



E-ISSN: 2320-7078

P-ISSN: 2349-6800

www.entomoljournal.com

JEZS 2020; 8(3): 1967-1972

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Received: 03-03-2020

Accepted: 06-04-2020

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In vivo histopathological and *in vitro* cell culture studies upon *Canine parvovirus* infection

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Abstract

In the present study *in vivo* and *in vitro* studies were carried out on Canine parvovirus (CPV). Crandell rees feline kidney and Madin darby canine kidney cell lines were used for the propagation of the virus in the laboratory and tissues from the parvo affected dogs were collected after the post mortem and histopathological changes were noticed. A total of 9 carcasses infected with parvovirus were used for the *in vivo* studies and important infected organs like intestines and heart histopathological sections were done to identify the changes produced by canine parvovirus. Out of 342 clinically suspected fecal samples collected from the affected parvoviral cases of which 34 fecal samples confirmed of CPV by haemagglutination test (HA) and Polymerase chain reaction (PCR) were propagated in two different cell lines to study the variations in the cytopathic effects caused by CPV. Thirty four samples were propagated in CRFK and only 16 were able to produce minimal cytopathic effect whereas 9 nine samples were infected in MDCK and 5 produced marked cytopathic effect.

Keywords: Canine parvovirus, cell cultures, histopathological studies, *In vivo* and *In vitro* studies

Introduction

Canine parvovirus (CPV-2) is one of the most important enteric viral pathogen affecting canines. Widespread outbreaks of canine hemorrhagic enteritis occurred throughout the world and CPV emerged as an important zoonosis of dogs (Zhao *et al.*, 2016) [27]. Canine parvovirus, 40 years after its emergence disseminated in nearly 42 countries all over the world (Miranda and Thompson 2016) [10].

Canine parvovirus symptoms vary clinically depending upon the age group of the affected dogs. Canine parvovirus has more affinity towards the mitotically active tissues mainly lymphoid tissue, intestinal epithelium, bone marrow and in neonatal pups cardiac tissue is also affected (Mylonakis *et al.*, 2016) [11]. The disease is characterized by vomitions, bloody diarrhea, myocarditis and leucopenia (Streck *et al.*, 2009) [23]. The infection leads to rapid loss in condition of the animal if not treated timely (Kaur *et al.*, 2016) [8]. Thus diagnosis of the CPV infection is of great concern. In the present study, growth variations of CPV in *in vitro* cells like CRFK and MDCK cell lines were taken up and focused on the post cytopathic effects caused by the virus. The various pathological changes occurred in the dead carcasses infected with CPV were carried out by the histopathological studies followed by H&E staining.

Materials and Methods

Three hundred and forty two fecal samples suspected of CPV were collected from 11 districts of Andhra Pradesh during the months of June 2017 to December 2018. The fecal material was collected with the help of sterilized swabs from dogs from Super Speciality Veterinary Hospital, Vijayawada, Teaching Veterinary Clinical Complex, NTR College of Veterinary Science, Gannavaram and College of Veterinary Science, Tirupati and from various Veterinary Polyclinics across different districts of Andhra Pradesh. Canine parvovirus affecting the dogs has more affinity to intestinal epithelium and in pups, the myocardium. To carry out the *in vivo* studies of the CPV upon post mortem tissues were selectively collected from intestines and heart depending upon the age of the dogs. CRFK (Crandell Rees Feline Kidney cell line) and MDCK (Madin-Darby Canine Kidney cell line) were used for the *in vitro* growth variation studies of CPV. The cell culture works and histopathological studies were carried out at NTR College of Veterinary Science, Gannavaram, Andhra Pradesh.

