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Development and Standardization of Sandwich ELISA for the Detection of Canine Parvovirus Infection

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Abstract

Canine parvovirus (CPV) is a highly contagious viral pathogen affecting dogs aged 6-21 weeks primarily targeting rapidly dividing cells of GI tract, which leads to haemorrhagic gastroenteritis and symptoms such as fever, vomiting, in appetence and abdominal pain. Several variants include CPV-2, CPV-2b and CPV-2c. CPV can be prevented by timely and complete vaccination and can be treated effectively if timely diagnosis is done. It can be diagnosed by effective methods such as PCR, fecal ELISA, HA and HI, the most effective being ELISA and PCR. Therefore, the present research was aimed at developing and standardizing a Double Antibody Sandwich ELISA for the detection of CPV by using rabbit hyperimmune sera as primary antibody and mice hyperimmune sera as secondary antibody.

Keywords: Canine parvovirus; ELISA; Hyperimmune sera; PCR.

1. Introduction

Canine parvovirus (CPV) a highly contagious viral pathogen that affects dogs aged 6-21 weeks mainly and poses a significant threat to canine health worldwide. The virus primarily targets the rapidly dividing cells of gastrointestinal tract and can lead to severe gastroenteritis. CPV continues to be a top concern for both pet owners and veterinary experts due to its quick transmission and adaptability in the environment [1]. The effective therapy and prevention of disease depends on the quick and precise identification of CPV. To detect CPV infections, a variety of diagnostic techniques have been used, such as PCR, ELISA, virus isolation, and haemagglutination testing [2].

CPV-2 belongs to the Parvoviridae family and is a linear, single-stranded, negative-sense virus. Two structural proteins, VP-1 and VP-2, as well as two nonstructural proteins, NS-1 and NS-2, are encoded by the 5323 bases of single-stranded DNA that make up the genome (Reed *et al.*, 1988) In the 5323 base CPV genome, the VP-1 gene is found between 2285 and 4537 (2253 bases) and the VP-2 gene between 2783 and 4537 (1755 bases).

It has been discovered that dogs of all sexes, ages, and breeds are vulnerable to CPV-2 infection [3, 4]. During the acute phase of infection, the virus is shed in the feces of infected dogs (more than 10^9 virus particles/gram of feces), which is primary source of infection.

The virus can spread to people' hands, shoes, clothing, food bowls, and other objects, which can then infect dogs [5]. The virus spreads either directly or indirectly among the dog population and is quite stable in the environment, kennels, and veterinary offices.

Indirect ELISA can identify IgM antiviral antibodies, a sign of a recent CPV infection. In comparison to other serological tests like the HA test, dot-ELISA has also been proven to be more sensitive and specific [6]. To identify CPV antigen in faecal samples, a double antibody sandwich ELISA test was developed. Its sensitivity and specificity are 87% and 100%, respectively, as against 63% and 87% for the HA test [7]. The sensitivity and specificity of an ELISA is considered to be better after PCR. Therefore, this study was aimed to develop a double antibody sandwich ELISA to detect Canine Parvovirus infection and to make the results available in few hours, since all the kits used at present are imported and no indigenous kits are available.

2. Materials and Methods

The sandwich ELISA included two antibodies one raised in rabbit and other in mice. For this rabbit hyperimmune serum was available in the Department of Veterinary Microbiology, COVS, Guru Angad Dev

Veterinary and Animal Sciences University, Ludhiana. The mice hyperimmune serum was raised in this study using standard protocol with ethical approval under the protocol no GADVASU/2022/ IAEC/65/04 on 11 October 2022. The serum was separated from blood collected from mice and an indirect ELISA was performed by the guidelines mentioned to know the antibody titre.

2.1. Development and Validation of an ELISA for Canine Parvovirus Diagnosis

To standardize the ELISA, chemicals and reagents used were as given in Table 1.

