

Isolation of Canine Parvovirus from Rectal Swabs of Suspected Dogs from Various Regions in Northern India

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Abstract

Canine parvovirus (CPV) is a highly contagious pathogen causing acute gastro-intestinal illness among the canine population. The study aimed to isolate canine parvovirus in a Madin-Darby Canine Kidney (MDCK) cell line from rectal swabs of dogs having signs of gastroenteritis. A total of 45 rectal swabs were collected from dogs exhibiting clinical signs of canine parvovirus infection from various regions of northern India, viz., Punjab, Assam, Delhi, Chandigarh and Jammu. The samples were processed and subjected to MDCK cell isolation, and isolates that exhibited cytopathic effects (CPE) like rounding of cells, clumping and detachment of cells were confirmed by PCR and nPCR. Out of 45 samples, 12 (26.66%) isolates showed CPE on MDCK. Further, it was observed that out of these 12 isolates, three isolates were from dogs which had history of vaccination for the disease.

Keywords: Canine parvovirus, MDCK cell line, isolation, Northern India, cytopathic effects.

Резюме

Кучешкият парвовирус (*Canine parvovirus*, CPV) е силно заразен патоген, причиняващ остро стомашно-чревно заболяване сред кучешката популация. Проучването има за цел да изолира кучешки парвовирус в клетъчна линия Madin-Darby Canine Kidney (MDCK) от ректални тампони, взети от кучета с признаци на гастроентерит. Събрани са общо 45 ректални тампона от кучета, показващи клинични признаци на кучешка парвовирусна инфекция от различни региони на Северна Индия, а именно Пенджаб, Асам, Делхи, Чандигарх и Джаму. Пробите са обработени и изолирани в MDCK клетъчна линия. Изолатите, които проявяват цитопатични ефекти (CPE) като закръгляване, струпване и отделяне на клетките са потвърдени чрез PCR и nPCR. От 45 проби ректални тампона на предполагаеми за носителство кучета, 12 изолата (26.66%) показват CPE върху клетки MDCK. От тези 12 изолата, три изолата са от кучета, които са с данни за ваксинация срещу болестта.

Introduction

Canine parvovirus (CPV) is the leading cause of highly contagious and a relatively common cause of acute, infectious gastro-intestinal illness, especially in young/unvaccinated dogs. Canine parvovirus is one of the smallest known viruses (~25nm in diameter) belonging to the genus *Protoparvovirus* of the family *Parvoviridae* (Appel, 1978; Berns, 1990). CPV is a non-enveloped icosahedral, linear, non-segmented, negative-sense, single-stranded DNA genome of 5,323 bases which encodes for three structural proteins (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2). VP2 is the major structural protein of capsid and its key antigenic determinant. The earliest known CPV positive sera were reported in Greece in 1974. CPV

was first identified in the mid-1970s as an emerging pathogen (Appel, 1978; Appel *et al.*, 1979) and was referred to as CPV-2 to distinguish it from CPV-1 (Parrish and Carmichael, 1983). In India, CPV-2 was first reported from Madras by Balu and Thangaraj (1981) and it was first isolated in India by Ramadass and Khader (1982). A few amino-acid substitutions in the VP2 gene sequence can lead to mutations in CPV and are responsible for different antigenic variants of the virus (Phromnoi *et al.*, 2010; Kaur *et al.*, 2016). Presently, there are four known antigenic types of CPV circulating throughout the world, viz., CPV-2, CPV-2a, CPV-2b and CPV-2c. As the knowledge regarding the current antigenic types of CPV in northern India is still

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limited, more seroprevalence data and detection of ongoing mutations in the VP2 gene would be of great help in the early detection and identification of the possible new emerging CPV strains. Thus, the present study was designed to isolate CPV from the rectal swabs of suspected dogs.

