RESEARCH ARTICLE

Antigenic Relationship of Canine Parvovirus Type 2c with other Variants and Vaccine Strain using *In Vitro* Cross Neutralization Assay

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Abstract

Canine parvovirus-2 (CPV-2) is known to cause acute viral infection in domestic canines. Dogs of any age group may invariably be affected by CPV which can cause up to 100% morbidity and 10-91% mortality. The difference in amino acid sequences over the capsid protein VP2 of CPV-2 has resulted in the emergence of several variants, namely CPV-2a, CPV-2b, and CPV-2c. Despite the in-depth vaccination program adopted worldwide, CPV infections are one of the major causes of infection and death in young dogs. The recent trends of vaccine failure throughout the world have raised questions about the effectiveness of the old type vaccine (CPV-2 based) against all the antigenically different strains, especially CPV-2c. The present study aimed at establishing the antigenic relationship of CPV-2c and other variants by *in vitro* Cross Neutralization Assay. The results indicated that the serum of CPV 2c could neutralize the homologous type of virus at a much higher titre than the heterologous virus types. The vaccine strain also showed less titre against CPV-2c virus type.

Key words: Antigenic relationship, Canine Parvovirus type 2c, CPV-2 variant, Neutralization assay, Vaccine strain.

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Introduction

The Canine Parvovirus is a small DNA virus belonging to Parvoviridae family and known for high mortality and morbidity in canine population for number of years (Voorhees *et al.,* 2019). Since its occurrence in 1978; it is still continuously evolving, and is one of the most common pathogens of the canine population causing gastroenteritis and often myocarditis in young pups. The disease is characterized by foul-smelling diarrhoea, vomiting, and dehydration, which in extreme cases lead to shock and death. CPV-2 virus can affect canines irrespective of breed, sex, or age, however, young pups from 6 weeks to 6 months appear to be particularly susceptible.

Though CPV-2 is a DNA virus, its nucleotide substitution rate is similar to that of RNA viruses (Shackelton *et al*., 2005). Another troublesome fact associated with this virus is that despite its vaccine being included in the core vaccination program, cases are still being reported in both vaccinated as well as unvaccinated dogs (Singh *et al.,* 2021). There is apprehension that the old type of vaccines (CPV-2-based) may become less effective due to the antigenic changes from the currently circulating field strains, especially CPV-2c (Pratelli *et al*., 2001). The incidence of parvoviral illness, primarily brought on by CPV-2c, in dogs that had received CPV-2 vaccinations is being documented in an increasing number of case reports. Therefore, the present study was aimed to evaluating the antigenic relationship of CPV-2c with other prevalent variants in India and knowing the effectiveness of vaccine strain against CPV-2c.

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Materials and Methods

Ethical approval- IAEC permission vide number GADVASU/2021/IAEC/60/07 was taken for the work on laboratory animals.

The Madin Darby Canine Kidney (MDCK) cell line procured from the College of Animal Biotechnology, GADVASU, Ludhiana was used to produce virus in bulk. The processed fecal sample was used as virus inoculum for CPV 2c confirmed by PCR sequencing (Buonavoglia *et al.,* 2001). Approximately 50 mL of the virus was produced and later concentrated by ultracentrifugation at 1,00,000 *g* for 2 h at 4°C. The pellet was resuspended in 15 mL of PBS (pH 7.4) and aliquots of the virus were made and stored at -20°C till further use. The

Tissue Culture Infective Dose 50 ($TCID_{50}$) of the concentrated virus was estimated using the Reed and Muench method, TCID50 = Log of dilution above 50% + (Proportionate distance × dilution factor), where Proportionate distance = (CPE above 50%- 50)/(CPE above 50%- CPE below 50%).

The hyperimmune serum against the CPV-2c variant was raised in a male rabbit of one year of age belonging to a non-descript breed. Initial dose of 2.0 mL of the concentrated virus was injected intravenously and subsequent doses of 1.0 mL each through the intramuscular route were administered at weekly interval for three weeks. After checking the rabbit serum for the anti-CPV-2 antibodies by an indirect ELISA kit, Ingezim Parvo Canino 15.CPV.K1 kit (Eurofins Technologies), the rabbit was bled and serum was separated, and stored at -20°C for the cross-neutralization experiment.