Table-1. Solutions and chemicals used to develop sandwich ELISA

S. No.	Solution	Components	Quantity
I.	PBS (pH 7.4)	PBS tablet (SIGMA) Distilled water	1 tablet dissolved in 100 ml distilled water
II.	Coating Buffer 0.5 M Carbonate-bicarbonate buffer (pH 9.6)	Sodium Carbonate Sodium Bicarbonate Distilled water	1.59 gm 2.93 gm 1000 ml
III.	Blocking Buffer (to be made fresh)	5% Skimmed Milk Powder, Or 3% Bovine Serum Albumin (BSA)	5 gm skimmed milk powder in 100 ml Distilled Water 3 gm BSA in 100 ml PBS
IV.	Substrate Solution (to be made fresh)	1. OPD (o-phenylenediamine dihydrochloride) 6% H_2O_2 (Hydrogen Peroxide) Substrate solution (Phosphate-Citrate Buffer) 2. TMB solution (3,3',5,5'-Tetramethylbenzidine)	4 gm 2 µl 10 ml Procured from ThermoFisher should come in front of TMB solution
V.	0.05 M Phoshpahte-Citrate Buffer (to be made fresh)	a) $Na_2HPO_4, 2H_2O$ b) Citric Acid (hydrous)	0.93 g in 26 ml distilled water 0.53 g in 25 ml distilled water 25.7 ml of a. + 24.3 ml of b. + 50 ml of distilled water were mixed and pH adjusted to 5.0
VI.	Stopping Solution (3 M H_2SO_4)	Conc. H_2SO_4 Distilled Water	294 ml 1000 ml
VII.	Washing Buffer	10X Washing solution (PBS-Tween-20) (TAKARA-BIO) Distilled Water	5 ml 95 ml

2.2. Standardisation of Antibody

2.2.1. Dilutions of Antibodies from 1:250 to 1:16000

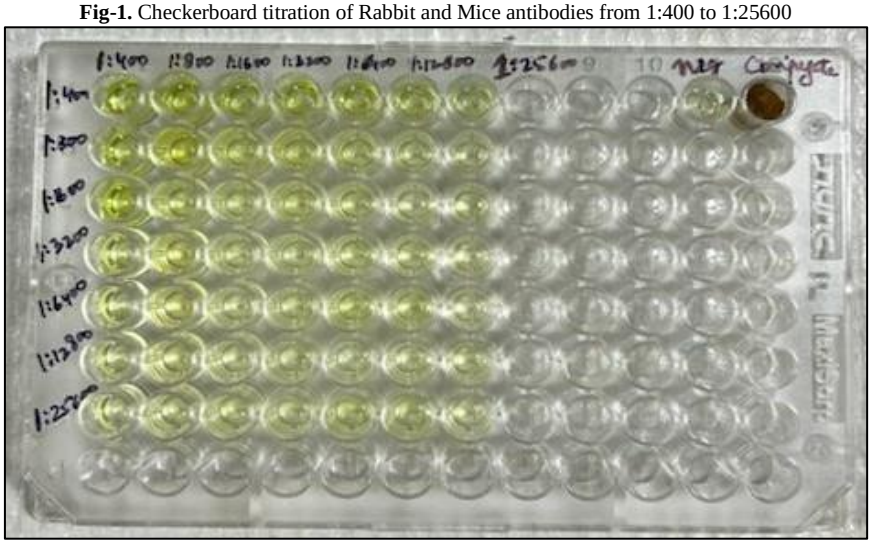
Rabbit and mice hyperimmune sera were used for checkerboard titration to standardize the dilution of each antibody. The antibody titre for both CPV 2a and CPV 2b was calculated by indirect ELISA and was found to be 99606.24 and 86940.95, respectively Karman [8]. CPV 2a and CPV 2b available in the department of veterinary microbiology were used as the antigen in the ELISA. $100TCID_{50}$ for both CPV 2a and 2b was calculated to be 10^3/mL and $10^{2.53}$/mL Karman [8].

The rabbit hyperimmune sera was standardized along the vertical axis of ELISA plate as one row would be for CPV 2a (positive control) and the second row would be for negative sample (fecal sample from healthy dog), while the mice hyperimmune sera was standardized in single wells along the horizontal axis of ELISA plate.

For 1:250 dilution, 2.49 mL coating buffer was added in all eppendorfs. 10 µL of rabbit serum was added to first tube, mixed and 10 µL of 1:250 dilution was added in 1:500 and so on. 10 µL of 1:16000 dilution was discarded in the end 50 µL of each dilution was added in duplicate wells, except the last eight wells of H row, which were left for the blank and conjugate control. The plate was sealed and incubated at 37° C for 6 hrs. After that washing was done 3 times washing buffer 300 µL/well. 200 µL of blocking buffer was added in each well (except for the conjugate control wells), plate sealed and incubated at 37° C for 2 hrs. Again plates were washed three times and then the antigen (CPV 2a) was diluted in blocking buffer in the ratio 1:2 and 50 µL/well was added. 50 µL PBS was added in four wells kept as blank. The plate was sealed and incubated at 37° C for 2 hrs and then washed three times.

