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Faecal shedding of Canine parvovirus in clinically healthy vaccinated pups

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ABSTRACT

Canine parvovirus (CPV-2) is an important dog pathogen that causes severe hemorrhagic enteritis. The incidence of vaccinated, clinically healthy puppies shedding either the field strain or the vaccine strain of CPV is not fully understood. Moreover, little is known about the duration and extent of CPV vaccine virus shedding in puppies. To explore this phenomenon further, the current study aimed to understand the dynamics of faecal shedding of CPV in clinically healthy, vaccinated puppies. In this study, 351 faecal swabs were collected from twenty-seven clinically healthy pups at intervals over six months post-vaccination. Samples were analyzed for canine parvovirus shedding using hemagglutination (HA), PCR and qPCR assays. Two of the 351 samples screened demonstrated an HA titer 1: 2 on the 3rd and 7th day following the primary vaccination. In contrast, the PCR assay identified fifteen positive samples on various days post-vaccination. Among 60 randomly analyzed fecal swabs, 55 tested positive using real-time PCR. Sequence analysis of both conventional PCR and real-time PCR products provided clear evidence of subclinical shedding of both the vaccine strain and the field strain of CPV among the vaccinated puppies. The study concludes that vaccinated puppies shedding the vaccine strain (CPV-2) in their faeces may help to provide herd immunity. Additionally, healthy vaccinated puppies that shed the field virus strain suggest local intestinal multiplication of the virus, which could be a source of CPV infection for unvaccinated puppies.

Introduction

Canine parvovirus (CPV-2) is one of the most important pathogenic viruses causing acute haemorrhagic enteritis and myocarditis in dogs. It is recognized as a significant enteric pathogen affecting dogs globally, with a morbidity rate of 100% and a mortality rate of up to 10% [1]. After the emergence of canine parvovirus in the late 1970s, due to rapid evolution, new antigenic types were evolved as CPV-2a, CPV-2b and CPV-2c, which have entirely replaced the original CPV-2 [2].

In India, the previously predominant strain in the dog population was CPV-2a, along with a few co-circulating CPV-2b and CPV-2c strains [3, 4, 5, 6, 7]. However, a recent study showed that the CPV-2c (N426E) variant had almost completely replaced the previously dominant CPV-2a variant (N426) in India [8]. The original CPV-2 was not found in the dog population but is present only in vaccine formulations [2, 9].

In many countries the puppies are first vaccinated with a multivalent vaccine against canine parvovirus (CPV-2), canine distemper virus (CDV), canine adenovirus type (CAV-2), canine parainfluenza virus (CPi) and leptospirosis when they are between six and eight weeks of age, with booster vaccinations being given every three to four weeks until the age of 16 weeks and possibly 24 weeks in high-risk breeds. All dogs should

receive a booster one year after completion of the initial series, followed by a booster every 3 years [10, 11].

The immune response generated by modified live vaccines closely resembles that triggered by natural infections. CPV vaccine strains can replicate in lymphoid tissues and the intestinal mucosa, leading to viraemia and a short duration of fecal shedding [12]. The amount of excreted virus is sufficient to immunise other susceptible contact dogs [13]. Vaccination can yield positive results in nucleic acid amplification assays and fecal antigen tests, depending on the viral load excreted [14]. Thus, vaccine virus shedding can lead to misdiagnosis of parvovirus infection in the post-vaccination period. This presents a significant diagnostic challenge, particularly in puppies with acute gastroenteritis, shortly after their initial vaccination.

The attenuated pathogens in modified live virus (MLV) vaccines typically do not cause disease. However, diarrhoea that occurs shortly after vaccination has raised concerns among veterinarians and dog owners regarding the possibility of reversion to virulence. However, a study on dogs with parvovirus-like disease after vaccination with modified-live CPV showed that most cases were linked to infections with CPV field strains or other pathogens [15].

Similarly, little is known about the duration and extent of CPV vaccine virus shedding from the field condition. The incidence of healthy pups shedding the field strain of CPV due to subclinical infection also remains unclear [16]. Despite protective titre after vaccination and protection against the disease after challenge, the puppies were found susceptible to infection and shed the challenged virus without clinical disease [17]. Therefore, the present study was undertaken to understand the dynamics of faecal shedding of CPV in clinically healthy vaccinated pups.

Materials and methods

Ethical approval

Ethical approval was not required for this study. However, samples were collected according to standard procedure without harming or stressing the animals.

