistan is new CPV-2a, although CPV-2 was detected in one sample. It is striking that the variant 2b and 2c were not detected in any of the sequenced samples. This information contradicted the information provided by Towakal et al. (2010) who claimed that CPV-2 and CPV-2b are present in Pakistan. The reason behind this contradiction may be the method of identification used in both studies.

This new CPV-2a variant was identified in China and Uruguay between 2006 and 2009 and in 2010, respectively (Pérez et al., 2012). This is the first detection of this new CPV-2a in Pakistan. Nevertheless, this new CPV-2a variant freshly appeared in Uruguay and experienced clonal expansion. Substitution of amino acid 324 (Tyr → Ile) has been detected in Korea (Yoon et al. 2009), China (Zhong et al., 2014), Thailand (Phromnoi et al., 2010), Uruguay (Pérez et al., 2014), Japan (Soma et al., 2013), Taiwan (Lin et al., 2014), and India (Mittal et al., 2014).

The major circulating strain we identified was new CPV-2a (6/7, 85.7%) replacing threonine with alanine at residue 440 which is considered as a significant factor for antigenicity (Decaro et al., 2009). It was observed that severe diarrhea was seen in those six samples. So it is important to conduct more studies in this regard in order to comprehend the link

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between severity of clinical signs and these residues. The other mutations in amino acid residues in circulating strains in Pakistan were found at the VP2 gene (positions 297,324, 370 and 426) with reference to M38245 strain. Another substitution of position 4176 & 4177 (TA to AT) in addition with 3954 (A to G) variation should also be considered for further investigation and research.

According to Pakistan veterinary legislation, the age for the first vaccination for puppies is specified to be as early as 6 to 8 weeks of age; we sequenced one of the most commonly used vaccines in use to control parvovirus infection in dogs, and we were surprised to interpret that this vaccine contains CPV-2 strain. So this could be one of the reasons that some vaccinated puppies are also infected with this lethal virus as prevailing strains differ from the strain against which animal is protected by vaccine. However, this could also be another aspect of further studies to investigate whether the vaccine on the market with CPV-2 gives cross protective immunity against other variants or not. I would be careful suggesting CPV-2 vaccines won't protect against "newer" variants like 2a and 2b. There is no evidence for that, and several experimental trials have shown type 2 vaccines still work.

It was also noted that only our CPV-2 strain branched independently from other Pakistani as well as reference strains. In this study, the Pakistani new CPV-2a strain from dogs assembled together in an entirely different branch of the phylogenetic tree than the vaccine strains, except for one sample that showed CPV-2. Even if CPV-2a variant was determined as a circulating field strain, it is not present in commercial vaccine strains. This outcome can be used further for comparisons of vaccine and field strains at more advanced levels. It was also noted that our only CPV-2 strain branched independently from other Pakistani as well as reference strains levels, and it may become beneficial for further vaccine development studies (Decaro et al., 2007; Nandi et al., 2010). Furthermore, the typical variants of specific strains in a region or a country should be elucidated before vaccine selection.

The preventive measures for the control of CPV infections in Pakistan can be enhanced by conducting more frequent and sporadic molecular level investigations covering a larger population size and area selection.

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Author's Contribution

This work was done under the supervision of TY and MK. UF collected samples. AM reviewed the literature and evaluated the results. MA did data and statistical analysis.

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