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Sub culturing of CRFK and MDCK Cell lines

Crandell rees feline kidney cells and Madin darby canine kidney cells were maintained routinely in tissue culture flasks. The cells were subcultured with a split ratio of 1:3 and 1:2, respectively. For CRFK, after obtaining confluent monolayer, the growth medium was completely drained and monolayer was washed with warm calcium- magnesium free-PBS but in case of MDCK, warm CMF-PBS of about 2 ml was poured into the flask and placed at 37°C in an incubator for 15 min. Later, CMF-PBS was drained out and pre-warmed trypsin of 0.5 ml was added to the monolayer and spread over the monolayer by tilting the bottles for 30 sec until cell sheet was detached in CRFK and for MDCK cell lines the flasks were placed at 37°C in an incubator for 3 to 5 min until there was complete rounding of cells. The cells were gently flushed down the surface with 3 ml of growth medium. Then the cells were suspended in growth medium and distributed in the split ratio of 1:3 and 1:2 respectively and incubated at 37°C in carbon dioxide incubator at 5% CO₂ level until the confluent monolayer was formed.

Propagation of CPV in CRFK and MDCK Cell lines

Thirty four processed fecal samples were propagated in CRFK and nine samples in MDCK having high HA titre of 1 in 32 and above, followed by polymerase chain reaction confirmation were selected.

Isolation of CPV

Virus isolation was carried out as per the procedure recommended by Hirayama *et al.* (2005) [7]. Each 25cm² tissue culture flask showing 70 -75 % monolayer was washed with 0.01M PBS of pH 7.4 and infected with 0.5 ml of the processed sample. Virus adsorption was facilitated by incubating the infected tissue culture flask at 37°C for 1h with intermittent manual rotation. The inoculum was discarded and the infected cell culture was replenished subsequently with 6 ml maintenance medium and maintained at 37°C for 7 days. Every day the infected flasks were monitored and the changes in the cells were recorded. An uninfected flask of CRFK and MDCK with maintenance medium was used as a negative control.

Cytopathic Effect (CPE)

The infected cultures were observed for CPE seven days post inoculation and compared with uninfected control cultures. Cover slip cultures were also used for the purpose and haematoxylin and eosin staining was carried out.

Quantitation of CPV in cell culture supernatants by TCID₅₀

The Quantitation of CPV in cell culture supernatants was done by TCID₅₀ as per the method described by Reed and Muench method, (1938) [19].

Harvesting of the Virus

After 72 hrs of post infection, cells showing rounding, granulation and detachment indicative of Cytopathic effect were breezed and thawed thrice and later clarified at 5000 rpm for 10 min at 4°C. The resultant supernatant was collected in a micro centrifuge tube and stored at -80°C.

Confirmation of Virus

The presence of virus at each passage level was confirmed by PCR using H primers

specific for VP2 gene.

DNA extraction from cell culture fluid

The DNA was isolated from cell culture fluid as per Vieira *et al.* (2008). Processed cell culture fluid of 100µl was used for template DNA preparation. The samples were boiled at 96°C for 10 min and immediately chilled in crushed ice. Then the samples were centrifuged at 12,000 × g for 10 min at 4°C. The DNA extracted was used for confirmation of virus in cell culture fluid with primer pair H_{for} and H_{rev} to amplify the capsid protein encoding gene of CPV. Primers used were H_{for}

CAGGTGATGAATTTGCTACA, H_{rev} –
CATTGGATAAACTGGTGGT. The cyclical conditions were as follows. Initial denaturation at 95°C for 5 min, denaturation at 95°C for 30 sec, annealing 56°C for 2 min, extension at 72°C for 2 min and final extension at 72°C for 10 min. The cycle was carried for 35 cycles.

In vivo studies

In the present study, detailed post mortem examination was conducted on nine CPV positive dogs that died during the course of disease.

Histopathology

Tissue samples collected for the histopathological studies were processed by routine paraffin embedding technique and sections of 4 to 5µm thickness were cut and stained by Harris Haematoxylin and Eosin method (Luna, 1968) [9].

