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# Study on Host Predisposing Factors and Diagnostic Tests for Canine Parvovirus (CPV-2) Infection in Dogs

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**ABSTRACT**

In the present study, a total 61 faecal samples from dogs, showing symptom of Canine Parvovirus infection, were collected from in and around Navsari district. The samples were screened for CPV by different diagnostic methods *viz.* Immuno Chromatographic Assay (ICA), Haemagglutination (HA) test, Enzyme linked immuno sorbant assay (ELISA) Polymerase chain reaction (PCR) and Isolation of CPV by culture of virus on Madin Darby Canine Kidney (MDCK) cells. Out of five assays tested, PCR was found to be the most sensitive (62.29%) in detecting CPV followed by HA test (50.82%), virus isolation (50.00%), ICA (37.70%) and sandwich ELISA (31.14%). Considering PCR as most sensitive test, overall prevalence rate was found as 62.29%. Along with samples clinical history of animals was collected to determine the host predisposing factors. Majority of dogs affected were in the age group of 0-3 months (47.37%), then 3-6 months (39.47 %) and were males (24/38; 63.16%). Breed wise study revealed that desi/local breeds (26.32 %) were more susceptible followed by Labrador retriever (18.42%) and German shepherd (15.79%). Non-vaccinated dogs (71.05 %) were more victimized to CPV infection than the vaccinated dogs. Season wise incidence showed that cases were almost exclusively occurred in winter (97.37%) followed by monsoon (2.63%).

**Keywords:** Canine parvovirus**,** Immuno chromatographic assay, Haemagglutination test, Sandwich ELISA, MDCK cell line, Polymerase chain reaction

Canine Parvovirus (CPV) causes haemorrhagic or non-hemorrhagic gastroenteritis with vomiting and diarrhoea in dogs of all ages. The virus causes high morbidity (100%) and frequent mortality up to 10% in adults and 91% in pups and it was supposedly originated as a host range variant from Feline Panleukopenia Virus (FPV) (Appel *et al.,* 1979) and is different from minute virus of canine (CPV-1). CPV belongs to genus *Erythroparvovirus* of *Parvoviridae* family, with a single-stranded DNA genome of 5.2 kilobases (kb) in length. CPV has two promoters resulting in the expression of five peptides through alternate splicing of the viral mRNAs. Among them VP2 (64 kDa) is an $NH_2$-terminally truncated form of VP1 (84 kDa) and is the major component of the non-enveloped icosahedral capsid of CPV and plays a very important role in determining antigenicity and host range specificity of CPV (Srinivas *et al.,* 2013).

In India first confirmation of CPV-2 was from Madras (Now Chennai), since then a large number of incidences of CPV-2 or variant strains have been reported from different states of India (Nandi and Kumar, 2010). CPV cause rapid fatality in patients, therefore, prompt and accurate diagnosis must be carried out. Over the years many diagnostic tests have been developed to detect CPV. The routinely available disease diagnosis methods are virus isolation, haem agglutination (HA) test and its inhibition (HI), enzyme linked immuno sorbant assay (ELISA), polymerase chain reaction (PCR) and Real-time polymerase chain reaction (RT-PCR). Application of each test varies in its sensitivity, specificity, rapidity, economics, ease of conduction and clinical uses (Desario *et al*., 2005).

There are certain host related predisposing factors which

determine the susceptibility of infection. The important factors are breed, sex, vaccination status and season of occurrence (Houston *et al.,* 1996; Nandi and Kumar, 2010). Therefore, host predisposing factors and evaluation of different diagnostic tests for CPV carries great value in the benefit of pet and its owners and described in present communication.

## MATERIALS AND METHODS

### Study design and collection of samples

The research work was done in and around Navsari, during the period of October 2016 to April 2017. The faecal samples/rectal swabs from dogs, suggestive of CPV infection, were collected directly from the rectum of affected animals in screw capped sample vials containing 3 ml Hank's balanced salt solution (HBSS) with Kanamycin. These were brought to laboratory under refrigerated condition on ice packs and processed either immediately or stored at -80˚C temperature for later investigation. Samples were made bacteria free by filtration with 0.22 µm syringe filter (Millex Merck). Cotton swabs were used for ICA and bacteria free filtrates (BFF) were used for all other tests.

