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Screening of Faecal Samples of Pet Dogs for the Presence of Canine Parvoviral Infection by using Haemagglutination Assay and Polymerase Chain Reaction Test

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ABSTRACT

Background: Canine parvovirus-2 causes inflammation of the gastrointestinal tract in dogs. Dogs from Mhow and Indore area suffering from symptoms related to gastroenteritis were investigated for the presence of Canine parvovirus-2 infection.

Methods: A total of 50 faecal samples from dogs (33 male and 17 female) were collected individually in 5 ml of phosphate buffer saline solution in sterile containers. These samples were tested in haemagglutination assay using pig red blood cells for the presence of Canine parvovirus-2 specific antigen and in a molecular test, polymerase chain reaction, using primers to amplify Canine parvovirus-2 specific product of 681 base pairs.

Result: A total of 5 faecal samples (10%) tested positive in haemagglutination assay indicative of presence of Canine parvoviral-2 antigen in the faecal material. Haemagglutinating titre for positive samples ranged from 32 to 1024. Only one faecal sample found positive in polymerase chain reaction test for amplification of Canine parvovirus-2 specific product. The results of the present study indicate presence of Canine parvoviral-2 infection in pet dogs suffering from gastroenteritis, however at a low level.

Key words: Canine parvovirus-2, Faecal samples, Haemagglutination, Polymerase chain reaction.

INTRODUCTION

Canine parvovirus-2 (CPV-2) is highly infectious and contagious virus in the genus *Parvovirus* of the *Parvoviridae* family which affects dogs that cause gastroenteritis. Previously, the virus was implicated in causing heavy mortality in pups due to myocarditis worldwide. Monoclonal antibodies were used to detect newer antigenic variants of CPV-2. This variant was known as CPV-2a. New variants of CPV, known as CPV-2b and CPV-2c were also reported (Parrish *et al.*, 1985; Parrish *et al.*, 1991; Nakamura *et al.*, 2001; Chinchkar *et al.*, 2014). In India, CPV was isolated in 1982 for the first time (Ramadass *et al.*, 1982). Thereafter, it was reported from various states of the country (Biswas *et al.*, 2006; Nandi *et al.*, 2006; Sagar *et al.*, 2008; Khare *et al.*, 2019).

The main transmission of CPV-2 is through faecal-oral route. Dogs that develop the disease show symptoms of illness within 3 to 10 days. The symptoms include lethargy, vomiting, fever and diarrhea (usually bloody). Quick diagnosis of CPV-2 infection in dogs is essential for effective treatment. The present study reports results of screening of faecal samples of pet dogs (from Mhow and Indore area) suffering from gastroenteritis for the presence of CPV-2 infection by using haemagglutination assay (HA) and polymerase chain reaction (PCR), a molecular test.

MATERIALS AND METHODS

Sources of samples, history and place of investigation

A total number of 50 faecal samples (from May 2018 to May 2019, 3 in Monsoon and 47 from Summer season) were collected from dogs suffering from diarrhea, vomiting and gastroenteritis problems brought to the Teaching Veterinary Clinic Complex (TVCC) of the Veterinary College campus, Dr. Ambedkar Nagar-Mhow (47 samples; 34 males, 13 female) and private veterinary clinic, situated in Indore (3 samples; 2 male and 1 female) (Table 1). The faecal samples were collected in phosphate buffered saline solution (pH 7.2) and stored at -20°C. History of pet dogs selected for the sample collection was noted (Table 1). The present investigation was conducted at Department of Veterinary

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Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Dr. Ambedkar Nagar-Mhow, Indore, Madhya Pradesh, India. The investigation plan for the present study was approved by the institutional level ethical committee.

Vaccine

Canishot-K5 vaccine manufactured by Choong Ang Vaccine Laboratories Company Limited (Korea) used as positive control. The combined freeze-dried vaccine contained Live Canine distemper virus, Canine adenovirus type 2, Canine parvovirus and Canine parainfluenza virus.

Pig red blood cells

A total of 10 ml of blood from a healthy adult swine (Middle white Yorkshire crossbred reared at Piggery unit, Livestock Farm complex of the College) was collected in Alsever's solution (1:1, v/v), kept at 4°C and used within one week of collection, whenever required. After removal of plasma and buffy coat, pig red blood cells were washed thrice in phosphate buffered saline solution (PBS), pH 7.2, then stored at 4°C and used within 3 days.

