

Original Research Article

<https://doi.org/10.20546/ijcmas.2020.909.458>

Standardization of SYBR Green Based Real Time PCR for Detection of Canine Parvovirus-2 (CPV-2)

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ABSTRACT

Keywords

Real time PCR, Canine parvovirus, Conventional PCR, SYBR green dye, Phenol chloroform method, DNA extraction

Article Info

Accepted:
28 August 2020
Available Online:
10 September 2020

CPV-2 infection in dogs is recognized as one of the most common diseases especially in young puppies of less than a year age. Keeping in view of frequency of the occurrence of infection, sometimes even in vaccinated animals, the present study was taken up with the objective of standardizing the Real time PCR for easy and rapid diagnosis of the CPV-2 virus. In the present investigation a total of 100 faecal samples/rectal swabs were collected from dogs suffering with gastrointestinal disorders and showing the symptoms of canine parvovirus, like bloody diarrhoea, vomiting and pyrexia. The samples were tested initially with SYBR green dye based real time PCR with different combinations of the cycles and the results obtained were further confirmed by conventional PCR. We could able to standardize the real time PCR which was more helpful in screening of the faecal samples further in large numbers.

Introduction

Gastroenteritis, resulting in vomiting and diarrhea is one of the major diseases commonly encountered in dogs. This can occur quite quickly, be very dangerous to the pet's health and, if left untreated, leads to death - especially in young animals. Various causative agents of canine viral enteritis include canine parvovirus (CPV), canine

distemper virus, canine adenovirus-1, canine coronavirus, canine rotavirus etc. (Decaro *et al.*, 2007a).

One of the most devastating viral diseases of canine population is CPV enteritis which is highly contagious worldwide and is still evolving (Shackelton *et al.*, 2005). Since the initial parvovirus enteritis pandemic following the emergence of CPV type 2

(CPV-2), three variants, namely CPV-2a, CPV-2b and CPV-2c completely replaced the original type-2 viruses (Buonavoglia *et al.*, 2001; Filipov *et al.*, 2011). Canine parvovirus infection is a serious threat to canine population and has been reported from many parts of the world (Carmichael, 2005). This infection is an emerging disease in India and the incidence is showing an increasing trend (Chinchkar *et al.*, 2006; Sanjukta *et al.*, 2011; Wazir *et al.*, 2011). Clinical diagnosis of CPV infection is difficult, because the main clinical sign of the disease are common to other enteric diseases as well (Castro *et al.*, 2011). However, polymerase chain reaction (PCR) technique has been widely applied for early and confirmatory laboratory diagnosis of the disease due to high sensitivity and specificity (Nandi *et al.*, 2010a). But the advanced Real time PCR is more sensitive and less time consuming than the conventional PCR. Keeping in view of this we have standardized the Real time PCR in our laboratory for rapid diagnosis of the CPV-2 virus.

Materials and Methods

Collection and processing of samples

A total of 100 faecal samples/rectal swabs were collected from dogs exhibiting clinical signs viz., gastroenteritis, haemorrhagic enteritis, pyrexia etc. from teaching Veterinary Hospital, College of Veterinary Science, Rajendranagar, Hyderabad and various Private clinics in Hyderabad. These faecal samples were used in the present study. The faecal samples/rectal swabs obtained from the suspected dogs were emulsified in 1 mL of 0.1 M PBS (pH 7.4) containing antibiotics (100 IU/mL Benzyl Penicillin, 100 µg/mL Streptomycin sulphate) centrifuged at 6000 rpm for 10 min at 4°C. The supernatant was collected and filtered through 0.22µ syringe filter and stored at -20°C until further use.

Extraction of DNA

The following 2 different procedures was followed and standardized according to the convenience and ease of doing, initially the standard phenol chloroform extraction method was followed as given below

One ml of the fecal sample was spun in a microcentrifuge for 2 min, or until a compact pellet was formed. The supernatant was discarded and the pellet was resuspended in 567 µl TE buffer by repeated pipetting. Then 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K were added to give a final concentration of 100 µg/ml proteinase K in 0.5% SDS. Mixed thoroughly and incubated 1 hr at 37°C. The solution become viscous as the detergent lyses the bacterial cell walls. Then add 100 µl of 5 M NaCl, mix thoroughly and add 80 µl of CTAB/NaCl solution and mix thoroughly and incubate 10 min at 65°C.

Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol (24:1) mix thoroughly, and spin 4 to 5 min in a microcentrifuge. This extraction removes CTAB–protein/polysaccharide complexes. A white interface will be visible after centrifugation.

Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), mix thoroughly, and spin in a microcentrifuge for 5 min.

Transfer the supernatant to a fresh tube. Add 0.6 vol isopropanol to precipitate the nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. The precipitate can be pelleted by spinning briefly at room temperature. If no stringy DNA precipitate forms in the above

step, this implies that the DNA has sheared into relatively low-molecular-weight pieces.

Wash the DNA with 70% ethanol to remove residual CTAB and respin for 5 min at room temperature to repellet it. Carefully remove the supernatant and briefly dry the pellet. Redissolve the pellet in 100 µl TE buffer.

The second protocol followed was detailed below (Decaro *et al.*, 2007b)

100 µL of faecal sample was used for template DNA preparation.

The samples were boiled at 96⁰C for 10 min and immediately chilled in crushed ice.

The samples were then centrifuged at 12,000 rpm for 10 min at 4⁰C using cooling centrifuge (Centrifuge 5810 R, Eppendorf).

The supernatant was then diluted 1:5 in nuclease free water to reduce residual inhibitors of DNA polymerase activity and used as template for PCR.

Positive control

Commercially available live attenuated vaccine for CPV-2 (Canigen DHPPi) served as positive control. The vaccine DNA was extracted using the same procedures mentioned above.

Real-time PCR

Real-time PCR was carried out using SYBR[®] Premix Ex Taq[™] PCR Master Mix (Cat.No.RR420A; TaKaRa) with CPV-2 primer set (Table 1), in StepOnePlus Real-time PCR system (instrument) from Applied Biosystems (Kumar M and Nandi S 2010). Primers were reconstituted with nuclease free water to obtain 100 pmol/µL stocks. Stocks were made into 10 pmol/µL working solution and used for PCR. Real-time PCR was standardized for the primer set of CPV-2 as

reported by Decaro *et al.*, (2007b)

SYBR[®] Premix Ex Taq[™] PCR Master Mix (2X concentration) contains SYBR[®] Green I Dye, AmpliTaq Gold[®] DNA polymerase, dNTPs, Passive reference and Optimized buffer components. The SYBR Green dye binds to DNA, thus providing a fluorescent signal that reflects the amount of ds DNA product generated during PCR.

The SYBR[®] Premix Ex Taq[™] PCR Master Mix and the working solution of primer set were allowed to thaw completely. Then were mixed gently and spun briefly.

PCR master mix was prepared by scaling the volumes listed below to the desired number of PCR reactions.

| Reaction Component | Volume |
|---|--------|
| Power SYBR [®] Green PCR Master Mix (2X) | 5 µL |
| Forward primer (10 µM) | 0.5 µL |
| Reverse primer (10 µM) | 0.5 µL |
| Template DNA | 1 µL |
| ROX dye | 1 µL |
| Nuclease-free water | 2 µL |
| Total | 10 µL |

The PCR mixture was mixed gently and spun briefly.

Then PCR reaction plate was prepared by distributing 9 µL of PCR mixture into each well of MicroAmp[®] Fast 96-Well Reaction Plate, to that 1 µL of template DNA was added, according to template sheet including No Template Control and Positive Template Control.

The Multiwell Plate was transferred into the plate holder of the StepOnePlus Real-time PCR system Thermal Cycling block. Then the programmed plate-document was run according to following cycling conditions for

amplification and melt curve generation.

Reaction conditions of real time PCR

To standardize the reaction conditions for the CPV-2 primer set, we have tried with 3 different combinations of denaturation, annealing and extension and the reaction conditions are listed in the tables (2,3 & 4).

Results and Discussion

In order to test more number of samples for canine parvovirus and other enteric viral diseases, we have standardized the Real time PCR in place of conventional PCR in order to save time and laborious process of agarose gel preparation and viewing under the UV transilluminator.

Initially we have screened 100 DNA samples by Real-time PCR (Fig.I & II) using CPV-2 primers and 43 samples tested positive. The positive samples were further confirmed by conventional PCR (data not shown) using CPV-2ab primers and almost same number of samples (a few lesser) were tested positive in conventional PCR suggesting the high

sensitivity of the Real time PCR (Kaur *et al.*, 2016). For DNA extraction first we have followed the standard phenol chloroform method and since it is time consuming and laborious process we shifted to boiling method to extract DNA as suggested by Deacaro *et al.*, and the results when compared to the standard phenol chloroform method was identical and hence we have shifted to the physical method of DNA extraction to all our samples. To optimize the reaction conditions for the given primer set, we have done 3 different combinations of the temperatures for denaturation, Annealing and extension. Finally we have arrived at the conclusion, that the 3rd set of combination trial was good in amplifying our products when compared to the previous 2 trials.

