RESEARCH ARTICLE Indian Journal of Animal Research, Volume 60 Issue 3: 528-533 (March 2026)

Comparative Efficacy of Conventional PCR and SYBR Green-based qPCR Assay for Detection of *Canine parvovirus-2* in Diarrheic Pups from Nagpur

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10.18805/IJAR.B-5449

ABSTRACT

Background: *Canine parvovirus* (CPV) infection is an infectious and contagious viral disease of canines especially puppies of age under 6 months. CPV-2-infected dogs are characterized by a gastroenteritis disorder with clinical signs of anorexia, lethargy, vomiting, fever and diarrhoea. The present study was planned for the development of SY BR -Green-based real-time polymerase chain reaction for detection and quantitation of canine parvovirus type 2 in faecal samples of dogs and compare the efficacy with conventional PCR.

Methods: A total of (n=200) samples from clinically ill dogs for parvovirus were tested by utilizing conventional PCR and qPCR based on SYBR green chemistry. A standard curve was plotted using a 10-fold serial dilution of standard DNA and Ct value. The standard curve was found linear over a 10^{-8} dilutions. The real-time PCR results were expressed as the number of DNA copies of CPV 2 per mg of faecal samples.

Result: The results showed that out of 200 samples, 64 (32%) and 96(48%) samples were found positive by conventional PCR and SYBR green-based real-time PCR respectively. The qPCR showed range of 1.0×10^{2} to 7.0×10^{8}. The present study suggested that qPCR was more sensitive and specific in the detection of CPV-2 as compared to conventional PCR.

Key words: *Canine parvovirus*, Conventional PCR, qPCR, Sensitivity, Specificity.

INTRODUCTION

Canine parvovirus-2 infection is a highly contagious disease of dogs, seen worldwide and characterized by acute, fibrinous, necrotic or hemorrhagic enteritis (Appel *et al.,* 1979; Buonavoglia *et al.,* 2000). *Canine parvovirus-2* is a member of the *Parvoviridae* family, *Parvovirinae* subfamily, *Protoparvovirus* genus and *Carnivore Protoparvovirus* 1 species. CPV-2 was first described as the prime cause of gastroenteritis in groups of dogs in the year 1977 (Pollock *et al.,* 1993). Despite being a non-enveloped DNA virus, previous studies have indicated the emergence of new variants worldwide (2a/2b/2c). The newly emerged virus variant CPV-2c was first described in 2000 in Italy (Buonavoglia *et al.,* 2000). The genome of *Canine parvovirus* is described to be 5200 nucleotides long, single-stranded, comprising of two open reading frames (ORFs). The first ORF1 encodes the non-structural proteins NS1 and NS2 and is located at the 3′ end of the genome while the ORF2 encoding structural proteins VP1, VP2 and VP3 is located at the 5′ end of the genome (Tsao *et al.,* 1991; Nandi and Kumar, 2010; de Oliveira *et al.,* 2019). The VP2 gene is regarded as the important major antigenic capsid protein that is involved in the determination of tissue selectivity and host range of the virus (Liu *et al.,* 2021). Furthermore, VP2 protein has high variability and is thus used as a major target for the characterization of CPV-2 in dogs (Mira *et al.,* 2018; Inthong *et al.,* 2020). Surveillance tools for diagnosis of CPV-2 with high sensitivity and specificity are necessary to be adopted for prophylaxis and control of the disease. Earlier conventional tools such as Haemagglutination tests, virus isolation by cell culture and indirect fluorescent antibody tests have been used to detect CPV-2 but are often lacking in sensitivity. Molecular tools such as conventional PCR and quantitative PCR (qPCR) have demonstrated high sensitivity for the detection of pathogens and among them, qPCR is a much more efficient diagnostic tool for the detection and

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How to cite this article: Nair, S.B., Warke, S., Kolangath, S. and Gupta, V. (2026). Comparative Efficacy of Conventional PCR and SYBR Green-based qPCR Assay for Detection of *Canine parvovirus-2* in Diarrheic Pups from Nagpur. *Indian Journal of Animal Research*. **60(3):** 528-533. doi: 10.18805/IJAR.B-5449.