Results and Discussion

Isolation and Characterization of CPV in cell lines

Crandell rees feline kidney cell lines and Madin darby canine kidney were successfully used for the isolation of CPV virus. Out of thirty four processed clinical fecal samples, CRFK infected flasks had no consistent CPE in the first four passages. However there was minimal CPE from fifth passage onwards after 72 h post infection (PI) (Fig.1). Only 16 samples were able to produce CPE and was characterised by increased granularity, rounding of cells and degenerative changes. The infected monolayers were harvested on the third day of PI with or without CPE by three cycles of alternative freezing and thawing. Reports of minimal cytopathic effect and need of more number of passages in CRFK cell line was noticed. CPV could be isolated using CRFK cell lines after three blind passages and could observe the mild cytopathic effect like rounding, aggregation and detachment after 72 h PI after third passage (Parthiban *et al.*, 2011 and Srinivas *et al.*, 2013) [16, 22]. Raj *et al.* (2010) [18] also isolated CPV in CRFK cell line and mild CPE in the form of rounding, increased granularity and detached cells could be seen 3-4 days PI at third passage level. Deka *et al.* (2015b) [5] attempted to isolate fifteen CPV fecal samples in CRFK cell lines and found an increase in the number of samples 8,10 and 11 of CPV positive in first, second and third passages, respectively. Moderate isolation rate (47.05 %) may be attributed to the presence of antibodies in the intestinal lumen of dogs which may bind CPV virions and prevent viral attachment to cell receptors. Similar observations were also made by Srinivas *et al.* (2013) [22] and Parthiban *et al.* (2011) [16] who observed that isolation could be done only few days after post infection.

Out of nine, in MDCK cell lines only 5 samples (55.55%) were able to bind to MDCK cell lines and there was a characteristic CPE in the third passage after 72 h PI. The CPE

at third passage level was characterised by rounding, aggregation and slight detachment of cells at 48 h PI (Fig. 2) and was followed by complete detachment at 72 h PI (Fig. 3). There was no consistent CPE in first and second passage. The concentration of virus in MDCK cell line was measured by TCID₅₀ and found to be 10^{5.47} per millilitre of cell culture fluid. Similar results were reported by Nandi *et al.* (2009) [12] was able to adapt only one sample out of 5 after blindly passing three or more times in MDCK cell lines with characteristic CPE like rounding, aggregation of cells and granulation etc. Similar results were reported by Kaur *et al.* (2015) [8] and Sharma *et al.* (2016) [21] of the CPE produced in MDCK after passing for three times.

The cell culture adapted fluid is quite useful for the biochemical and molecular characterization of the CPV virus. Both the CRFK and MDCK cell lines supported the growth of the virus and characteristic CPE could be observed in MDCK rather than in CRFK cell lines.

The morphological changes in the both the cell lines was similar but the difference in the CPE observed during the process of adaptation in CRFK and MDCK cell lines was characteristic. Mild and a very minimal cytopathic effect was seen in CRFK at fifth passage level after 72 h PI whereas marked rounding and aggregation of cells after 48 h and total detachment of the infected monolayer after 72 h PI was marked in MDCK cell lines. The reason might be, MDCK cell lines were originated from canine kidney cell lines thus facilitating the CPV for prominent morphological changes.

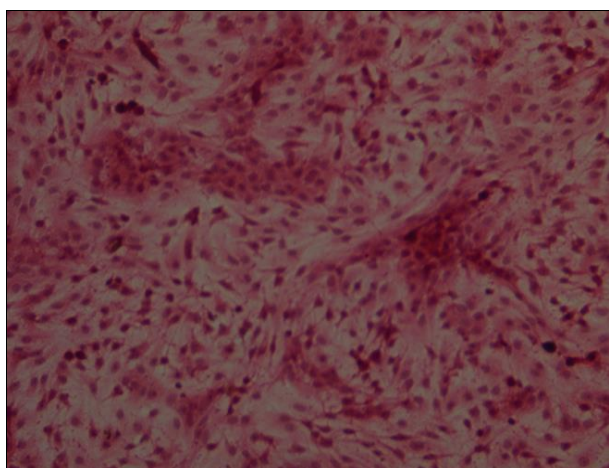


Fig 1: Cytopathic effect of CPV in CRFK cell monolayer in fifth passage 72 h PI. H&E stained (200X)

