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Original Research

Canine Parvovirus Isolates of India and the Relevance of Canine Parvovirus Type-2 Vaccines

Shankar R. Chinchkar, Bhaskaran Mohana Subramanian, Hariprasad Naidu, Dorairajan Thiagarajan, Villuppanoor A. Srinivasan*

Research and Development Centre, Indian Immunologicals Limited, Gachibowli, Hyderabad-500032, India

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Abstract

A study was conducted to characterise the field isolates of canine parvovirus (CPV) and an *in vitro* cross neutralisation assay was performed against the vaccinated dog sera. Out of 45 faecal samples processed for virus isolation, 27 samples showed cytopathic effect (CPE) at first passage, which were confirmed positive by CPV variant types specific PCR. The CPV type 2 was not detected in any of the clinical samples. Of these 27 positive samples only 23 samples showed CPE and were further confirmed as CPV by haemagglutination inhibition test, ELISA and immuno-chromatographic strip test. Antigenic typing performed using a panel of monoclonal antibodies revealed that four of the 23 isolates were CPV 2b type and the remaining 19 isolates were typed as CPV 2a. The antigenic typing results obtained using the monoclonal antibodies corroborated the sequencing results reported by our group earlier. The cross neutralization study with polyclonal sera revealed that the sera of original antigenic type CPV 2 can neutralize the antigenic variants 2a and 2b effectively. Thus we conclude that the vaccines containing CPV type 2 virus can be used to immunise the dogs against the prevalent CPV 2a and CPV 2b infection. A live virus challenge study in dogs may further confirm this observation.

Keywords: Canine parvovirus; Isolation; Typing; Vaccine; Neutralisation; India

Introduction

During the 1970s, a new infectious disease of pups characterized by either gastro-enteritis or myocarditis was observed worldwide and the etiological agent was identified as canine parvovirus type 2 (CPV 2) (Appel *et al*., 1979; Burtonboy *et al*., 1979). Canine parvovirus is a highly contagious disease in dogs, characterized by hemorrhagic gastroenteritis, vomiting and high temperature. Though the disease can affect dogs of any age, the disease is often fatal in pups. The CPV type 2 virus underwent genetic and antigenic drift to become CPV type 2a, subsequently to type 2b and type 2c (Parrish *et al*., 1991; Buonavoglia *et al*., 2001; Nakamura *et al*., 2004). Incidence of the original CPV type 2 virus was not reported later and was replaced with the variant types (2a, 2b and 2c).

Most of the available vaccines contain attenuated CPV type 2.When *in vitro* cross neutralization experiments were performed using CPV 2 vaccinated sera against CPV type 2, 2a, 2b and 2c virus, the neutralization titers were significantly lesser with heterologous type virus (2a, 2b or 2c) compared to the homologous type 2 virus (Pratelli *et al*., 2001; Cavalli *et al*., 2008).Though the interference from maternal antibodies and low antibody titer were the common factors responsible for vaccine failure, antigenic variation between the available vaccine strains and the prevalent virus types was also indicated as a possible reason for vaccine failure (Decaro *et al*., 2008). However, Spibey *et al*. (2008) reported that the CPV type 2 protected vaccinated dogs against the experimental challenge with CPV type 2c. The present study was undertaken to evaluate the antigenic relationships of the

*Corresponding author: V.A. Srinivasan

E-mail address: vasrini@nddb.coop

Indian field canine parvoviral isolates with the CPV type 2 vaccine strain *in vitro*.

Materials and methods

Faecal samples of dogs suspected to have CPV infection were collected from seven states of India. Genetic and antigenic typing along with cross neutralization study was performed for all the isolates.
Clinical samples, Cells, sera and reference virus
Virus isolation was performed using faecal samples, which were collected from dogs having the clinical symptoms of gastro-enteritis. These samples were obtained from seven different states of India (Table 1). A72 cells were procured from American Type Culture Collection (ATCC) (University Boulevard, Manassas, VA 20110 USA), and the cell line was used for virus isolation. Type specific polyclonal sera were raised in CPV sero-negative dogs using CPV type 2 vaccine strain as well as inactivated antigens of CPV field isolates, P-24 (CPV 2a, Gene Bank Accession no - DQ182624) or P-25 (CPV 2b, Gene Bank Accession no - DQ182625) separately. Type 2 vaccine strain of the virus was used as positive controls in various assays described in this report.

