RESEARCH ARTICLE

Molecular Detection and Phylogenetic Analysis of *Canine Parvovirus*-2 in Dogs

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Abstract

Canine Parvovirus (CPV) is a most significant viral disease causing acute haemorrhagic gastroenteritis and myocarditis in puppies at the age of 3-4 months. It causes serious morbidity and mortality, in spite of the effective vaccination. Inadequate immunity and modern practices of early weaning in the kennels have predisposed the young dogs to CPV. In the present study, total 50 fecal samples were collected from dogs suspected for CPV-2 from Nagpur, India and screened by Ubioquick® VET lateral flow assay kit and Polymerase Chain Reaction (PCR) followed by the sequencing and phylogenetic analysis of VP2 gene. Out of 50 samples, 10 (20%) were found positive by antigen detection test, 18 (36%) by Conventional PCR method. Sequence analysis revealed 98-99% homology with sequences of CPV-2b antigenic type reported in dogs from India and Sri Lanka.

Key words: Antigen detection test, *Canine Parvovirus*, Polymerase chain reaction (PCR), Sequencing and phylogeny.

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Introduction

Canine Parvovirus caused by Canine Parvo Virus -2, is one of the highly contagious viral diseases of dogs, mainly occurring under six months of age. It causes haemorrhagic gastroenteritis and myocarditis in puppies (Kaur *et al.,*2016; Sheikh *et al.,* 2017). *Canine Parvovirus* is a widespread viral disease contributing to 27% of all diarrhoeic cases in canines (Raj *et al.,* 2010). CPV-2 comprises two Open Reading Frames (ORF) which encodes two structural protein VP1 and VP2 and non-structural proteins NS1 and NS2. VP2 is a major capsid protein and influences the antigenicity and host range of CPV-2. Vaccination has proven to be effective in the control of Parvovirus in companion animals. However, recent studies have shown that the CPV-2C causes canine Parvovirus disease. The virus attacks cells in intestine of dogs and stop from being able to absorb vital nutrients. Maternal antibodies provide innate protection against CPV-2; however, recent trend of commercial kennels to wean puppies early for sale has led to increased incidence of CPV-2 (Miranda *et al*., 2015).There are arrays of tests for the detection of CPV-2 in clinical samples including the electron microscopy (Finlaison,1995). Polymerase Chain Reaction (PCR,Mochizuki *et al.,* 1993), Multiplex PCR (Kaur *et al.,* 2016), qPCR (Quantitative Polymerase Chain Reaction) (McKnight *et al.,* 2007), Nested PCR (Kumar *et al*., 2011) and antigen based lateral flow assays (Nahat *et al.,* 2015). The detection of parvoviruses is complicated owing to the drawbacks like suboptimal conditions in samples and PCR inhibitors in faecal samples. The present study was aimed to identify CPV virus and to compare the sensitivity of antigen based lateral flow assays, PCR and phylogenetic analysis.

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Materials and Methods

Sample Collection and Antigen Detection Test

A total of 50 canine fecal samples were collected from various clinics located in Nagpur city, exhibiting clinical signs like vomiting, high temperature, haemorrhagic gastroenteritis and anorexia. The collected fecal samples were emulsified in 1 mL of 0.1 M phosphate buffered saline (pH 7.2) and the suspension was centrifuged to remove cellular debris at 10,000 x g for 10 min. The supernatant was collected and stored at -20°C until further analysis. Ubioquick^{VET} *Canine Parvovirus* antigen rapid test kit, was used in the study for screening of samples. The samples were processed as per manufacturer's instructions.

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Extraction of DNA and Polymerase Chain Reaction

The DNA was extracted from fecal samples by using DNA extraction kit (Promega kit,USA) as per manufacturer's instructions.