β method of cross-neutralization assay, the method in which serial dilutions of serum are made and the virus is kept constant, was used for establishing the antigenic relationship of various variants of CPV with CPV-2c. For this, seven sets of serum-virus combinations were used and the same procedure was followed for each set. Serum was initially diluted to 1:10 and further two-fold serial dilutions of serum were prepared (1:2 to 1:32768) in PBS (pH 7.4). 100 $TCID_{50}$ for each of the four CPV types used. Sera against virus type CPV-2a, CPV-2b, and CPV-2 (vaccine type) and $TCID_{50}$ of the virus types CPV-2a, CPV-2b, and CPV-2 were already present in the Department of Veterinary Microbiology, GADVASU, Ludhiana. One mL of each dilution of serum was mixed with one mL of 100 $TCID_{50}$ of the virus, as per the combination set, and incubated for 2 h at 37°C and inoculated in monolayer of MDCK cells in 24-well cell culture plates. The 200 μl of each dilution of serum-virus combination was inoculated in the five wells of the cell culture plate. The three controls used in the assay were; virus control (100 $TCID_{50}$ of the respective CPV type), serum control (1:10 dilution of the serum type) and negative control (MM-DMEM). The plate was incubated for 1.5 h at 37°C with 5% CO_2. Later, the serum-virus mixture was discarded and 500 μl of maintenance media (MM-DMEM) was added and the plates were further incubated at 37°C with 5% CO_2. The plates were observed daily for cytopathic effects. The neutralization titre of the serum for the respective CPV type was calculated as the reciprocal of the greatest dilution of serum that did not demonstrate cytopathic effects.

Results and Discussion

The MDCK cells formed the complete monolayer within 48 to 72 h. The observations made post-subculture are shown in Table 1. This was similar to the study of Dorlikar *et al*. (2019) who also obtained a complete monolayer of MDCK cells within 48 to 72 h.

Scaled-up MDCK cells when infected in bulk exhibited peculiar cytopathic effect within three days. The observations recorded post-infection are shown in Table 2. A similar pattern was observed by Kumari *et al*. (2020) with rounding and aggregation of cells at 48 h and complete detachment by 72 h. Kaur *et al*. (2015) observed rounding of cells within 24 h, clumping within 48 h, and detachment by 72 h. Contrary to our study, Dorlikar *et al*. (2019) found a detachment of cells even after 96 h. PCR was used to check the supernatant before the cells were harvested on the third day.

The hyperimmune sera raised in rabbit using concentrated virus was successfully evaluated and verified by Indirect ELISA. Since rabbits are not a natural host of CPV-2 and might not have previously been «primed» by this virus, they were used to raise hyperimmune sera in order to elicit a monospecific serological response (Cavalli *et al*., 2008). The $TCID_{50}$ of the pure virus determined using the Reed and Muench formula came to be log $10^{3.67}$/mL. Therefore, 100 $TCID_{50}$ (log $10^{1.67}$/mL) was used in cross-neutralization assay. As this method needs values just above and below 50% for measuring the median dose, it is also possible to narrow the dose range (Ramakrishnan and Muthuchelvan, 2018). Cavalli *et al*. (2008) evaluated the antigenic relationships among the CPV-2 variants and revealed that the results were more appreciable by serum neutralization (SN) than by haemagglutination inhibition (HI). Pratelli *et al*. (2001) also concluded through their results that the genuine immunological status of dogs with regard to CPV may not always be adequately assessed

Table 1: Observations made post-subculture

Time after sub-culture	Observations
Day 0 (immediately after subculture)	Round cells, some in clumps floating in the media, few started adhering
Day 1 (24 h)	Cells adherent, dividing cells as evident by tailing morphology, shiny in appearance
Day 2 (48 h)	Adherent cells, epitheloid in shape, few areas of gap
Day 3 (72 h)	Healthy, shiny cells with no gaps, complete monolayer formed