### Immuno Chromatographic Assay

ICA was carried out with a commercial rapid CPV antigen test kit (Anigen, Korea), following the manufacturer's instructions. Briefly, faecal sample was emulsified in 1 ml of assay diluent. Then four drops of emulsion were dispensed in to the sample well of test cassette. Colour band development within 5-10 min was considered positive.

### Haemagglutination test

Haemagglutination test was performed upon 1:2 diluted BFF against 1% suspension of porcine RBC in PB S(pH-6.5) at 4 ˚C for 4 hours (Mochizuki *et al*., 1993).

### Enzyme linked immuno sorbant assay

The CPV antigen was detected by sandwich ELISA as per the protocol mentioned in INGEZIM CPV-DAS kit, Spain. First 100 µl of test samples along with controls were added to each wells. Plate was sealed and kept for incubation at 37˚ C for 1 h and washed for 4 times with washing buffer. 100 µl of Conjugate I was put in to each well and incubated for 1 h at 37˚ C and washed. Further, 100 µl of Conjugate II was added and washing was applied after 15 min incubation. After that 100 µl of substrate solution was added to each well and the absorbance was taken at 405 nm.

### Genomic DNA extraction and PCR amplification of CPV-2

The genomic DNA from the fecal samples was extracted by the phenol chloroform method (Kumar *et al*., 2011). In PCR assay, primer pairs Pab sense (5′-GAAGAGTGGTTGTAAATAATA-3′) and Pab anti-sense (5′-CCTATATCACCAAAGTTAGTAG-3′) were used to amplify the partial VP1/VP2 gene of the CPV to yield amplicon of 681bp (Pereira *et al.,* 2000). The PCR was performed in a thermal cycler for 30 cycles, each consisting of denaturation at 94˚ C for 30 s, annealing at 55˚ C for 2 min and extension at 72˚ C for 2 min. PCR products were electrophoresed on 1.5% agarose gels, gels were stained with ethidium bromide and then visualized and documented under gel documentation system.

### Isolation on MDCK cell lines

Ten random samples which were positive by all the above tests were subjected to isolation in MDCK cell line. The supernatant of faecal homogenate was inoculated onto freshly trypsinised MDCK cells grown in Dulbecco's minimal essential medium containing 2% foetal bovine serum (FBS). After an incubation period of 3 days at 37 ˚C, inoculated cells were observed for cytopathic effect (CPE).

## RESULTS AND DISCUSSION

Out of 61 faecal samples, 23 (37.70%) were found positive by ICA (Fig. 1). Previously Chinchkar *et al.* (2014) observed higher positive percentage than the present findings. On the other hand, lower positivity was recorded by Reddy *et al.* (2015). Overall, ICA can be used in rapid detection of CPV antigen in faeces and could be useful for routine applications in kennels with large number of

puppies at risk and can be performed by veterinarians as well by owners (Esfandiari and Klingeborn, 2000).

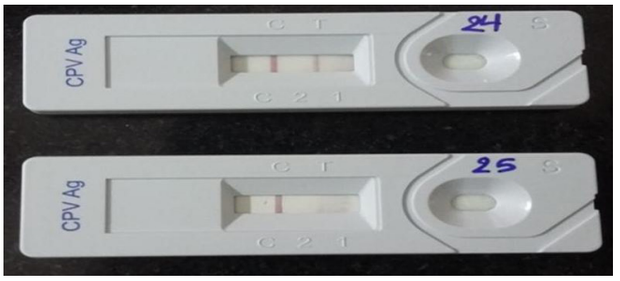


**Fig. 1:** Screening of CPV infection with ICA, samples upper cassette showing positive and lower cassette with negative results

