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Pawar Purushotham
Department of Veterinary Microbiology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India

Kalyani Putty
Department of Veterinary Biotechnology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India

Vishweshwar Kumar Ganji
Department of Veterinary Biotechnology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India

Dhanalakshmi K
Department of Veterinary Microbiology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
Pawar Purushotham
Department of Veterinary Microbiology, College of Veterinary Science, PVNRTVU, Rajendranagar, Hyderabad, Telangana, India

Isolation of canine parvovirus-2, from clinical cases of dogs in Hyderabad

Pawar Purushotham, Kalyani Putty, Vishweshwar Kumar Ganji and Dhanalakshmi K

Abstract
The canine parvovirus-2 (CPV-2) is a member of the Parvoviridae family and is responsible for severe and fatal viral enteritis in canines. It has been observed that several outbreaks of this virus have occurred due to the emergence of new variants, such as CPV-2a, CPV-2b, and CPV-2c. The objective of this study was to rapidly identify and isolate the CPV-2 in clinical cases using real-time PCR and growth in cell cultures. Out of the 100 rectal/fecal swab samples analyzed, 67 were positive for CPV-2 in Real-time PCR. In conventional PCR, 50 samples were typed as CPV-2b type whereas other 17 samples were typed as CPV-2a type. The isolates were recovered 57 samples in Crandell-rees canine kidney cells (CRFK) and 10 samples in Madin-darby canine kidney cells (MDCK). These isolates can serve as a basis for study of variations in CPV-2 and formulating vaccines with circulating variant types.

Keywords: Canine parvovirus, isolation, real-time PCR

Introduction
Canine Parvovirus is a major canine viral illness. It was first observed in 1967 in the faeces of normal dogs [2]. and was called the minute virus of canines (MVC). The second virus emerged in 1978 and was termed as CPV-2 to differentiate it from the MVC which was also called CPV-1 [1]. CPV-1 was less pathogenic and serologically unrelated to CPV-2 [5]. Since its emergence in 1978, CPV-2 has well established itself as an enteric pathogen of canines and several wild carnivore species globally, with high morbidity and mortality. In 1979, a variant virus CPV-2a was first detected which became the predominant type of CPV. The variant viruses CPV-2b and CPV-2c were reported in later on [4].
Several methods are in use for detection of virus, namely virus isolation in CRFK, MDCK and A-72 cell lines, haemagglutination test (HA), haemagglutination inhibition test (HI), agar gel precipitation test (AGPT), electron microscopy, indirect fluorescent antibody Test (IFT), ELISA and PCR [11]. Both conventional and Real-time PCR (RT-PCR) are the most sensitive techniques for demonstrating the presence of CPV DNA. Real-time PCR for CPV-2a, 2b and 2c in dog faeces allows precise DNA quantification from 10^2 to 10^9 copies of viral DNA [7].
Owing to the importance of the disease in canine gastrointestinal cases and the variants being reported, regular screening and isolation of CPV from canine gastrointestinal cases is needed. Hence the current study is taken up to identify Canine Parvovirus from clinical gastroenteritis cases in Hyderabad using PCR and to isolate the virus from PCR-positive samples using specific cell lines.

Materials and Methods
Samples and cells
CRFK and MDCK cell lines used for the present research purpose were obtained from College of Veterinary Science, Gannavaram, Vijayawada and maintained at the Department of Veterinary Biotechnology, College of Veterinary Science, Rajendranagar, Hyderabad in MEM with 1% FBS and 1X antibiotic solution (100µg/mL Streptomycin sulphate, 100 IU/mL Benzyl Penicillin). The 100 faecal samples/rectal swabs were obtained from dogs showing clinical signs of CPV such as gastroenteritis, haemorrhagic enteritis, pyrexia (Fig. 1) from Teaching Veterinary Clinical Complex, College of Veterinary Science, Rajendranagar and various private clinics in Hyderabad.