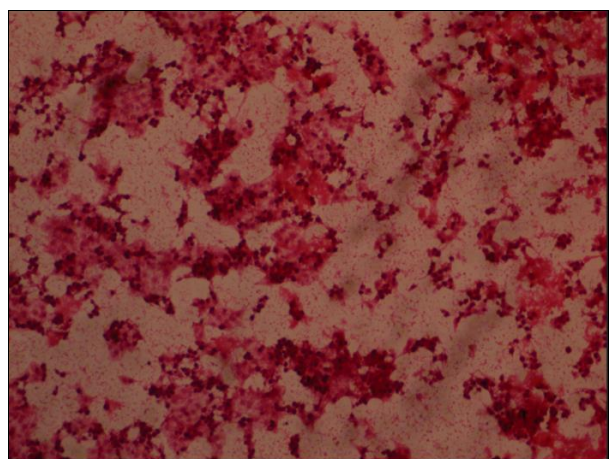


Fig 2: CPV infected MDCK cell monolayer at third passage 48 h of PI H&E stained (100X)

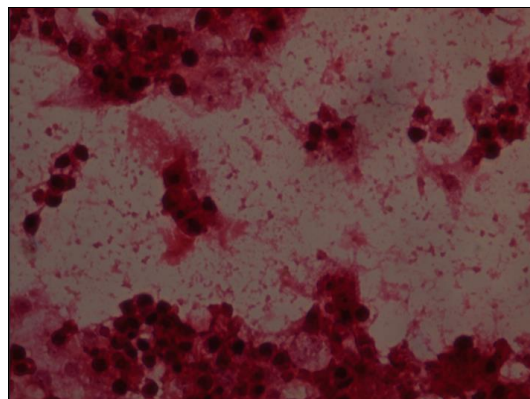


Fig 3: CPV infected MDCK cell monolayer at third passage 72 h of PI H&E stained (200X)

Confirmation of CPV in cell culture lysates

The presence of virus at each passage level was confirmed by PCR using H primers specific to VP2 gene and positive samples of CPV produced an amplicon size of 630 bp (Fig. 4).

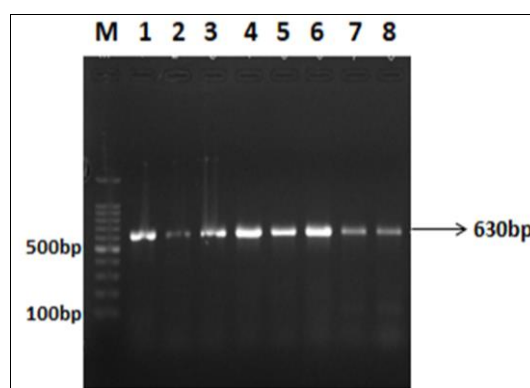


Fig 4: Confirmation of CPV in cell culture lysates at different passage levels using H_{for}/H_{rev} primers

M 100 bp ladder

Lane 1 – 8 Positive samples

In vivo Studies

The carcasses of the dogs affected with CPV were cachectic, dehydrated and showed blood tinged perianal region on external examination. Lesions were noticed in the intestine, heart, stomach, spleen, lung, liver and kidneys (Fig 5, 6 and 7). Microscopically, sections of intestines revealed haemorrhages in the lamina propria, submucosa, muscularis and the subserosa (Fig. 8) Villous atrophy, fusion and necrosis of intestine, goblet cell hyperplasia, cystic enlargement of the intestinal crypts filled with mucous and cellular debris was noticed (Fig. 9 and 10). Diphtheritic membrane composed of fibrin and mononuclear cells, infiltration of mononuclear cells in lamina propria, lymphoid depletion in Payer's patches was noticed (Fig. 11 and 12). The microscopic findings were in accordance with the reports of Cooper *et al.* (1979) [3], Nivy *et al.* (2011) [13], Vural and Alcigir (2011) [25], Behera *et al.* (2014) [2] Umar *et al.* (2015) [24] and de Oliveira *et al.* (2018). The lesions resulting from CPV were determined primarily by the parvovirus which required actively dividing cells. Hence, viral replication occurs principally in tissues with high rates of cell turnover- the intestine, lymphoid tissues and bone marrow (Pollock and Coyne, 1993) [17]. The gastrointestinal form of CPV is common, because the intestinal epithelium actively

regenerates even in healthy animals (Parrish, 1995) [15]. The gross changes in the intestine and microscopic changes like cryptic dilatation and villus atrophy noticed in the present study might be due to the selective replication of the virus in the enterocytes as described by Behera *et al.* (2014) [2]. This tropism is related to their requirement that host cells pass through the DNA synthesis phase of mitotic division (Rohovsky and Griesemer, 1967) [20].



