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# First Isolation of Highly Cytopathic Canine Parvovirus-2 on Madin Darby Canine Kidney Cells from a Diarrheic Pet Dog of Indore in Madhya Pradesh and its Molecular Characterization as Canine Parvovirus-2a

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## ABSTRACT

**Background:** Canine parvoviral diarrhea is one of the most common viral infections in dogs. Canine parvoviral diarrhea is caused due to the virus, Canine parvovirus-2. Regular surveillance and knowledge of the emergence of new Canine parvovirus-2 viral variants in infected pet dogs is the prerequisite for the prevention and control of this disease.

**Methods:** Deoxyribonucleic acids extracted from processed faecal samples of 50 diarrheic pet dogs were used to amplify Canine parvovirus-2 specific gene (Partial nucleotide region) by using a polymerase chain reaction test. Viral inoculum prepared from the positive faecal sample in the above test was adsorbed on Madin Darby Canine Kidney cell line of epithelial origin. The isolated virus was further characterized by using a molecular test: amplification refractory mutation system-polymerase chain reaction.

**Result:** Deoxyribonucleic acid extracted from the processed faecal material of the dog tested positive for the presence of a Canine parvovirus 2-specific gene. The virus was successfully adapted to the Madin Darby Canine Kidney cell line and showed cytopathic effects on the very first passage. The isolated highly cytopathic virus Canine parvovirus-2 from the diarrheic pet dog was further characterized as Canine parvovirus-2a by using a molecular test.

**Key words:** Canine parvovirus-2a, German shepherd, Madin darby canine kidney cells, Veterinary clinical complex.

## INTRODUCTION

According to the State of Pet Homelessness Index Data, there were around 62 million stray dogs in India. The report also stated that 85% of companion animals in India were homeless (https://www.outlookindia.com/website/story/62-crore-dogs-and-91-lakh-cats-are-homeless-in-india/402674; Accessed on 30th June 2022). However, the Coronavirus disease (COVID)-19 pandemic scare led people to remain at home for longer duration in isolation. It resulted in a greater urge to have companionship. Rearing a dog as a pet animal became popular amongst Indians. The report suggested that 6 out of 10 citizens in India have a pet. The growth of the pet care industry is going up due to the adoption of pets by nuclear families having higher income levels and the ongoing pandemic. Around 28 million dogs were reared as pet animals in India in the year 2021 (https://www.cnbctv18.com/videos/lifestyle/study-finds-6-in-10-indians-now-have-a-pet-heres-whats-fuelling-the-pet-care-industry-11496972.htm; Accessed on 30th June 2022). Canine parvovirus-2 (CPV-2) is placed under the newly classified system for viruses designated by the International Committee on Taxonomy of Viruses in the year 2022 as per the following; Realm-*Monodnaviria*, Kingdom-*Shotokuvirae*, Phylum- *Cossaviricota*, Class-*Quintoviricetes*, Order-*Piccovirales*, Family-*Parvoviridae*, Subfamily-*Parvovirinae* and Genus-*Protoparvovirus* (https://ictv.global/news/taxonomy-2022; Accessed on 19th May 2023). The virus, CPV-2 cause one

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of the very important viral diseases of pet dogs, Canine parvoviral diarrhea. The disease is characterized mainly by haemorrhagic enteritis, myocarditis and lymphopenia in young pups or nonvaccinated dog population. The carrier dogs can excrete CPV-2 in faeces as the virus replicates in intestinal crypts besides lymphoid organs. The virus most commonly transmits through the oronasal route from infected to susceptible animal populations (Lin and Chiang, 2016). The affected dogs often appear very weak due to diarrhoea, if unattended. The CPV-2 infection can cause morbidity up to 100% and mortalities up to 10% in adult dogs and 91% in pups (Nandi *et al*., 2019). Canine parvoviral enteritis due to CPV-2 is diagnosed tentatively clinically (history of bloody diarrhea) and also definitively by various serological, molecular and virus isolation-based laboratory diagnostic methods (Minakshi *et al*., 2016). CPV-2 isolation is done by using different cell lines namely Madin Darby Canine Kidney cells (Sharma *et al*., 2016), A-72 cells (Chinchkar *et al*., 2014; Verma *et al*., 2016) and Crandell Feline Kidney cells (Parthiban *et al*., 2011). Apart from the original CPV-2, there are three different strains of CPV-2 (CPV-2a, CPV-2b and CPV-2c) circulating amongst the dog population. Recently newer CPV-2 variants have been reported, namely new CPV-2a, new CPV-2b and emergent CPV-2c due to genetic changes in the CPV-2 (Lin and Chiang, 2016; Abas *et al*., 2022). Regular testing of diarrheic pet dog population is essential to ascertain the cause of the disease which will subsequently help in preventing and controlling CPV-2 infection. In the present study, CPV-2 diagnosis was achieved by using molecular (nucleic acid-based PCR) and virus isolation methods (isolation on Madin Darby Canine Kidney cell line of epithelial origin) to confirm CPV-2 infection. Further, molecular characterization of the isolated virus strain in the study revealed the involvement of Canine parvovirus 2a.