In the first two trials the amplification curves were abnormal and the positive control whose Ct value was expected to be lower than the samples was a bit higher or equal or sometimes not even amplified. While in the third trial the amplification of the positive control was consistently good and the Ct value was lower than that of the samples.

Table.1 Real time PCR primers used for CPV-2 detection

| Forward and reverse primers | Primer Sequence (5'-3') direction | Tm value and fragment size |
|-----------------------------|-----------------------------------|----------------------------|
| CPV-2 (F) | AAACAGGAATTA ACTATACTAATATATTT | 60 ⁰ c |
| CPV-2 (R) | AAAATTTGACCATTGGATAAACT | 93 bp |

Table.2 Reaction conditions of real time PCR for detection of CPV-2 (trial 1)

| Enzyme Activation | PCR | | Melt Curve Stage | | |
|-------------------|--------------------|---------------------|-------------------|-------------------|-------------------|
| | Cycles (45 cycles) | | | | |
| Hold | Denaturation | Annealing/Extension | | | |
| 10 min | 15 sec | 1 min | 15 sec | 1 min | 15 sec |
| 95 ⁰ C | 95 ⁰ C | 60 ⁰ C | 95 ⁰ C | 60 ⁰ C | 95 ⁰ C |

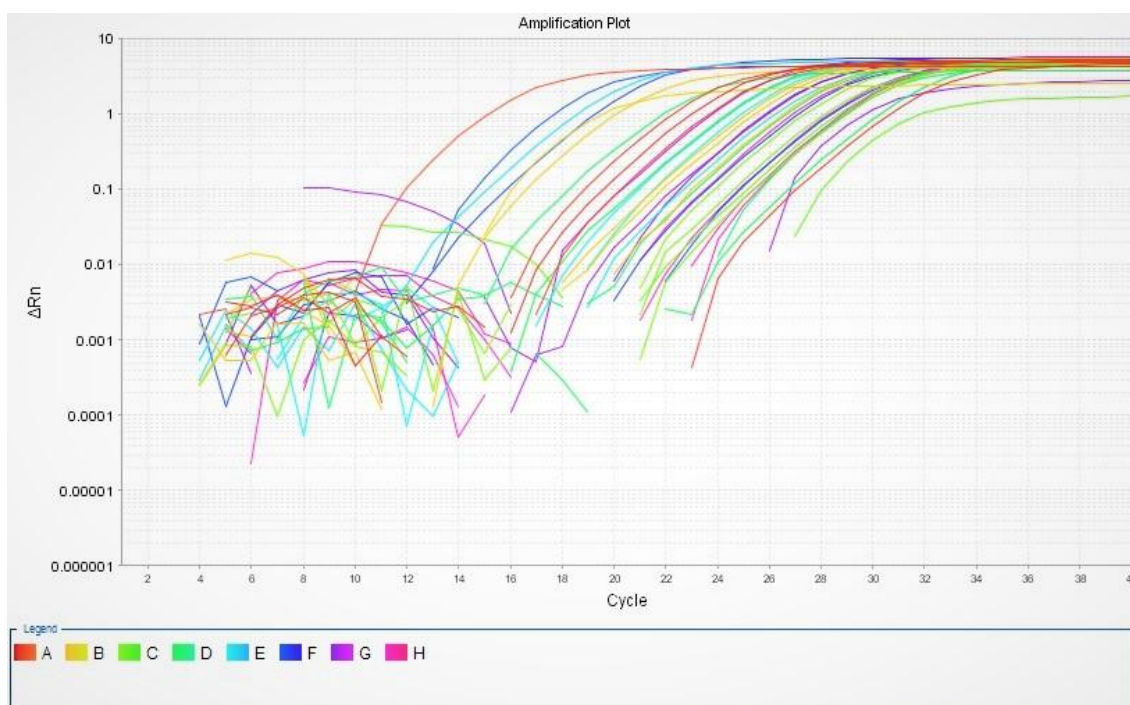
Table.3 Reaction conditions of real time PCR for detection of CPV-2 (trial 2)

| Enzyme Activation | PCR | | Melt Curve Stage | | |
|-------------------|--------------------|---------------------|-------------------|-------------------|-------------------|
| | Cycles (40 cycles) | | | | |
| Hold | Denaturation | Annealing/Extension | | | |
| 2 min | 15 sec | 2 min | 15 sec | 1 min | 15 sec |
| 95 ⁰ C | 95 ⁰ C | 60 ⁰ C | 95 ⁰ C | 60 ⁰ C | 95 ⁰ C |

