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Research Article

A COMPARATIVE ASSESSMENT OF HAEMAGGLUTINATION ASSAY AND POLYMERASE CHAIN REACTION IN DETECTING CANINE PARVOVIRUS FROM FECAL SAMPLES

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ABSTRACT: The present study was aimed to lay the basis for a comparative study of the diagnostic tests namely haemagglutination assay and polymerase chain reaction for their efficacy in detecting the Canine parvovirus (CPV-2) from fecal samples. A total of 342 samples (vaccinated 61 and unvaccinated 281) were used for the comparative assessment of the diagnostic assays. Out of 342 samples tested for haemagglutination assay, only 71 were positive and they were further confirmed by haemagglutination inhibition assay. Polymerase chain reaction detected 234 samples positive for Canine parvovirus indicating that PCR is more efficient than haemagglutinating test in detecting the parvovirus from the fecal samples.

Key words: Canine parvovirus, Polymerase chain reaction, Haemagglutination assay.

INTRODUCTION

Viral infections in canines are dreadful and one among them is caused by the enteric virus, Canine parvovirus (CPV-2), the smallest single stranded DNA virus next to Circovirus, belonging to the Parvoviridae family. CPV-2 emerged as a new virus in the year 1978, later spread worldwide and was responsible for severe enteritis in dogs (Appel 1979, Hoelzer 2010). Infected pups succumb to death very early once the myocardium is affected and when supportive treatment is not given in time. Early detection and differential diagnosis of the causative agent plays a vital role in giving an effective treatment and protecting the dogs from CPV-2 infection (Hirasawa *et al.*1994). Various methods, both conventional and molecular assays are quite useful in the detection of virus but with varying sensitivity and specificity (Nandi *et al.* 2019). Hence, the present study was taken as a comparative study between polymerase chain reaction and haemagglutination assay (HA) for their efficacy in detection the virions from fecal samples of CPV.

MATERIALS AND METHODS

Collection of fecal samples

In the present study, were collected for a period of one and half year during the months of June 2017 to December 2018 from dogs suffering with diarrhoea and vomition, suspected for CPV infection. A total of 342 samples (vaccinated 61 and unvaccinated 281) were collected from Super Specialty Veterinary Hospital, Vijayawada, Teaching Veterinary Clinical Complex, NTR College of Veterinary Science, Gannavaram, College of Veterinary Science, Tirupati and from various Veterinary Polyclinics across different districts of Andhra Pradesh. The fecal samples were collected in the form of a rectal swab using pre sterilized swabs containing PBS, neatly labeled and immediately transferred to - 20°C till further processing. The details of the samples collected are presented in Table 1.

Haemagglutination test

The haemagglutination test was done as per the method described by Carmichael (1980).The fecal samples / rectal swabs obtained from the suspected dogs were emulsified in 1 ml of 0.2M PBS of pH 7.4 and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was collected and stored at - 80°C until further use.

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Chloroform treatment of the clinical samples

Ninety microlitre of processed fecal sample was treated with 10ìl of chloroform and mixed well. The mixture was kept at 4°C for 10 min and centrifuged at 10000 rpm at 4°C for 10 min. The supernatant was collected and used for haemagglutination test.

Preparation of swine RBC

Packed pig erythrocytes of 0.8 ml were suspended in 100 ml of 0.2M Sorenson's phosphate buffered saline of pH 7.0, after three washings with the same solution.

Protocol

Two-fold serial dilutions of 50 µl of the chloroform treated samples were made in 0.2M Sorenson's PBS of pH 7.0 in a 96 well 'U' bottom microtitre plates. To each well, 50 µl of 0.8 percent pig erythrocytes was added, mixed gently and allowed to settle at 4°C for 4 h. One well, added with 50 µl of 0.2 M Sorenson's PBS of pH 7.0 and 50 µl of 0.8 percent pig erythrocytes, served as RBC control. The highest dilution of the sample showing complete haemagglutination was considered as the haemagglutination titer.

