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## Isolation and identification of canine parvovirus infection from around Nagpur region

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### Abstract

Diarrhea is a very common condition in dogs caused by various viral and bacterial causative agents. It has economic implications when a large number of dogs are affected in quick succession. It is multifactorial and the origin of *Canine parvovirus* (CPV) though is not absolutely clear but phylogenetically originated from feline panleukopenia virus or a very closely related carnivore parvovirus of feral canids like foxes and mink. The current study was designated for isolation and identification of parvovirus infections in dogs as one of the most important and main viral infection causing diarrhea in and around Nagpur by testing 91 fecal samples of diarrheic dogs below three months of age from September 2017 till March 2018. The samples were inoculated in MDCK cells revealed successful isolation of rotavirus in five samples with characteristic cytopathic effects. Out of 91 faecal samples 41(45.05%) samples were found positive with a uniform band of 681 bp. Among all PCR positive samples, one sample randomly selected for virus isolation in MDCK cell line, virus gets adopted after 3 passages. Characteristic cytopathic effect was noticed after 24 hours of post infection in shape of rounding of cell, followed by complete rounding of cell after 48 hours, clumping in 72 hours of post infection and eventually detachment of cell after 96 hrs. by using polymerase chain reaction (PCR).

**Keywords:** *Canine parvovirus*, Madin Darby Canine Kidney cell line (MDCK), polymerase chain reaction

### Introduction

Canine parvovirus -2 is a highly infectious viral disease of dogs and one of the serious concerns to pet owners, practicing veterinarians and scientists due to its high morbidity and mortality rate. Canine parvovirus belongs to family *Parvoviridae* comprising two subfamilies *Parvovirinae* and *Densovirinae*. *Parvovirinae* contain virus of vertebrates and *Densovirinae* contain virus of insects. There are 3 genera in sub family *Parvovirinae* viz, *Parvovirus Erythrovirus* and *Dependovirus*. (Murphy *et al.*, 1999) [7]. Canine parvovirus-2 are non enveloped having 25nm in diameter with icosahedral symmetry. Genome consist of single stranded negative sense DNA virus having size of 5.2 kb in length (Murphy *et al.*, 1999) [7]. CPV strains have undergone a series of evolutionary selections in nature, which was globally distributed as new variants which have completely replaced the original CPV-2. Recently, CPV has three main antigenic variants viz. 2a, 2b and 2c which are distributed among the dog population throughout the world. (Decaro *et al.*, 2006) [3]. VP2 which is a major capsid protein of CPV, play a major role in the diagnosis of host range and antigenicity of CPV. Mutation which affect VP2 gene are predominantly responsible for evolving different antigenic variant of CPV-2. (Phromnoi *et al.*, 2010) [8], (Kaur *et al.*, 2015) [5]. It has been observed that the canine parvovirus serotypes circulating in the Indian landmass is completely different from that included in the vaccine and may lead to decrease in the efficacy of the CPV-2 build vaccines. (Greenwood *et al.* 1995, Yule *et al.* 1997, Pratelli *et al.*, 2001) [4, 14, 9], could be the one reason for the infection in the vaccinated pups (Savi *et al.*, 2009) [10].

### Material and Methods

#### Collection of samples

Total 91 faecal samples/rectal swabs were collected from a dog exhibiting clinical sign like, vomition, anorexia, high temperature, depression, and gastroenteritis, hemorrhagic enteritis, from various clinics located in and around Nagpur.

### Isolation of Canine parvovirus-2

Virus Isolation is done by using Madin Darby Canine Kidney (MDCK) Cell Line which was procured from National Centre of Cell Science, Pune (India). The fecal sample positive for canine parvovirus by PCR used for virus isolation in MDCK cell line.

### Cell line and culture

MDCK cell line was procured from National Centre of Cell Science, Pune (India) and was maintained in the Department of Veterinary Microbiology and Animal Biotechnology T & R Cell, Nagpur Veterinary Collage, Nagpur. The cell culture flask having a MDCK cell line had been transported in maintenance medium as monolayer in cell culture flask. The cell culture flask (25 cm<sup>2</sup> cell culture flask) was kept in incubator at 37 °C with 5% CO<sub>2</sub>. Cells were observed under inverted microscope.