Fig 5: Hyperemic small intestines



Fig 6: Haemorrhages in the intestinal mucosa

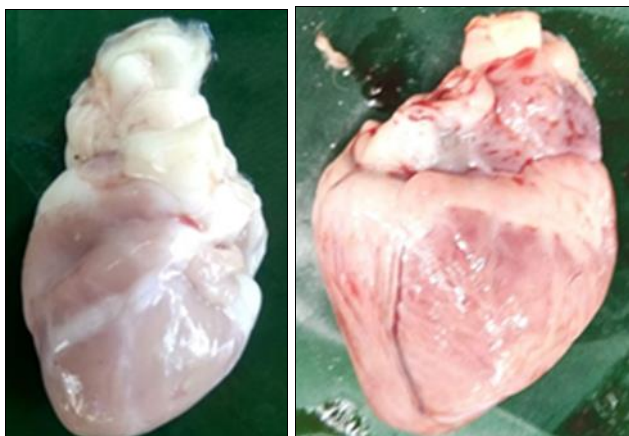


Fig 7: Pale and mottled appearance of the heart.

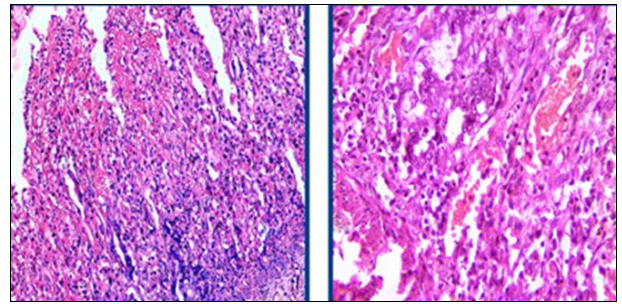


Fig 8: Haemorrhages in the intestine H&E × 100.

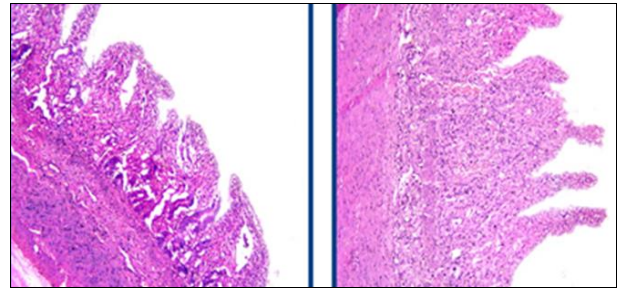


Fig 9: Intestines showing villi atrophy and fusion of atrophy H&E × 100.

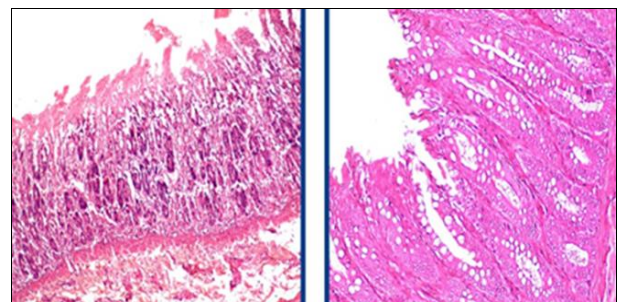


Fig 10: Necrosis of villous epithelium and goblet cell hyperplasia H&E × 400.

