Characterization of Iranian isolates of canine parvovirus in fecal samples using polymerase chain reaction assay

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Abstract
Despite the widespread prevalence of canine parvovirus disease (CPV) in Iranian dog population, molecular diagnosis of CPV variants, and investigation of the trends of its genetic changes is a new effort. In this study 50 samples from dogs suspicious of infection with clinical signs of diarrhea and vomiting, and 25 samples from dogs suspected of infection with general symptoms such as depression and anorexia were collected from dogs presented to the veterinary clinic of Shiraz University, Shiraz, Iran. Viral DNA was extracted from feces. Three specific pairs of primers, P2, Pab, and Pb, were used in a PCR assay for differential diagnosis of the virus type. Pab primer pairs detect the new type-strains, CPV-2a and 2b. The primer pairs P2 and Pb detect CPV types 2 and 2b, respectively. Our results showed that 44 individuals with clinical signs of diarrhea and vomiting were positive for CPV-2. 39 individuals (89%) were positive for CPV-2b and 5 individuals (11%) for CPV-2a. Therefore, the CPV-2b was identified as the predominant virus type. All dogs without symptoms of diarrhea and vomiting were CPV-negative. The relationship of breed, age and sex with PCR results was not significant (P>0.05). For the first time in the country, the causative agent of CPV-2 was identified, and presence of new antigenic variants, CPV-2a and CPV-2b was confirmed.

Keywords**:** Polymerase chain reaction; Canine parvovirus; Dog; Iran

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INTRODUCTION

Canine parvovirus (CPV), a member of the *Parvoviridae* family first identified in 1978, is an epidemic enteric pathogen of dog worldwide (Guy, 1986). It has a single-stranded DNA genome of about 5,200 nucleotides length, with two promoters. The virus expresses two structural (VP1 and VP2) and two non-structural (NS1 and NS2) proteins through alternative splicing of viral mRNAs (Notomi *et al*., 2000). In 1980s, two antigenic variants of CPV-2, distinguishable using monoclonal antibodies (MoAbs), emerged almost simultaneously and were termed CPV type 2a and type 2b (Parrish *et al*., 1991). Although a few years after its introduction, the original variant of CPV-2 was completely replaced by the antigenic variants, the original CPV type 2 is still used in most commercial vaccines (Martella *et al*., 2005b).

The CPV viruses cause a highly contagious disease and rapidly spread through dogs' population. Early and rapid diagnosis is necessary so that infected dogs can be isolated and supportive treatment can be administered to reduce morbidity and mortality (Cho *et al*., 2006). Clinical diagnosis of CPV infection is difficult, because the main clinical signs of the disease (vomiting and diarrhea) are common to other enteric diseases as well (de Castro *et al*., 2007).

Polymerase Chain Reaction (PCR) technique has been widely applied for laboratory diagnosis of the diseases, due to high sensitivity and specificity (Schunck *et al*., 1995). The assay is also conveniently

rapid, requiring only two to four hours of time for detection of viral nucleic acid. However, it can only be performed in a diagnostic or commercial laboratory, with access to specialized equipment not common to all veterinary clinics.

Proportions of CPV-2, the new antigenic types, vary in different countries. CPV-2b is the predominant virus type responsible for most outbreaks of CPV infection in US (Truyen, 2006; Parrish *et al*., 1991), Japan (Hirayama *et al*., 2005), Brazil (Pereira *et al*., 2000), Switzerland (Truyen *et al*., 2000), Taiwan (Wang *et al*., 2005), and South Africa (Steinel *et al*., 1998). CPV-2a is the predominant strain in Italy (Martella *et al*., 2006), India (Chinchkar *et al*., 2006), and Korea (Kang *et al*., 2008). Both CPV-2a and CPV-2b are present in UK (Greenwood *et al*., 1996), Germany (Truyen *et al*., 2000), Spain (de Ybanez *et al*., 1995), and Australia (Meers *et al*., 2007), with similar frequencies of isolation. However, CPV-2 still causes many cases of acute infectious diarrhea in dogs in Japan (Hirasawa *et al*., 1996). This study is the first to describe the molecular epidemiology of canine parvovirus in Iran. The first isolation of CPV in Iran using MDCK cell culture and electron microscopy was performed by Hemmatzadeh and Jamshidi (2002).