Now, the dilutions for mice hyperimmune sera were made in blocking buffer similar to rabbit hyperimmune sera and 50 µL of each dilution was added in the wells (except conjugate wells). The plate was sealed, incubated for 2 hrs at 37° C and washed three times. Now, anti-mice conjugate with HRP was diluted in blocking buffer in the ratio 1:5000 and added 50 µL/well, sealed and incubated for 2 hrs at 37° C. Washing was done three times and 50 µL TMB substrate solution was added in each well and kept for 10 min at room temperature. Reaction was stopped by adding 50 µL/well stopping solution and the plate was read at 450 nm on ELISA reader (Thermo Fisher). All possible dilutions were assessed from 1:50 to 1:250 and 1:400 to 1:25600 (Fig. 1).

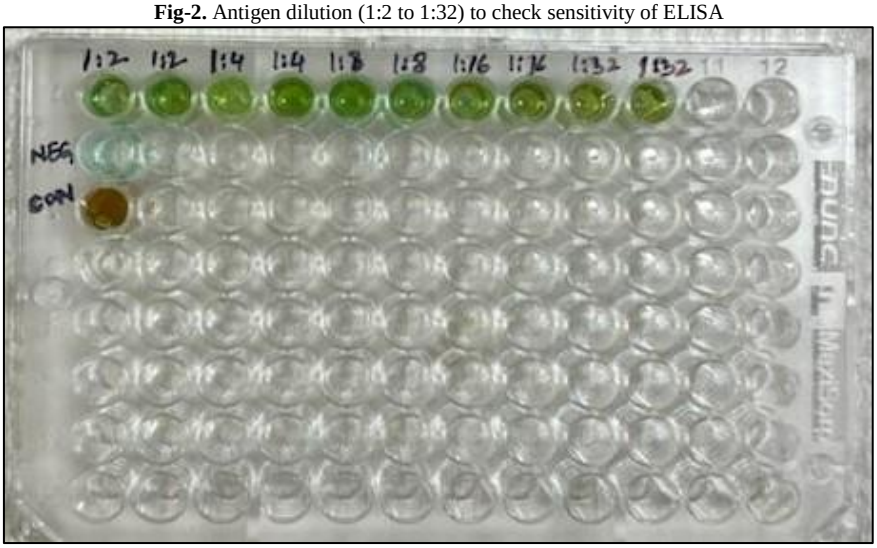
Fig-1. Checkerboard titration of Rabbit and Mice antibodies from 1:400 to 1:25600



2.3. Standardisation of Antigen

For standardizing the antigen, we used 1:250 dilution of rabbit hyperimmune sera and 1:50 dilution of mice hyperimmune sera. Dilutions were assessed from 1:2 to 1:32 (Fig. 2) and 1:64 to 1:1024 and the steps for ELISA were followed as mentioned above.

Fig-2. Antigen dilution (1:2 to 1:32) to check sensitivity of ELISA



2.4. To decide the Primary and Secondary Antibody

ELISA was performed to compare amongst using rabbit and mice hyperimmune sera (HIS) as primary/capture antibody wherein anti-rabbit conjugate and anti-mice conjugate were used, respectively. The ELISA was performed following the same procedure using 1:250 dilution of rabbit HIS, 1:50 dilution of mice HIS and 1:32 dilution of the antigen as shown in flow charts A and B.

A.

Substrate (OPD)
↑
Conjugate (Anti-rabbit)
↑
1:250 dilution of antibody (Rabbit HIS)
↑
Antigen (1:32 CPV 2a)
↑
Coating with 1:50 dilution of antibody (Mice HIS)

B.