In HA test 31/61 (50.82 %) samples displayed haemgglutination with porcine erythrocytes. The titer of the samples was in range of 16- 512 HA unit (Fig. 2; Table 1). The finding corroborated wherein the virus was detected to be excreted in 45.30% faecal samples as reported by Archana *et al.* (2009). Higher values *i.e.* 71.42% was reported earlier by Kumar *et al.* (2004). While lower values *i.e.* 36.71% and 36.37% were reported earlier by Parthiban *et al.* (2011) and Joshi *et al.* (2012), respectively. For a clear reading of the HA test, good quality erythrocytes should be ensured since the test is affected by an altered coefficient of erythrocyte sedimentation which may occur in case of stress or disease of the donor pig (Desario *et al.,* 2005).

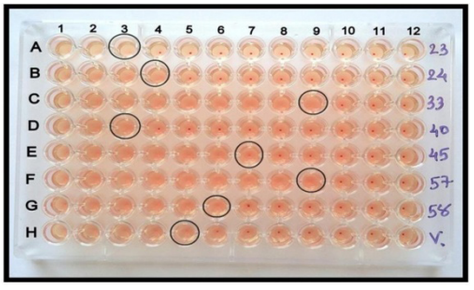


**Fig. 2:** Microtiter plate showing haemagglutination of porcine erythrocyte by CPV

CPV vaccine (V) as positive control. Encircled well is titer of the test.

Sandwich ELISA was applied for the detection of CPV antigen in total of 61 faecal samples. Nineteen (31.14%) samples were positive for canine parvovirus antigen (Fig. 3). Nearby observation (45.39%) was reported earlier by Kumar *et al.* (2010). The exceptional number of negative results was recorded in ELISA. However, false negative faecal antigen ELISA result has been analysed earlier by Proksch *et al.* (2015). According to them low faecal CPV load and presence of antibodies binding to CPV antigen in faeces could be the important reasons for false negative faecal antigen ELISA results.

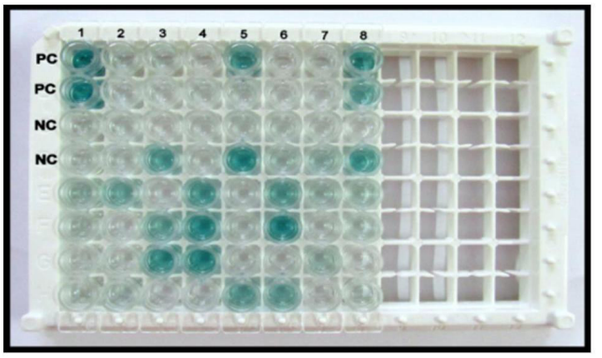


**Fig. 3:** An ELISA module showing positive (blue colour) and negative reactions, (colourless) for CPV antigen by sandwich ELISA. Well A1 and B1 as positive control, Well C1 and D1 as Negative control

A total of 38 out of 61 samples (62.29%) were found positive by P2ab (681 bp) primer set (Fig. 4). These 38 samples showed CPV-2 infection which might belong to any genotype. Similar findings were reported previously by Mohanraj *et al.* (2010), Mukhopadhyay *et al.* (2012), and Miranda *et al.* (2016) such as 66.23%, 57.85% and 64.1%, respectively.

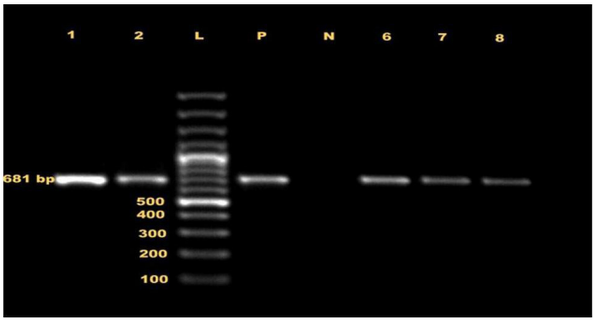


**Fig. 4:** Agarose gel electrophoresis picture showing amplicon of 681 bp, L is 100 bp ladder, P and N are positive and negative control sample, respectively