Raising of Canine parvovirus hyper-immune serum

Hyper-immune serum against CPV was raised in adult male rabbits and the protocol was approved by Institutional Animal Ethics Committee (IAEC) with No. 6/IAEC/NTR CVSc./2018 dated 1/9/18. Hyper immune serum against CPV was raised as per Deepa and Saseendranath (2000) by concentrating the CPV infected MDCK cell culture fluid with saturated ammonium sulphate. A healthy adult rabbit was injected intramuscularly with a suspension of 50 ml of concentrated CPV diluted in 450 ml of PBS mixed with 500 ml of Freund's complete adjuvant (FCA). Another two injections of the freshly prepared antigen were given intramuscularly at an interval of 7 days with Freund's incomplete adjuvant (FIA). About 7 ml of blood was collected from the heart of the rabbit after seven days of the last injection and the serum was separated. The hyper-immune serum was inactivated at 56°C for 30 min in a water bath. The serum was centrifuged at 3000 rpm for 5 min and tested for CPV antibodies by Haemagglutination inhibition test using CPV standard antigen.

The tested serum was used for the confirmation of CPV antigen. The serum was stored at - 80^0C.

Confirmation of CPV in fecal samples by Haemagglutination Inhibition (HI) Test

The CPV positive fecal samples confirmed by HA test were further confirmed by HI as per the method described by Carmichael (1980).

Test procedure

Two fold serial dilutions of 50 µl of the serum was made in 0.2M Sorenson's PBS of pH 7.0 in a 96 well 'U' bottom microtiter plates. To each well, 50 µl of 4 HA units of fecal sample to be tested was added. Later, to each well 50 µl of 0.8 percent pig erythrocytes was added, mixed gently and allowed to settle at 4°C for 2 hrs. One well, added with 50 µl of 0.2 M Sorenson's PBS of pH 7.0 and 50 µl of 0.8 percent pig erythrocytes, served as RBC control. As virus control, one well was added with 50 µl of 0.2 M Sorenson's PBS of pH 7.0, 50 µl of CPV antigen and 50 µl of 0.8 percent pig erythrocytes. As serum control, 50 µl of 0.2 M Sorenson's PBS of pH 7.0, 50 µl of CPV antigen, 50 µl of positive serum and 50 µl of 0.8 percent pig erythrocytes.

Detection of CPV by Polymerase Chain Reaction targeting VP2 gene

DNA extraction from prepared fecal samples

The DNA was isolated from fecal samples as per Vieira *et al.* (2008). The samples were boiled at 96ºC for 10 min and immediately chilled in crushed ice (Schunck *et al*.1995, Uwatoko *et al*.1995 and Decaro *et al*. 2005b). Then the samples were centrifuged at 12,000 × g for 10 min at 4°C. The supernatants were diluted 1:5 in distilled water to reduce residual inhibitors of DNA polymerase activity (Decaro *et al.* 2006a) and 100µl was used as template DNA for PCR. The concentration of DNA was measured with Nanodrop 200C (Thermoscientific, USA) at 260/280 Å. The PCR was put up using 2X master mix (Gotaq green) with the following conditions in a thermocycler.

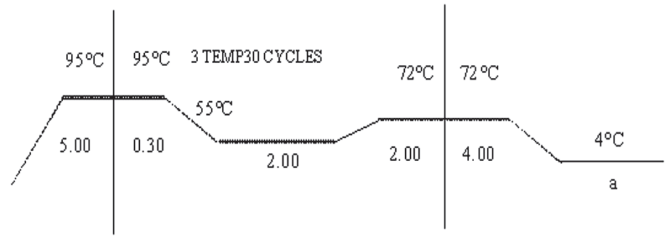

Table 1. Details of the fecal samples collected from various districts of Andhra Pradesh.