Virus isolation in cell culture

Virus isolation was performed using A72 cells as per the method described by Pollock (1981) with some modifications. Briefly, the faecal samples were diluted (1:10 w/v) in chilled minimum essential medium (MEM) and homogenized using vortex mixture. The homogenate was centrifuged at 3000g for 15 minutes and treated with 0.1% (v/v) chloroform. Then, the samples were filtered through 0.2µ pore size syringe filter. An aliquot of 0.2 ml of the filtrate was used to inoculate the A72 cell monolayer in 25 cm^2 flask. The flasks were incubated at 37ºC and were observed for cytopathic effect (CPE) at 12 hourly intervals. Rounding and detachment of the cells were considered as the indication of CPE. When the CPE was more than 70%, the virus was harvested by freeze-thawing the cells. In the absence of any pronounced CPE, the culture filtrates were harvested five days post inoculation. All the samples were passaged in A72 cells for five times, irrespective of the presence of CPE. Samples showing no CPE after five passages were declared negative for virus.

Virus identification

Presence of CPV in the A72 culture harvest was identified by Haemagglutination (HA) test using porcine red blood cells (RBCs). The identification of the virus was further confirmed by haemagglutination inhibition test using CPV specific polyclonal dog sera, immuno-chromatographic strip test and ELISA.The HA test was performed as described by Pollock (1981) in 'U' bottom 96 well microtitrer plate (Laxbro, India). The HA titer was defined as the reciprocal of highest dilution that caused haemagglutination as indicated by mat formation. The tissue culture fluid of fifth passage harvest, which was positive for HA test with pig RBC was subjected to haemagglutination inhibition (HI) test using CPV vaccinated dog sera. The test was carried out as per the method described by Pollock (1981). The samples showing complete inhibition of haemagglutination activity (no mat formation) were declared as CPV positive.

Immuno-chromatography test kit, which detects all the CPV types (IngezimParvocromTM kit- INGENASA, Spain), was used to confirm the virus from A72 cell culture passages. The test was carried out as per the method described by the manu-

Table 1. Source of the clinical samples used in the study.

S. No.	State	Place	Type of sample	No. of samples
1	Maharashtra	Parbhani	Faecal	13
		Mumbai	Faecal	3
		Nagpur	Faecal	7
2	Gujarat	Anand	Faecal	1
3	Andhra Pradesh	Hyderabad	Faecal	12
4	Kerala	Trichur	Faecal	1
5	Uttar Pradesh	Mathura	Faecal	3
6	New Delhi	New Delhi	Faecal	3
7	Haryana	Ambala	Faecal	2

facturer. Briefly, a drop of tissue culture supernatant was used for the test. Appearance of a purple line in the test window no: 3 (control line) indicated the validity of test. Presence of CPV in the sample was indicated by the appearance of purple line in test window no: 2.

All the tissue culture fluid harvests from A72 cell passage were subjected to double antibody sandwich ELISA (DAS ELISA kit- INGENASA, Spain) for the identification of CPV. The test was carried out as per the method suggested by the manufacture. The absorbance of each well was recorded by a spectrophotometer at 405 nm. Samples with OD value higher than the cut off value were considered positive and samples with OD value lower than the cut off values were considered negative.

CPV typing using Polymerase chain reaction (PCR)

All the processed clinical samples and tissue culture fluid harvest from A72 cell passage were subjected to PCR. DNA extraction was carried out using DNAzol reagent (Invitrogen, USA) following the manufacturer's instructions. The CPV types were identified from the extracted total DNA using PCR. Two independent PCRs were performed to identify CPV2 and its variants. The primer pair P2s/P2as described by Senda *et al*. (1995) was used to identify CPV type 2 whereas primer pairs Pabs/Pabas described by Pereira *et al*. (2000) were used to identify the variant types. The samples which were positive in Pabs/Pabas PCR were again subjected to Pbs/Pbas PCR (Pereira *et al*., 2000) to identify the CPV type 2b virus.

