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# Molecular typing of *Canine parvovirus*-2 occuring in Nagpur by multiplex PCR

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**Abstract**
*Canine parvo*virus is one of the major causes of haemorrhagic enteritis especially in young dog's leads to high morbidity and fatality. The present study was designated for identification of *Canine parvovirus* in diarrhoeic dogs by Antigen detection kit, PCR and antigenic typing by employing Multiplex PCR.A total of 50 faecal samples from diarrheic dogs were screened for CPV antigen by Antigen detection test kit. Out of 50 samples 11 (22%) were found positive by Antigen detection kit test. All the 11 samples were found positive by Conventional PCR showing uniform band of 681 bp. All the 11 samples found positive by PCR were subjected to multiplex PCR assay with CPV-2, CPV-2a, CPV-2B AND CPV-2 c. It was inferred that CPV-2a were predominant antigenic type followed by CPV-2c and CPV-2 and negligible prevalence of CPV-2b.

**Keywords:** Canine parvovirus, polymerase chain reaction, multiplex PCR

## Introduction
*Canine Parvovirus* (CPV) is highly infectious, cause of Haemorrhagic gastroenteritis and myocarditis in young dogs. Mostly, CPV-2 in young dogs leads to high morbidity and Mortality. *Canine parvovirus* (CPV) is advised by many investigators as the main cause of diarrhea in dogs under 6-month-old (Hackett and Lappin, 2003) [7] Prittie, (2014) [16]. Canine animal virus belongs to the family Parvoviridae comprising 2 subfamilies: the taxonomic category Parvovirinae that contains viruses infecting vertebrates and taxonomic category Densovirinae contains viruses that infect insects. There square measure three genera within the taxonomic category Parvovirinae *viz*. Parvovirus, Erythrovirus and Dependovirus (Murphy *et al.*1999) [11]. Parvovirus is non-enveloped, diameter of 25 nm and has icosahedral symmetry containing approximately 5.2kb of a single-stranded negative-sense DNA (Parrish *et al*. 1991) [14]. The genome comprises 2 open reading frames (ORF), one coding for structural viral proteins (VP) and another coding for non-structural proteins (NS) (Reed *et al.*1988) [18]. 2 non-structural proteins NS 1 and NS 2 and two structural proteins VP1 and VP2 are encoded by the genome. The VP2 protein is the main capsid protein and plays a crucial role in virus pathogenicity (Mochizuki *et al*. 1996) [9]. Evolution of distinct antigenic variant of CPV-2 can occur if any mutation which affects VP2 gene. Recently, it has been noted that the serotype of CPV circulating in the Indian sub-continent is fully different from that included in the conventional vaccine. This leads to the decreased potency of the CPV-2 based vaccines (Greenwood *et al.* 1995 [6]. Yule *et al*. 1997 [21]. Pratelli *et al*. 2001) [15]. It was observed that (Nandi *et al*. 2010) [12] the majority of the vaccines used in India are based on the strains that were isolated about 25 to 30 years ago. Thus, it becomes terribly crucial to discover the precise substance sort of CPV that is current within the landmass in order that these specific substance varieties may be enclosed within the vaccines for manufacturing effective protection. Early detection in conjunction with the data of genetic variations of VP2 may be of vast facilitate in distinguishing rising CPV strains in order that this information may be used for the event of immunizing agent. Keeping in view the above points this study was undertaken to develop a multiplex PCR that could be used in identification of the CPV antigenic types and also to study the prevalence of CPV.

## Materials and Methods

### Collection of samples

Total 50 rectal swab/faecal swab were collected for detection of virus from dog showing clinical signs like, emesis, anorexia, depression, haemorrhagic enteritis. Collections of Samples were done from various clinics located in Nagpur City**.** Samples along with the history *viz*. breed, age, sex and vaccination status of the animal were collected. Sterile swabs were used for collection of samples. Collected samples were stored at -20 $^0$C for further processing.