### Subculturing of MDCK cells

Observed the cell under inverted microscope for confluent monolayer of MDCK cell line in cell culture flask was subjected to subculturing. The GM DMEM in the cell culture flask was discarded and monolayer of MDCK cells was given two washings with PBS (pH 7.2). Then, 1 ml of TPGV solution was added to cover the surface of cells and the flask was kept at 37 °C for 30 seconds for the detachment of the cells. Again, 2 ml of TPGV was put in to flask and kept for 1 min, then medium was removed and a film of TPGV was left in the flask. Flask was kept at incubator at 37°C with 5% CO<sub>2</sub> for 5 min. Detachment of cell monolayer could be visualized against the tube light. Once the cell detachment was observed under inverted microscope, GM DMEM was added to neutralise the effect of trypsin. Vigorous pipetting was done for effective detachment and new flasks were seeded with these cells at the split ratio of 1:2. The new flasks were supplemented with 5 ml of GM DMEM each and incubated at 37 °C for obtaining a complete monolayer.

### Preparation of virus inoculums/ sample processing

The collected fecal sample were suspended in 10% (W/V) phosphate buffered saline (PBS, pH 7.2), was centrifuged at 10000 rpm for 10 minutes rpm at 4 °C to collect the supernatant. Collected supernatant then filter through 0.45 membrane syringe filter, and these filtrated mixed with equal volume of DMEM medium contain 2% FBS and 10 ug/ml crystalline trypsin. After proper mixing incubate it at 37 °C for 60 min. after incubation 1 ml of mixture inoculated into cell culture flask with 80-90% confluent monolayer of MDCK. Kept it for 1 hour of incubation in CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub> for complete adsorption. Washed the cells 3 times with plain DMEM. After washing over layered the cells with maintenance medium contain 1 ug/ml crystalline trypsin and placed the flask in CO<sub>2</sub> incubator at 37 °C with 5% CO<sub>2</sub> and examined under inverted microscope daily up to 5 days for the appearance of cytopathic effects (CPE). One un-infected monolayer cell culture flask was kept as normal cell control by adding maintenance media for maintenance of the MDCK cells.

### Harvesting of the virus

After incubation for 5 days for the CPE, the cell line irrespective of whether CPE appeared or not was subjected to two cycles of alternative freezing and thawing. The samples which did not exhibit CPE in first passage were further

subjected to 2<sup>nd</sup> and 3<sup>rd</sup> passage. The cell culture fluid was collected in micro centrifuge tube and stored at -20 °C to be used for further passaging of the samples.

### Polymerase chain reaction for detection of CPV-2 and cell culture passage samples

The DNA was extracted from CPE positive cell culture fluid by using DNA extraction kit. (Promega kit,USA).Detection of Parvovirus VP2 gene amplification using CPV-2ab-F and CPV-2 ab-R set of primers was used and conditions were optimized (Sheikh *et al.*, 2017) [12].