Submitted: 17-07-2024 **Accepted:** 29-11-2024 **Online:** 10-01-2025

quantification of viruses (Giler *et al.,* 2024). Moreover, early diagnosis of the infection enhances the likelihood of treatment and quantitative molecular diagnostic tools such as real-time PCR allows the precise detection of CPV-2a, 2b and 2c in dog faeces with quantification ranging from 10^2 to 10^9 copies of viral DNA (DeCaro *et al.,* 2005). A greater understanding of *Canine parvovirus* ecology in clinical cases, along with rapid, standardized detection and typing technologies, would give important tools and knowledge for the development and verification of improved *Canine parvovirus* control strategies. Hence, the present study was designed to detect CPV-2 in the faeces of diarrheic dogs of Nagpur employing molecular tools such as conventional PCR and real-time PCR (qPCR) and compare their efficacy for the detection of *Canine parvovirus-2.*

MATERIALS AND METHODS

Sample collection and DNA extraction

The faecal samples/rectal swabs (n=200) were collected from dogs exhibiting clinical symptoms such as gastroenteritis, haemorrhagic enteritis, vomiting, anorexia, high temperature and depression from clinics in and around Nagpur during the year 2023-2024. The vaccination status of the puppies was also noted, along with history, age, sex and breed. The collected faecal samples was suspended in Hank's balanced salt solution (HBSS) containing streptomycin (100 mg/l), it was filtered (0.45 um) and then centrifuged at 2000 rpm for 10 minutes to remove debris. The supernatant was collected and stored at -20°C for subsequent laboratory examination. Viral DNA was extracted from clinical samples and pure CPV using the Qiagen Viral DNA Kit according to the manufacturer's protocol. The viral DNA extract was kept at a temperature of -20°C.

Conventional PCR amplifying VP2 gene fragment

The conventional Polymerase chain reaction was performed as per the method described by Sheikh *et al.* (2017). The forward and reverse primers used for the amplification of the VP2 gene fragment were as follows: forward 5′-GAAGAGTGGTTGTAAATAATT-3′ and reverse 5′CCTATATAACCAAAGTTAGTAC-3′. A reaction of 25 µl was set up comprising 12.5 µl of 2X PCR master mix, 1 µl (10 pmol/µl) of each forward and reverse primer, 8.5 µl of nuclease-free water and 2 µl of template DNA was added at the end of the reaction. The cycle conditions for amplification of the VP2 gene fragment started with an initial denaturation at 94°C for 5 minutes and was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, extension at 72°C for 2 minutes and a final extension step of 10 minutes at 72°C. The PCR products obtained were then run on 1% agarose gel and visualized under a UV transilluminator (Syngene G box, UK) in a gel documentation system.

Dilution of vaccine strain and construction of standard curve

Serial dilution of DNA of the CPV vaccine to obtain a ten-fold final dilution containing 10^8 to 10 copies per 10 µl was made to generate a standard curve. The standard curve was plotted with a known copy number of target DNA having corresponding Ct values (Fig 1).

SYBR Green based Real-time PCR (qPCR) for amplification of VP2 gene fragment

The VP2 gene fragment of viral DNA was amplified using specific primers, *viz*., 5′ TACATYTAAATATGCCAGAA 3′ (Forward) and 5′ GACCAAGGTGTTACMATTTG 3′ (Reverse) targeting 124 bp region (Lin *et al.,* 2014).

SYBR Green-based real-time PCR assays were performed using the Light Cycler Nano (Roche Diagnostics, Mannheim, Germany). Each 10 µl reaction mixture contained 0.5 µl each of forward and reverse primers (50 pm/l) and 2 µl of DNA extract. Sybr green master mix 5 µl and nuclease free water 2 µl. Each run included serial 10-fold dilutions of the standard vaccine DNA as a positive control and for the construction of a standard curve. A negative control without the DNA template was included to detect possible cross-contamination.