Processing of faecal samples and haemagglutination assay

On thawing, samples were centrifuged at 6,000 revolutions per minutes (rpm) for 15 minutes in the sterile tube (Eppendorf) and supernatant was transferred to another tube. Tubes with supernatants were stored at -20°C till it's use in haemagglutination (HA) assay. Before HA, the supernatants were heat inactivated at 56°C in temperature-controlled water bath for 30 minutes and treated with 10% Chloroform by keeping at 4°C for 10 minutes and then centrifuged at 10,000 rpm for 10 minutes at 4°C. Final supernatant resulted after Chloroform treatment was used for HA (Parthiban *et al.*, 2011). 25 µl of virus diluents was dispensed in each well of 96 well 'V' bottom microtitre plate (Laxbro) (Dahiya and Kulkarni, 2004). The processed faecal sample was serially diluted two-fold in virus diluent (Phosphate buffered saline solution, pH 6.0) and equal quantity (25 µl) of 1% pig red blood cells in the diluent [Phosphate buffered saline solution, 0.3 M phosphates, pH 6.0 containing 3% foetal calf serum (FCS)] was added to the each well. Controls were also kept and the plate was incubated at 4°C for overnight duration and the results were recorded.

Polymerase chain reaction test

Primers (Forward: 5′-GAAGAGTGGTTGTAAATAATT-3′ and Reverse: 5′-CCTATATCACCAAAGTTAGTAG-3′) were used to amplify partial VP1/VP2 gene of 681 base pairs specific for CPV-2 in faecal samples (Nandi *et al.*, 2006; Singh *et al.*, 2014). PCR (25 µl) was performed in 0.2 ml sterile nuclease free tubes (12.5 µl of PCR mastermix (Fermentas), 3 µl of forward and reverse primers, 3 µl of faecal sample and 0.5 µl of enzyme Taq polymerase of Genei™ and 6.5 µl of nuclease free water). Thermocyclic conditions used for amplification of the target DNA as follows (initial denaturation: 95°C, 5 minutes, followed by 30 cycles of a) denaturation: 94°C, 30 seconds, b) primer annealing: 55°C, 2 minutes, c) extension: 72°C, 30 seconds and final extension: 72°C, 10 minutes). Positive (Vaccine) and negative (No template) controls were also kept during the reaction. PCR products were mixed with 6X bromophenol blue loading dye and loaded into a well along with a DNA marker and electrophoresed (Genei®, Bangalore) in 1.5% agarose gel in Tris borate ethylenediaminetetraacetic acid (TBE) buffer for 90 volts/cm for an hour. Ethidium bromide (10 mg/ml) stain to a final concentration of 0.005 µg/ml was added in the gel. Bands in the gel were visualized, digitally recorded by gel documentation system (Alphalmager, USA) and analysed.

RESULTS AND DISCUSSION

Haemagglutination assay

CPV-2 haemagglutinates pig red blood cells. Haemagglutination assay (HA) carried out in a 96-well plate format allows rapid processing of many samples (Desario *et al.*, 2005). So, in the study 50 processed faecal samples from individual dogs suffering from gastroenteritis were screened by using HA (Table 2, Fig 1, Fig 2) for knowing the status of CPV-2 infection. Out of 47 dogs (25 days to 14 years of age from TVCC of the College, 30 were male and 17 were female dogs of various breeds (Non-descript (17; 11 male, 6 female); German Shepherd (11; 8 male, 3 female); Labrador (11; 6 male, 5 female); Pomeranian (4; 2 male, 2 female); (Pug 2; 1 male, 1 female); Poodle (1 male) and Cross breed (1 male). Out of 3 dogs of below 6 months of age from private veterinary clinic, Sudama Nagar, Indore, all were male animals (1 non-descript, 1 Dachshund and 1 Cross breed). HA titres of tested faecal samples ranged in between 32 to 1024. These findings are very similar to a finding which reported titres upto 1280 (Puentes *et al.*, 2012). 5 samples (4 male, 1 female with clear matt formation) showing HA titres of 32 or above were treated as positive for CPV-2 infection (Parthiban *et al.*, 2011). Results of the HA study indicate presence of CPV-2 infection in pet dog population in the region, however at lower magnitude. Two dogs (1 non descript female of 1.5 years of age and 1 Cross breed male of 3 months of age) from private veterinary clinic, Indore tested positive in HA test with HA titres of 1:32 and 1:512, respectively. Other three male dogs between 2 months to 6 months of age (2 German Shepherd and 1 Labrador) tested positive in HA. Labrador recorded highest HA titre 1:1024 than German Shepherds (1:32 and 1:256). Overall, per cent positivity for CPV-2 infection in the dog population was 10% as per HA test results. Varying percentages of CPV-2 positivity in HA test were reported earlier (Mathys *et al.*, 1983; Rai *et al.*, 1994). Five CPV-2 positive dogs in the present study were ranging from 2 months to 1.5 years of age. However, from these five positive dogs for CPV-2 infection in HA, 3 grouped in the age group in between 3-6 months of age. However, more cases of CPV-2 infection were recorded

Table 1: History of pet dogs investigated for Canine parvovirus-2 infection.