### Antigen detection kit

Faecal swabs were screened for presence of CPV antigen by Ubioquickvet *Canine parvovirus* antigen rapid test kit. The faecal sample was advised positive for the presence of CPV antigen if two red bands appear one in the control line (C) and the other in the test line (T). The faecal sample was advised negative for the presence of CPV antigen if one red band appears one in the control line (C) with no apparent band in the test line (T).

### DNA extraction

DNA was extracted from faecal samples and commercially available vaccine *viz* Vanguard 5L4) using DNA extraction kit (Promega) following manufacturer's instructions. Sambrook, J., Russel, D. W. (2001) [19].

### Polymerase chain reaction

The Oligonucleotide primers used for Conventional PCR to amplify VP2 gene of CPV-2 virus (Sheikh *et al.* 2017) [2]. The primer sequences and nucleotide position of oligonucleotide primers are shown in Table 1.

**Table 1:** Details of primers specific for VP2 gene of CPV used in PCR

| Name of Primer | Primer sequence | Productsize | Reference |
|---|---|---|---|
| Cpv-2ab-f | GAAGAGTGGTTGTAAATAATT | 681 BP | Sheikh *et al.* (2017) [2] |
| Cpv-2ab-r | CCTATATAACCAAAGTTAGTAC | | |

The extracted viral DNA was amplified by PCR assay using VP2 gene-specific primers and reaction mixture consisted of 2 µl Template DNA,12.5 µl PCR Master Mix,2 µl Forward Primer, 2 µl Reverse Primer, 6.5 µl Nuclease free water to make the volume of 25 µl. all the ingredients mixed properly by vortex ting. The PCR was programmed as Initial Denaturation at 94 $^0$C for 5 min followed by 30 cycles of 94 $^0$C for 1 min, annealing at 50 $^0$C for 2 min, extension at 72 $^0$C for 2 min and a final extension at 72 $^0$C for 10 min.

### Antigenic typing of CPV by multiplex PCR

For antigenic characterization, primers used for multiplex PCR were four antigenic types of CPV *viz*. CPV-2, CPV-2a, CPV-2b and CPV-2c mixed together in a single reaction. The Oligonucleotide primers sequence and product size used for Multiplex PCR (Kaur *et al.* 2014) [8] is given in Table-2

**Table 2:** Details of primers of multiplex PCR for CPV

| Sr. no | Antigenic Type of CPV | primer | Primer sequence | Product size |
|---|---|---|---|---|
| 1 | 2 | CPV-2GM F | 5'CTGCTACTCAGCCACCAACT-3' | 719 |
| | | CPV-2GM R | 5'AGGTGTTTCTCCTGTTGTGGT-3' | |
| 2 | 2a | CPV-2aGM F | 5'-AGAGCATTGGGCTTACCACC-3' | 379 |
| | | CPV-2aGM R | 5'ATCTTCCTGTATCTTGATGTGCT-3' | |
| 3 | 2b | CPV-2bGM F | 5'-TGTATTGCTACCAACAGATCCA-3' | 178 |
| | | CPV-2bGM R | 5'TGGTGCATTTACATGAAGTCTTGG-3' | |
| 4 | 2c | CPV-2cGM F | 5'-GTGGTTCTGGGGGTGTGG-3' | 470 |
| | | CPV-2cGM R | 5'-AGCTGCTGGAGTAAATGGCA-3' | |

The reaction mixture consisted for identifying various antigenic types by Multiplex PCR and reaction was set of 5 µl Template DNA, 12.5 µl PCR Master Mix, 0.8 µl Forward Primer, 0.8 µl Reverse Primer, 5.9 µl Nuclease free water to make the final volume of 25 µl. all the ingredients mixed properly by vortexting. The Multiplex PCR was programmed as Denaturation at 94 $^0$C for 60 sec followed by 35 cycles of annealing at 60 $^0$C for 60 sec, extension at 72 $^0$C for 150 sec and a single cycle of final extension at 72 $^0$C for 10 min.