The reaction mixture was subjected to amplification with initial denaturation at 95°C for 10 min followed by 3-step cycling for 40 cycles which includes denaturation at 95°C for 10 sec, annealing at 52°C for 20 sec and extension at 72°C for 20 sec. The end of the cycle is completed with a final extension at 72°C for 20 sec. The PCR end products were removed from the LightCycler Nano (Roche Diagnostics, Mannheim, Germany) and results were analysed.

Data analysis

For data analysis, Ct (cycle threshold) values were calculated in Microsoft Excel in statistical mode.

Limit of detection

The limit of detection is defined as the lowest concentration of viral DNA present in the ten-fold dilutions detected by the assay.

Sensitivity and specificity of molecular assays

The sensitivity and specificity of both assays were determined using the following formulas:

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False positive}} \times 100$$

$$\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \times 100$$

RESULTS AND DISCUSSION

Conventional PCR amplifying VP2 gene

Out of a total of 200 samples subjected to conventional PCR amplifying VP2 gene fragment, 64 (32%) were found

to be positive for CPV-2 producing an amplicon of 681 bp (Fig 2). The present study showed results similar to those obtained by Magar *et al.* (2020) who reported that 11(22%) out of 50 infected dogs were positive for CPV-2 infection by conventional PCR. Similarly, a study by Karapinar *et al.* (2023) reported that 09 (56.25%) out of 16 rectal swabs were positive for *Canine parvovirus-2* by PCR. Likewise, study by Purushotham *et al.* (2023) reported that out of 67 samples screened 50 (74.62%) samples were found to be positive for CPV-2 by conventional PCR.

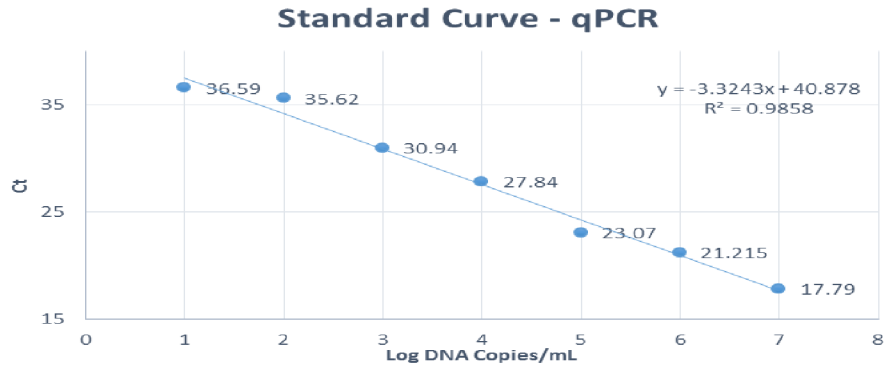
Sensitivity and specificity of PCR

The sensitivity and specificity of the conventional PCR used to amplify the VP2 gene for the detection of CPV-2 were determined to be 66.66% and 100% respectively.

qPCR amplifying VP2 gene

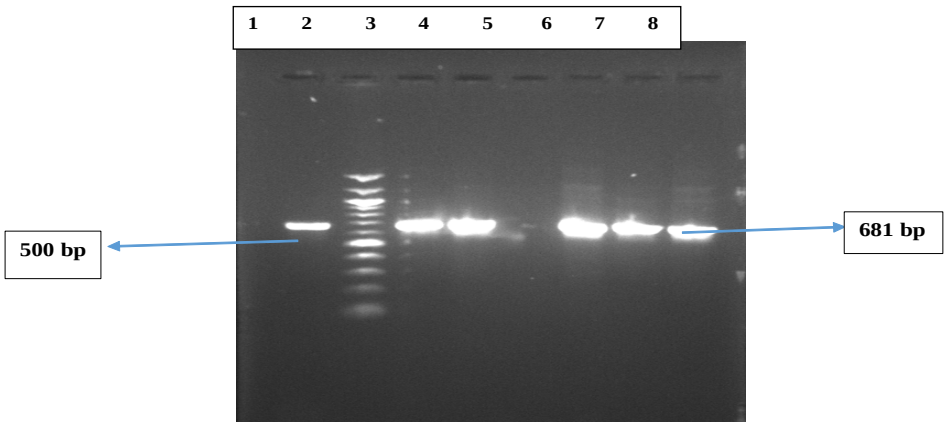
In the present study, the standard curve generated using pure CPV DNA (2 TCID50) had a linear range of eight orders of magnitude (10^0 to 10^8 copies/L). The real-time PCR efficiency was determined to be 99.89%, whereas the correlation efficient (R^2) between the number of quantification cycles (Cq) or threshold cycles (Ct) and the logarithm of the pure CPV DNA copy number was equal to 0.9858. The slope value of -3.3243 indicated the reproducibility and the detection limit of the assay (Fig 1). The detection limit was found to be 10 copies /10ul. The analytical sensitivity was also estimated with CPV2 having a titre of 10^5 $TCID_{50}$/ml. The overall detection limit was shown to be equivalent to 0.05$TCID_{50}$ per reaction.

