



MOLECULAR DETECTION OF CANINE PARVOVIRUS 2 SUBTYPES FROM CLINICAL CASES IN IRAN

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Summary

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Canine parvovirus type 2 (CPV2) is a major infectious agent in dogs that targets rapidly dividing cells in the gastrointestinal tract leading to leukopenia and highly contagious gastroenteritis with high mortality rates. The virus includes several subtypes including CPV2a, CPV2b, and CPV2c. This study focuses on detecting and distinguishing circulating CPV2 subtypes in 47 diarrhoeic dog cases in Iran. Three PCR sets using 555, *Pab*, and *Pb* primer pairs, were used to detect CPV2a and CPV2b, and a RFLP, through the application of *Mbo*II restrictive enzyme was used to detect CPV2c. A total of 47 cases were indicated as positive cases using immunochromatography kits. Subsequently, 28 out of the 47 cases were confirmed as CPV2-positive with PCR. Among these, five cases were infected with CPV2c (17.9%), two cases with CPV2a (7.1%), and 21 cases with CPV2b (75%). This research identified the presence of three CPV2 subtypes in dog populations in Iran. Findings of this research further confirm the reported prevalence results from previous studies in Iran.

Key words: CPV2, Iran, PCR, RFLP, vaccine failure

INTRODUCTION

Canine parvovirus type 2 (CPV2) causes one of the most prevalent fatal viral diseases in dogs, resulting in leukopenia and highly transmissible gastroenteritis with significant mortality rates in dogs (Zhou *et al.*, 2024). At present, there are three distinct CPV2 subtypes – CPV2a, CPV2b, and CPV2c – that cause disease in dogs globally, with no cases attributed to the original CPV2 (Chen *et al.*, 2021). CPV2 is a small, non-enveloped virus with a

single-stranded DNA genome consisting of about 5000 nucleotides. The virus has an icosahedral capsid symmetry composed of 54 VP2 copies and 6 VP1 copies (Umar *et al.*, 2024). In spite of CPV2 being a DNA virus, it has a nucleotide substitution rate resembling an RNA virus, with 10^{-4} substitutions per site per year (Voorhees *et al.*, 2019).

CPV2 was first detected in 1978 and rapidly spread worldwide from 1978 to 1979. CPV2 is speculated to be derived

from feline panleukopenia virus (FPV). The original CPV2 is distinct from FPV by six amino acids (AA) in the VP2 protein. These changes are likely to have increased the affinity of the virus for canine transferrin receptors (Tfr) inducing a shift from FPV to CPV2, while enhancing the transmission of the virus among dogs (Li *et al.*, 2022). The mutated AAs in the VP2 protein include residues 80, 93, 103, 323, 564, 568, and more recently, increasing within the CPV2c subtype population, AA residue 370, rendering CPV2 a more rapidly modified virus compared to FPV (Sarabandi & Pourtaghi, 2023). Later to the original CPV2, two new antigenic variants, CPV2a and CPV2b, appeared and have majorly taken over the original CPV2 in circulation. CPV2a and CPV2b are distinct from the original CPV2 by five to six AA residues in the VP2 protein, specifically at the sites 87, 101, 297, 300, 305, and 426. CPV2c was first reported in Italy in the year 2000. The subtypes are distinguished by AA substitutions at site 426 in the VP2 protein, for which original CPV2 and CPV2a possess N, CPV2b possesses N426D, and CPV2c possesses N426E (Chen *et al.*, 2021; Umar *et al.*, 2024). Currently, the novel variations of CPV2 a, b, and c, are predominant in vast regions globally (Sarabandi & Pourtaghi, 2023). Moreover, it is reported that the original CPV2 is no longer circulating in dogs but still appears in a vast proportion of vaccines (Day *et al.*, 2016; Zhou *et al.*, 2016; Umar *et al.*, 2024). CPV2c has been found to affect adults as well as vaccinated dogs and cats (Charoenkul *et al.*, 2019). The worldwide spread of CPV2 subtypes shows that CPV2a is the most common subtype in Asia, whereas CPV2c is more prevalent in South America and Europe (Zhou *et al.*, 2017). Earlier research reports CPV2b as the subtype more

common in Iran and the US (Firoozjahi *et al.*, 2011; Miranda & Thompson, 2016; Nikbakht *et al.*, 2018).

The aim of this study was detection and distinguishing of circulating CPV2 variants in the dog population in Tehran and Alborz provinces of Iran by using three PCR and RFLP assays.