## Results and Discussion

Ubioquick$^{vet}$ Canine parvovirus antigen rapid test kit revealed out of 50 samples 11 (22%) samples found positive by antigen detection kit (Fig 1) Dorlikar *et al.* (2019) [5]. screened 91 faecal swabs by Ubioquick VET *Canine Parvovirus* antigen rapid kit from dogs. Out of 91 rectal swabs 34(37.36%) samples were found positive for CPV antigen.

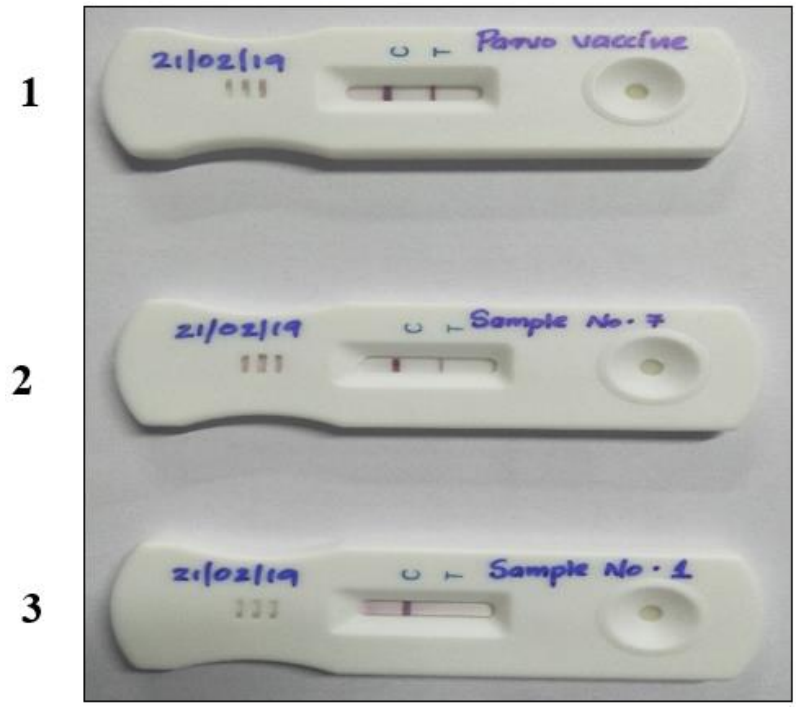
By employing conventional PCR it was found that out of 50 samples, 11 (22%) were found positive by polymerase chain reaction with uniform band of 681 BP (Fig 2). Results were resembled by Reddy *et al*. (2015) [17] who reported, out of 217 faecal sample tested, 72 (33.17%) were positive. These finding are in agreement with the findings of Sankalp *et al.* (2018) reported 18% prevalence of CPV by PCR in Madhya Pradesh.

Age wise distribution by conventional PCR showed that maximum positive samples refer to age group of 0-4 (54.54%,) followed by 4-8 (18.18%) and then 8-11/2 (27.27%). (Fig. 3). Prevalence observes highest in male dog (63.63%). (Fig. 4). As per breed study highest occurrence reported in Cross breed (63.63%) followed by German shepherd (18.18%), Labrador (9.09%) and Golden retriever (9.09%). (Fig.5). 2 samples were positive with prevalence of (18.18%) in vaccinated and (81.81%) in non-vaccinated dogs. The results are shown in (Fig.6). Hence the maximum dogs infected by CPV were of 0-4 month of age similar results were also concordance by (Tagorti. 2018 [21]. and Nookala *et al.* 2016) [13] who specified that it is known the increased intestinal epithelial turnover caused by changes in the microflora and diminishing maternal antibody level are the reason of CPV infection in pups. Sex-wise prevalence result in the present study was similar to the result obtained by and Behera *et al*. (2015) [3] and Agnihotri *et al*. (2018) [1] who observed high prevalence of CPV in male dogs and the reason