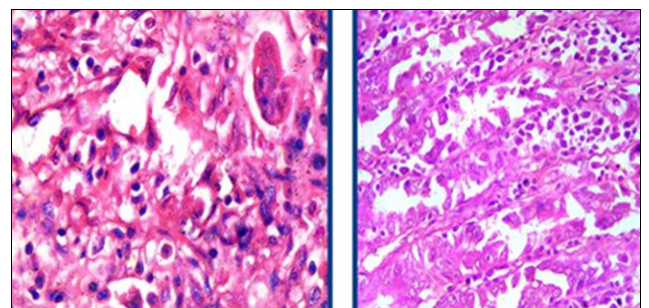


Fig 11: Syncytia formation and mononuclear cell infiltration in the intestines H&E × 400.

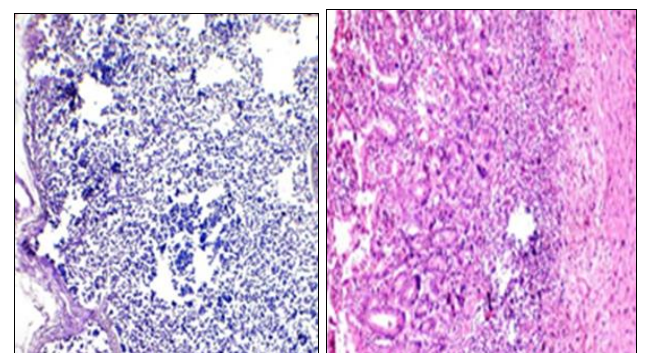


Fig 12: Lymphoid depletion in mesenteric nodes and Peyer's patches H&E × 40.

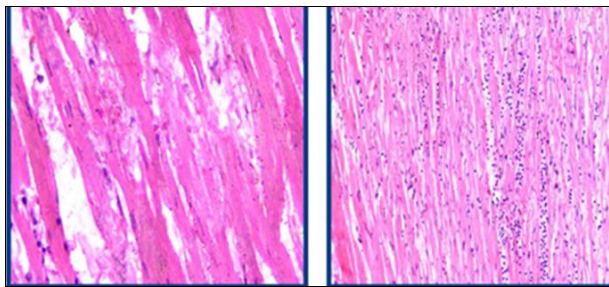


Fig 13: Pup heart showing necrosis of cardiac muscle fibers and inflammatory cell infiltration H&E \times 400.

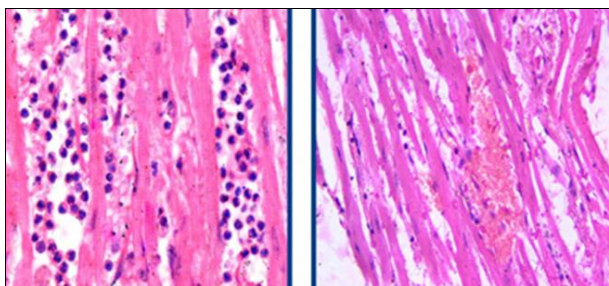


Fig 14: Pup heart showing infiltration with polymorphs and few mononuclear cells and haemorrhages between cardiac muscle fibers H&E \times 400.

Heart

Microscopically, sections revealed necrosis of cardiac muscle along with varying degrees of interstitial myocarditis with infiltration of mononuclear and polymorphonuclear cells. Multifocal haemorrhages were also observed in between the cardiac muscle fibres (Fig. 13 and 14). Similar histopathological lesions were described in the heart of dogs with CPV by Harcourt *et al.* (1980)^[6] and Umar *et al.* (2015)^[24]. The microscopic findings were in accordance with the earlier reports made by Nho *et al.* (1997)^[14], Agungpriyono *et al.* (1999)^[1], Nivy *et al.* (2011)^[13] and Behera *et al.* (2014)^[2]. However, inclusion bodies in muscle fibers reported by Harcourt *et al.* (1980)^[6] and Agungpriyono *et al.* (1999)^[1] were not noticed in the present study.

Conclusion

Virus isolation was successfully carried out in feline (CRFK) and canine (MDCK) cell lines. Marked morphological changes could be well noticed in MDCK rather than in CRFK cell lines. Maintenance, growth and sub culturing of MDCK cell lines was easy when compared to CRFK. Histopathological studies revealed characteristic features of CPV and microscopic lesions were more prominent in the intestines for all age groups and myocardial lesions were only noticed in pups rather than in dogs.

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