Sample no.	Breed of dogs	Age	Sex	Day***	History
1	Non descript*	1 Year	Female	5th	+++
2	Poodle*	1 Year	Male	4th	++
3	Non descript*#	14 Years	Male	3rd	+++
4	Non descript	5 Months	Male	3rd	+++
5	Dachshund**	4 Months	Male	4th	+++
6	Non descript**	1.5 Year	Female	2nd	+++
7	Cross breed**	3 Months	Male	3rd	+++
8	Non descript*	1 Month	Male	2nd	+++
9	German Shepherd*	2.5 Months	Male	2nd	+++
10	Non descript*	3 Months	Female	3rd	+++
11	German Shepherd*	3 Months	Male	6th	+++
12	Labrador*	5 Months	Male	4th	+++
13	Non descript*	8 Months	Male	1st	+++
14	Non descript*	8 Months	Female	2nd	+
15	Non descript*#	1 Year	Male	4th	++++
16	Labrador*	4 Months	Female	3rd	+++
17	Cross breed*#	4 Years	Male	6th	+++
18	Labrador*	2 Months	Female	3rd	+++
19	German Shepherd*	1 Month	Female	4th	+++
20	Labrador*##	2 Months	Male	2nd	+
21	Pug*##	11 Years	Male	3rd	++++
22	Non descript*	5 Months	Female	3rd	+
23	German Shepherd*^#	6 Months	Male	3rd	+++
24	Pomeranian*##	2 Years	Male	4th	+
25	Non descript*	8 Months	Male	3rd	+++
26	Non descript*#	12 Years	Male	4th	++++
27	Pomeranian*##	13 Years	Female	3rd	++
28	Labrador*	25 Days	Male	2nd	++
29	Labrador*	2 Months	Male	2nd	+++
30	Non descript*	1 Year	Male	5th	+++
31	Non descript*	3 Months	Male	4th	+++
32	Non descript*#	14 Years	Male	5th	+++
33	German Shepherd *	1 Month	Female	1st	+++
34	German Shepherd *	3 Months	Male	2nd	+++
35	Labrador*	2 Months	Female	3rd	+++
36	Labrador*#	2 Months	Male	2nd	+
37	Labrador*	4 Months	Female	4th	+++
38	Pomeranian##	2 Years	Female	2nd	+++
39	German Shepherd *	3 Months	Male	5th	+++
40	Pomeranian*##	2 Years	Male	2nd	++
41	Labrador*	3 Months	Male	3rd	+++
42	Non descript*	3 Months	Female	4th	+++
43	Non descript*	1 Month	Male	3rd	+++
44	Labrador*	2 Months	Female	4th	+
45	German Shepherd*	3 Months	Female	2nd	++
46	German Shepherd*	4 Years	Male	3rd	+++
47	German Shepherd*	3 Months	Male	5th	+++
48	German Shepherd*	3 Months	Male	4th	+++
49	Pug	13 Years	Female	4th	+++
50	Non descript	5 Months	Male	3rd	+++

***: Of sample collection after first clinical signs appeared, *: TVCC, Mhow, **: Private Veterinary Clinic, Indore, +: Diarrhea or Vomiting, ++; Diarrhea along with blood, +++: Diarrhea and Vomiting, ++++: Melena, ^: Sample was collected on 2nd day after appearance of ciinical signs also, #: Non vaccinated, ##: Vaccinated

Table 2: Distribution of faecal samples on the basis of haemagglutinating titres.

Titre	Faecal sample number	Total no. of samples
>1:2	1,2,3,4,5,8,9,10,12,14,16,20,21, 22,24,25,26,27,28,29,30,31,32, 33,34,35,34, 35,37,38,39,40,41, 41,43,45,46,48,49,50	38
1:2	17,18,19	3
1:4	13,23,44	3
1:8	15	1
1:16	-	0
1:32	6,11	2
1:64	-	0
1:128	-	0
1:256	47	1
1:512	7	1
1:1024	36	1
1:2048	-	0
1:4096	-	0