Sample collection and processing

A total of 351 faecal samples/ rectal swabs were collected from twenty-seven vaccinated pups whose primary vaccination was carried out at the age of 42nd to 45th day (considered as 0 days) followed by the 21st day (1st booster) and the 42nd (2nd booster) day post primary vaccination (DPPV). The samples were collected on 0 day (the day of primary vaccination) followed by 3rd, 7th, 21st, 24th, 42nd, 45th, 63rd, 84th, 105th, 126th, 147th and 168th dppv. Faecal samples or rectal swabs obtained from the pups were emulsified in 1ml of 0.1M PBS (pH 7.4) and transported to the laboratory under refrigeration (4°C). The emulsion was centrifuged at 6000 rpm for 15 minutes at 4°C, and the supernatant was stored at -40°C. The dogs were also monitored for any gastrointestinal disorders and other complications during the entire period of study.

Screening of faecal samples for CPV

Haemagglutination assay

Two-fold serial dilutions of the processed faecal samples were made in 0.2M Sorenson's PBS of pH 7.0 in a microtitre plate (50µl each well). To each well, 50µl of 0.65% pig erythrocytes was added, mixed gently and allowed to settle at 4°C for 4hrs. One well was added with 50µl of 0.2M Sorenson's PBS and 50µl of 0.65% pig erythrocytes to serve as cell control. The highest dilution of the sample showing complete haemagglutination (HA) was considered the HA titre [18].

PCR assay

The processed faecal samples were screened for CPV VP2 gene encoding capsid protein using primer pair H_{for}/H_{rev} (Table 1) that amplified a 630bp fragment amplicon [19]. The PCR reactions were conducted in a 50 µL volume using a 2X Taq DNA polymerase Red master mix (Ampliqon, Odense, Denmark). Other components include 0.2µM each primer (H_{For} & H_{Rev}), 2µL template DNA and nuclease free water (NFW) to make up the volume. The PCR amplification was performed on the automated thermal cycler (Eppendorf Master Cycler, Germany). The PCR reaction included an initial denaturation at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 45 seconds. Finally, there was a final extension at 72°C for 10 minutes. The PCR products were resolved in 1.5 per cent agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer (1X) and visualized under UV transilluminator (Syngene).

Real-time PCR assay

Generation of standards for Real-time PCR

To develop a standard DNA for real-time PCR, we amplified a 630-bp PCR product that covers the VP2 region from CPV-2 DNA using H_{for} and H_{rev} primers (Table 1). The amplified product was purified using the DNA Miniprep Kit (Sangon Biotech Co.) and quantified with a NanoDrop spectrophotometer (Qiagen), resulting in a 78.8 ng/µL concentration. The number of DNA molecules was calculated using the formula: amount (copies/µL) = [DNA concentration (g/µL) / (plasmid length in base pairs × 660)] × 6.02 × 10^23. The calculated copy number was 1.22 x 10^{11} DNA copies. The amplified PCR product was 10-fold serially diluted to achieve DNA concentrations ranging from 10^7 to 10^1 copies/µL, which were used as the standard DNA for the CPV-2 real-time PCR assay. The threshold time was plotted against the number of

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molecules detected. Using the Cycle Threshold (CT) value, a standard curve was generated to calculate the DNA copies of CPV in the fecal samples of the vaccinated puppy.

Table 1. Oligonucleotide primer Sequences for PCR and Real-time PCR assay for CPV-2

Primer name	Primer Sequence 5'→ 3' direction	Position of the genome	Amplicon size (bp)
H $_{For}$	CAGGTGATGAATTTGCTACA	3556 - 3575	630 bp [19]
H $_{Rev}$	CATTTGGATAAACTGGTGGT	4185 - 4166	
VP2-qPCR $_{For}$	CACAACAGGAGAAACACCTGAG	3953 - 3974	144 bp [20]
VP2-qPCR $_{Rev}$	CCAATTGGATCTGTTGGTAGC	4096 - 4076	

***Both primer pair sequences were 100% identical to their target sequences of the CPV-2a, 2b and 2c genomic DNA.*

Screening of faecal sample by Real-time PCR

Sixty faecal samples from 12 vaccinated pups collected on 0, 3, 7, 21 and 24th day post-vaccination were randomly selected for Real-time PCR assay to detect and quantify CPV-2 using the primers as mentioned in Table 1. The real-time PCR reactions were conducted in a 25µL volume using 2X SYBR Green Mix (Qiagen). Other components include 0.2µM each primer (VP2-qPCR$_{For}$ & VP2-qPCR$_{Rev}$), 4µL template DNA and NFW to make up the volume. Real-time PCR specific for CPV-2 was conducted using the Rotor-Gene Q real-time PCR system (Qiagen) described previously with some modifications [15]. The real-time PCR reaction was conducted as follows: 95°C for 5 minutes, followed by 40 cycles of 94°C for 10 seconds (denaturation), 56°C for 10 seconds (annealing), and 72°C for 10 seconds (extension). Finally, the Ramp (Raising by 0.5 deg each step) at 65ºC to 95ºC (5 sec each step) was performed for the melt-curve analysis.