A standard curve was plotted over a range of target DNA concentrations (1.0×10^8 to 1.0×10 copies of DNA per 10 µl reaction). The linear portion of the standard curve was found to span from 1.0×10^8 to 1.0×10, therefore, a lower detection limit (or cutoff) of 10 copies per 10 µl reaction was established. There was a cut-off value corresponding to a threshold cycle (Ct) of 31.45 and was



The vaccine DNA copy number is indicated on the x-axis, the Ct values (threshold cycles) on the y-axis. The R2 (correlation efficient) was 0.9858 (slope= -3.3243).

Fig 1: Standard curve of the Ct values against the input copy number of the standard vaccine DNA in SYBR Green-based real-time PCR.



Lane 1:- Sample No.32, Lane 2:- Ladder 100 bp,Lane 3:- Sample No.13, Lane 4:- Sample No.34, Lane 5:- Negative control, Lane 6:- Positive control (Vaccine), Lane 7:- Sample No.23, Lane 8:- Sample No.20.

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

applied to test samples. The samples with a Ct >31.45 were taken as not quantifiable (*i.e*. below the detection limit of the test) for CPV 2 DNA and 104 samples were considered negative. The dilution factor was taken into consideration when calculating the copies of CPV 2 in the sample. The results were expressed as the number of copies of CPV 2 genomes per mg of faecal sample. The 96 samples found positive in this study had 1.0×10^{2} to 7.0×10^{8} copies of viral DNA per mg of stool samples in real-time PCR. The results similar to the findings of (Decaro *et al*., 2005), who showed virus titre ranging from 1.0×10^{3} to 7.43×10^{11} copies/mg of faeces by real-time PCR.

The results revealed that out of 200 faecal samples, 96 samples (48%) were found positive by SYBR green-based real-time PCR. Specific PCR products were then identified by melting curve analysis and a reproducible distinct melting point (Tm) of 73.5° was observed (Fig 3 and Fig 4). Real-time PCR showed the mean ΔCt values of 25.65±1.39 for faecal samples (P=0.2) by amplification curve analysis (Fig 5).

The findings obtained in our study were similar to those reported by Giler *et al.* (2024) who revealed that 136/200 (68%) samples were positive for CPV-2 by real-time PCR and showed an efficiency of 99.9%. Manchikatla *et al.* (2020) reported that 43/100 (43%) samples were positive for CPV-2 by real-time PCR. Moreover, studies by Gonuguntla *et al.* (2016) and Purushotham *et al.* (2023) revealed that 22 (36.66%) out of 60 samples and 67 (67%) out of 100 samples were positive for *Canine parvovirus-2* by SYBR green-based real-time PCR assay.

