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Optimization and Comparison of Real-time Reverse Transcription PCR with Conventional PCR for detection of Canine Distemper Virus

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ABSTRACT

Canine distemper virus is a single-stranded RNA virus of the genus *Morbillivirus* family *Paramyxoviridae*. It causes a serious, highly contagious, often fatal, disease of dogs and many carnivores resulting in respiratory and gastro-intestinal disease with frequent involvement of the central nervous system. Although vaccination against CDV disease has been widely used for many decades, this infection still represents an important disease of dogs. Though conventional RT-PCR is widely used for the diagnosis of CDV, Real-time RT-PCR because of its ease of performance and high sensitivity gained more attention. In this study we have optimized SYBR green based Real-time RT-PCR for detection of CDV and also compared its performance with conventional PCR. A total of 228 fecal samples/rectal swabs were collected and tested for CDV using Real-time RT-PCR targeting N gene and conventional RT-PCR targeting partial H and N genes. In real time RT-PCR 24 samples were found positive while only 17 were positive by conventional RT-PCR.

HIGHLIGHTS

- qRT-PCR for detection of CDV was standardized.
- qRT-PCR was more sensitive and easy in screening large number of samples.

Keywords: Canine Distemper virus, Real time RT-PCR, SYBR green dye, Conventional RT-PCR

Canine distemper is caused by a single-stranded RNA virus of genus *Morbillivirus* and family *Paramyxoviridae*. The disease is responsible for high mortality rates in dogs worldwide. It causes a highly systemic infection with prominent respiratory, gastrointestinal and nervous signs in dogs (Bi *et al.,* 2015). The disease is characterized by a rapid onset of severe leucopenia and loss of lymphocyte proliferation ability. The resulting immune-suppression increases the host's susceptibility to opportunistic infections which are the main cause of distemper-associated deaths. The broad spectrum of clinical signs, similar to the signs observed in other respiratory and enteric diseases of dogs, hampers clinical diagnosis of canine distemper (Hornsey *et al.,* 2019; Zhao and Yanrong, 2022) infection and renders necessary laboratory confirmation. Accordingly, a sensitive, specific and rapid method is desirable to detect even small amounts of virus early in infection. Reverse-transcriptase polymerase chain reaction (RT-PCR) has been applied successfully for the diagnosis of CDV in the clinical samples (Namroodi *et al.,* 2013; Wang *et al.,* 2020; Ricci *et al.,* 2021). In India, CDV infections are quite common and serological surveys using dot ELISA have been done (Latha *et al.,* 2007; Swati *et al.,* 2015; Ashmi *et al.,* 2017). The disease has been controlled by the use of attenuated live virus vaccines. However, several CDV vaccinated dogs also suffered from the clinical disease worldwide (Feijóo *et al.,* 2021;

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Yilmaz *et al.,* 2022). These findings suggested that there are genomic/virulence differences between the wild-type CDV and vaccine strains (Feijóo *et al.,* 2021). Partial N gene sequencing of the RT-PCR positive samples and the vaccine virus revealed that the CD field isolate was highly divergent from the vaccine virus and other field isolates (Pawar *et al.* 2011). Keeping in view of the diagnostic importance of the disease, we have optimized a SYBR green based Real-time RT-PCR for detection of CDV by targeting N gene and also compared the results with conventional RT-PCR.

MATERIALS AND METHODS

Collection and processing of samples

A total of 228 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 located in Hyderabad. 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 -20ºC until further use.