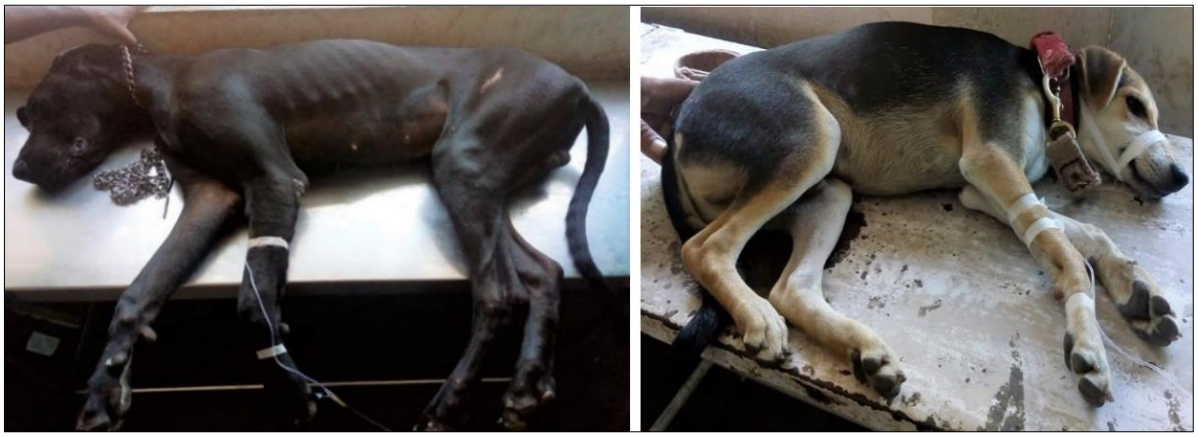


Fig 1: Dogs showing symptoms of CPV infection with severe diarrhoea and vomiting undergoing treatment

Extraction of viral nucleic acid (Template DNA preparation)

The faecal samples/rectal swabs were immersed in 1 mL of PBS (0.1 M, pH 7.4) containing antibiotics and centrifuged at 4º C for 10 min at 6000 rpm. The supernatant was filtered through a 0.22µ syringe filter and stored at -20 ºC until further use. The samples were boiled at 96 ºC for 10 min and immediately placed in crushed ice. The samples were then centrifuged at 12,000 rpm for 10 min at 4 ºC. finally supernatant was diluted 1:5 in nuclease-free water before using as the template for PCR. Commercially available live attenuated vaccines for CPV-2 Megavac and Canigen DHPPi were included as the positive control.

Real-time PCR

Real-time PCR was performed using the Power SYBR® Green PCR Master Mix with Viral DNA extract as template (1 µl) and the primers (200 nM) mentioned in Table 1 targeting VP2 region using the conditions as mentioned by Decaro *et al*. (2007) [8]. The reaction was setup in StepOnePlus Real-time PCR system (Applied Biosystems). The reaction conditions were set with 95 ºC for 10 min as initial denaturation, 40 cycles of 95 ºC for 15 sec and 60 ºC for 1 min followed by melt curve starting with 95 ºC for 15 sec, 60 ºC for 1 min and to 95 ºC with a ramp speed of 1%

Polymerase chain reaction (PCR)

PCR was performed using Emerald Amp® GT PCR Master Mix with Viral DNA extract (5 µl) as template and primers (200 nM) mentioned in Table 1 targeting the *VP2* gene segment. PCR reaction was set for CPV-2ab and CPV-2b as mentioned by Nandi *et al*. (2010a) [12] and for CPV-555 as mentioned by Buonavoglia *et al*. (2001) [4].

