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Study on antigenic relationship of Canine Parvovirus types with vaccine strain using *in-vitro* cross-neutralization assay

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ABSTRACT

Canine Parvovirus-2 causes hemorrhagic gastroenteritis in dogs and spreads rapidly in dog population. The virus sheds in large numbers in the feces and thus, feces serve as a source of infection. The disease condition has been complicated further due to emergence of variants over the years. The present study was conducted to know whether the antigenic types of CPV cross protect each other, cross neutralization assay was performed for vaccine strain (CPV 2) and other strains of virus CPV 2a and CPV 2b. Hyperimmune serum was successfully raised in rabbits for the vaccine strain and confirmed by Indirect ELISA. TCID50 was found to be 102.49/ml for CPV 2. Cross-neutralization assay was carried out using 100 TCID50 of virus types and two fold serial dilution of hyperimmune serum in various combinations of virus and serum types. The maximum dilution at which serum against CPV 2 type could neutralize CPV 2a and CPV 2b virus was 1:2048 and the dilution at which serum against CPV 2a could neutralize CPV 2 virus type was 1:4096. Similarly, the maximum dilution at which serum against CPV 2b could neutralize CPV 2 virus type was 1:4096. The titre of the serum CPV 2 for the homologous virus type is higher i.e. 8192 as compared to heterologous virus type.

Keywords: Canine Parvovirus types; vaccine strain; antigenic relationship; Cross neutralization assay; TCID50; hyperimmune serum

HIGHLIGHTS

- CPV causes haemorrhagic gastroenteritis
- Number of variants of virus
- Neutralization assay to analyse cross protection efficacy of variants

INTRODUCTION

Parvoviruses are smallest viruses of 23-28nm in diameter having non-segmented, single stranded DNA genome of 5200bp [1]. Genome codes for the structural and non-structural proteins namely VP1, VP2, VP3 and NS1 and NS2 respectively. The most part of the capsid is formed by VP2 and is the most important protein antigenically and immunogenically. There are continuous changes in the genome of CPV-2 due to the nucleotide substitutions. In the early 1970s, an antigenic type of CPV has aroused and that had spread to different regions of a country and different countries of a world within in short span of time. Then antigenic drift occurred in the genome of CPV 2 which led to the emergence of CPV 2a; a mutant strain of CPV. When compared to CPV type 2 original strain, CPV 2a strain had five substitutions in the sequence of VP2 capsid protein and the changes in the amino acid residues are at 87th position of a VP2 from methionine to leucine, change at 300th position from alanine to glycine, and change at 305th position from aspartate to tyrosine [2] and was known to cause infection in cats too [3]. A variant of CPV 2a; CPV 2b was recognized in 1984, and it differed in an antigenic epitope as a result of the substitution at 426th position of VP2 from asparagine to aspartate and at 555th position from isoleucine to valine [4]. The two strains CPV-2a and CPV-2b are still the most prevalent strains noticed in different breeds of dogs and cause severe enteritis in canine worldwide [2,3]. Thereafter in early 20th century, a new mutant CPV-2c which differed from CPV 2b was found in dogs in the regions of Italy. This mutant had a change in the amino acid at the 426th position of genome of CPV 2b from aspartic acid to glutamic acid. Later this mutant was found in various countries [5] Vietnam, Spain, United Kingdom, South America, North America, Portugal and India [6-8].

Due to minor changes in the amino acids in the genomes of feline panleucopenia virus, canine parvovirus and its antigenic types it has led to the difference in the antigenic features of the virus and change in its host range [3,9,10], the interactions with the cellular receptor, the transferrin protein [11-13] and the virulence [14]. Also, there is discussion among the researchers working on Parvovirus going on that the current vaccines against canine parvovirus infection may not elicit complete protection against these new antigenic types [15]. So the present study was carried out to elucidate the antigenic relationship of Canine Parvovirus types with vaccine strain and to establish its protective efficacy.

MATERIALS AND METHODS

Raising of hyperimmune sera in rabbit

A rabbit was purchased from the paradise Rabbit farm, Kurukshetra to raise the hyperimmune serum for the CPV-2 virus type which was considered as vaccine strain. The purchased rabbit was one year old, male and was of non-descript breed. The rabbit was kept at small animal house, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

The rabbit was injected with CPV-2 type using vaccine (Nobivac DHPPI, Intervet, Pvt. Ltd) for raising hyper immune serum. At first, 2.0 ml of vaccine was injected intramuscularly into the rabbit. Subsequently, three injections of vaccine 1ml each were given intravenously at weekly interval. Blood was collected from rabbit after one week of last injection of vaccine. The serum was separated from blood and kept at -20°C. The antibodies in the hyperimmune serum were confirmed by indirect ELISA using the INGEZIM PARVO CANINO 15.CPV.K1 Kit. The procedure was followed as per the manufacturer's instructions with few modifications, instead of anti-canine conjugate provided in the kit, we had used anti-rabbit conjugate which was available in the Department of Veterinary Microbiology, GADVASU, Ludhiana. After confirmation, the hyperimmune serum was used in in vitro cross neutralization assay.