behind it is incidence of roaming and dominance aggression toward other dogs is high in males. Breed-wise prevalence result in the present study was resembling with la Torre *et al*. (2018) and Mokhtari *et al*. (2017) [10] reason behind this might due to higher population of cross breeds and absence of vaccination. In the present study, Prevalence result of CPV in non-vaccinated dogs is quite high, whereas vaccinated dogs also affected by CPV infection which is similar finding to Singh *et al.* (2013) [20] it might be because of irregular vaccination or incomplete vaccination or it might be possible that vaccination of pups against CPV is not conferring immunity against CPV. This might be due to the mismatching of vaccine strain and the CPV strain causing infection in dogs. In the present study, the antigenic characterization of CPV was also done using the primers for identification of CPV-2, CPV-2a, CPV-2b and CPV-2c individually (Fig. 7). The 11 rectal swabs which came positive by conventional PCR were subjected to Multiplex PCR and results revealed (Fig 3) that all the samples were found positive for CPV-2a. With product size of 379 bp. 6 (54.54%) faecal samples were positive for CPV-2c with product size of 470 BP and 2 samples were positive for CPV-2 with product size of 719 bp. While 6 (54.54%) faecal samples were positive for CPV-2a and CPV-2c. CPV-2b was absent in all the faecal samples and 2 (18.18%) samples was found positive for the three antigenic types such as CPV-2, CPV-2a and CPV-2c. Positive control Vaccine (Vanguard 5L4) found positive for only CPV-2a. The result of the present study discloses that CPV-2a were predominant antigenic type followed by CPV-2c and CPV-2 whereas negligible prevalence of CPV-2b.These findings are similar with Decaro *et al*. (2013) [4] according to them CPV-2a was the predominant strain worldwide, followed by CPV-2c and CPV-2b. Kaur *et al*. (2016) [8] screened 40 faecal sample by differential PCR, out of which 7 (17.5%) were found positive for antigenic type CPV2a with 379 bp amplicon, 3 (7.5%) positive for antigenic type CPV-2b with 178 bp amplicon and 1 (2.5%) was positive for antigenic type CPV-2c with 470 bp amplicon. (Table 3)

**Table 3:** Overall result of CPV infected dogs.

| Sample No. | Age | Breed | Vaccination status | Antigen kit | PCR positive | Multiplex PCR | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | 2 | 2a | 2b | 2c |
| 3 | 3 M | Labrador | NV | + | + | - | + | - | + |
| 4 | 4 M | German Shepherd | NV | + | + | - | + | - | - |
| 5 | 4 M | Cross Breed | NV | + | + | - | + | - | + |
| 6 | 11/2 Year | Cross Breed | NV | + | + | - | + | - | + |
| 7 | 2 M | Cross Breed | NV | + | + | + | + | - | - |
| 9 | 41/2M | German Shepherd | V | + | + | - | + | - | - |
| 10 | 1 Year | Cross Breed | NV | + | + | + | + | - | + |
| 12 | 3 M | Cross breed | NV | + | + | - | + | - | - |
| 16 | 3 M | Golden retriever | V | + | + | - | + | - | + |
| 18 | 9 M | Cross breed | NV | + | + | - | + | - | + |
| 20 | 5 M | Cross breed | NV | + | + | - | + | - | - |