MATERIALS AND METHODS

Sample collection

A total of 47 faecal samples from vaccinated and unvaccinated dogs were collected. Information about the age, breed, gender, and vaccination status of dogs exhibiting clinical symptoms was gathered from owners through questionnaires. Dogs were aged 2 to 11 months, all expressed clinical signs such as vomiting and diarrhoea, and had positive IC kit test results. Samples were collected using sterile swabs from patients in seven veterinary clinics in Alborz and Tehran provinces of Iran and were sent to the Faculty of Veterinary Medicine in the Karaj Islamic Azad University.

DNA extraction, PCR amplification and RFLP

DNA was extracted from the faecal samples using DynaBio™ DNA extraction kit by Takapozist Co. in accordance with manufacturer instructions. Sequences for PCR primers were selected from variable regions in the VP1/VP2 capsid gene, similar to published nucleotide sequences for distinguishing CPV2, CPV2a, and CPV2b (Pereira *et al.*, 2000; Buonavoglia *et al.*, 2001). All primer pairs were synthesised by Cinna-Gene, Iran. In total, three different pairs of primers associated with distinct regions of the VP2 gene were used to distinguish CPV2 and its subtypes. Ini-

tially, the primer pair *555 for* (5'-CAGGAAGATATCCAGAAGGA-3') and *555 rev* (5'-GGTGCTAGTTGATATGTAATAAACA-3'), which amplify a 583 bp fragment, was used to detect CPV2 positive samples. Then, PCR products from these positive samples were digested using the restriction enzyme *MboII* (Thermo scientific, *MboII*, Lithuania) to detect the CPV2c subtype (Buonavoglia *et al.*, 2001). Following *MboII* RFLP, the primer pair *Pab for* (5'-GAAGAGTGGTTGTAAATAATT-3') and *Pab rev* (5'-CCTATATAACCAAA GTTAGTAC-3'), that amplify a 681 bp fragment, was used to detect both CPV2a and CPV2b subtypes on PCR positive samples. Consequently, the primer pair *Pb for* (5'-CTTTAACCTTCCTGTAACAG-3') and *Pb rev* (5'-CATAGTTAAATT GGTATCTAC-3'), that amplify a 427 bp fragment, was subsequently used on *Pab* pair amplified products to distinguish the CPV2b subtype from CPV2a (Pereira *et al.*, 2000). PCR amplifications were performed in a total volume of 25 μ L containing 250 μ M dNTP, 0.5 mM MgCl₂, 2.5 μ L of 10 \times PCR buffer, 1 μ M of each primer, and 0.2 U Taq DNA polymerase. The conditions for primer pair *555* amplification consisted of 94 $^{\circ}$ C for 5 min for initial denaturation, then 35 cycles at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 45 s, and 72 $^{\circ}$ C for 60 s, followed by 72 $^{\circ}$ C for 5 min for final extension.

The primer pairs *Pab* and *Pb* were applied in the following setting: 94 $^{\circ}$ C for 5 min initial denaturation cycle, then 30 cycles at 94 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 2 min, and 72 $^{\circ}$ C for 2 min, followed by a cycle at 72 $^{\circ}$ C for 5 min for final extension. All three procedures included a final cool-down cycle at 4 $^{\circ}$ C for 10 min.

Finally, the visualisation of PCR products was done by electrophoresis in 1.5%

agarose gel prepared in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). To determine molecular weight, 100 bp DNA ladder was used.

Statistical analysis

The determination of 95% confidence limits was performed with the statistical software GraphPad InStat v. 3.00 104 (GraphPad Software Inc., La Jolla, CA).

RESULTS

Fig. 1 to 4 show the results of PCR gel electrophoresis and RFLP. The amplification of 583 bp fragments using primer pair *555* confirmed that 28 of the 47 collected faecal samples contained CPV2 DNA. In *MboII* enzyme restriction on positive PCR products by primer pair *555*, five samples were digested and produced a 500 bp product, thus confirming CPV2c presence. All remaining samples amplified 681 bp DNA fragments using the *Pab* primer pair, thus indicating CPV2a or CPV2b. Using the *Pb* primer pair, 21 samples had a 472 bp PCR product, so

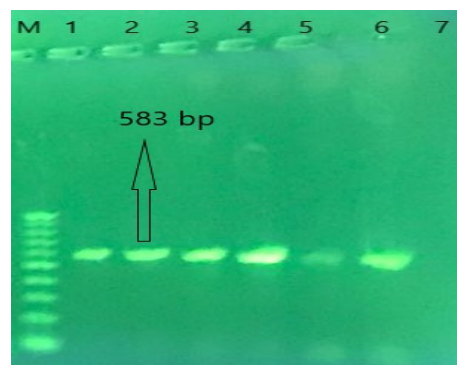


Fig. 1. Agarose gel electrophoresis of some CPV2 samples using *555* primer pairs that were positive using IC kit. Lane M: 100 bp DNA ladder; lanes 1 to 5: positive isolates with 583 bp PCR product size; lane 6: positive control; lane 7: negative control.