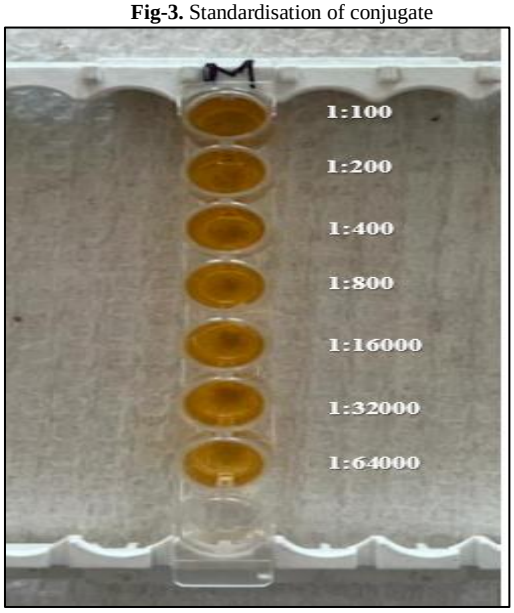
Substrate (OPD)
↑
Conjugate (Anti-Mice)
↑
1:50 dilution of antibody (Mice HIS)
↑

Antigen (1:32 CPV 2a)
↑
Coating with 1:250 dilution of antibody (Rabbit HIS)

2.5. Standardisation of Conjugate

After standardization of antigen and antibody, we standardized the conjugate as well. The dilutions used for the standardization of conjugate ranged from 1:1000 to 1:64000 (Fig. 3). The substrate used for this experiment was OPD.

Fig-3. Standardisation of conjugate



3. Results

3.1. Calculating Antibody Titre of Mice Hyperimmune Serum (raised) by Indirect ELISA

The antibody titre of the sample was calculated as:

Y (titre) = 54 (e^{4x})

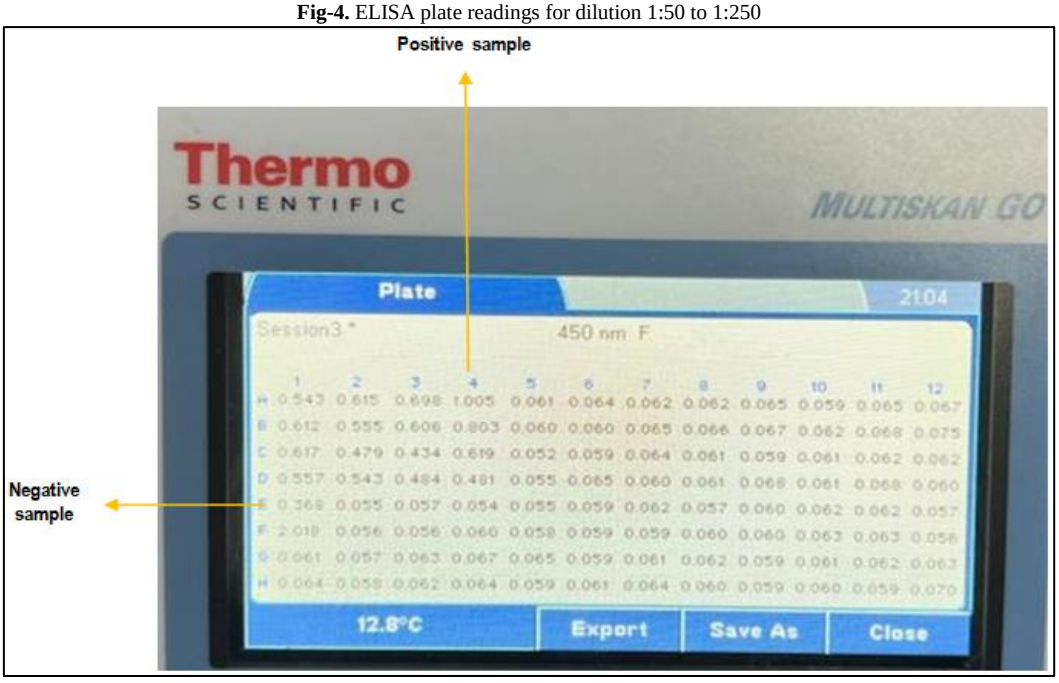
= 54 ($2.718282^{4*1.654}$)