Table 1: Primers used for CPV-2 detection

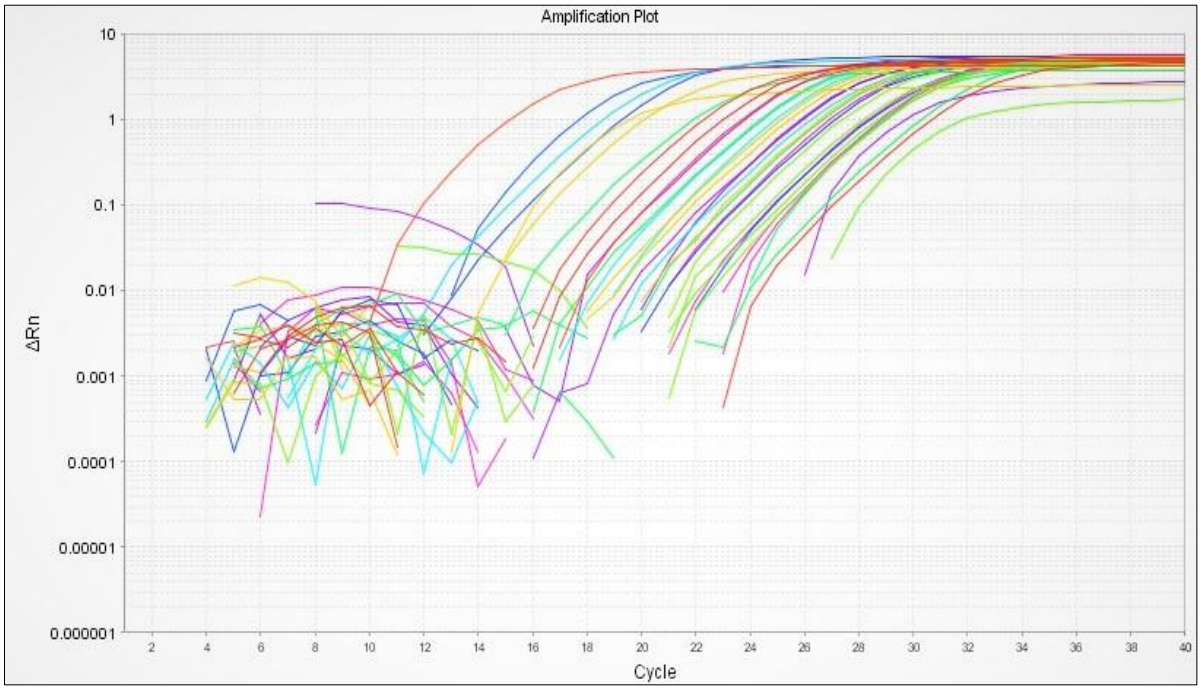
S. No.	Forward and reverse primers	Primer Sequence (5'-3') direction	CPV type amplified	Position of the genome	Annealing temperature and product size
1	CPV-2ab(F) CPV-2ab(R)	GAAGAGTGGTTGTAAATAATT CCTATATAACCAAAGTTAGTAC	Both CPV-2a and CPV-2b	3025-3045 3685-3706	56 ºC 681 bp
2	CPV-2b(F) CPV-2b(R)	CTTTAACCTTCCTGTAACAG CATAGTTAAATTGGTTATCTAC	CPV-2b	4043-4062 4449-4470	56 ºC 427 bp
3	CPV-2 (F) CPV-2 (R)	AAACAGGAATTAACTATACTAATATATTTA AAATTTGACCATTTGGATAAACT	CPV-2 For Real-time PCR	-	60 ºC 93 bp

Result and Discussion

CPV is one of the most important viral disease that is more often reported in clinical cases of canine gastroenteritis and was highly variable. Hence, regular monitoring and isolation of CPV is much important to understand the viral aetiology.