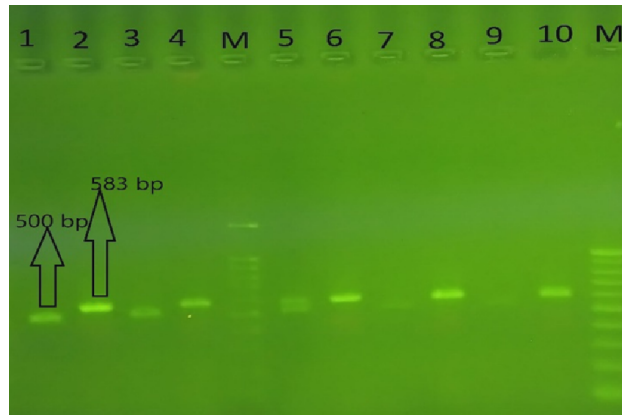


Fig. 2. Agarose gel electrophoresis of CPV2c positive samples using 555 primer pairs products digested using *Mbo*II enzyme in RFLP. Lanes M: 100 bp DNA ladder; lanes 1, 3, 5, 7 and 9: the positive samples after digestion; lanes 2, 4, 6, 8 and 10: the same samples before digestion.

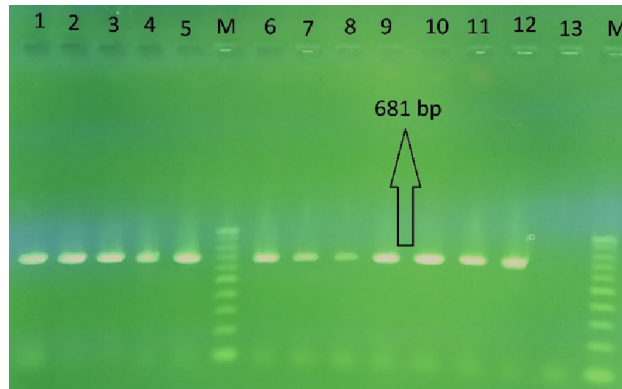


Fig. 3. Agarose gel electrophoresis of CPV2 positive samples further investigated using *Pab* primer pairs. Lanes M: 100 bp DNA ladder; lanes 1 to 11: positive samples that were either CPV2a or CPV2b; lane 12: positive control; lane 13: negative control.

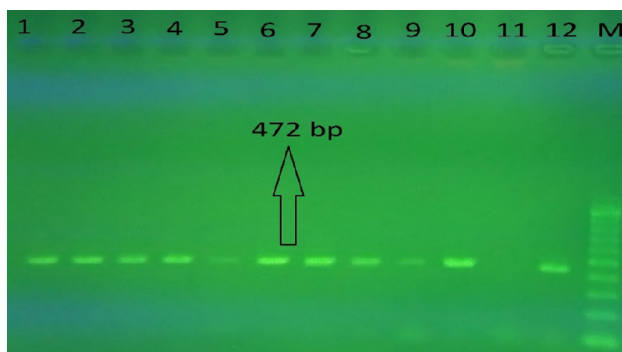


Fig. 4. Agarose gel electrophoresis of CPV2 positive samples further investigated using *Pb* primer pairs. Lane M: 100 bp DNA ladder; lanes 1 to 10: positive samples that were CPV2b; lane 11: negative control; lane 12: positive control.

Table 1. CPV2 positive case profiles, including breed, age, vaccination status, and individual results for involved CPV2 subtypes after each step of PCR, and RFLP testing

Breed	Age (months)	Vaccination status	PCR by 555 primer pair	RFLP	PCR by <i>Pab</i> primer pair	PCR by <i>Pb</i> primer pair
Shih Tzu	5	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	6	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed	4	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed Terrier	3	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Chihuahua	9	+	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed Terrier	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	3	-	CPV2	CPV2c	-	-
German Shepherd	3	-	CPV2	CPV2c	-	-
Mixed	3	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed Terrier	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Rottweiler	3	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2a
German Shepherd	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Pomeranian	3	+	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Shih Tzu	4	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed Terrier	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	4	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2a
German Shepherd	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Pomeranian	3	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Pomeranian	9	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed	7	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Mixed Terrier	6	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	10	-	CPV2	CPV2c	-	-
Dobermann	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
Dobermann	2	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	3	-	CPV2	CPV2a or CPV2b	CPV2a or CPV2b	CPV2b
German Shepherd	6	-	CPV2	CPV2c	-	-
German Shepherd	6	-	CPV2	CPV2c	-	-

they were confirmed as CPV2b. The remaining two samples that had no PCR product were identified as CPV2a using *Pb* primer pair.