Polymerase chain reaction was carried out following the method of Sheikh *et al.* (2017). The PCR amplified a fragment of 681 bp from the VP2 gene, using the primers forward 5' GAAGAGTGGTTGTAAATAATT 3' and Reverse 5' CCTATATAACCAAAGTTAGTAC 3' For PCR amplification, 25 µL reaction mixture contained 12.5 µL master mix, 1 µL each forward and reverse primer (20µM), 8.5 µL nuclease free water and 2 µL template. The reaction conditions were: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 2 min and extension at 72 °C for 2 min and a final extension step of 10 min at 72 °C. Commercially available vaccine Megavac-6 was used as positive control. The amplified PCR products were run on 1% agarose gel.

Sequencing and Phylogenetic Analysis

The amplicon (681 bp) was purified as per the manufacturer's instructions using QIAquick PCR Purification Kit (Mfg. Qiagen Inc, MD, USA) and the purified amplicon was sequenced using the forward and reverse primers with the help of ABI 3130 automated DNA Sequencer, (Mfg. Applied Biosystems, CA, USA). A systematic phylogenetic investigation was carried out preferentially including the CPV-2 sequences reported from India and neighbouring countries (Srinivas *et al.,* 2013). Maximum Likelihood tree was constructed using the bootstrap method with bootstrap values of 1000 replications and analysed using Mega XI software (Kumar *et al.*, 2018). VP2 gene of CPV-20 from Besa, Nagpur and CPV-25 from Gaddigodam, Nagpur and the sequence of CPV vaccine strain (Megavac) and 30 randomly selected sequences from India and other countries were retrieved from NCBI included in phylogenetic analysis.

Results and Discussion

In the present study, out of 50 fecal samples screened for the presence of CPV antigen by Ubioquick^{VET} Rapid Antigen Detection Test Kit, a total of 10 samples were found positive during the screening (Fig.1).The findings corroborate with the reports of Tanwar *et al.*(2020), Hasan *et al.* (2016), Magar (2019) and Baidya *et al.* (2016) with rapid test kit. Whereas PCR assay confirmed 36 % (18/ 50), positive for CPV producing an amplicon of 681 bp corresponding to the VP2 gene fragment targeted using the forward and reverse primers (Fig.2). CPV-2 has been reported throughout the Indian subcontinent in stray and domesticated dogs. The prevalence of CPV by PCR differs region to region. At the same time 40.84 % and 45.9% infection of CPV-2 was reported by Behera *et al.* (2015) and Soma *et al.*(2013) respectively. A little higher (52% and 58%) prevalence of CPV2 was reported by Thomas *et al.* (2014) and Mukhopadhyay *et al.* (2014) while 31.2% from Dhaka, Bangladesh (Hasan *et al.*, 2016) and 22.7 % from Lahore, Pakistan was reported (Umar *et al.*, 2015). A rapid diagnosis of CPV-2 infection is important in kennels and shelters to isolate infected dogs and prevent transmission to susceptible contact animals. Stray population has significant influence on the incidence of CPV-2 among dog population by acting as reservoirs of the infection (Nandi and Kumar, 2010). PCR is a specific, sensitive and simple method for detecting *Canine Parvovirus* in the faeces of infected dogs (Weiquan, 2001).

Two representative samples (CPV-20 from Besa, Nagpur and CPV-25 from Gaddigodam, Nagpur) were selected randomly and amplicon submitted to National Centre for Biotechnology Information (NCBI) through GenBank. The sequences were allotted accession numbers LC646118 and LC646119 for CPV-20 from Besa, Nagpur and CPV-25 from Gaddigodam, Nagpur respectively (Fig.3)