Subsequently, widespread outbreaks of canine hemorrhagic enteritis with high morbidity and mortality occurred over the whole country, although there has been no information concerning the antigenic types of CPV prevailing in Iran. In this study, we carry out PCR assay to type CPV strains, based on genetic markers of the three antigenic variants, and make use of this assay to characterize Iranian CPV isolates.

MATERIALS AND METHODS

Samples: A total of 75 fecal samples from vaccinated and unvaccinated domestic dogs suspected of having CPV were collected from March 2006 to January 2008. The samples came from diarrheic and vomiting dogs (50 individuals), and dogs suspected of CPV only with general symptoms such as depression and anorexia (25 individuals), presented to the Veterinary Clinic of Shiraz University in Shiraz, southern Iran. The animals' ages ranged from 1 month to 3 years.

DNA extraction: Fecal samples were collected with a sterile swab and stored at -70ºC until used. A CPV-2 vaccine strain was used as a positive control. DNA was extracted from the fecal samples and from positive control using the AccuPrep® stool DNA extraction kit (Bioneer Co., Korea), according to the manufacturer's instructions.

Differential primers for PCR: Sequences of all PCR primers (Table 1) were selected from variable regions in the VP1/VP2 capsid genes, according to published nucleotide sequences of CPV-2, 2a, and 2b (Parrish *et al*., 1991; Reed *et al*., 1988). One of the primer pairs called Pab, which can detect the new type-strains (CPV-2a and 2b), has been described in a previous report (Senda *et al*., 1995). The primer pairs P2 and Pb to detect CPV types 2 and 2b, respectively, were designed by Pereira *et al*. (2000). Primer pairs Pab and P2 (Table 1), recognizing the variants and the original type, respectively, were selected at overlapping positions, so that the nucleotide difference between the primers is restricted to one base at the 3′-end of each primer. Since the PCR products were of the same size, the primers were used in separate sets of reactions. Differential amplification of CPV type 2b is ensured by single nucleotide variations in the very 3′ end of each primer, and these nucleotide variations (A→G at 4062, and G→A at 4449) correspond to the strategic amino acid changes Asn-426 to Asp and Ile-555 to Val, that determine the antigenic variation from type 2a to type 2b. Therefore, CPV type 2a is recognized by the

Table 1. Sequences of PCR primers selected from variable regions in the VP1/VP2 capsid genes of canine parvovirus (CPV).

CPV type	Primers	Nucleotide sequence	Location	Amplicon length (bp)
CPV-2	P2s*	5′-GAAGAGTGGTTGTAAATAATA-3′	3025-3045	681
	P2as	5′-CCTATATCACCAAAGTTAGTAG-3′	3685-3706	
CPV-2b	Pbs	5′-CTTTAACCTTCCTGTAACAG-3′	4043-4062	427
	Pbas	5′-CATAGTTAAATTGGTTATCTAC-3′	4449-4470	
CPV-2a and CPV-2b	Pabs	5′-GAAGAGTGGTTGTAAATAATT-3′	3025-3045	427
	Pabas	5′-CCTATATAACCAAAGTTAGTAC-3′	3685-3706	

*s: sense; as: anti-sense.

Table 2. PCR results of the fecal samples of dogs presented to the veterinary clinic for detection of canine parvovirus (CPV).

	CPV type	CPV positive (%)	CPV negative (%)	Total
Diarrheic dog		44 (58.7)	6 (8)	50
Primer pairs Pab	CPV-2b and CPV-2a	44 (58.7)		
Primer pairs Pb	CPV-2b	39 (52)		
	CPV-2a	5 (6.7)		
Primer pairs P2	CPV-2	13 (17.3)		
Suspected dog		0 (0)	25 (33.3)	25
Total		44 (58.7)	31 (41.3)	75

primer pair Pab only, while CPV type 2b is recognized by both primer pairs Pab and Pb. The primers for PCR were provided from Bioneer Co., Korea, and other solutions from Cinnagen Co., Tehran, Iran.