Materials and Methods

Sample collection and preparation of virus inoculum

A total of 45 rectal swabs in two ml of phosphate buffer saline (pH=7.2) from dogs exhibiting clinical signs of parvoviral enteritis like gastroenteritis/hemorrhagic enteritis with pyrexia, vomiting, dehydration etc. were collected from New Delhi (n=11), Chandigarh (n=6), Jammu (n=3), Punjab (n=24) and Assam (n=1). The rectal swabs were squeezed around the walls of the collection tube and centrifuged at 3000 rpm for 15 minutes to collect supernatant and 0.1 ml of antibiotic and antimycotic 100x solution was added and kept for incubation at 37°C for 30 minutes. Then it was stored at -20°C till further use.

Virus isolation

Dulbecco's Modified Eagle's Medium (DMEM) growth medium (GM) with 10% fetal bovine serum (FBS) was used for virus isolation in Madin-Darby Canine Kidney (MDCK) cell line. After a complete monolayer of MDCK cells was obtained on 12 -well culture plate, 0.1 ml of prepared viral inoculum was added to each well and culture plates were kept for incubation at 37°C for 1.5 h for the adsorption of the virus onto cells in each well. Then after washing each well, 1ml DMEM maintenance media (MM) with 2% FBS was added and the culture plates were incubated at 37°C with 5% CO₂ for 3-7 days to observe CPE. The cell culture fluid was collected after three cycles of freezing and thawing from the wells that showed CPE.

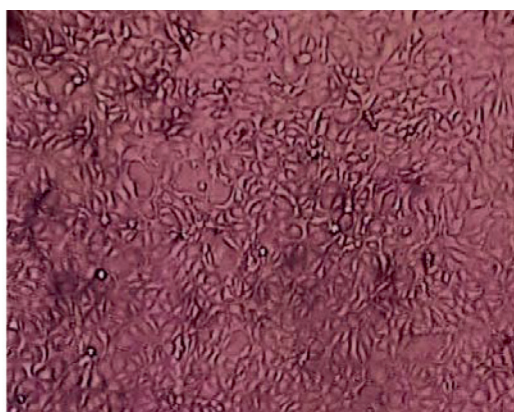


Fig. 1. Negative control having monolayer of MDCK cells at 10x magnification

Confirmation of isolates

DNA was extracted from the cell culture fluid showing CPE (Russell and Sambrook, 2001) and was subjected to PCR and NPCR. For PCR and NPCR, primers were used as per Mizak and Rzezutka (1999). For PCR, the reaction mixture was prepared by adding 15µl of the template DNA, 5.0 µl of 10x PCR buffer (with 15 mM MgCl₂), 1.0 µl of forward and reverse primer (20 pm/µl) each, 1.0 µl of dNTPs mix (10 mM), 0.2µl Taq DNA polymerase 5 units/µl and the final reaction mixture volume was made up to 50 µl using nuclease-free water. For further confirmation by NPCR, the reaction mixture was prepared by adding 5 µl of the PCR product (from above reaction), 2.5 µl of 10x PCR buffer (with 15 mM MgCl₂), 1.0 µl each of forward and reverse primer (20 pm/µl), 1.0 µl of dNTPs (10 mM), 0.2 µl Taq DNA polymerase (5 units/µl) and the final volume was made up to 25 µl by adding nuclease-free water.

Both PCR and NPCR reactions were set at thermocycling parameters with 35 cycles of denaturation at 94°C for 60s, annealing at 55°C for 60s, elongation at 72°C for 150s and a final elongation at 72°C for 10 min. Both PCR and NPCR products (10 µl each) were subjected to Agarose Gel Electrophoresis by using 1% agarose at the rate of 5 volts/cm with GeneRuler Ladder plus 100 bp (New England Biolabs, USA). The products on gel were visualized and documented using Gel documentation system (Syngene, USA).