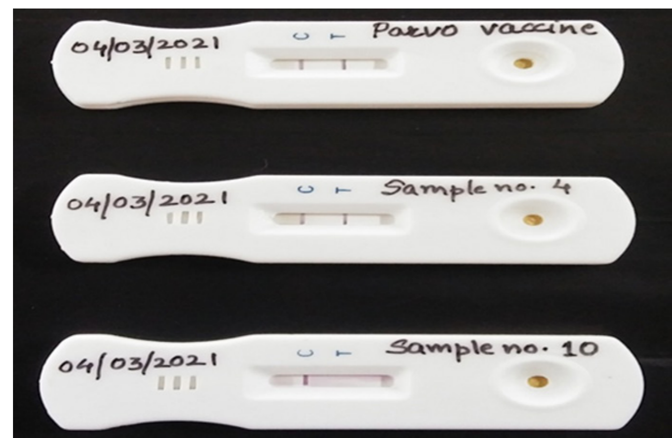

Fig. 1: Antigen Based Lateral Flow Assay for the detection of Canine Parvovirus-2 (CPV-2) from faecal samples from clinical dog samples. A. Parvovirus vaccine (Positive control):- Positive; Sample no.4 :- Positive; Sample no.10 :- Negative

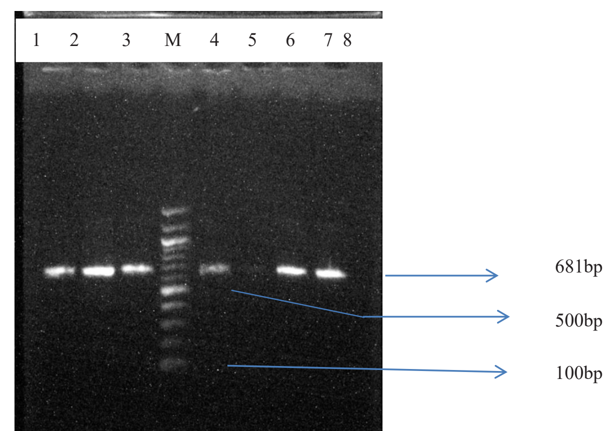

Fig. 2: Agarose Gel Electrophoresis (1.5% gel) with ethidium bromide staining exhibiting an amplicon of 681 bp which is positive for VP2 gene using targeted primers. Lane 1:-Sample no.20; Lane 2:-Positive control (Vaccine); Lane 3:-Sample no.25; Lane M:- Ladder 100 bp; Lane 4:-Sample no.37; Lane 5:- Negative control; Lane 6:-Sample no.21; Lane 7:-Sample no.50.