PCR: The PCR assay was performed as described by Pereira *et al*. (2000). Briefly, the reaction mixture (20 µl) consisted of PCR buffer 1x (KCl 50 mM, Tris-HCl 10 mM, pH 8.3), $MgCl_2$ 1.5 mM for primers P2 and Pab, and 2 mM for primer Pb, 200 µM of each deoxynucleotide (dATP, dCTP, dGTP, dTTP), 10 pmole of each primers, 1 U of Taq DNA polymerase, and 5 µl of template DNA. PCR was performed in an Eppendorf thermal cycler (Eppendorf, AG, Hamburg, Germany) for 30 cycles, each consisting of denaturation at 94ºC for 30 s, annealing at 55ºC for 2 min and polymerization at 72ºC for 2 min. PCR products were electrophoresed on 1.2% agarose gels. The gels were stained with ethidium bromide and then visualized with ultraviolet light (Figs. 1, 2 and 3). The strategy used to type the samples was to submit each sample to an initial screening with primers P2 and Pab. After that, if the sample was positive with primer Pab, it would be amplified with Pb, to obtain type-specific results.

Statistical analysis: Data analysis was performed with SPSS v.11.5 for Windows (SPSS Inc, Chicago, IL, USA). Fisher's exact test was performed to investigate differences in PCR results based on sex, age and breed. Statistical significance $P< 0.05$ was adopted.

RESULTS

Results of molecular typing by PCR carried out on 75 isolates collected showed that the predominant strain during the study was CPV-2b (39/44 positive dogs). Five dogs showed CPV-2a. We also found 13 original type 2 viruses (vaccine type) in this period, which

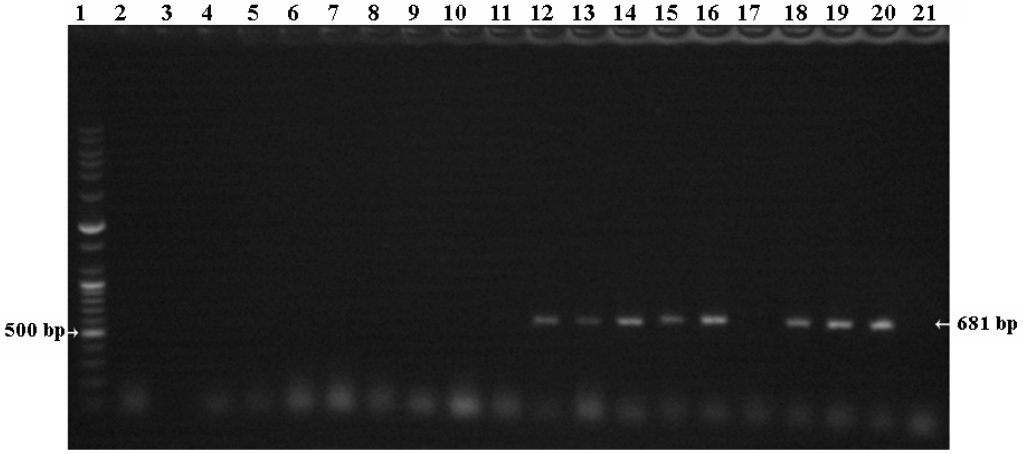

Figure 1. Agarose gel electrophoresis and ethidium bromide-fluorescence of PCR-amplified DNA in fecal samples of dogs presented to the Veterinary Clinic of Shiraz University, showing CPV-2, using primer pairs P2. Lane 1) molecular weight markers: 100 bp DNA ladder; lanes 2, 4-11 and 17) CPV-2 negative samples; lane 3) blank; lanes 12-16, 18 and 19) CPV-2 positive samples; lane 20) vaccine strain using primers P2; lane 21) negative control (healthy dog).