Results and Discussion

Out of the 45 samples subjected to virus isolation, 12 (26.66%) samples exhibited cytopathic effects (CPE) in the MDCK cell line. The CPE observed were rounding of cells within 24 h (Fig. 1), clumping of cells within 48 h (Fig. 2), and detachment of cells within 72 h of inoculation (Fig. 3,

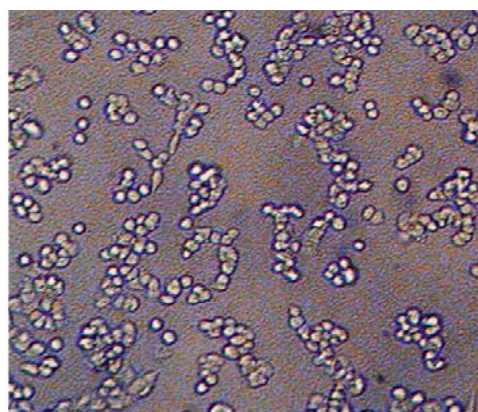


Fig. 2. The cytopathic effect: Rounding of MDCK cells by CPV at 20x magnification

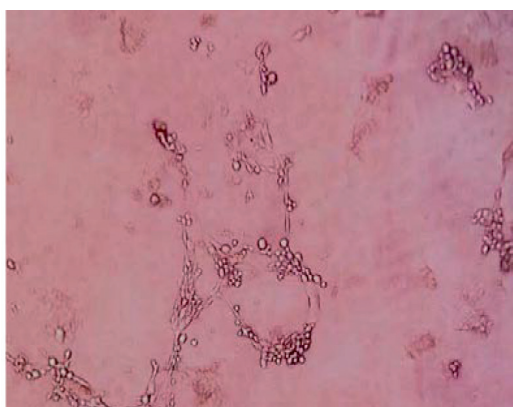


Fig. 3. The cytopathic effect: Clumping of MDCK cells by CPV at 10x magnification

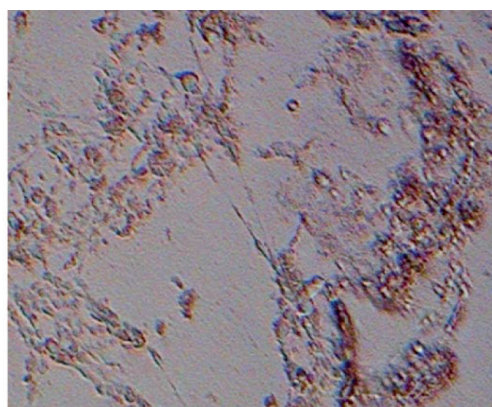


Fig. 4. The cytopathic effect: Detachment of MDCK cells by CPV at 20x magnification

Fig. 4). Out of the 12 samples that exhibited CPE in MDCK cell line, eleven samples (D4, P4, P5, P15, P16, C3, C6, J1, G28, G30, A35) showed CPE in the first passage and one sample (D5) showed CPE in the 2nd passage, whereas the remaining samples did not exhibit any CPE even after the 3rd passage (Table 1).

In PCR and NPCR, 1198bp product and 548bp product were amplified respectively, which confirmed the presence of CPV. Casewise analysis revealed that four CPE positive samples (D4, D8, P15 and P16) were from the dogs having haemorrhagic gastroenteritis; three CPE positive samples (P4, P5 and C6) were from vaccinated dogs, and