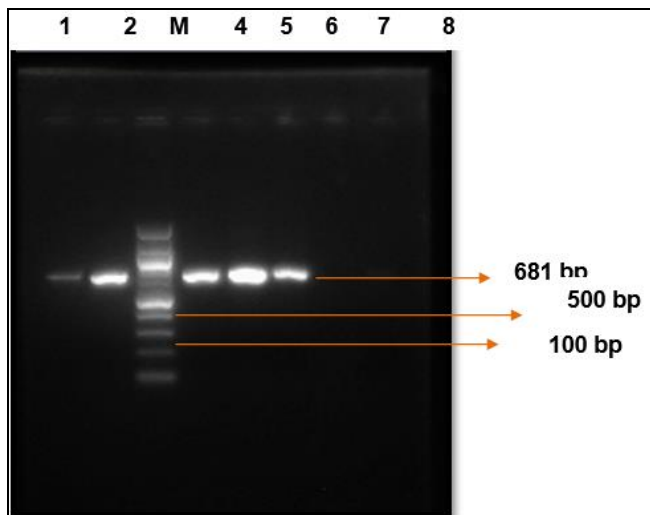
The extracted viral DNA was amplified by PCR assay using VP2 gene specific primers. The reaction mixture consisted of 2 µl Template DNA, 10 µl 5X PCR buffer, 3 µl MgCl<sub>2</sub>, 1 µl dNTPs, 1 µl Taq polymerase, 2 µl Forward Primer, 2 µl Reverse Primer, 29 µl Nuclease free water to make volume of 50 µl. All these ingredients were mix properly by vortexing. The PCR was programmed as Initial Denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 1 min, annealing at 50 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min. 5 µl of PCR product was then mixed with 3 µl of bromophenol blue (6X) and was run on gel electrophoresis and visualized by using UV transilluminator (Syngene G box, Uk).

### Results and Discussion

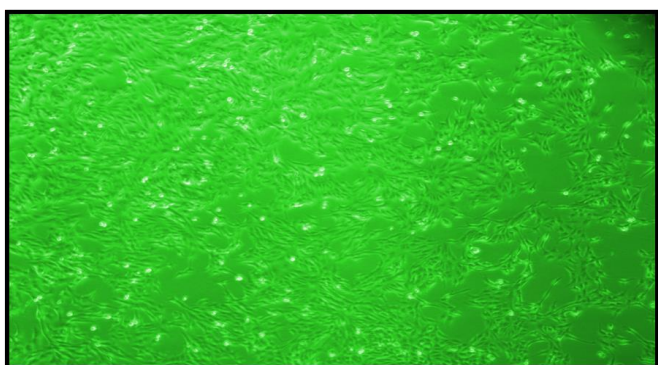
Out of 91 faecal samples 41(45.05%) samples were found positive by Polymerase chain Reaction with uniform band of 681 bp. (fig.1). These findings are in agreement with the findings of Sharma *et al.*, (2016) [11] who confirmed 50% (52/102) of the samples were found to be positive with a CPV-2ab PCR assay that detects newer variants of CPV circulating in the field and PCR positive isolate of CPV-2b was adapted to grow in MDCK cells and produced a characteristic cytopathic effect after 5th passage.

Among all PCR positive samples, one sample randomly selected for virus isolation in MDCK cell line, virus gets adopted after 3 passages. After the inoculation virus get adopted in cell. The complete monolayer was obtained within 48-72 hours Virus inoculated in MDCK cell line and show the cytopathic effect in the form of rounding of cells (fig. 2) within 24 hr of post infection and after 48 hrs there was complete rounding of cell was observed, (fig. 3) CPE has been marked after 72 hr in the form of clumping of cells (fig.4). The cytopathic effect remain continued after 96 hrs with detachment of cell. (fig. 5) These changes were typical of parvovirus infection in MDCK cell line and were also reported by Kaur *et al.* (2015) [5] who examined 60 samples selected for isolation, only five NPCR positive samples exhibited cytopathic effects (CPE) in the MDCK cell line. The CPE observed in the shape of rounding of cells within 24 hr, clumping of cells within 48 hr and detachment of cells within 72 hr of inoculation. Zhao *et al.* (2013) [15] examined fecal samples, isolated the type CPV-2a from three fecal samples, among three samples, one isolate CPV-2 and with second blind passages, typical CPE as rounding, increased granularity and detached cells recorded to be appearing in the contaminated F81 cells. Kaur *et al.* (2015a) [6], isolated canine parvovirus-2 by using MDCK cell line, samples show cytopathic effect in the form of rounding, clumping and detachment of cell, complet destruction of cell structure after 96 hours of PI. Brindalakshmi *et al.* (2016) [2], isolated virus in CRFK cell line, rounding as well as degenerative changes were observed as cytopathic effect at third passage level of 72