Table.4 Reaction conditions of real time PCR for detection of CPV-2 (trial 3)

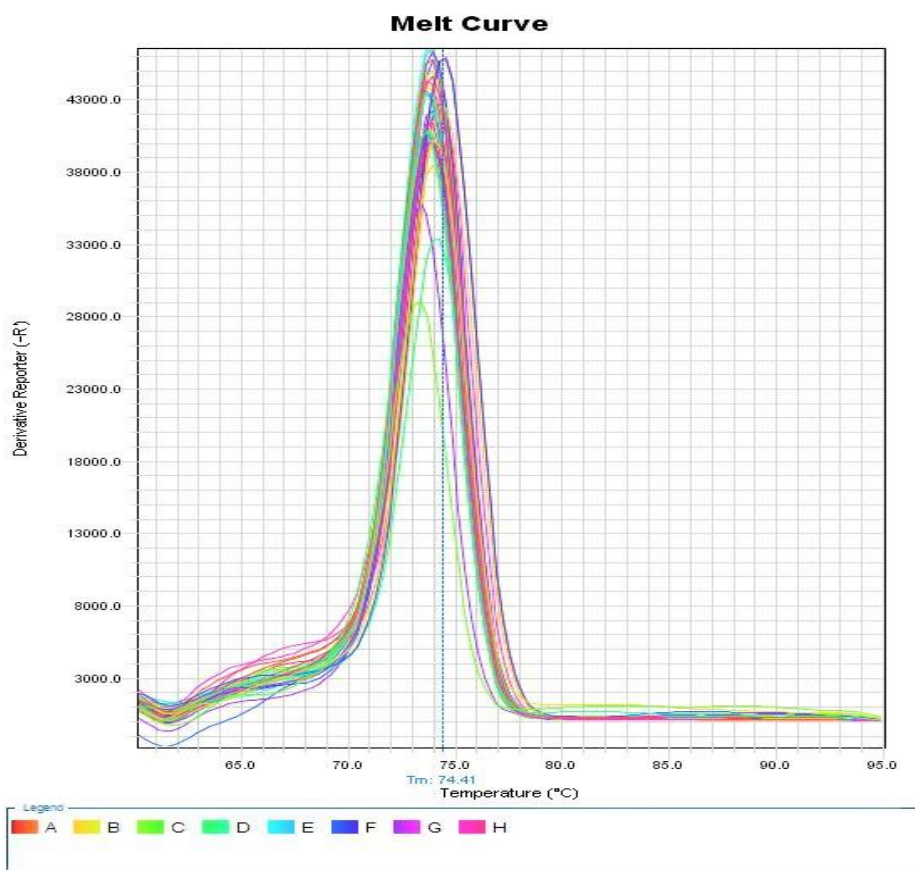
| Enzyme Activation | PCR | | | Melt Curve Stage | | |
|-------------------|--------------------|-------------------|-------------------|-------------------|-------------------|--|
| | Cycles (40 cycles) | | | | | |
| Hold | Denaturation | Annealing | Extension | | | |
| 30 sec | 05 sec | 1 min | 1 min | 15 sec | 1 min | |
| 95 ⁰ C | 95 ⁰ C | 48 ⁰ C | 60 ⁰ C | 95 ⁰ C | 60 ⁰ C | |

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



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

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

Once we have standardized the real time PCR for CPV-2, we have further screened more than 400 samples in our laboratory and were much easier and less laborious than conventional PCR which was followed earlier.

In conclusion the Real time PCR for screening of fecal samples for CPV-2 was standardized and was even more convenient than conventional PCR and can be replaced with suitable equipment.

Acknowledgements

I am highly thankful to the principal investigator, project on viral agents involved

in canine gastroenteritis sponsored by Department of Biotechnology, Ministry of Science & Technology, Govt. of India.

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How to cite this article:

Srinivas Manchikatla¹, Narasimha Reddy Yella¹ and Kalyani Putty. 2020. Standardization of SYBR Green Based Real Time PCR for Detection of Canine Parvovirus-2 (CPV-2). *Int.J.Curr.Microbiol.App.Sci*. 9(09): 3716-3722. doi: <https://doi.org/10.20546/ijcmas.2020.909.458>