Antigenic typing

Antigenic typing was carried out for all the CPV isolates and two reference vaccine strains of CPV Type 2 (strain 1 and strain 2) using a panel of four monoclonal antibodies (mAbs; kindly provided by Dr Y. Tohya, University of Tokyo, Bunkyo-ku, Tokyo). The HI test was carried out with the monoclonal antibodies against 8 HA units of tissue culture adapted CPV isolates and reference CPV type 2 virus. The comparative titers were evaluated for antigenic typing of the virus. Mouse monoclonal antibody 19D7 detects all the CPV types, mink enteritis virus and feline panleukopenia virus. The virus isolates, which showed high reactivity with mAbs 21C3, 20G4 and 2G5 were typed as CPV 2b. The virus isolates having high reactivity with mAbs 20G4 and 2G5 and less reactivity with mAb 21C3 were typed as CPV 2a. The CPV isolates that showed low reactivity with mAbs 20G4, 2G5 and 21C3 and high reactivity with mAb 19D7 were classified as CPV type 2.

Antigenic characterization by cross neutralization with specific polyclonal sera

Polyclonal dog sera against CPV type 2, type 2a and type 2b respectively were prepared as described above. All the virus isolates were subjected to cross reactivity test using the type specific polyclonal sera in a HI test. The test was carried out using 8 HA units of tissue culture adapted CPV isolates. The reciprocal of highest dilution of serum showing inhibition was considered as HI titer. The HI titer with homologous as well as heterologous virus was recorded.

Results

Virus isolation in cell culture

Virus isolation was attempted for 45 faecal samples collected from different parts of India. After the first passage in A72 cells, 27 out of 45 samples showed CPE and on subsequent passages only 23 samples showed distinct CPE such as rounding, development of multinucleated cells and detachment of the cells from monolayer. Similar kind of CPE was reported earlier also by Pollock (1981). The HA titer of these samples was ≤ 4 HA units on second passage onwards.

Identification of virus

Hemagglutination and HI test

All the 45 samples were subjected to HA test using porcine RBC's after every passage on A72 cells. Samples producing distinct CPE in cell culture also showed ≥4 HA units/50 µl of the sample. Out of the 45 samples processed for virus isolation, 27 samples showed titers ≥ 4 HA units per 50µl of tissue culture harvest on first passage (Table 2). The 23 isolates were passaged five times in A72 cells and HI test was performed on the culture super-

natant of the fifth passage material. The complete inhibition of haemagglutination activity of all 23 virus isolates was observed using CPV specific serum (CPV vaccinated dog sera). The HI titer values ranged between 128 and 1024 HI units (Table 2). Clear inhibition of HA activity of CPV specific serum against the virus isolates confirmed the identity of the isolates as CPV.

Immunochromatography (INGEZIM PARVO CROM kit) and Double antibody sandwich ELISA (DAS ELISA): The 23 CPV isolates were further confirmed in immuno-chromatographic strip test and double antibody sandwich ELISA (INGEZIM PARVO CROM kit and DAS ELISA kit). All the 23 isolates tested positive in both the assays and these results confirmed the isolation of CPV from clinical material (Table 2).

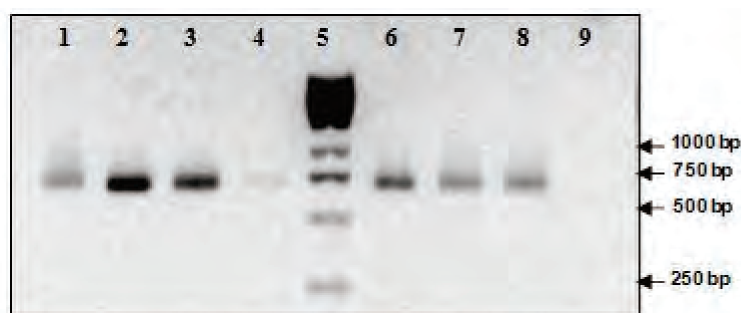

Fig. 1. Gel picture shows the results of PCR with the DNA from few of the tissue culture supernatants. The PCR was performed with Pabs and Pabas primers to identify the CPV 2 variants -CPV 2a and CPV-2b. 1. Isolate P-1; 2. Isolate P-5;3. Isolate P-8; 4. Isolate P-14; 5.1 Kb ladder; 6.Isolate P-17; 7. Isolate P-20; 8. Isolate P-25; 9. Negative control.

Table 2. Identification of CPV isolates using various laboratory tests.27 of the 45 clinical samples were positive by PCR. Of the 27 samples, virus isolation was successful for 23 samples. The isolates were confirmed using ELISA, immuno-chromatographic strip test and HI after five passages in A72 cell.