The following procedure was followed for RNA extraction:

1. Five mL of freeze/thawed infected vero cell culture harvest was centrifuged at 4000 rpm for 15 min at 4°C to obtain cell pellet (Centrifuge 5810 R, Eppendorf).
2. The pellet was collected into 1.5 mL microfuge tube along with 200 µL of supernatant, to which 750 µL of TRIZOL® reagent (Ref:15596018; Ambion®) was added or 750 µL of TRIZOL® reagent was added directly to 500 µl of fecal sample.
3. The mixture was vortexed for 1 minute and incubated at room temperature for 5 minute.
4. 250 µL of chloroform was added to the above mixture.
5. The above mixture was hand mixed to avoid any cellular DNA contamination and incubated on ice for 10 minutes.
6. The mixture was then centrifuged at 13,200 rpm for 15 min at 4ºC using cooling centrifuge.
7. The aqueous phase was collected carefully to another RNase free 1.5 mL microfuge tube without touching the protein layer.
8. Equal amount of ice-cold isopropyl alcohol was added to the collected aqueous phase.
9. This mixture was incubated at -20ºC for overnight.
10. The following day, the mixture was centrifuged at 13,200 rpm for 15 min at 4ºC.
11. Supernatant was discarded leaving the small pellet (sometimes invisible).
12. One mL of 70% ethyl alcohol was added to the pellet and centrifuged at 13,200 rpm for 10 min at 4ºC.
13. Supernatant was discarded and the pellet was air dried.
14. The pellet was then dissolved in 25 µL of nuclease free water.

Positive control

Commercially available live attenuated vaccine for CDV (Canishot K5) served as positive control. The same vaccine RNA was extracted and converted into c-DNA and was used as positive control for CDV.

Reverse Transcription Polymerase Chain Reaction

RT-PCR was done in two steps. First cDNA synthesis was carried out and the synthesized cDNA was used as template for PCR.

Reverse transcription

The reaction was carried out using PrimeScript™ 1st strand cDNA Synthesis kit (Cat No.6110A; TaKaRa). A 20 µL of RNA of each sample was converted to 40 µL of cDNA, in following steps.

The reagents were allowed to thaw completely. Then were mixed gently and spun briefly. The master mix was prepared by scaling up based on the volumes listed below to the desired number of reactions.

The following mixture was prepared in a microfuge tube.

Reagent	Volume (µL)
Random hexamers	2.0
dNTP Mixture	2.0
Template RNA	20.0
RNase free dH_2O	6.0
Total	**30.0**

This template RNA-Primer Mixture was incubated for 5 min at 65ºC and then snap cooled on ice.

The following reaction mixture was prepared.

Reagent	Volume (µL)
Template RNA Primer Mixture	30.0
5X PrimeScripit Buffer	8.0
RNase Inhibitor	1.0
PrimeScript RTase	1.0
Total	40.0

This reaction mixture was added into the processed template RNA-Primer Mixture by mixing gently and then incubated at following conditions in thermal cycler (Prima-Duo, HiMedia).

Temperature	Time
25ºC	10 min
42ºC	60 min
70ºC	10 min (for enzyme inactivation)
4ºC	∞

Once the c-DNA is synthesized, using it as template the PCR was carried out by preparing PCR mixture as follows. The specific primers for partial N and H genes were listed in table 1.

Reagent	Volume (µL)
EmeraldAmp GT PCR Master Mix (2X Premix)	6.25
Template c-DNA	2.0
Forward Primer (10 pmol)	0.5
Reverse Primer (10 pmol)	0.5
dH_2O (sterile distilled water)	3.25
Total	**12.5**

Following cycling conditions were used for amplification of CDV primer set.

Initial denaturation	96ºC/1 min	
Denaturation	94ºC/10 sec	35 cycles
Annealing	53ºC/40 sec	
Extension	68ºC/2.15 min	
Final extension	68ºC/10 min	
Hold	4ºC/∞	

Agarose gel electrophoresis

1% agarose gel was prepared in TAE buffer, the wells were loaded with the PCR product and electrophoresis was run at 80 volts for 1 hour.

The gel was transferred to gel documentation system (Gene flash, Syngene bio imaging) having UV trans-illuminator and observed under medium wavelength for the desired bands.