Sequencing and phylogenetic analysis

The primer pair H_{for}/H_{rev} helps identify the informative nucleotides like 3675, 3684, 3699, 3753, 3909 and 4064 considered important for strain differentiation of CPV during sequencing [19]. The amplified PCR product (630 bp) of seven randomly selected faecal samples was gel extracted and single pass sequence analysed to know the types/strains of CPV excreted by the vaccinated pups. Similarly, the Real-time PCR product (144 bp) of six randomly selected clinical samples, along with two standards (10^7 and 10^3 dilutions), was also sent for sequencing to confirm the CPV sequence. Custom sequencing was conducted in both directions (5'-3' & 3'-5') using the Applied Biosystems 3100 automated sequencer. The specificity of the obtained sequences, along with the nucleotide and amino acid variations related to the VP2 gene sequence of canine parvovirus, was determined using BLAST [Basic Local Alignment Search Tool] (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The query nucleotide sequences were aligned with corresponding sequences available in GenBank using a multiple alignment program, Clustal Omega (http://www.ebi.ac.uk/clustalomega/). From the aligned sequences of clinical samples, the phylogenetic tree was constructed with the CPV sequences obtained in the study and CPV sequences from various parts of the world (GenBank) using the Maximum likelihood method in the MEGA7 program.

Results and discussion

Screening of Faecal Samples for CPV

Haemagglutination assay

Out of the 351 faecal samples collected from 27 healthy vaccinated pups at different days post-vaccination, only two pups showed HA titre of 1:2 on the 3rd and 7th day PPV, respectively. As a very high quantity of virus excreted through faeces (>10^7-10^9 virus particles/g) is required for appreciable HA, most of the vaccinated pups in the study group might have excreted a very low titre of virus similar to a subclinical infection. There were also reports that HA was poorly sensitive since high viral quantities were required to determine a visible CPV-2-induced haemagglutination [21]. None of the vaccinated dogs showed any gastrointestinal disorders or other complications during the entire study period.

PCR assay

Similarly, among 351 samples screened for CPV by PCR assay employing H_{for}/H_{rev} primers, fifteen samples at various days post-vaccination from 12 puppies yielded a specific amplicon of 630 bp. The relatively higher sensitivity of PCR over HA could be due to its ability to detect a much lower titre of virus in faeces (upto 10^3 PFU/g of faeces) compared to the HA test [22, 23]. The sequence analysis of PCR product from clinical samples revealed that five sequences had genomes identical to the CPV-2 vaccine strain, and two genomes were identical to the CPV-2a field strain (Table 2). The aligned sequences of CPV VP2 genes from 7 randomly selected faecal samples under this study were submitted to Genbank for allotment of accession numbers (MK193008, MK193009, MK193010, MK193011, MK193012, MK193013, MK193014). Phylogenetic analysis revealed that the five sequences clustered with the clade of the vaccine strain (CPV-2) (Fig. 1), but the other two query sequences clustered along with the CPV field strain (CPV-2a) clade.

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Table 2. Amino acid residues in the VP2 gene of the CPV reference strains, vaccines and test sample sequenced in this study

Type	GenBank Accession ID	Amino acid residues in the VP2 capsid protein gene of the CPV					Strain
		297	300	305	375	426	
CPV-2	M38245	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
CPV-2a	KP071945	Ala / GCT	Gly / GGT	Tyr / TAT	Asp/ GAT	Asn/ AAT	**Field Strain**
CPV-2b	KP071955	Ala / GCT	Gly / GGT	Tyr / TAT	Asp/ GAT	Asp/ GAT	**Field Strain**
CPV-2c	KP071956	Ala / GCT	Gly / GGT	Tyr / TAT	Asp/ GAT	Glu/ GAA	**Field Strain**
Sample 1 (7917)	MK193008	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
Sample 2 (8218)	MK193009	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
Sample 3 (1130)	MK193010	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
Sample 4 (7970)	MK193011	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
Sample 5 (350)	MK193012	Ser / TCT	Ala / GCT	Asp / GAT	Asn / AAT	Asn / AAT	**Vaccine Strain**
Sample 6 (905)	MK193013	Ala / GCT	Gly / GGT	Tyr / TAT	Asp/ GAT	Asn/ AAT	**Field Strain (CPV-2a)**
Sample 7 (715)	MK193014	Ala / GCT	Gly / GGT	Tyr / TAT	Asp/ GAT	Asn/ AAT	**Field Strain (CPV-2a)**