Table 1. CPV samples positive with cytopathic effects

S. No.	Sample No.	Age (Months)	Sex	Breed	Diarrhoea	CPE (I Passage)	CPE (II Passage)	Vaccination/Booster Status
1	D4	1.5	M	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
2	D8	2	F	Mongrel	Hemorrhagic	No CPE	Rounding and Clumping	✗
3	P4	4	M	Indian Bully	Non-Hemorrhagic	Rounding and Detachment	...	✓
4	P5	6	M	Pitbull	Non-Hemorrhagic	Rounding and Detachment	...	✓✓
5	P15	2.5	M	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
6	P16	1.5	F	GSD	Hemorrhagic	Clumping and Detachment	...	✗
7	C3	2.5	M	Labrador Retriever	Hemorrhagic	Clumping and Detachment	...	✗
8	C6	4	F	Cocker Spaniel	Non-Hemorrhagic	Rounding	...	✓✓
9	J1	4	F	Mongrel	Hemorrhagic	Clumping and Detachment	...	✗
10	G28	2	F	Mongrel	Non-Hemorrhagic	Rounding and Clumping	...	✗
11	G30	3	F	Labrador Retriever	Non-Hemorrhagic	Rounding and Clumping	...	✗
12	A35	2.5	M	Mongrel	Non-Hemorrhagic	Rounding and Clumping	...	✗

eight CPE positive samples (D4, D8, P15, P16, C3, G28, G30, A35) were from dogs below 3.5 months of age.

CPV causes a highly contagious form of acute gastro-intestinal illness often leading to high mortality among dogs all around the globe. CPV has been isolated from many regions all over the world and is known to continually evolve into its various antigenic variants due to high mutation rates, which has raised a concern for potential CPV enteritis outbreaks, hence exposing the health of dogs to risk. Thus, studying the ongoing status of CPV geographical prevalence of any new antigenic types is of key importance in order to predict and ensure the good health of dogs in future.

Parvovirus is a fastidious pathogen and difficult to isolate, since replication is hostcell dependent and takes place only in actively dividing S-phase of cells, where cellular DNA polymerase is synthesized abundantly (Tattersall, 1972; Berns, 1990). Isolation of CPV on cell lines was reported by various workers in the past. Canine parvovirus was first isolated in the year 1978 by Appel *et al.* (1979), where they used primary cultures of canine foetal lung, kidney as well as feline foetal lung and kidney, which had given consistent results. A-72 cell line was used by Sagazio *et al.* (1998) for isolation of CPV-2 using co-cultivation method. The CPE was observed for 3-4 days. Indirect fluorescent antibody test (IFAT) using a canine serum with antibodies to CPV-2 was used to confirm the virus.

In another study, Nandi *et al.* (2010) isolated CPV on MDCK cell line grown in DMEM containing 10% fetal calf serum and observed CPE up to 72 hours post infection. Further, out of five samples, only one showed cytopathic changes, including rounding of cells, granulation, aggregation of cells similar to the cytopathic changes observed in the present study. In another study, Kumar *et al.* (2010) subjected 25 samples for the isolation of CPV using MDCK cell line and reported that 9 samples developed CPE in the MDCK cell line.

Raj *et al.* (2010) observed that out of fifty-one samples passaged in CRFK cells, sixteen isolates were confirmed by PCR. Similarly, isolation was attempted from Puducherry, South India, in 2010 (Parthiban *et al.*, 2010), in which CPV was isolated from rectal swabs from suspected cases of dogs similar to the present study. Thus, isolation of CPV has been done by various researchers from India and across the world over and they had similar observations of the cytopathic effects produced by canine parvovirus in MDCK or CRFK cell line (Zhao

et al., 2013; Deka *et al.*, 2015; Kaur *et al.*, 2015; Wang *et al.*, 2016; Vieira *et al.*, 2017; Jaune *et al.*, 2019; Abdel-Rehman *et al.*, 2019; Chen *et al.*, 2019). Thus, from the study it could be concluded that isolation of CPV from rectal swabs of dogs was successful. Further, these isolates can be subjected to sequencing of VP2 in order to study variation.

Conclusions

Canine parvovirus could be isolated in MDCK cells from rectal swabs collected from clinical cases of dogs in the regions of Punjab, New Delhi, Chandigarh, Jammu and Assam. Twelve isolates were obtained which can be used to gain wider insights on the possible mutations and prevailing antigenic types of CPV in different geographical regions of India.

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