Estimation of TCID50 of the purified virus (CPV 2)

The Tissue Culture Infective Dose 50 (TCID50) of the purified virus i.e. CPV 2 type available in the Department of Veterinary Microbiology, GADVASU, Ludhiana was calculated by Reed and Muench Formula.

Ten fold serial dilution of the virus isolate was used in madin darby canine kidney cells (MDCK cells) for the evaluation of TCID50. Two wells in the cell culture plate were designated as positive and negative control in which undiluted virus and maintenance media were added respectively. The plates were then incubated at 37°C with 5% CO2 and observed daily for development of cytopathic effects. The results were read when cytopathic effects were observed in positive control well.

In-Vitro cross neutralization test

In cross neutralization test virus was kept constant (100 TCID50) and two fold serial dilutions of serum were used. The following five sets of serum-virus combinations were used in the assay.

	SET I	SET II	SET III	SET IV	SET V
SERUM	CPV-2	2	2	2a	2b
VIRUS	CPV-2	2a	2b	2	2

The cross neutralization test was carried for three CPV types viz., CPV 2, CPV 2a and CPV 2b. The hyperimmune serum for CPV 2a and CPV 2b raised in rabbits and the 100 TCID 50 of the virus for CPV 2a and CPV 2b used in the experiment was available in the Department of Veterinary Microbiology, GADVASU, Ludhiana. For each set of serum-virus combination the following procedure was followed.

First the 1:10 dilution of hyper immune serum was two- fold serially diluted up to 1:16384. The 100 TCID 50 of the each virus type was prepared in PBS using the purified virus. Serum-virus mixtures were prepared by mixing 1.0ml of each dilution of serum with 1.0ml of the TCID50 of CPV virus type. These mixtures were incubated for 2 hours at 37°C. A 24-well cell culture plate with monolayer of MDCK cells was used for inoculation of serum-virus combination. Each serum-virus dilution for each dilution of serum was inoculated 0.2 ml in wells of the cell culture plate i.e. five wells per dilution of serum. The plates were incubated at 37°C with 5% CO2 for one and a half hour. After incubation the serum-virus mixture was discarded and 500µl of maintenance media was added in each well. The plates were incubated at 37°C with 5% CO2. Three controls were used in the experiment are virus control, serum control and negative control. The plates were observed daily for the development of cytopathic effects. The results were noted when cytopathic effects was observed in the virus control.

RESULTS AND DISCUSSION

To perform cross neutralization test among CPV types and vaccine strain hyperimmune serum was raised in rabbit using vaccine and confirmed by ELISA (Figure 1). The results were validated as OD 450 for positive control is higher than 1.2 and OD 450 for negative control is lower than 0.40.

Ratio S/P = OD of Sample/ OD of Postive Control = 3.6015/ 3.0625= 1.176

The titre was calculated using the formula; Y (titre) = 54 (e4x) = 54 (2.7182824*1.176) where e= 2.718282 and x= S/P ratio of sample.

Titre = 54 (2.7182824.704) = 54 x 110.387 = 5960.898

Figure 1. Confirmation of antibody titre of hyperimmune serum by Indirect ELISA.

Wells A and B: Positive control; Wells C and D: Negative control; Wells G and H: Hyperimmune serum to be tested.

Estimation of TCID50 of the purified virus (CPV 2)

The TCID50 of the CPV 2 virus was calculated by Reed and Muench formula (Table 1) in the 12 well cell culture plate. The CPV 2 isolate was available in the Department of Veterinary Microbiology, COVS, GADVASU. For this tenfold serial dilution of the virus was done and four dilutions were used 101 to 104. Each dilution of the virus was inoculated in 5 wells of MDCK cells to observe the CPE produced.