S.No	Place of Collection	Number of Samples	Vaccination status	
			Vaccinated	Unvaccinated
1	Srikakulam	14	1	13
2	Visakhapatnam	26	5	21
3	East Godavari (Kakinada, Rajamundry)	52	11	41
4	West Godavari (Tanuku)	13	2	11
5	Krishna*	59	7	52
6	Guntur	44	9	35
7	Nellore	51	11	40
8	Tirupati	26	6	20
9	Kadapa	14	3	11
10	Kurnool	43	6	37
	Total	342	61	281

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Table 2. Primers used for CPV by Polymerase Chain Reaction.

Forward and reverse primers	Primer sequence 5'-3' direction	Amplicon size	Position of the genome	Reference
CPV-2ab(F) CPV- 2ab(R)	GAAGAGTGGTTGTAAATAATT CCTATATAACCAAAGTTAGTAC	681 bp	3025-3045 3685-3706	Senda *et al*. (1995)
555 (F) 555 (R)	AGGAAGATATCCAGAAGGA GGTGCTAGTTGATATGTAATAAACA	583 bp	4003-4022 4585-4561	Buonavoglia *et al*. (2001)

RESULTS AND DISCUSSION

Screening of fecal samples by Haemagglutination test

A total of 342 clinical fecal samples from diarrheic dogs for Canine parvovirus and commercially available vaccine for CPV (CANIGEN) as a positive control were included in the present study and processed for virus detection, isolation and molecular characterization. As a preliminary test, HA was carried out for all the 342 samples of which 71(20.76%) samples agglutinated pig RBC with HA titers ranging from 1 in 32 to 1 in 512 and hence considered positive for the presence of CPV (Fig. 1). Fecal samples with < 1 in 32 are considered to be negative. Out of 71 HA positive, 23 samples were highly positive (HA titer 1:64 and above) and 48 samples were weakly positive (HA titer 1:32). Eighty-one fecal samples had a titer ranging from 1 in 2 to 1 in 16 and remaining 190 could not haemagglutinate the swine RBC.

Detection of CPV by Polymerase Chain Reaction targeting the VP2 gene

Out of 342 samples, 233 producing an amplicon product size of 681 bp with CPV-2ab primer (Fig. 2) and one sample with $555_{for}/555_{rev}$ primer produced a product size of 583 bp. The details of positive samples obtained are presented in Table 3. The vaccine strain also reacted with the primers specific to VP2 gene. The size of the PCR products specific to partial VP2 gene by 1.5% electrophoresis was 681 bp. The fecal sample from a healthy dog was used as negative control and was unresponsive to primer pair.

CPV causes severe gastroenteritis in dogs of all age groups characterized by bloody diarrhea, foul odor, volition, dehydration and finally leading to death if associated with myocarditis (Miranda and Thompson 2016). An easy method and early detection of CPV could be detected by performing heamagglutination test and later it can be further confirmed by molecular based VP2 gene detection using PCR.

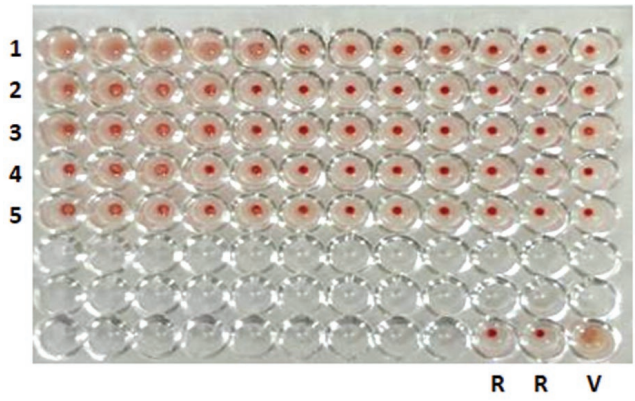

Fig. 1. Preliminary screening of fecal samples by Haemagglutination assay.
[1-5 = Fecal samples, 1 = HA titer 1 in 32, 2 – 5 = HA titer 1 in 16, R = RBC cell control, V = Virus control].