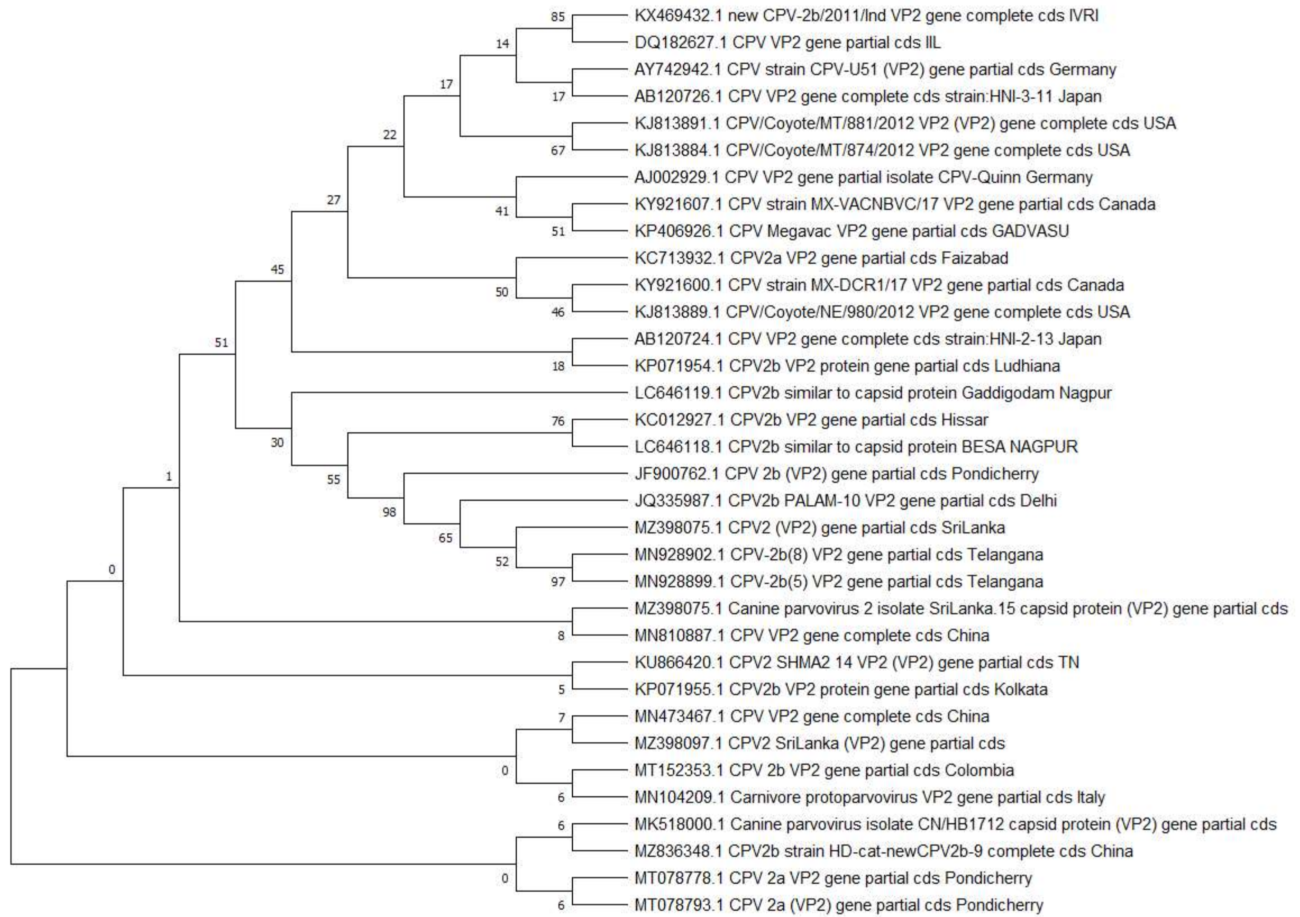

Fig. 3: Phylogenetic analysis of partial gene of CPV samples with Indian and world isolates

Phylogenetic analysis of nucleotide sequences from sample no. CPV20 (LC646118) revealed that the organism was closely related with Acc. No. KC012927 from Hissar, India. All the other isolates/ sequences from India were placed in different Clads and thus were distantly located from CPV-20 (LC646118). CPV 25 (LC646119) was located close to MZ398097 from Sri Lanka, although it was placed on a separate node. It was found to be distantly located to CPV-20. LC646119 was found 99.22 % identical to VP2 reported in dogs from Sri Lanka (Accession No. MZ398097). Similarly, sequence LC646118 was found to be 98.30% identical to CPV-2 sequences reported from Hissar (Accession No. KC012927).Both the strains CPV-20(LC646118) and CPV-25 (LC646119) were placed on separate nodes from those of Indian isolates and other isolates from different countries of the world.The sequences were found to be identical to sequences of CPV- 2b reported from India and neighbouring countries. A similar finding was observed by Yang *et al*. (2015) who determined the nucleotide sequence of VP2 genes of QIACP1403 and QIACP1404 and framed to translation in amino acid sequences and further analysed by molecular weight and found to be of CPV-2b type. Similarly Sheikh *et al*. (2017) reported eight nucleotide of field virus sequences showed homology in the range 95-99%, which conclude that CPV-2b was currently the only virus circulating in Suleimani/Iraq.

The Inhibitors present in the feces can be a major hurdle in obtaining error free results during the diagnosis of CPV-2 in dogs. In the present study PCR method was found superior over commercially available Lateral flow kit.

Conclusion

The study reveals that PCR is a sensitive tool for the detection of CPV-2 from clinical fecal samples of infected dogs. The overall prevalence of CPV-2 in Nagpur city was significantly high (36%). In the present study PCR method was found superior over commercially available Lateral flow kit.

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