Table 1: $TCID_{50}$ for CPV 2

Dilutions	CPE positive	CPE negative	Cumulative Frequency		Ratio	Percentage
10^1	5	0	15	0	15/15	100%
10^2	5	0	10	0	10/10	100%
10^3	3	2	5	2	5/7	71.4%
10^4	2	3	2	5	2/7	28.5%

According to REED and MUENCH FORMULA:

$$\text{Proportionaldistance(E)} = \frac{\text{mortality above 50\% - 50}}{\text{Mortality above 50\% - below 50\%}} = \frac{71.4 - 50}{71.4 - 28.5} = 0.498$$

= Log of dilution above 50% + (e * dilution factor)
= 3 + (0.49*1) = 3 + 0.49 = 3.49
= 1 $TCID_{50}$ = log $10^{3.49}$/ 0.1ml
= 1 $TCID_{50}$ = log $10_{4.49}/_{1}$ ml = 100 $TCID_{50}$ = log $10^{2.49}$/ml
100$TCID_{50}$ for CPV 2a used was 10^3 /ml and 100 $TCID_{50}$ for CPV 2b used was log $10_{2.53}$/ml which was already done previously in Department of Veterinary Microbiology. TCID50 experiments using CPV have also been performed by various researchers [16-19] for different purposes.

Cross Neutralization Test

The results were noted when cytopathic effects were observed in the virus control for each set of serum-virus (Table 2). It was observed that the maximum dilution at which serum against CPV 2 type could neutralize CPV 2a and CPV 2b virus was 1:2048. The maximum dilution at which serum against CPV 2a could neutralize CPV 2 virus type was 1:4096. Similarly, the maximum dilution at which serum against CPV 2b could neutralize CPV 2 virus type was 1:4096 (Table 3). The titre of the serum is the reciprocal of the dilution of the serum. Therefore, the titre of the serum CPV 2 at which it could neutralize virus CPV 2a and CPV 2b was 2048. The titre of serum 2a at which it could neutralize virus CPV 2 was 4096 and the titre of the serum 2b at which it could neutralize virus CPV 2 was 4096. The titre of the serum CPV 2 for the homologous virus type was higher 8192.

Table 2: Depicting the maximum dilution of serum at which it can neutralize the CPV

Sets of serum-virus mixture	**Twofold serial dilutions of serum**				
	1:2 to 1:1024	**1:2048**	**1:4096**	**1:8192**	**1:16384**
SET I	**No CPE**				**CPE observed**
SET II	**No CPE**				
SET III	**No CPE**				
SET IV	**No CPE**				
SET V	**No CPE**				

Table 3: Titre of serum at which it neutralizes the virus completely

	SET I	SET II	SET III	SET IV	SET V
Serum	2	2	2	2a	2b
Virus	2	2a	2b	2	2
Titre of serum	8192	2048	2048	4096	4096

Neutralization experiments have been conducted by many researchers working on CPV for various purposes. For example, in an experiment conducted by Teramoto et al [20] to compare Enzyme-Linked Immunosorbent Assay, DNA Hybridization, Hemagglutination, and Electron Microscopy for detection of Canine Parvovirus infections, neutralization titers were also determined in the procedure by incubating antibodies diluted in minimal essential medium with 100 to 500 50% tissue culture infective doses of parvovirus. The neutralization titer was expressed as the highest dilution of antibody at which no infected cells were detected. Similarly, Reitzenstein et al [21] assessed the serological response to vaccination by quantification of CPV neutralizing antibodies. The neutralization titre was determined in a micro-titre serum neutralization test for the detection of neutralizing antibodies to CPV-2. The sera were tested in a SN test using dog kidney (DK) cells and 50 to 300 TCID50 of the CPV-2 as the reference virus. SN titers were calculated using the Spearman-Karber formula.

Further, in a study by Gamoh et al [22], an FPLV-based vaccine was able to cross-protect against a challenge with a virulent CPV-2b strain. According to Parrish et al [2] and Nakamura et al [23] using cross-neutralisation studies, the antigenic differences between FPLV vaccines and CPV-2 variants appear to be much more marked, reflecting the number of mutations scattered throughout the VP2 protein [24].

Cavalli et al [25] evaluated the antigenic relationships among the original canine parvovirus type 2 (CPV-2) and the variants CPV-2a, -2b, and -2c. Cross-antigenic evaluation revealed clear differences among the CPV variants, which were appreciable by serum neutralization (SN). Antigenic differences were found mostly between the original CPV-2 and the variants, but they were also observed among the variants CPV-2a, -2b, and -2c.

Cross-neutralization assay was done by Karman [26] random isolates of CPV 2a and CPV 2b were grown in bulk in MDCK cell line. The virus was then purified by ultracentrifugation and hyperimmune sera for both CPV 2a and CPV 2b was raised in Rabbits. After confirming the hyperimmune sera by indirect ELISA, 100TCID50 was calculated for both CPV 2a and CPV 2b to be used in cross-neutralisation assay. It was concluded that the titre of serum 2a at which it can neutralise CPV 2b was 4096 and the titre of serum 2b at which it could neutralise CPV 2a was 2048. The titre of both the serum for the homologous virus type was found to be higher i.e., 8192.

CONCLUSION

It can be concluded from the cross neutralization study conducted that homologous serum virus type was neutralized better as compared to heterologous type.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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