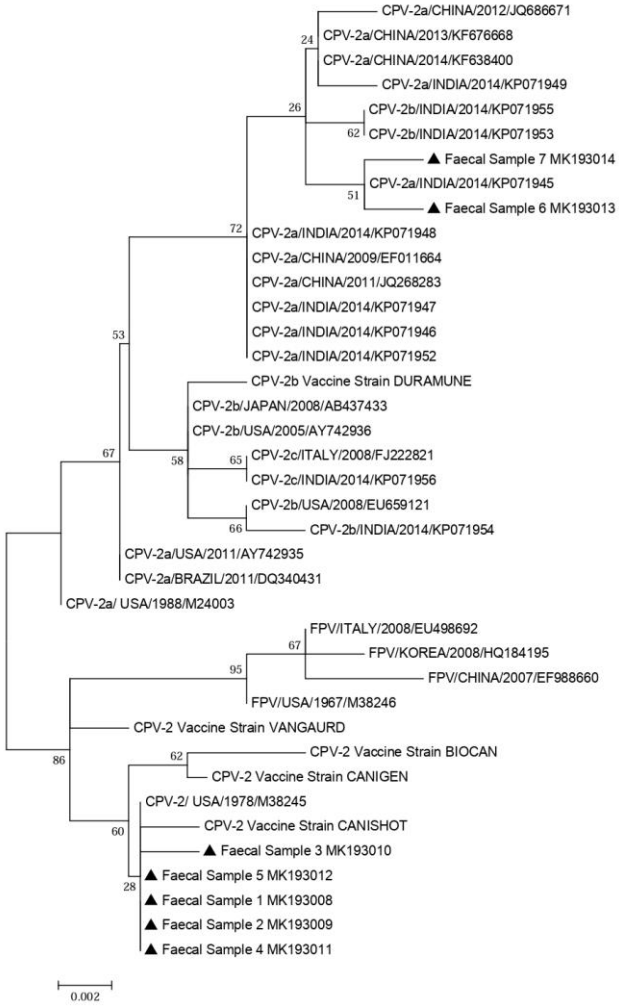

Fig 1. Maximum likelihood tree depicting phylogenetic relationship among canine parvovirus clinical samples. Parvovirus strains obtained from dogs sequenced in this study are shown with solid circles respectively, the vaccine strains are shown with solid Square and the various field strains CPV are shown with solid triangles

The sequencing and phylogenetic analysis results provided ample evidence of subclinical shedding of vaccine strain and field strain of CPV in the field by the vaccinated pups. The result indicated that not only dogs with clinical signs transmit CPV infection, but healthy dogs could also serve as a source of environmental contamination. Diagnosing diseases linked to canine parvovirus (CPV) can be challenging, as detecting CPV in fecal samples doesn't necessarily mean the dog is ill. The study also found that periodic exposure to the CPV vaccine strain boosts and maintains dogs' immunity, highlighting the need for continuous vaccination efforts. The findings also suggested that the natural CPV infection in dogs with pre-existing systemic immunity was most likely restricted to transient subclinical viral shedding. Compared to natural infection, vaccination with MLV was reported to induce shedding of a much lower quantity of CPV particles in the faeces [24, 25].

Real-time PCR for detection of parvoviral load

Out of 60 faecal samples tested, 55 were found to be positive by real-time PCR (Table 3). The Melt curve analysis suggested that these amplified real-time PCR products had a melting temperature of ~78.5ºC, which matches the CPV standards (Fig. 2). The sequence analysis of the real-time PCR product (144 bp) for six clinical samples and two standards (10^7 and 10^3 dilutions) was highly specific to the CPV-2/CPV-2a sequence by 'BLAST' analysis. The real-time PCR product of six clinical samples, along with two standards (10^7 and 10^3 dilutions), were allotted with the Genbank accession numbers (MK193015, MK193016, MK193017, MK193018, MK193019, MK193020, MK193021, MK193022). Real-time PCR was very sensitive and could detect even low-titred virus shedding (10^3-10^6 copies/ 100µl sample), common during subclinical shedding. Low levels of viral replication could explain this in the face of pre-existing systemic or local antibodies in the intestinal mucosa. The findings are consistent with a study on specific pathogen-free (SPF) puppies, which demonstrated that even though the puppies had protective antibody levels after vaccination, they remained susceptible to infection following a challenge. They shed the challenged CPV strains without showing any signs of illness [17].