Isolate ID	After a single A72 cell passage		After five A72 cell passages		
	HA titer	PCR[a]	immuno-chromatographic strip test	ELISA	HI titer
P-1	8	Positive	Positive	Positive	512
P-2	4	Positive	Positive	Positive	512
P-3	4	Positive	Positive	Positive	512
P-4	16	Positive	Positive	Positive	256
P-5	4	Positive	Positive	Positive	1024
P-7	32	Positive	Positive	Positive	512
P-9	8	Positive	Positive	Positive	1024
P-10	8	Positive	Positive	Positive	512
P-11	8	Positive	Positive	Positive	128
P-12	16	Positive	Positive	Positive	256
P-13	8	Positive	Positive	Positive	256
P-14	4	Positive	Positive	Positive	512
P-15	8	Positive	Positive	Positive	512
P-17	4	Positive	Positive	Positive	256
P-18	4	Positive	Positive	Positive	256
P-19	4	Positive	Positive	Positive	512
P-20	8	Positive	Positive	Positive	1024
P-21	16	Positive	Positive	Positive	512
P-22	16	Positive	Positive	Positive	512
P-23	4	Positive	Positive	Positive	512
P-24	16	Positive	Positive	Positive	512
P-25	32	Positive	Positive	Positive	512
P-27	4	Positive	Positive	Positive	256
P-6	4	Positive	Positive	Positive	Not done
P-8	4	Positive	Positive	Positive	Not done
P-26	4	Positive	Positive	Positive	Not done
P-28	4	Positive	Positive	Positive	Not done

[a]All the 27 isolates were positive when the PCR was performed directly from the clinical material.

Virus identification and typing by polymerase chain reaction

All 45 clinical samples were screened in PCR using P2s/P2as and Pabs/Pabas primers. We observed that 27 out of 45 samples produced a band at 680 bp with Pabs/Pabas primers confirming the presence of CPV variant types in these clinical samples (Fig. 1). None of the clinical samples produced amplicons of designated size with P2s/P2as (Fig. 2). except CPV type 2 vaccine strains, which was used as positive control. Our results indicate the absence of CPV type 2 in the clinical samples; all the virus isolates were of variant types. Out of the 27 isolates only 23 samples turned positive when the PCR was conducted using culture supernatant from the fifth

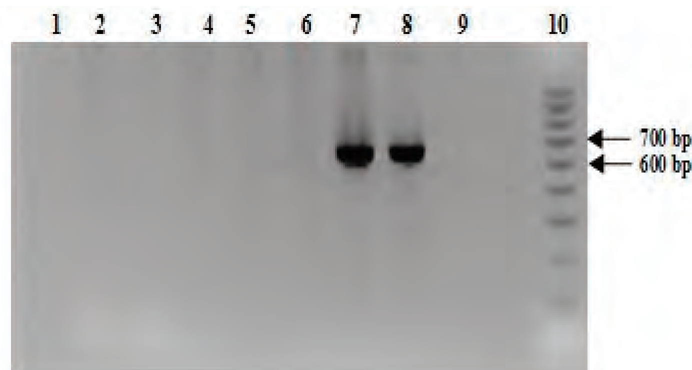

Fig. 2. Gel picture shows the results of PCR using the primer pairs P2s and P2as.This primer pair was used to identify the CPV type 2. None of the field isolates showed positive amplification and both the vaccine strains were positive in the PCR.1. Isolate P-1;2. Isolate P-5;3. Isolate P-8; 4. Isolate P-14; 5.Isolate P-17; 6. Isolate P-20; 7. CPV2 vaccine-strain 1; 8.CPV2 vaccine-strain 2; 9.Negative control; 10.100 bp ladder.

Table 3. Haemagglutination inhibition titersusing type specific monoclonal antibodies.Based on the HI titers, four of the 23 isolates were typed as CPV 2b and the remaining 19 isolates were typed as CPV 2a. The typing results using the mAbs matched 100% with that of sequencing results (genetic typing).