Sensitivity and specificity of qPCR

The sensitivity of the qPCR used to amplify the VP2 gene for the detection of CPV-2 was determined to be 100%. The samples were also tested for Canine distemper, Canine adenovirus and Canine rotavirus and were detected to be negative for all validating the assay's specificity. The present study reported that qPCR assay was more sensitive in the detection of CPV-2 than conventional PCR. The findings obtained in our study were similar to those reported by Kumar and Nandi (2010a) who studied the

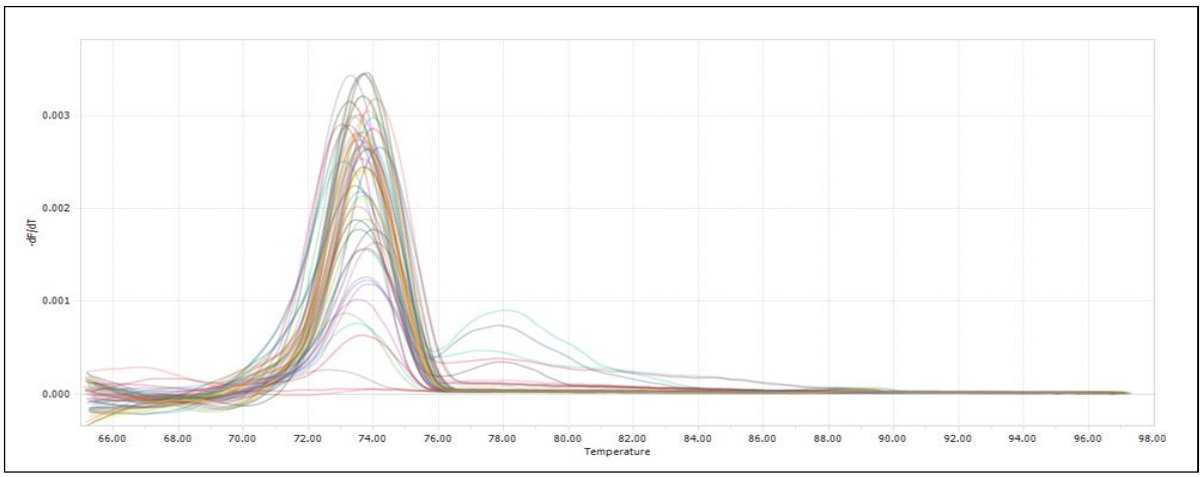


Fig 3: Melting peak of pure CPV with positive and negative samples for *Canine parvovirus.*

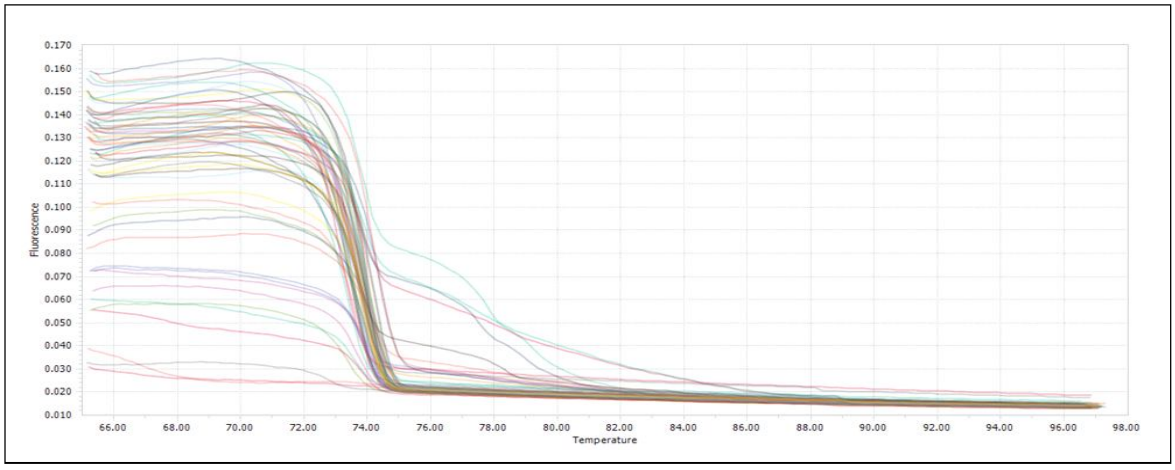


Fig 4: Melting curve analysis of pure CPV with positive and negative faecal samples for *Canine parvovirus.*