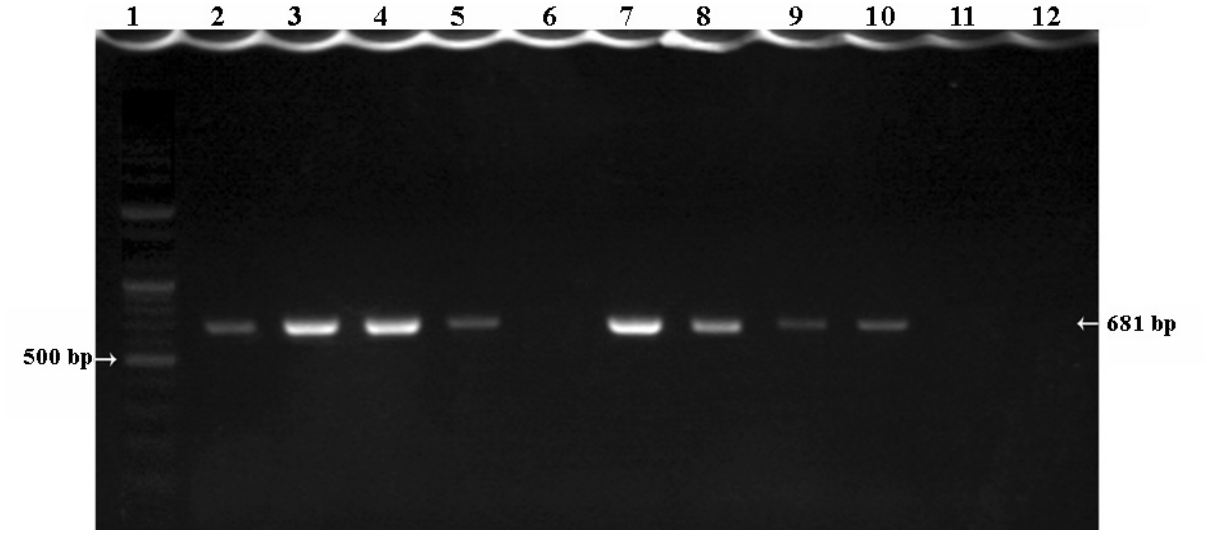

Figure 2. Agarose gel electrophoresis and ethidium bromide-fluorescence of PCR-amplified DNA in fecal samples of dogs presented to the Veterinary Clinic of Shiraz University, Shiraz, Iran, showing CPV-2a/b, using primer pairs Pab. Lane 1) molecular weight markers: 100 bp DNA ladder; lane 2-5 and 7-10) CPV-2a/b positive samples; lane 6) vaccine strain; lane 7) vaccine strain using primers P2; lane 11) blank; lane 12) negative control (healthy dog).

were accompanied by CPV-2a and CPV-2b strains, as shown in Table 2. Our results showed that 44 individuals among those with clinical signs of diarrhea and vomiting were positive for CPV-2 (Table 2). 39 individuals (89%) were positive for CPV-2b and five individuals (11%) for CPV-2a. Therefore, CPV-2b was identified as the predominant virus type. All dogs without symptoms of diarrhea and vomiting were CPV-negative. The mean age of CPV-positive dogs was 5.19 months, with 31 of 44 dogs between 1.5 and 6 months old (Table 3). In terms of breed, 22 of 44 positive dogs were German Shepherds, nine dogs were Terriers, eight dogs were of mixed breed, and the remaining five dogs were from other pure breeds (Table 3). There is a significant relationship between occurrence of diarrhea and detection of CPV in the feces (P=0.001), but the relationship of breed, age and sex with PCR results was not significant (P>0.05).

Table 3. Classification of PCR results for 50 fecal samples of diarrheic dogs presented to the veterinary clinic, for detection of canine parvovirus (CPV) according to age, breed and sex.

	CPV positive	CPV negative	Total
Age			
≤ 1.5 months	6	1	7
1.5-6 months	31	2	33
≥ 6 months	7	3	10
Breed			
German shepherd	22	4	26
Terrier	9	0	9
Mixed	8	2	10
Other	5	0	5
Sex			
Male	25	3	28
Female	19	3	22
Total	44	6	50

DISCUSSION

Canine parvovirus strains are able to replicate in many organs including heart, kidney, liver, tonsils, thymus, peripheral and mesenteric lymph nodes, different parts of small intestine, and in lower cases in colon (Nho *et al*., 1997). Tongue epithelium has been also shown to be a replication site of the virus (McKnight *et al*., 2007). The virus enters target cells via a dynamin-dependent, clathrin-mediated endocytic pathway, after capsid interactions with the transferrin receptors (Parker *et al*., 2001). Parvoviruses can only replicate in the nucleus of dividing cells. Parvovirus genome does not encode a DNA polymerase, an enzyme necessary for the initial step of parvovirus DNA replication. As cellular DNA polymerase is only expressed during mitosis, the first and crucial step of parvovirus replication, therefore, requires the dividing cell (Steinel *et al.*, 2001). The virus shedding in feces can occur from five to 52 days after infection (Decaro *et al*., 2005). Laboratory tests like hemagglutination, virus isolation on cell cultures, PCR, and real-time PCR are useful to