Table 1 provides details on the breed, age, vaccination status, and subtype populations of CPV2. As shown, only two out of the 28 samples reported positive by PCR belonged to vaccinated dogs, both of which infected only by the CPV2b subtype. A statistical report on the relationship between vaccination and infection with CPV2 types is provided in Table 2.

DISCUSSION

The findings from this research revealed a notable prevalence of CPV2 subtypes (59.5%) among dogs exhibiting gastroenteritis. Our study confirmed the co-circulation of all three CPV2 subtypes in Iran. CPV2b was the most common subtype (75%), followed by CPV2c (17.9%) and CPV2b (7.1%). These findings are in line with previous studies, which reported CPV2b as the most prevalent subtype in Iran (Firoozjahi *et al.*, 2011; Nikbakht *et al.*, 2018). Our results differ from the studies across Asia identifying CPV2a as

the predominant subtype (Zhao *et al.*, 2013; Yi *et al.*, 2016; Zhou *et al.*, 2017). The presence of CPV2c is announced as emerging across the world in countries such as China, India, Taiwan, and Vietnam in Asia (Charoenkul *et al.*, 2019). CPV2c is the predominant subtype in America and Europe except for the USA (Hong *et al.*, 2007; Calderón *et al.*, 2011; Zhou *et al.*, 2017), which suggests that ecology can impact the CPV2 geographical distribution. As of 2019, CPV2b and CPV2a subtypes are present in Iran as well as accounts of CPV2c (Saei *et al.*, 2017; Nikbakht *et al.*, 2018). There are, however, no reported cases of original CPV2 collected from diarrhoeic dogs in Iran.

Canine parvovirus (CPV) gastroenteritis is known for its high morbidity and fatality rates in dogs, demanding early diagnosis for better treatment and control over its spread. Recently, various commercial immunochromatographic (IC) test kits have been made available for rapid diagnosis, which raises the question of their reliability. All 47 samples tested positive with IC kits but only 28 (59.7%) were positive with initial PCR testing. These findings suggest that IC kits do

Table 2. Responsible CPV2 subtypes and abundance with respect to the vaccination profile of participating dogs

Vaccination	PCR			Total
	CPV2b (95% CI)	CPV2c (95% CI)	CPV2a (95% CI)	
Negative	19 73.1 (52.2÷89.4)	5 19.2 (6.6÷39.4)	2 7.7 (1.0÷25.1)	26
Positive	2 100.0 (15.8÷100.0)	0 0 (0.0÷84.2)	0 0 (0.0÷84.2)	2
Total	21 75 (55.1÷89.3)	5 17.9 (6.1÷36.9)	2 7.1 (8.8÷23.5)	28

CI=confidence interval.

not show reliable results compared to PCR supplementary tests. However, false positive results in IC kits may also be induced by the effect of vaccination around the course of sample collection, or contamination during sampling.

Vaccination is recognised as an effective method in protecting dogs against infection with CPV2 (Day *et al.*, 2016). However, maternal antibodies can result in vaccine failure, and undermine the development of a proper immune response (Chastant & Mila, 2019). One case was nine months old at the time of infection. As the case history indicates, it had received two vaccine doses, with the last being 20 days prior to infection. Infection despite vaccine administration can hint at vaccine failure, as well as the emergence of a novel mutation. Due to diminished titres of maternally derived antibodies in dogs with more than 16 weeks of age, no interference with vaccines is expected. Therefore, a single dose of CPV MLV vaccine should be sufficient to induce an adequate immune response (Day *et al.*, 2016; Decaro *et al.*, 2020). Another possible explanation could be host-related immunisation failure. For instance, some cases where dogs did not develop a protective immune response after vaccination, even at ages older than 16 weeks might be considered genetically immunological non-responders (Day *et al.*, 2016; Decaro *et al.*, 2020). Yet a third potential reason could be the T440A mutation. According to some studies, T440A mutation is a potential cause of vaccination failure, and isolates with 440A may become the new CPV2 sub-variant (Zhou *et al.*, 2017). Therefore, sequencing of PCR products is suggested.

In summary, this research identified the presence of three CPV2 variants in dog populations in Iran. Therefore, con-

sistent assessment of CPV2 subtypes in domestic dogs should be carried out, ideally on a larger scale, to more accurately assess prevalent CPV2 variants and their regional spread, as well as the dynamics of the virus in the Middle East. Sequencing of whole genome of all CPV2 subtypes is crucial in identifying substitutions that could potentially contribute to vaccine failure.

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