Y= 40,335.354

3.2. Development of ELISA

3.2.1. Standardisation of Antibody

Fig-4. ELISA plate readings for dilution 1:50 to 1:250

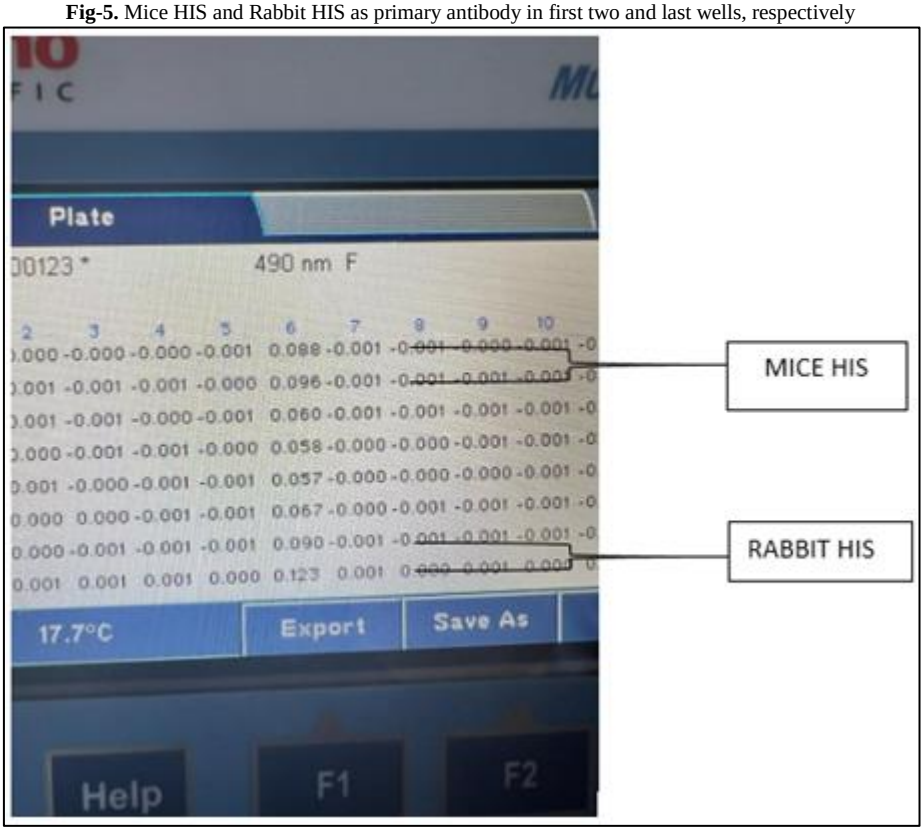


According to these results, the best results were found at dilution 1:250 for rabbit hyperimmune sera and at dilution 1:50 for mice hyperimmune sera *i.e.* Positive sample - 1.005, Negative sample - 0.368 (Fig. 4). Therefore, the antibodies were standardized at dilutions, 1:250 for rabbit hyperimmune sera and 1:50 for mice hyperimmune sera.

3.2.2.1. To Decide the Primary and Secondary Antibody

The antigen used was at the dilution 0f 1:2 in all experiments. Rabbit HIS and Mice HIS were both used as primary antibody in two experiments performed simultaneously. Results of the indirect ELISA were as follows:

Fig-5. Mice HIS and Rabbit HIS as primary antibody in first two and last wells, respectively



Readings for Mice HIS - 0.088 & 0.096; Readings for Rabbit HIS - 0.090 & 0.123 (Fig.5). Rabbit HIS was therefore used as the primary antibody.

3.3. Standardisation of Conjugate

A range of dilutions from 1:1000 to 1:64000 was chosen to standardize the conjugate and all the dilutions from 1:1000 to 1:64000 gave similar readings with very little difference, therefore, we chose the median dilution and set 1:5000 dilution as the standard for all the experiments.

The indigenous ELISA kits for detection of Canine Parvovirus in dogs are not available and the commercial kits that are available are expensive and per sample cost goes very high for the dog owners. Therefore, an attempt was made to standardize an ELISA for the detection of CPV in dogs which can detect large number of samples at one time and could be cheaper for dog owners.

4. Conclusions

Canine Parvovirus causing gastroenteritis in dogs is highly prevalent in dog population and is also responsible for heavy mortality in young dogs. Numbers of diagnostic tests are available for CPV, but indigenous ELISA for detection of CPV antigen in the rectal swabs of dogs is not available. The study was undertaken to develop a Sandwich ELISA for detection of Canine Parvovirus in dogs. For this rabbit hyperimmune serum was used as primary antibody at the dilution of 1:250 and mice hyperimmune serum was used as secondary antibody at the dilution of 1:50. Mice hyperimmune serum was raised in the present study. Various dilutions of hyperimmune serum were used and the dilutions showing best results were selected for the ELISA. Similarly further standardizing the ELISA antigen dilution, conjugate dilution, blocking buffer selection were all standardized.

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Conflict of Interest: None

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