in 0-2 months of age group in dogs by other investigators (Khan *et al*., 2006). Out of five positive dogs in the study, 2 dogs were of German Shepherd breed. Similar higher level of CPV-2 infection in German Shepherd breed of 84% was also reported (Khan *et al*., 2006). Four male dogs (HA titres ranging from 32-1024) were positive for CPV-2 infection in our study as compared to only single female dog (HA titre of 32) which was positive in HA test. In HA test, 12.12% male (4 positive out of 33) and 5.88% female (1 positive out of 17) tested positive. HA titres ranging from 1:40 to 1:20,480 were also reported from dogs (Mohan *et al*., 1992) which corresponds to findings of the present investigation. The remaining 45 faecal samples, that were negative in HA test for CPV-2 could possibly be due to i). absence of CPV-2 infection, ii). sedimentation of virus in the faecal samples having low viral titres during centrifugation and iii). viral neutralization or aggregation by copro-antibodies (Carmicheal *et al*., 1980). The haemagglutinaton inhibition (HI) activity was noticed in HA positive samples by using anti-serum from CPV-2 vaccinated dog confirming the involvement of CPV-2 infection (results are not shown) in pet dog population suffering from gastroenteritis.

Fig 1: A German Shepherd dog exhibiting weakness due to gastroenteritis.

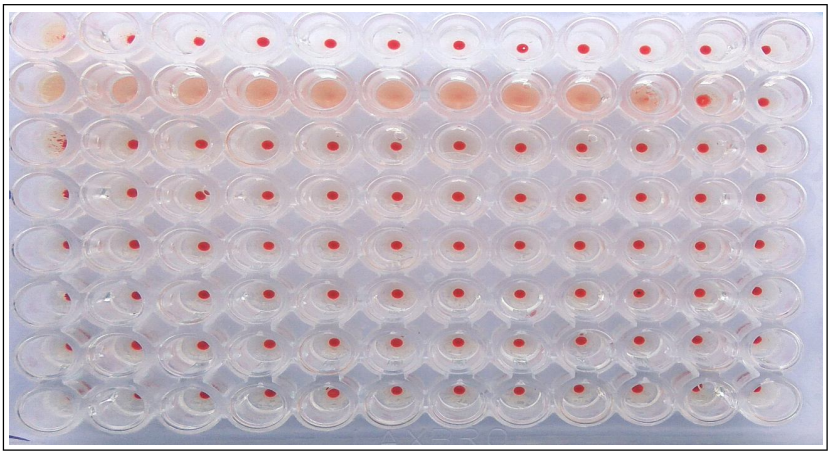

Fig 2: Microtiter plate showing haemagglutination assay results (1st well to last well dilutions - 1:2 to 1:4096).

Polymerase chain reaction test

Initially, PCR conditions were tested successfully by amplification of 681 base pairs deoxyribonucleic acid (DNA) product of partial VP1/VP2 gene specific for CPV-2 by using the Canine parvovirus-2 contained in the vaccine (Canishot-K5), directly. No nucleic acid extraction protocols were followed because of positive PCR results on using Vaccine virus without any prior treatments. Positive HA results indicate involvement of CPV-2 infection, however, few workers reported on CPV-2 strains that lacked HA activity (Parrish *et al.,* 1988; Cavalli *et al.,* 2001). So, 12 pet dogs tested positive (4) and negative (8) in HA were randomly selected and processed individual faecal samples from these dogs were directly used as template in PCR as per Nandi *et al.* (2006). These samples tested negative in PCR. Because of the above facts and to save the PCR reagents, rest of the faecal samples were pooled (5 faecal samples at the most) and tested negative in PCR. Majority of faecal samples tested negative in PCR in the study can be due to excretion of viruses in faecal material to the undetectable level, presence of PCR inhibitors (Oikarinen and Hyoty 2009) in faeces or unexplained reasons/errors. However, these faecal samples will be used in future studies to extract parvoviral DNA and that will be used in PCR/nested set PCR. A German Shepherd dog (Sample no 23) whose faecal material was collected twice on 2nd (tested negative in PCR) and 3rd day (tested positive in PCR, may be due to excretion of virus in huge amounts in the faecal material) after the notice of first clinical signs of gastroenteritis. Besides Canine pavovirus-2 molecular detection of other viral infections that cause gastroenteritis in dog population were also reported (Agnihotri *et al.*, 2017). The findings of the study will be helpful in understanding the epidemiology of infectious diseases that cause gastroenteritis in pet dogs of the area and its management, prevention and control.

CONCLUSION

The results of the present study indicated presence of Canine parvovirus-2 infection at lower magnitude in pet dogs suffering from gastroenteritis in the region. However, it is essential to test these cases for involvement of other parasitic infestations, viral and bacterial infectious agents causing gastroenteritis in pet dogs in the region.

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Conflict of interest: None.

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