## MATERIALS AND METHODS

### Cell line and its maintenance, growth and observation

Madin Darby Canine Kidney (MDCK) epithelial cell line, suitable cell culture medium ready to use Minimum Essential Medium (MEM) Eagle with Earle's salts, L-Glutamine, Sodium pyruvate and Sodium bicarbonate from Hi Media, readymade solution for trypsinization and foetal bovine serum (FBS) of commercial origin was transported to Department of Veterinary Microbiology, College of Veterinary Science and Animal Husbandry, Nanaji Deshmukh Veterinary Science University, Dr. Ambedkar Nagar-Mhow - 453 446 (Place A for the study), Indore, Madhya Pradesh, India from Veterinary Type Culture Collection (VTCC), Division of Biological Standardization, Indian Council of Agricultural Research, Indian Veterinary Research Institute, Izatnagar - 243 122 (Place B for the study), Bareilly, Uttar Pradesh, India in the month of November 2019. Subsequent cell culture passaging, growth and maintenance of MDCK cells was conducted at Place A. The normal epithelial morphology of the MDCK cells and cytopathic effects produced due to Canine parvovirus-2 infection were observed by using an inverted microscope (Metzer) available at the Central Instrumentation Laboratory of the college at Place A. On 90% of monolayer formation at passage level 84th, it was further given 2 more passages in the cell culture flasks (Schott Duran) at the split level of 1:3 to stabilize these cells in the incubating conditions.

### Canine population and faecal samples

The detailed history of the pet dogs used in the present study was published previously (Kanesh *et al*., 2022).

### Processing of faecal sample for preparation of inoculum in the virus isolation

The faecal sample was diluted in 10 ml sterile phosphate-buffered saline solution and homogenized. The mixture was further centrifuged at 3,000 revolutions per minute (rpm) for 10 minutes in the refrigerated centrifuge machine. A total of 5 ml of the supernatant was further clarified by using a sterile 0.44 µm polyvinylidene fluoride (PVDF) syringe filter.

### Adsorption of canine parvovirus-2

A total of 100 µl of clarified viral inoculum was layered over 75% confluent MDCK cells (monolayer) in the cell culture flask for 1 hour at 37°C in the incubator for adsorption. Thereafter, the remaining viral inoculum was discarded and the monolayer was rinsed with 2 ml of sterile cell culture media. Now, a maintenance medium containing 2% FBS was added to the cell culture flask. These flasks were kept in the $CO_2$ incubator and observed for at least 5 consecutive days or till the cytopathic effects (CPE) developed. Negative controls were also kept in the study.

### Nucleic acid extraction from faecal samples/canine parvovirus-2 infected cell culture fluid

Deoxyribonucleic acid (DNA) was extracted from faecal samples and virus-inoculated cell culture fluid in the study by the following method. In a sterile tube, 200 µl of 10% suspension of cells/cell culture fluid/faecal samples was taken. The equal quantity of Tris saturated phenol (pH 8.8) was added in the suspension. The mixture was centrifuged at 10,000 rpm for 3 minutes. Now, after drawing out 200 µl of the resulting supernatant into a separate tube, 100 µl of Tris saturated Phenol (pH-8.8) and 100 µl of Chloroform was added. This mixture was centrifuged at 10,000 rpm for 3 minutes. Again, 200 µl of supernatant was taken into a separate tube and into it, 200 µl of Chloroform was added. The resulting mixture was centrifuged at 10,000 rpm for 3 minutes. 20 µl of Sodium acetate (pH-5.5) was added to 200 µl of the supernatant and 1 ml of absolute ethanol was added. The tube was kept at the deep-freezing conditions at -20°C. The next day, in the morning the tube was centrifuged at 10,000 rpm for 10 minutes. After discarding the supernatant, resulting pellet was dried. Finally, the pellet was dissolved in 50 µl of nuclease-free water.