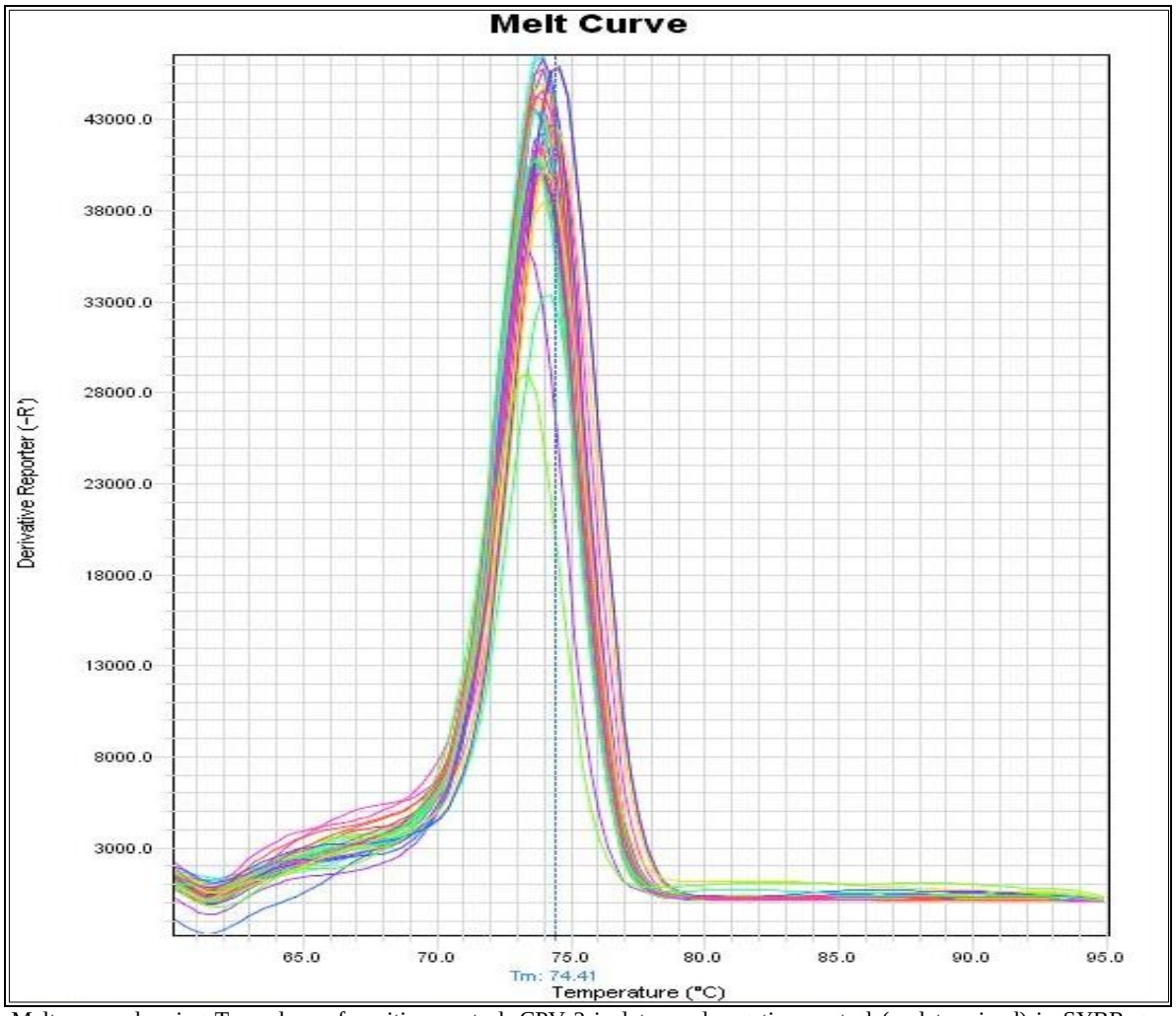
Molecular Detection of CPV-2 by Real-time and Conventional PCR: PCR is highly sensitive and specific for detection of viral pathogens from clinical samples. In the current study 100 faecal or rectal swabs were collected and the DNA was extracted as mentioned earlier. The DNA samples were initially screened by Real-time PCR and 67 of them tested positive for CPV-2. Fig. 2 and 3 show the amplification plot and melt curve of the representative clinical samples along with positive and negative controls. Similarly, Kumar and Nandi (2010) [10] reported higher prevalence of CPV-2 when screened the faecal samples by SYBR Green-based real-time PCR.

Further, to understand the variants of CPV-2, CPV-2a and CPV-2b, a conventional PCR was performed using CPV-2ab and CPV-2b primer pairs [12]. Of the 67 samples screened, 50 of them amplified 681 bp and 427 bp products respectively when screened using CPV-2ab and CPV-2b primer pairs confirming as CPV-2b type where other 17 samples amplified only 681 bp product were confirmed as CPV-2a type. We observed higher prevalence of CPV-2b over CPV-2a which was in agreement with previous reports by Pereira *et al*. (2000) [14], Chinchkar *et al*. (2006) [6], Zhang *et al*. (2010) [16] and Nandi *et al*. (2010) [12] all of them reporting CPV-2b dominant over CPV-2a.



Amplification curve showing CT values of positive control (first curve to the left), CPV-2 isolates and negative control (undetermined) as a template using CPV-2 primers and SYBR green dye-based detection of the amplicon to determine the serotype of the CPV-2 isolates obtained in the study.

Fig 2: Amplification plot of Real-time PCR of suspected faecal samples with CPV-2 primers.