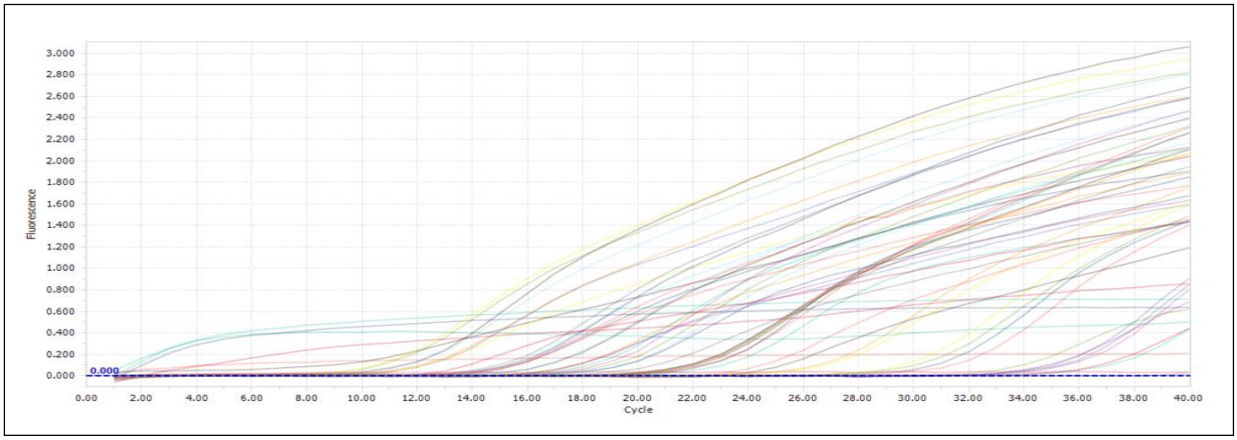


Fig 5: Amplification curve of Pure CPV with positive and negative faecal samples for *Canine parvovirus.*

SYBR Green-based real-time polymerase chain reaction (real-time PCR) for detection and quantitation of *Canine parvovirus* type 2 (CPV 2) in faecal samples of dogs based on nucleotide sequence of VP2 gene of CPV 2. Faecal samples (47) from dogs suspected of CPV 2 infection were analyzed by real-time PCR, haemagglutination (HA) assay and by a conventional PCR and 24, 20 and 22 samples were found positive for CPV 2, respectively. A comparison between the results of three different assays revealed that real-time PCR is more sensitive than HA and conventional PCR and allows the detection of low titers of CPV 2 in infected dogs.

The TaqMan probe-based assays are relatively more popular owing to their higher sensitivity and specificity in comparison to SYBR green assays, the SYBR green assays are relatively more cost-effective and easy to use because no primer designing and probe synthesis is involved. They possess practical use in laboratory studies in developing countries with a shortage of resources and requisite infrastructure. Furthermore, previous data suggests that the SYBR green assay can be optimized such that its performance and quality could be comparable to the TaqMan method (Tajadini *et al.,* 2014; Tao *et al.,* 2022). Similarly, Lin *et al*. (2014) reported that SYBR Green-based real-time PCR assay was sensitive, specific and reliable for the amplification of CPV 2, FPV and PPV DNA, with a reproducible limit of detection of as few as10 copies/ L of target DNA per reaction.

CONCLUSION

The present study concluded that conventional PCR and qPCR were sensitive tools for the detection of CPV-2 in diarrhoeic pups. The overall prevalence of CPV-2 in Nagpur was found to be 64 (32%) and 96 (48%) by conventional and qPCR respectively. These findings suggested that SYBR green-based qPCR assay was more sensitive than conventional PCR assay and could be utilized as a rapid, sensitive and specific approach for the detection of CPV-2 in infected dogs.

ACKNOWLEDGEMENT

The authors are thankful to the Associate Dean, Nagpur Veterinary College, Nagpur. Maharashtra Animal and Fishery Sciences University, Seminary Hills, Nagpur, India for provision of a research facility during this study.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare that they have no conflict of interest.

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