1. Parvovirus vaccine: Positive
2. Sample No.7: Positive
3. Sample No.1: Negative

**Fig 1:** Ubioquick[vet] canine parvovirus antigen rapid test kit

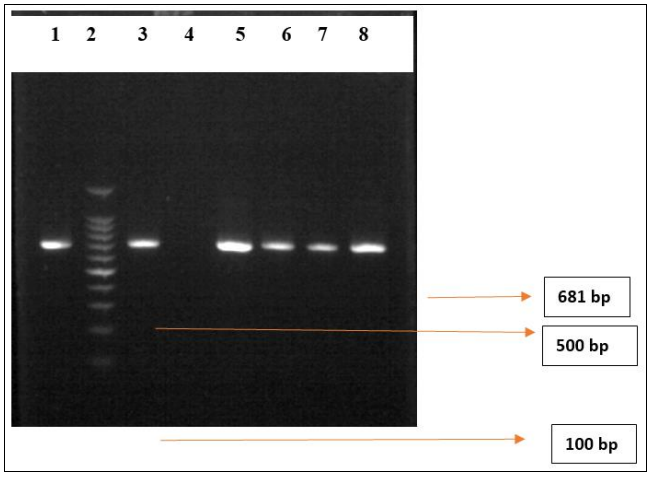
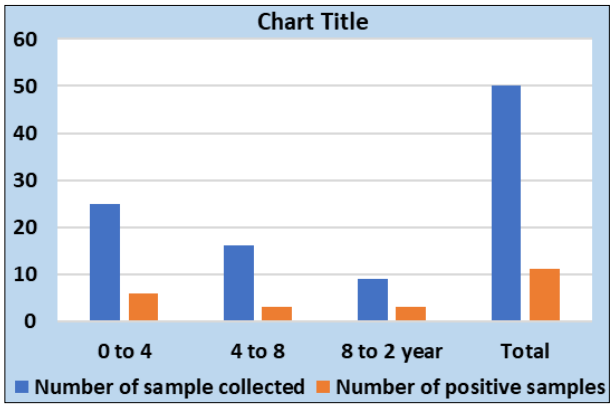
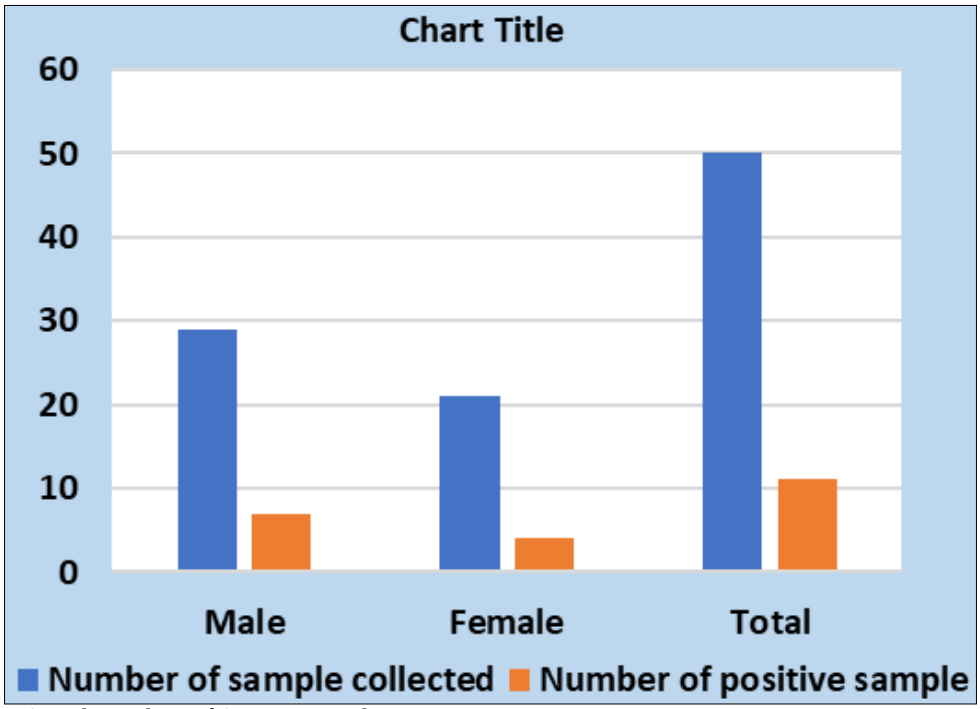