Isolate ID	Genetic Typing results[a]	HI units against each mAbs (specificity)				Antigenic Typing results
		19D7 (All types)	20G4 (2a, 2b, 2c)	2G5 (2a, 2b)	21C3 (2b)	
P1	CPV 2a	51200	25600	51200	400	CPV 2a
P2	CPV 2a	25600	12800	25600	1600	CPV 2a
P3	CPV 2a	25600	12800	25600	800	CPV 2a
P4	CPV 2a	12800	6400	12800	1600	CPV 2a
P5	CPV 2a	6400	6400	25600	800	CPV 2a
P7	CPV 2a	25600	12800	25600	400	CPV 2a
P9	CPV 2a	51200	25600	51200	1600	CPV 2a
P10	CPV 2a	25600	12800	25600	400	CPV 2a
P11	CPV 2a	6400	6400	12800	800	CPV 2a
P12	CPV 2a	25600	12800	25600	800	CPV 2a
P13	CPV 2a	6400	6400	12800	800	CPV 2a
P14	CPV 2a	25600	12800	25600	800	CPV 2a
P15	CPV 2a	51200	25600	51200	1600	CPV 2a
P17	CPV 2a	25600	12800	25600	400	CPV 2a
P18	CPV 2b	25600	12800	25600	25600	CPV 2b
P19	CPV 2a	25600	12800	25600	800	CPV 2a
P20	CPV 2b	12800	6400	12800	12800	CPV 2b
P21	CPV 2a	6400	6400	12800	800	CPV 2a
P22	CPV 2a	25600	12800	25600	800	CPV 2a
P23	CPV 2b	25600	12800	25600	12800	CPV 2b
P24	CPV 2a	51200	25600	51200	400	CPV 2a
P25	CPV 2b	102400	51200	102400	12800	CPV 2b
P27	CPV 2a	51200	25600	51200	1600	CPV 2a
CPV2 vaccine-strain 1	CPV 2	25600	200	800	100	CPV 2
CPV2 vaccine-strain 2	CPV 2	25600	100	800	Nil	CPV 2

[a]Chinchkar *et al.* (2006)

Table 4. Results of the haemagglutination inhibition test using polyclonal sera to study antigenic cross reactivity between CPV-2, CPV-2a and CPV-2b.

Virus	CPV Type	HI titer using the sera		
		P-24 (CPV-2a)	P-25 (CPV-2b)	CPV2 vaccine-strain 1
Isolate P2	2a	2560	1280	640
Isolate P4	2a	5120	2560	1280
Isolate P5	2a	2560	640	320
Isolate P12	2a	5120	2560	1280
Isolate P15	2a	1280	640	320
Isolate P23	2b	5120	2560	640
Isolate P24	2a	2560	640	320
Isolate P25	2b	2560	1280	640
CPV2 vaccine-strain 1	2	320	80	1280
CPV2 vaccine-strain 2	2	320	40	640

passage material. These 23 samples were subjected to PCR with CPV 2b specific primers (Pbs/Pbas) and few of the samples produced a PCR product of 420 bp. However, the 2b specific primers showed ambiguous results when the PCR was repeated.

Antigenic typing using a panel of monoclonal antibodies

For antigenic typing of the CPV isolates, a panel of four mAbs (19D7, 21C3, 20G4 and 2G5) were used. All 23 virus isolates showed equal reactivity with mAb 19D7, which confirmed the presence of CPV in the sample. The reactivity of all isolates was almost equal with mAbs 20G4 and 2G5, ranging from 6700 to 51200 HI titers. These two mAbs detects CPV variant types. The reactivity of four virus isolates was high with mAb 21C3 which detects CPV 2b specifically and the titers were ranging from 12800 to 25600 HI units. The other 19 isolates showed relatively low reactivity (400 to 1600 HI units) with mAb 21C3 and confirmed as type CPV 2a. Two reference canine parvovirus vaccine strains showed very low reactivity with mAbs 21C3, 20G4 and 2G5, whereas the reactivity of the vaccine strains was high using mAb 19D7. The results were summarized in Table 3. The antigenic typing results were on the same line of genetic typing (Chinchkar *et al*., 2006).

Antigenic characterization by cross neutralization with specific polyclonal sera

Cross neutralization of the virus isolates was performed *in vitro* with six CPV 2a isolates, two CPV 2b isolates and two reference CPV type 2 vaccine strains using mono-specific polyclonal serum against CPV type 2 (vaccine strain), type 2a (P24) and type 2b (P25). The results were summarized in Table 4. Monospecific polyclonal serum against CPV type 2 virus did not show any significant difference in reactivity with homologous (CPV type 2 strain 1 and strain 2) as well as heterologous (CPV 2a and CPV 2b) virus types. The HI titers were ranging from 320 to 1280 HI units. The HI titers of CPV 2a monospecific polyclonal serum against CPV 2a and CPV 2b virus isolates did not vary much (1280 to 5120 HI units). The reactivity of CPV 2b monospecific polyclonal serum against CPV 2a and CPV 2b virus isolates was also in a similar range (640 to 2560 HI units). However, the CPV 2a and CPV 2b monospecific polyclonal serum showed low reactivity against type 2 viruses and the titers were ranging from 40 to 320 HI units, whereas the reactivity of these sera with homologous virus were comparatively high (640 to 5120 HI units).