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Table 3. Screening and Quantitation of CPV in the samples with SYBR green real-time PCR

Sample ID		Day post primary vaccination									
		0		3		7		21		23	
		CT Value	Copy number	CT Value	Copy number	CT Value	Copy number	CT Value	Copy number	CT Value	Copy number
DOG 1	**8063**	16.0	1.7E+04	0.0	Negative	0.0	Negative	20.2	1.8E+03	18.5	9.7E+03
DOG 2	**8064**	14.9	1.01E+05	15.3	5.12E+04	14.7	1.3E+05	15.2	2.68E+05	16.2	1.01E+05
DOG 3	**7970**	15.0	8.2E+04	12.9	2.7E+06	14.0	4.2E+05	18.1	1.5E+04	13.9	9.9E+05
DOG 4	**7917**	12.9	2.7E+06	15.6	2.9E+04	17.2	2.3E+03	15.4	2.09E+05	18.5	1.02E+04
DOG 5	**8218**	14.5	1.8E+05	16.1	1.4E+04	17.4	3E+04	15.3	2.46E+05	14.7	4.46E+05
DOG 6	**108**	0.0	Negative	0.0	Negative	16.0	1.19E+05	13.9	9.57E+05	17.1	4.15E+04
DOG 7	**71**	17.3	1.8E+03	15.5	3.6E+04	15.3	2.5E+05	17.0	4.46E+04	16.5	7.3E+04
DOG 8	**715**	14.3	2.8E+05	10.4	1.78E+08	13.6	1.26E+06	17.9	1.73E+04	17.7	2.2E+04
DOG 9	**716**	14.9	9.5E+04	12.8	3.13E+06	13.3	1.7E+06	18.4	1.06E+04	18.3	1.17E+04
DOG 10	**905**	16.8	4.5E+03	12.1	1.02E+07	12.5	3.7E+06	18.8	7.57E+03	14.6	4.6E+05
DOG 11	**350**	0.0	Negative	13.9	5.17E+05	17.5	2.7E+04	14.4	5.76E+05	17.1	4.15E+04
DOG 12	**1130**	17.2	2.3E+03	13.5	1.04E+06	17.2	3.7E+04	20.6	1.25E+03	17.9	1.8E+04

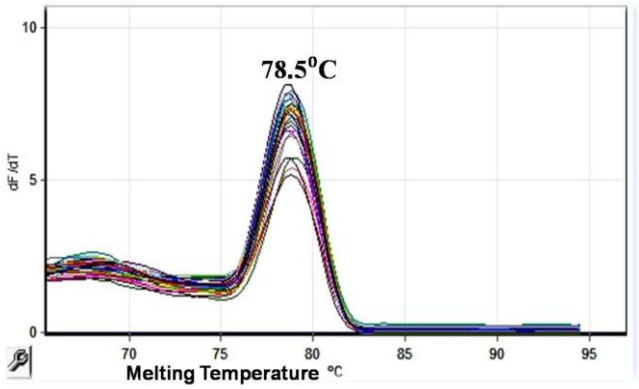

Fig 2. Melt curve analysis suggested that these amplified real-time PCR product had a melting temperature of ~78.5°C which matches with the CPV standards used.

Conclusion

Real-time PCR, PCR and HA detected faecal shedding of CPV during post-vaccination days. Real-time PCR demonstrated greater sensitivity compared to both conventional PCR and the haemagglutination assay. Real-time PCR could detect even low-titred virus shedding, which is common during subclinical shedding. Monitoring the faecal shedding of the virus using real-time PCR for an extended period after vaccination will be highly beneficial. Additionally, redesigning the real-time PCR primers should be considered so that they can distinguish between the CPV vaccine strain and the CPV field strain in future studies. Therefore, vaccinated puppies that shed the vaccine strain of canine parvovirus (CPV-2) in their faeces may contribute to herd immunity. However, when healthy vaccinated puppies shed the field virus strain, it indicates local intestinal replication, and they may serve as a source of CPV infection for unvaccinated puppies.

Disclosure statement

The author(s) reported no potential conflict of interest.

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