**Fig 2:** Amplification of VP2 gene of canine parvovirus-2 by PCR



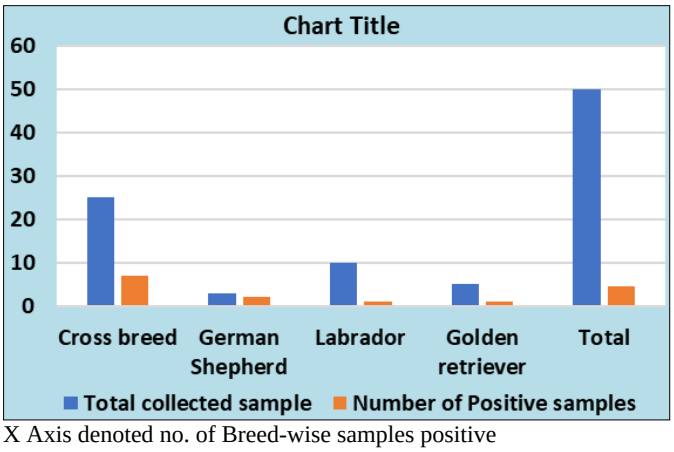

X Axis denoted no. of age wise samples positive
Y Axis denoted Total no. of samples collected

**Fig 3:** Age-wise status of *Canine parvovirus* by PCR



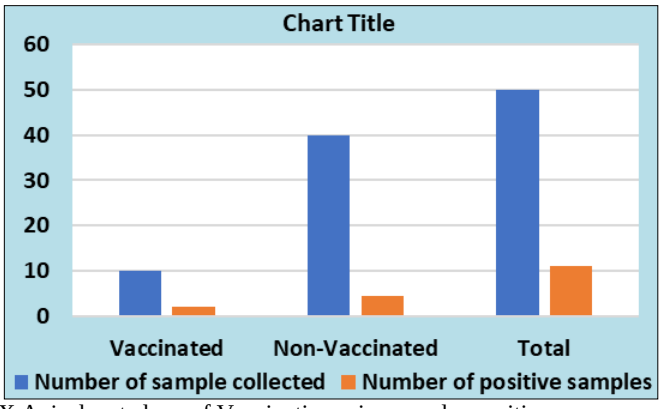

X Axis denoted no. of Sex wise samples positive
Y Axis denoted Total no. of samples collected

**Fig 4:** Sex-wise distribution of *Canine Parvovirus* by PCR




X Axis denoted no. of Breed-wise samples positive
Y Axis denoted total no. of samples collected

**Fig 5:** Breed- wise prevalence of *Canine Parvovirus* by PCR




X Axis denoted no. of Vaccination-wise samples positive
Y Axis denoted Total no. of samples collected

**Fig 6:** Prevalence of *Canine Parvovirus* by PCR based on the vaccination status

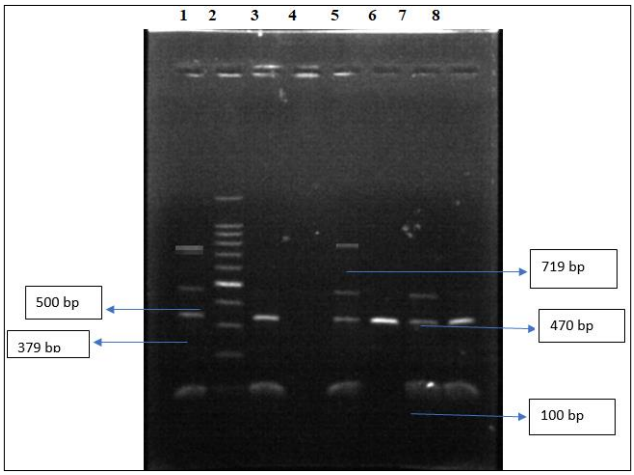



**Fig 7:** Multiplex PCR for the detection of CPV antigenic types

In the present study prevalence of age, sex and breed by Multiplex PCR is similar like that of PCR. But 2 samples were positive with prevalence of (18.18%) in vaccinated dogs. CPV infection in vaccinated dogs might be due to the difference of vaccine strain and the CPV strain which causing infection in dogs. There might be variation in the strain of CPV in conventional vaccines and the prevalent CPV strain in the field condition of India.

It can be concluded from the above study that canine parvovirus is prevalent in Nagpur and the most prevailing antigenic types are CPV-2a. The multiplex PCR was found to be highly efficient and quick as it was using only one cycle of PCR for detecting positive CPV. The results (age wise, breed wise, sex wise prevalence etc.) with the convectional PCR were similar to that of multiplex PCR.

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