Discussion

Parvoviral enteritis is one of the fatal diseases affecting pups worldwide. The disease is being controlled using vaccines. The variant types of CPV2 have emerged across the world. Though the variant types replaced the type 2 virus, most of the commercial vaccines contain the attenuated CPV type 2 strains.

We were successful in isolating CPV from 23 of the faecal samples of dogs showing enteritis. All the isolates were verified by HA and HI using CPV

specific sera. The HA and HI tests are routinely used methods for identification of CPV (Appel *et al*., 1979; Helfer-Baker *et al*., 1980; Kramer *et al*., 1980; Pollock, 1981). The HI test is considered to be the confirmatory test for identification of CPV virus and it has been used extensively by many investigators (Appel *et al*., 1979; Carmichael *et al*., 1980; Meunier *et al*., 1981). The isolates were further confirmed by Immunochromatography strip test and DAS ELISA.

The isolates were also subjected to PCR amplification using type specific primers. All the 23 isolates could be detected using primers specific for CPV variant strains. None of the virus isolated from the clinical samples were of CPV type 2. Our results are in agreement with the finding that suggested disappearance of CPV 2 in clinical specimens and replacement with its variant types (Parrish *et al*., 1991; Truyen *et al*., 1996; Steinel *et al*., 1998; Chinchkar *et al*., 2006; Mohan Raj *et al*., 2010). However, the PCR with 2b specific primers produced inconclusive results. The reason for the ambiguous PCR results with 2b specific primers was reported by our group earlier with the VP2 gene sequencing (Chinchkar *et al*., 2006). The primers in the PCR to differentiate CPV 2a from CPV 2b are designed based on the nucleotide changes at position 4062 (A→G) and 4449 (A→G). In general, CPV 2a has nucleotide A at position 4449 and CPV 2b has G at 4449. However, all the Indian CPV isolates had G at 4449, irrespective of the type (Chinchkar *et al*., 2006). Therefore, the CPV 2a and CPV 2b isolates had only one base difference in between them, rendering the PCR based differentiation difficult. Our work indicated that the PCR primers (Pereira *et al*., 2000) are irrelevant in differentiating CPV 2a from CPV 2b of India (Martella *et al*., 2006; Hariprasad Naidu *et al*., 2012). Antigenic typing using a panel of monoclonal antibodies demonstrated that 19 of the isolates were CPV type 2a and 4 of them were type 2b. The antigenic typing results matched 100% with the genetic typing which was published earlier (Chinchkar *et al*., 2006).

Polyclonal serum against CPV type 2 virus reacted with homologous (CPV type 2 strain1 and strain 2) as well as heterologous (CPV 2a and CPV 2b) virus types equally. This is in contrast to the observation made by Pratelli *et al*. (2001) and Cavalli *et al*. (2008). They reported significantly lower HI titer values against variant type viruses for the type 2 vaccinated dog sera. The CPV 2a and CPV 2b polyclonal serum showed low reactivity against type 2 viruses. Cavalli *et al*. (2008) also reported significantly lower HI titer against CPV 2 compared to the variant types when the sera from CPV 2b vaccinated dogs were tested.

The *in vitro* cross neutralization studies revealed that the sera developed using CPV type 2 virus reacted with the antigenic variants also with the HI titers similar to that of homologous virus. Therefore, the virus type used in the evaluation of CPV 2 vaccine titer might not influence the HI titer; furthermore, any one of the virus types can be used to assess the CPV 2 vaccine titer. Though the earlier reports published using the *in vitro* neutralisation tests suggests that the CPV 2 vaccine has lesser neutralisation titers against the variant types (Pratelli *et al*., 2001; Cavalli *et al*., 2008), our study did not show any significance difference in the HI titers using the CPV2 immune sera. Moreover, Spibey *et al*. (2008) performed a virus challenge study in CPV2 vaccinated dogs to conclude that the vaccine based on CPV2 strains could protect the dogs against CPV type 2c. Tough the in vitro cross neutralization study reported in this work indicated that the current vaccine strain can protect against prevailing antigenic variant CPV types, this finding must be confirmed using animal challenge studies.

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