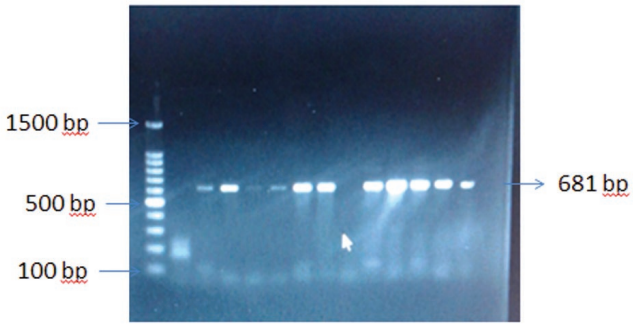

Fig. 2. Polymerase chain reaction for the confirmation of CPV in fecal samples.

Haemagglutination assay is considered as simple, sensitive, relatively inexpensive and easy to perform (Reddy *et al.* 2015). Hence, HA test was used for the preliminary detection of CPV in suspected samples. The test was considered positive and specific when the titer was 1 in 32 and above (Carmichael 1980). In the present study, fecal samples were initially screened by haemagglutination assay using 0.8 % swine RBC for the detection of virus. Out of 342 fecal samples from clinical cases, only 71 (20.76%) were positive with a titer varying from 1 in 32 to 1 in 512. The probable reasons for the low sensitivity of the HA test might be due to the pre-binding or sequestering of the CPV virions to the maternally derived antibodies present in the intestinal lumen thus preventing the haemagglutination reaction (Decaro *et al.* 2006b). Haemagglutinating activity in the fecal samples usually ceases 7 to 9 days post infection and less virions voided in the feces could not produce optimum HA reaction (Nandi and Kumar 2010) and naturally some CPV-2 strains lack the haemagglutination protein making the virus negative for HA test (Cavalli *et al.* 2001). Similar findings of low sensitivity of HA were also reported by Raj *et al.* (2010) and Parthiban *et al.* (2011). Akbar *et al.* (2015) could detect only 35 out of 50 samples by HA in which 30 % of the samples presenting bloody diarrhea were negative for HA. Fatima *et al.* (2017) reported that on simultaneous detection of CPV by HA and PCR, 30 % (30/50) and 38% (19/50) were positive, respectively. It was confirmed that HA was just a preliminary screening because of its less cost and

Table 3. Comparative assessment of PCR and HA of Canine parvovirus in different districts of Andhra Pradesh.

Sl. No.	District	Samples tested (Nos.)	Samples positive	
			PCR assay	HA test
1	Srikakulam	14	10 (71.42 %)	4 (28.57 %)
2	Visakhapatnam	26	20 (76.92%)	5 (20.00 %)
3	East Godavari (Kakinada, Rajamundry)	52	49 (94.23 %)	21 (40.38 %)
4	West Godavari (Tanuku, Eluru)	13	10 (76.92 %)	4 (21.05 %)
5	Krishna*	59	39 (66.10 %)	9 (16.66 %)
6	Guntur	44	35 (79.54 %)	17 (38.63 %)
7	Nellore	51	9 (17.64 %)	2 (3.92 %)
8	Tirupati	26	15 (57.69 %)	6 (23.07 %)
9	Kadapa	14	12 (85.71 %)	1 (7.14 %)
10	Kurnool	43	35 (81.39%)	4 (9.30 %)
	TOTAL	342	234 (68.42%)	71 (20.76%)

* Teaching Veterinary Clinical Complex, NTR CVSc., Gannavaram and Super Speciality Veterinary Hospital, Vijayawada.

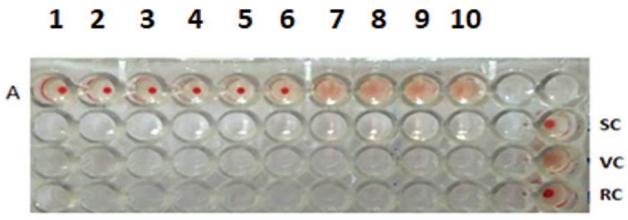

Fig. 3. Haemagglutination Inhibition assay of the hyper-immune serum.
[A = Hyper-immune serum – titer 1 in 64, SC = Positive serum control, VC = Virus control, RC = RBC control].