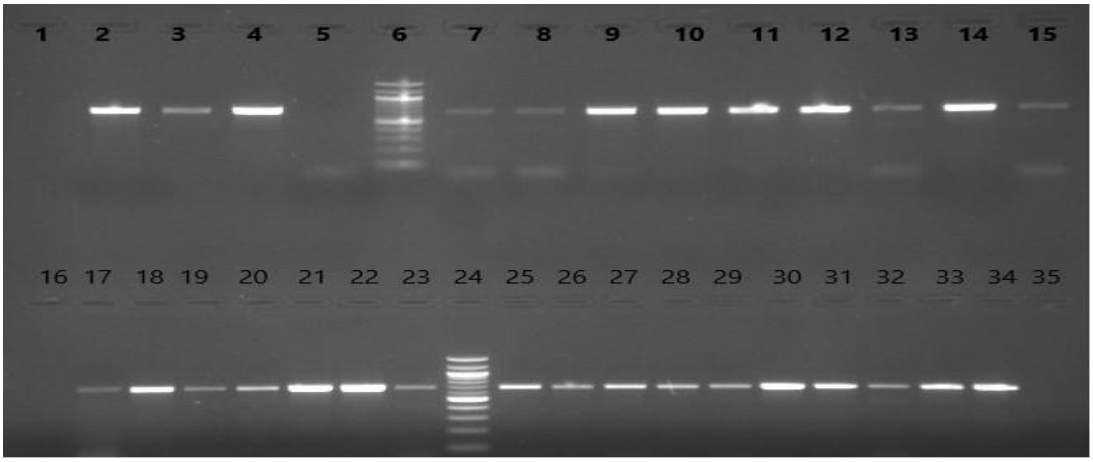


Melt curve showing Tm values of positive control, CPV-2 isolates and negative control (undetermined) in SYBR green dye-based detection of the amplicon in CPV-2 specific Real-time PCR suggesting the specificity of the reaction with no primer dimers.

Fig 3: Melt curve obtained in SYBR green dye-based Real-time PCR using CPV-2 primers.

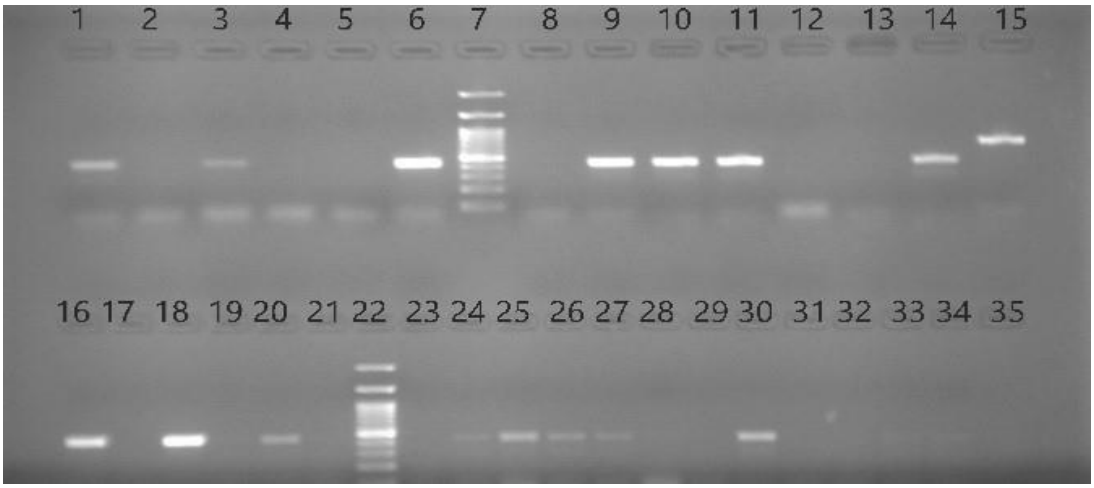
Isolation of CPV-2 in CRFK and MDCK cell lines

In this present study, attempts were made to isolate CPV-2 from the faecal samples by passaging in CRFK and MDCK cell lines. A characteristic cytopathic effect (CPE) for CPV-2 was observed after three passages in the CRFK cell line in the form of rounding off the cells, increased granularity and detachment (Fig. 6) were observed in 72-96 hours post-infection for 57 samples infected. Similar findings were reported by Raj *et al.* (2010) [15], Parthiban *et al.* (2011) [13] and Brindhalakshmi *et al.* (2016) [3] such as rounding and degenerative changes observed 72 h post-infection after third passage. Similar characteristic CPE for CPV-2 were also observed after three passages in the MDCK cell line in the form of rounding off the cells, increased granularity and detached cells (Fig. 7) within 72-96 hours post-infection for remaining 10 samples infected. Similar finding was reported by Kaur *et al.* (2015) [9] when isolated CPV-2.