Table 2: Observations made post-infection

Time after infection	Observations
Day 1 (24 h)	No apparent changes visible, except change in the morphology of cells a few places
Day 2 (48 h)	Change in the morphology of cells of an entire field, peculiar rounding of cells, cell clumping at few places
Day 3 (72 h)	Loss of cell morphology, extensive clumping and detachment of cells from the surface, cells seen floating in the media

Table 3: Titre of serum neutralizing the virus completely

	SET I	SET II	SET III	SET IV	SET V	SET VI	SET VII
Serum	CPV-2c	2c	2c	2c	2	2a	2b
Virus	CPV-2	2a	2b	2c	2c	2c	2c
Serum titre	4096	8192	8192	16384	2048	4096	4096

by the HI test. Therefore, the present study included the neutralization assay for evaluating the cross-protection among various variants of CPV-2.

The neutralization results showed that when compared to heterologous combinations, the homologous combinations or sets were found to have a considerably greater titre (Table 3). The highest dilution at which CPV-2c serum could neutralize CPV-2, CPV-2a, CPV-2b, and CPV-2c was 1:4096, 1: 8192, 1:8192, and 1:16384, respectively. The sera of CPV-2a and CPV-2b showed a similar neutralizing ability against the CPV-2c virus at 1: 4096, however, the vaccine strain neutralized the CPV-2c virus at 1: 2048 which was the lowest among all.

In the experimental studies conducted previously in our labs, the vaccine strain had shown similar lower neutralizing levels against the other heterologous variants too. The vaccine strain was found to neutralize both CPV-2a and CPV-2b at 1:2048 dilution, whereas the homologous set was neutralized at a higher titre of 8192. Hence, from the findings, it can be inferred that vaccine strain neutralizes the prevalent variants at a lower titre. Pratelli *et al*. (2001) also found out in their study that puppies given the CPV-2 vaccine had much greater neutralizing antibody titres to the homologous virus (CPV-2) than to the heterologous virus.

Though there were antigenic differences between the original CPV-2 and the variants, but there were also variances between CPV-2a, -2b, and -2c. The homologous sets showed higher protective values than the heterologous sets. These findings were similar to those produced by Cavalli *et al*. (2008) in reference to CPV-2b and other variants, wherein, with the CPV-2b vaccine the heterologous titres (against CPV-2a and -2c) were significantly lower than the homologous titre with CPV- 2b. However, their results showed an unusual pattern wherein the homologous sets with CPV-2c serum showed lower titre than the heterologous sets. But no such unusual pattern was observed in our study. On the contrary, the homologous set of CPV-2c serum and virus showed the highest dilution in the entire sets of experiments. Although these differences may not be accounted for the decreased protection against variants in dogs because antibody titres in young dogs appear to be significantly higher than the minimum levels required for protection against disease and infection after repeated immunizations but it is probable that these changes may allow young, unvaccinated pups to escape the limited antibody repertoire of maternal origin (Cavalli *et al*., 2008).

Conclusions

The present study clearly indicated the variations in the trend of neutralizing abilities of different variants of CPV-2 and found that the best protective or neutralizing potential is of CPV-2c. The most commonly used vaccine strain in our region is CPV-2, which showed the least potential in neutralizing heterologous variants, especially CPV-2c. Though the vaccine strain has been found to be within the protective range of titre but variations in the *in-vitro* and *in-vivo* conditions cannot be neglected. Perhaps, the efficacy of the current vaccine is further challenged by the different body conditions of the animal. Moreover, all of the CPV-2c positives found in the study were isolated from the vaccinated animals and this sits in accordance with the neutralization results where vaccine strain was found to be least effective against CPV-2c virus. But, the neutralizing abilities of CPV-2c serum against every heterologous variant were found to be higher than other variants used in the study supporting the hypothesis that this variant must be included in the vaccine being used to immunize pups against Canine Parvoviral infections.

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