Real-time PCR

Real-time PCR was carried out using SYBR® Premix Ex Taq™ PCR Master Mix (Cat.No.RR420A; TaKaRa) with CDV primer set (Table 2), in StepOnePlus Real-time PCR

Table 1: Conventional PCR primers used for amplification of partial N and H genes for identification of CDV

Sl. No.	Forward and reverse primers	Primer Sequence (5'-3') direction	Gene amplified	Position of the genome	Annealing temperature and product size
	CDV-N (F)	ACAGGATTGCTGA GGACCTAT		769-789	60ºC
1	CDV-N (R)	CAAGATAACCATG TACGGTGC	Partial N gene	1055-1035	287 bp
2	CDV- H(F)	TACTGAATGGAGA CGGTATGGAT		8379-8401	53°C
	CDV- H(R)	TCGATAGAATTGGTG ACATCACA	Partial H gene	8827-8805	449 bp

Table 2: Real time PCR primers used for CDV detection

Sl. No.	Forward and reverse primers	Primer Sequence (5'-3') direction	Position on the genome
1	CDV-N (F)	AGCTAGTTTCATCTTAACTATCAAATT	905-931
2	CDV-N (R)	TTAACTCTCCAGAAAACTCATGC	965-987

system (instrument) from Applied Biosystems. 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 CDV as reported by Gabriella *et al.* (2006) with slight modifications.

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 (given in table 2) were allowed to thaw completely, mixed gently and spun briefly.

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

Reaction Component	Volume (µL)
Power SYBR® Green PCR Master Mix (2X)	5.0
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
Template DNA	1.0
ROX dye	1.0
Nuclease-free water	2.0
Total	**10.0**

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 CDV primer set, various annealing temperatures were tried and the final reaction conditions are listed in the table 3.

Table 3: Reaction conditions of real time PCR for detection of CDV

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

RESULTS AND DISCUSSION

To optimize the reaction conditions for detection of CDV with the given primer set in real time PCR, several temperatures for annealing were tried and finally standardized the amplification conditions which are presented in the table 3.

After optimizing the amplification conditions, we have screened 228 fecal samples for canine distemper virus using both Real time and conventional PCR and also for other enteric viral diseases (data not submitted here), by targeting N gene in Real-time PCR. The results showed that 24 samples (~10%) were found positive by real time PCR (Fig. 1 and 2). Similarly Gabriella *et al.* (2006) also screened CDV in the clinical samples using Real-time PCR targeting N gene. Whereas Swati *et al.* (2015) used L gene and Agnihotri *et al.* (2017) used H gene as a target gene for detection of CDV. 24 samples which were positive in real time PCR were further amplified using conventional PCR targeting partial H and N genes and only 17 samples were

amplified with an amplicon size of 449 bp (Fig. 3) and 287 bp respectively (Fig. 4).

Eventhough there are many studies which have suggested the use of PCR for detection of CDV in fecal samples, few studies used realtime PCR for detection of CDV in fecal samples and its comparison with conventional PCR. The difference in detection of positive samples by Real time and conventional PCR clearly indicates the high sensitivity of Real time PCR in detecting very low concentrations of virus and similar type of results between conventional and Real time PCR were also observed by Gabriella et al., (2006).

In conclusion the Real time PCR for screening of fecal samples for CDV was even more convenient and is more sensitive than conventional PCR, hence it can be replaced with suitable equipment for routine screening of the fecal samples for CDV.