Cytopathic effect was observed in the form of rounding of cells, increased granularity, clumping and detachment of cell monolayer and were noted as typical CPE of CPV (Fig. 5A and 5B). Cell culture supernatant of 10 samples was collected and presence of virus in the harvested cell culture supernatant was reconfirmed in 5 samples (50.0%) by amplifying a 681 bp product in PCR. Similar moderate isolation rates 60.67%, 47.0% and 55.55% were also described by Desario *et al.* (2005), Decaro *et al.* (2005) and Srinivas *et al.* (2013) on MDCK and CRFK cell lines, respectively, who reported that the isolation of CPV can be done only for few days post-infection. Moderate isolation rate may be attributable to the presence of antibodies in the intestinal lumen of the infected dogs, which may bind virions and prevent viral attachment to cell receptors.

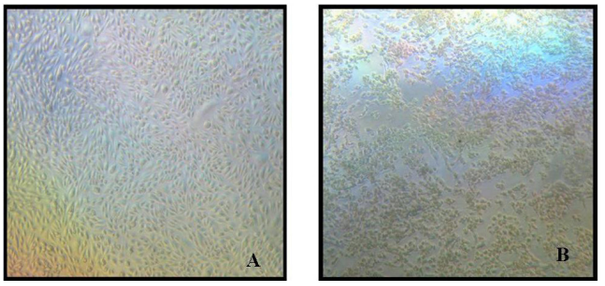


**Fig. 5:** Diagnosis of CPV on MDCK Cell line, (A) Normal MDCK cells monolayer. (B) Cells with cytopathic effects after positive sample inoculation

PCR was found to be the most sensitive (62.29%) in detecting CPV followed by HA test (50.81%), Virus isolation in cell culture (50.00%), ICA (37.70%) and sandwich ELISA (31.14%). The result resembled with the agreement of the Desario *et al.* (2005). It could be concluded the PCR to be highly sensitive (76.40%) assay for detection of CPV followed by virus isolation (60.67%), HA test (56.17%) and ICA (46.06%).

Further, out of 61 samples, 16 were found positive by all the above tests, while 21 were found negative by all the tests. Among remaining 24 samples 7, 15, 4 and 22 samples were detected positive by ICA, HA test, sandwich ELISA and PCR, respectively. Further, regarding specificity, PCR detected 6/7 ICA positive samples, 15/15 HA test positive samples and 3/4 sandwich ELISA positive samples. This indicates superiority of PCR assay among different applied diagnostic tests.PCR is reported to be sensitive assay over cell culture and HA test (Mochizuki *et al.,* 1993), ICA (Tinky *et al*., 2015) and ELISA (Kumar *et al.,* 2011). In contrast, Parthiban *et al.* (2012) reported ability of HA test to detect CPV higher than PCR, but later described them as false positive. These findings have allowed that HA test could be employed for the preliminary screening of the agent in field because of its less cost and rapid results but negative results from HA tests of suspected cases should be confirmed through molecular methods as there could be a possibility of presence of HA test negative CPV strain. Proksch *et al.* (2015) reported that 41/80 (51.3%) PCR positive samples had a false negative faecal antigen ELISA result. ELISA-negative dogs had a significantly lower faecal virus load, and higher serum antibody concentrations than ELISA-positive dogs. Result concludes that low faecal CPV load and antibodies binding to CPV antigen in faeces are likely to be important reasons for false negative faecal antigen ELISA/ICA results. Dogs with clinical signs of CPV infection should be retested by faecal PCR.