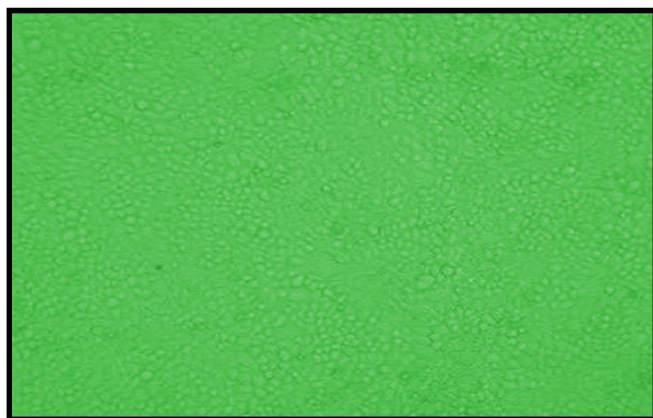
hours post infection. Vieira *et al.* (2017) [13] was isolated 67 out of 100 (67%) CPV samples. The cytopathic effect remained associated by cell rounding and/or partial or total lysis of the formed monolayer when checked with control. Berns (1990) [1] found that Parvoviruses are fastidious which are difficult to isolate in cell culture. Parvovirus replication is host-cell dependent and takes place only in actively dividing S-phase cells where cellular DNA polymerase is synthesized abundantly. The DNA extracted from the cell culture fluid from the samples exhibiting CPE was subjected to PCR confirmed CPV by amplifying 681 bp in PCR.



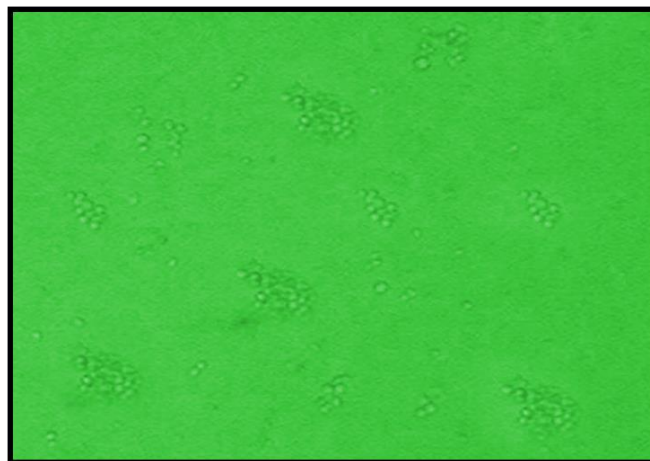
**Fig 1:** Amplification of VP2 gene of Canine parvovirus-2 by PCR.



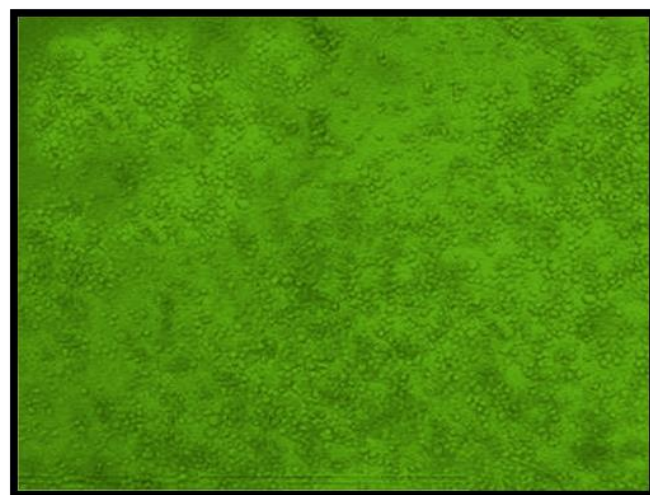
**Fig 2:** Rounding of cell start at 24 hours of post-infection at 10 x magnification



**Fig 3:** Complete rounding of cell after 48 hours of post-infection at 10 x magnification



**Fig 4:** Clumping of cell observed after 72 hours of post-infection at 10 x magnification



**Fig 5:** Detachment of cell observed after 96 hours of post-infection at 10 magnification

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