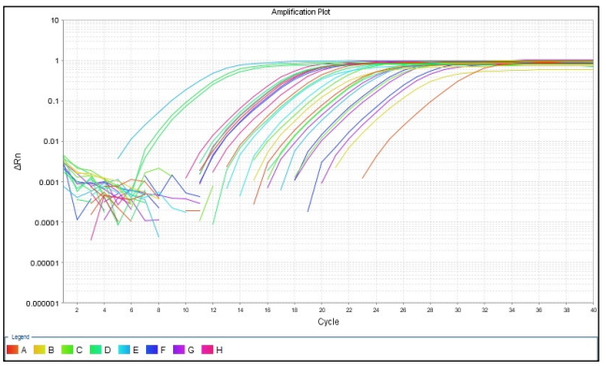

Fig. 1: Amplification plot of Real-time PCR of suspected faecal samples with CDV N gene primers

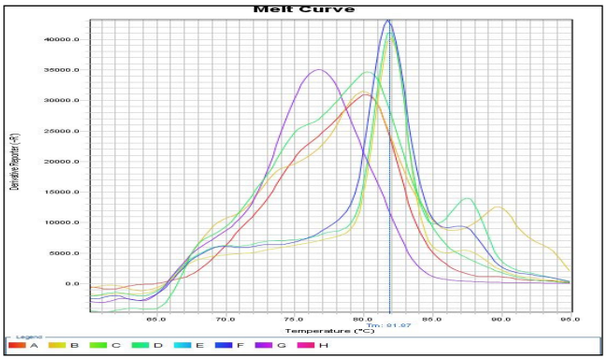

Fig. 2: Melt curve obtained in Real-time PCR suspected for CDV

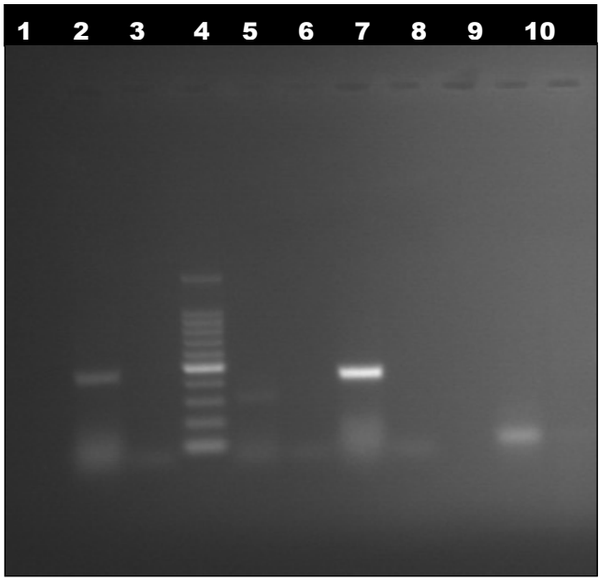

Fig. 3: Agarose gel electrophoresis pattern of CDV positive samples using partial H gene primers with amplicon size of 449 bp

Lane	Result
Lane 4	100 bp ladder
Lane 2,7	Positive samples
Lane 5, 6, 8, 10	Negative samples
Lane 3	Negative control
Lane 1,9	Empty wells

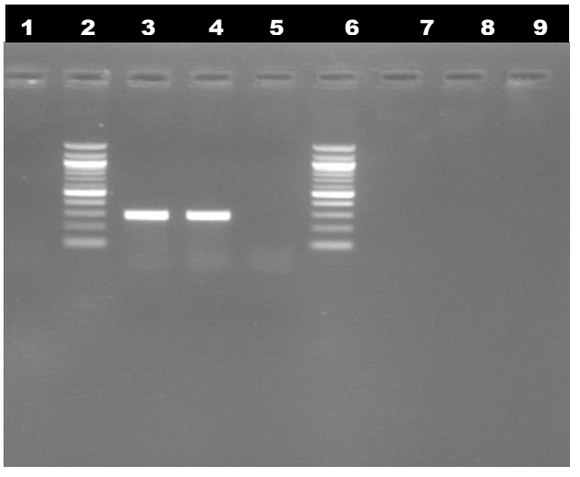

Fig. 4: Agarose gel electrophoresis pattern of CDV positive samples using partial N gene primers with amplicon size of 287 bp.

Lane	Result
Lane 2 & 6	100 bp ladder
Lane 3,4	Positive samples
Lane 1 & 5	Negative control
Lane 7,8,9	Empty wells

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