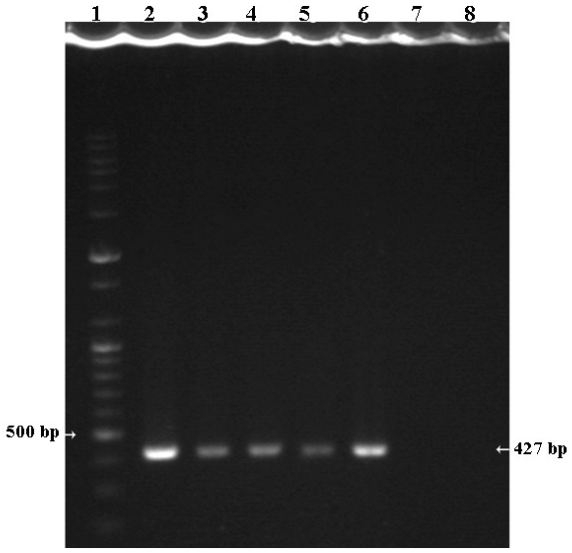

Figure 3. Agarose gel electrophoresis and ethidium bromide-fluorescence of PCR-amplified DNA in fecal samples of dogs showing CPV-2b, using primer pairs Pb. Lane 1) molecular weight markers: 100 bp DNA ladder; lane 2-6) CPV-2b positive samples; lane 7) negative control (healthy dog); lane 8) blank.

detect the virus shedding in the feces (Decaro *et al*., 2005). In this study PCR was applied for its sensitivity, specificity and easy accessibility. Although real-time PCR is the most sensitive test, current PCR strategy was chosen due to better cost-effectiveness.

A novel parvovirus was isolated both in canine and feline cell cultures, and was named CPV-2, to be distinguished from the previously described parvovirus canine minute virus (CMV or CPV-1), which is antigenically unrelated to CPV-2 (Carmichael *et al.*, 1994). The disease caused by MVC is mild in comparison to that caused by canine parvovirus-type 2 (Carmichael *et al*., 1994).

Most vaccines used in Iran and other parts of the world are based on the original type CPV-2, and vaccinal CPV is known to be shed in feces. Vaccination with current parvovirus vaccines is a potent way to prevent the disease and limit its spread in an area. During the last 30 years, the virus has generated different new types. Some reports show that there is cross-protection among heterologous types of the virus. However, occurrence of infections in puppies with maternal antibody indicates that protection with homologous types is a more efficient way to prevent the disease (Truyen, 2006). The current vaccine used in Iran contains the original type CPV-2, but according to the high occurrence of CPV-2b shown in this study, it seems that using new vaccines containing this virus type may be necessary. Although CPV-2a has a lower occurrence in different area of the world, management for protection against it is advisable. Meanwhile, Spibey *et al*. (2008) showed that CPV-2 can protect pets against the new type CPV-2c, which is being spread in some countries. Development of these new viruses may have benefited from replicative advantages in host cells, or more efficient virus shedding in feces. However, reasons to the different distribution of new antigenic types in various countries are unknown, and more studies will be required to address this question.

Another important concern in vaccination is attention to maternal antibody titer. In practice, immunization against CPV infection is usually based on repeated vaccination over a period of 6 to 18 weeks of age, without considering the maternal antibody titer. Hemagglutination inhibition (HI) and ELISA are the most useful tests to assess the antibody level before vaccination. Besides, clinicians can use these laboratory tests to assess immunization success or failure (Waner *et al*., 2006). However, Martella *et al*. (2005a) showed that intranasal administration of a modified live canine parvovirus type 2b vaccine in pups with considerable maternal antibody titer can protect them against the disease. Therefore, it seems using of intranasal vaccine can make a better immunization against CPV than the routine method without considering of maternal antibody. In the routine method it would be necessary to measure the antibody titer against CPV at least one month after the second vaccination.

Waner *et al*. (2006) reported that annual vaccination of dogs with CPV vaccine without considering their IgG titers may not be always beneficial, but sometimes even harmful. Therefore, they suggest that determining IgG levels to CPV using dot-ELISA test may be necessary before annual immunization. In the present study, we found that the infected dogs didn't have any history of vaccination. Therefore, more attention from veterinarians to animal immunization seems to be a priority in order for preventing and controlling the disease.

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