**Table 1:** Details of HA titer of positive samples by haemagglutination

| HA titer | No. of samples | Percent positive |
|---|---|---|
| Less than 16 | 06 | 9.84 |
| 16- 32 | 07 | 11.48 |
| 64- 128 | 8 | 13.11 |
| 256- 526 | 10 | 16.39 |
| Negative | 30 | — |
| **Total** | **61** | **50.82** |

Breed-wise distribution of CPV infection revealed that desi/local breeds (26.32%) were more prone to this infection than that of exotic breeds. Among exotic breeds Labrador (18.42%), German shepherd (15.79 %), Pug (10.52%), Doberman (7.9%), Spitz (7.9%) and others were included. Similar observations have been reported previously by Tajpara *et al.* (2009) and Behera *et al.* (2015) and reported 27.33% and 34.48%, incidence, respectively. In contrast to this, foreign breeds were found to be more susceptible than non-descript dogs and 69.20% and 31.64% incidence was reported by Kumar *et al.* (2011) and Nandi and Kumar (2010), respectively. More incidences in desi breeds might be due higher population density of this breed making their close proximity to spread the infection. In addition to this, poor vaccination

schedule being followed by the owners of desi breed due to lack of awareness among them. Among the exotic breeds, Labrador retriever and German shepherd were found to be more susceptible with incidences of 18.42% and 15.79%, respectively. It was reported earlier that medium and large breeds like Labrador and German shepherd are more susceptible to CPV infection (Kaur *et al.* (2014). Age-wise incidence was found to be more among the animals of 0-3 months (47.37%) (below 6 months) followed by 3-6 months (39.47%), 6-12 months (13.16%) and the least among above 12 month (Table 2).

**Table 2:** Age wise distribution of CPV samples

| Age | Samples tested | Positive samples | Percent positive |
|---|---|---|---|
| 0-3months | 27 | 18 | 47.37 |
| 3-6 months | 24 | 15 | 39.47 |
| 6-12 months | 08 | 05 | 13.16 |
| > 1 year | 02 | 00 | 00 |
| **Total** | **61** | **38** | **100** |

These findings corroborates with earlier reports where 67.5% (Kaur *et al.,* 2014) incidence of CPV infection between the age group of 0-3 months. On the other hand Srinivas *et al*. (2013) and Behera *et al.* (2015) found higher incidence between the age group of 3-6 months. The higher incidence of CPV below 6 months might be due to the affinity of the virus for rapidly multiplying intestinal crypt cells in weaning pups with higher mitotic index due to changes in bacterial flora as well as in the diet due to weaning (Deka *et al.,* 2013).

Above 1 year age, very less incidence is attributable to vaccination schedule practiced or due to accidental but sub clinical exposure of virus which lead to build up protective antibody titre in the host. Sex-wise incidence was analysed in which males 24/38 (63.16%) had shown more positivity to parvovirus infection than that of females 14/38 (36.84%) but the difference was found non significant and attributable to ownership practices rather than host itself. It was found that non-vaccinated dogs were more affected 27/38 (71.05%) than vaccinated ones 8/38 (21.05%). In 3/38 (7.90%) animals' vaccination history could not be recorded. Srinivas *et al.* (2013) and Kaur *et al.* (2014) similarly reported higher incidence of CPV infection in non-vaccinated dogs.

This fact indicates that current vaccines confer reasonably good protection, despite of heterologous genotype challenges. Though there are few reports of vaccinated animals coming down with CPV infection indicating vaccine failure (Mohanraj *et al.,* 2010; Parthiban *et al.,* 2011). The failure in vaccine is attributable to a fact that a window of susceptibility to infection remain open between to 8-12 weeks of age when maternal antibodies have waned but vaccine induced antibodies have not reached to protective level.

This fact also explains the age group related susceptibility of host to CPV infection. Incidence of CPV was found almost exclusively in winter months (97.37%) followed by monsoon months (2.63%). However against this finding Tajpara *et al.* (2009) found incidences of CPV more in summer months (27.77%) than in winter (17.24%) months. The difference may be explained as, in this region winter temperature does not fall below 8˚ C and provides ample opportunity for virus survival. Again this incidence may correspond to breeding season of dogs.

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