Lane 6 and 24 show 100 bp DNA ladder, Lane 2 show positive control with amplicon of size 681 bp, Lane 1 show negative control with no amplification, Lane 2,3,4,7-15,17-23,25-34 show samples positive for reaction with amplicon of size 681 bp, Lane 5,16 and 35 show samples negative for reaction with no amplification.

Fig 4: PCR amplification of VP2 region using CPV-2ab primers.



Lane 7 and 22 show 100 bp DNA ladder, Lane 1 show positive control with amplicon of size 427 bp, Lane 2 show negative control with no amplification, Lane 3,6,9-11,14,16,18,20,25-27,30,33 and 34 show samples positive for reaction with amplicon of size 427 bp, Lane 4,5,8,12,13,15,17,19,23,24,28,29,31 and 32 show samples negative for reaction with no amplification.

Fig 5: PCR amplification of VP2 region using CPV-2b primers.

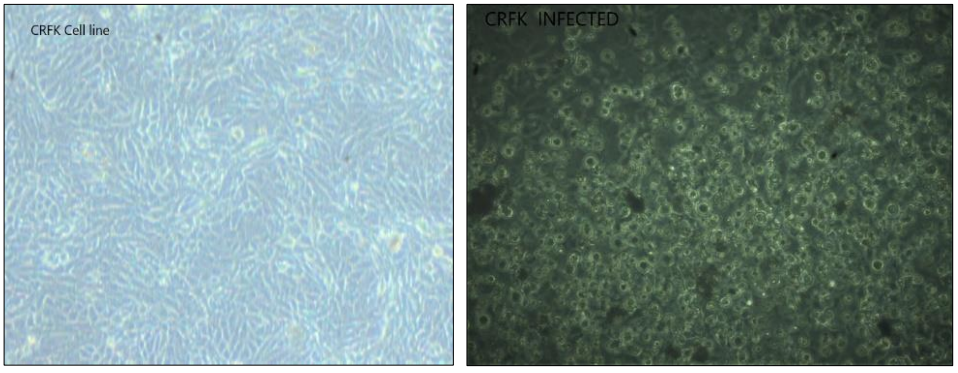


Fig 6: Representative image of CRFK cell line showing healthy cells on left and CPV-2 infected cells with CPE after 3rd passage on right.

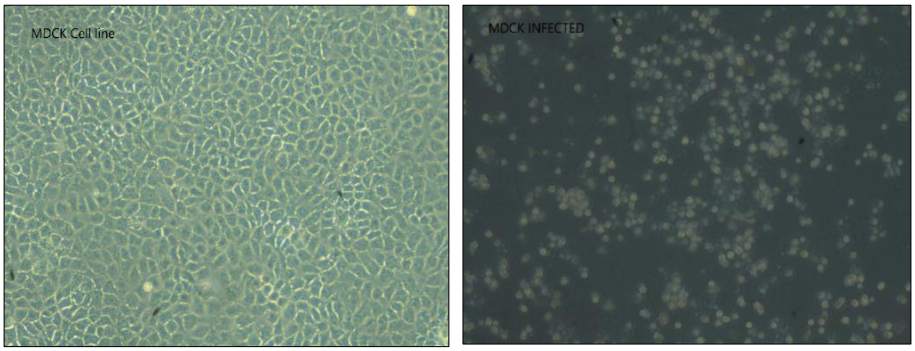


Fig 7: Representative image of MDCK cell line showing healthy cells on left and CPV-2 infected cells with CPE after 3rd passage on right.

Conclusion

In conclusion, a total of 67 samples were found positive for CPV-2 of which 50 samples were typed as CPV-2b type whereas other 17 samples were typed as CPV-2a type. Of the 67 samples, 57 samples were cultured in CRKF cell lines and remaining 10 samples were cultured in MDCK cell lines to recover isolates, both of which were found suitable for recovery of CPV-2. The isolates recovered can be used to study different variants circulating at a specified time and can be used for formulating vaccines with circulating variants.

Acknowledgments

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Competing Interest

No relevant financial or non-financial interests to disclose by the authors.

Ethics Approval

This study is an observational study. The Institutional Animal Ethics Committee (IAEC) has confirmed that no ethical approval is required.

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