**Polymerase chain reaction test**

The extracted nucleic acid was used in one of the molecular methods, the polymerase chain reaction test to amplify the Canine parvovirus-2 specific nucleotide product (part of the VP1/VP2 gene) (Nandi and Kumar, 2010; Singh *et al*., 2015; Kanesh *et al*., 2022). CPV-2 Forward primers-5′-GAA GAG TGG TTG TAA ATA ATA-3′ and Reverse primers: 5′-CCT ATA TCA CCA AAG TTA GTA G-3′ were used to amplify CPV-2 specific 681 bp of product in the test. Positive and negative controls were also kept in the study. The ethidium bromide-stained amplified products were visualized and documented upon agar gel electrophoresis (1.5% gel, Tris borate Ethylenediaminetetraacetic acid buffer).

## RESULTS AND DISCUSSION

In one of the previous studies, 50 processed faecal samples of diarrheic pet dogs were used directly without extracting nucleic acid in a PCR test (Kanesh *et al*., 2022) in which only a single faecal sample tested positive. So, these 50 faecal samples were sent to the Centre for Animal Disease Research and Diagnosis (CADRAD) at Place B for further confirmation. The submitted faecal samples were used first to extract nucleic acids by following the standard deoxyribonucleic acid (DNA) extraction protocols. The extracted DNA samples tested in PCR for specific amplification of VP2 gene specific product of CPV-2. Here also only one faecal sample tested positive in PCR. The PCR test is more sensitive than most of the other methods for the diagnosis of CPV-2, however, the low sensitivity of PCR to amplify specific gene products for CPV-2 even after using extracted DNA as a template in the present study can be due to the presence of PCR inhibitory substances in the faecal samples. There can be the involvement of other novel infectious agents in pet dogs that may cause diarrhea (Agnihotri *et al*., 2023). The faecal sample that tested positive in the PCR test from diarrheic German Shepherd pet dog was chosen for isolation of CPV-2 on the cell line to further characterization of CPV-2 at its molecular level. Hence, in the present investigation for isolation of CPV-2, Madin Darby Canine Kidney (MDCK) cell line was used. The viral inoculum prepared from PCR-positive faecal sample was used first in the adsorption of CPV-2 virus on cell surfaces of anchorage-dependent MDCK cells grown in the 25 $cm^2$ cell culture flask for an hour at 37°C. Thereafter, the excess viral inoculum was discarded and the cell culture flask was supplied with a maintenance medium (with 2% FBS). The inoculated cell culture flask was observed every 6-12 hours initially then daily. The cytopathic effects (CPE) like rounding of cells and detachment were observed 24 hours after initial adsorption in the viral inoculated flask. Similar CPE were observed by Zaher *et al*. (2020) on MDCK cells inoculated with CPV-2. The CPE was well over 75% within the next 24 hours. At this stage, the cell culture flask was kept in the freezer at -40°C. It was freeze-thawed twice and the resulting viral suspension was stored in cryovials. One such set of cryovials containing CPV-2 infected cell culture suspension was subsequently sent to CADRAD at Place B for confirmation of viral adaptation on MDCK cells. Sharma *et al.* (2016) reported CPE only after 5th passage on MDCK cells for an isolate of CPV-2b. In the present study, the CPV-2 viral isolate required only one passage on MDCK cells for its adaptation and production of CPE indicative of the highly cytopathic nature of isolated CPV-2. CPV-2 and its variants are being reported in India (Kaur *et al*., 2015; Sharma *et al*., 2018; Kumari *et al*., 2019). The highly cytopathic CPV-2 strain was further characterized at its molecular level as CPV-2a in the amplification refractory mutation system- polymerase chain reaction test (Chander *et al*., 2016) (National Centre for Biotechnology Information Accession Number: MZ209401).

## CONCLUSION

The results of the present study indicated involvement of Canine parvovirus-2 as a causative agent for gastroenetritis in a German Shephed dog of 6 months of age. The CPV-2 was readily adapted on Madin Darby Canine Kidney (MDCK) cells giving cytopathic effects post 24 hours of adding viral inoculum for adsorption. CPV-2 isolate was further identified as CPV-2a by using a multiplex amplification refractory mutation system - polymerase chain reaction test. The cell culture-adapted CPV-2 viral isolate in the present study from Dr. Ambedkar Nagar-Mhow, Indore, Madhya Pradesh was submitted to Virus Laboratory, Centre for Animal Disease Research and Diagnosis for conducting advanced studies related to nucleotide sequencing and phylogenetic analysis. Such studies will be helpful for understanding the molecular epidemiology of CPV-2 in India.

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