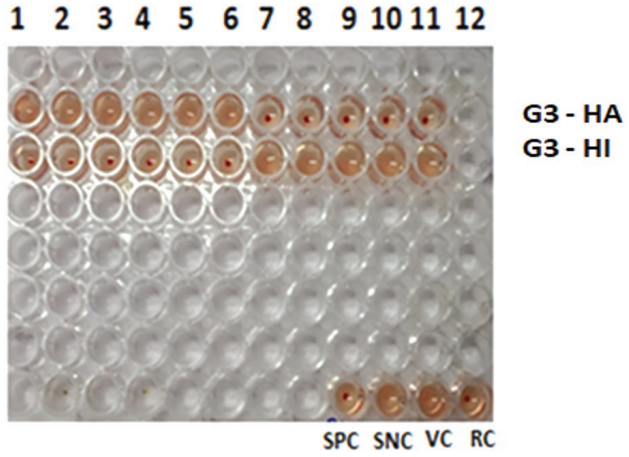

Fig. 4. Confirmation of CPV by Haemagglutination inhibition assay.
[G3-HI = Haemagglutination test 1 in 64, G3HA = Haemagglutination inhibition test 1 in 64, SPC = Serum positive control, VC = Virus control, RC = RBC control].

rapid results but negative results from HA test should be confirmed by molecular methods. Though HA test is simple, inexpensive and easy to perform, it has few disadvantages like continuous source of good quality of swine RBC and there is a need to monitor the specificity of the HA test by HI test for confirmation.

Confirmation of HA positive CPV samples by HI test

Of 342 suspected fecal samples screened by HA test, 71(20.76%) had a HA titer of 1 in 32 and above and hence were considered positive for the presence of CPV. The results of the haemaggluitnation test were further confirmed by performing haemaggluitnation inhibition test using hyper-immune serum raised in rabbits to avoid non-specific haemagglutinins which lead to false positive results (Carmichael *et al.*1980) .The haemaggluitnating activities of all the 71 samples were specifically inhibited in the HI test showing 100 % positivity (Fig. 4) and similar results were reported by Rai *et al.* (2004), Raj *et al.* (2010) and Reddy *et al*. (2015). Haemaggluitnation test followed by haemaggluitnation inhibition was found to be simple, economical, convenient and confirmatory method for the screening of fecal samples for the presence of CPV (Raj *et al.* 2010). The haemaagglutination inhibition titer of the hyper-immune serum was estimated to be 1 in 64 (Fig. 3).

Detection of CPV by PCR targeting VP2 gene

Polymerase chain reaction technique was known for its increased usage as a diagnostic tool for the detection and had been a rapid, sensitive and accurate test for the confirmation of CPV infection (Nandi and Kumar 2010). In the present study, all 342 fecal samples from clinically ill dogs when screened by PCR, 234 fecal samples were reacted with the primers specific to conserved VP2 region of the genome.

One hundred and sixty-three samples which were found to be negative by HA, were positive with PCR, thus suggesting the higher sensitivity of PCR assay over HA test. The PCR molecular assay is more preferable due to its ability to detect low levels of virus particles in the feces (10^3 PFU/gram of feces) (Shunck *et al.*1995) when compared to HA test which requires high quantities of viral particles to produce a visible haemagglutination reaction (Decaro *et al.* 2005). Out of two assays used for CPV detection, PCR was found to be the more sensitive than HA-HI tests yielding 163 samples (47.66 %) positive for CPV. The PCR assay protocol followed was found to be more convenient for routine screening of fecal samples. Sensitivity and specificity HA-HI with PCR were 20.76% and 68.42 %, respectively.

CONCLUSION

From the above studies, it could be concluded that PCR is 3.29 times more efficient than HA as the molecular technique was able to detect the presence of CPV in fecal samples of low number of virions whereas haemagglutinating assay could detect only from fecal samples of high titer.

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