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Molecular characterization of canine distemper virus from Tamil Nadu, India

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ABSTRACT

The focus of the present study was to detect canine distemper virus in clinically suspected dogs and its molecular characterization. The detection of CDV N gene was carried out using RT-PCR and Nested PCR. The N gene positive cDNA were used for the amplification of partial H and F genes. H gene positive products were subjected to restriction digestion with *NdeI*. Phylogenetic tree of partial CDV H and F gene nucleotide sequences were constructed using maximum likelihood method.Attempts were made to isolate CDV in vero cells expressing SLAM and MDCK cell lines. Out of the 90 samples collected from CDV suspected dogs, 19 samples (21%) were found positive by RT-PCR and 21 samples (23%) by nested PCR. When the N gene positive cDNA were subjected to PCR amplification of partial H and F gene sequences, 1 positive amplification each was obtained for H and F genes using the published primers and 2 H and F gene positive amplifications were obtained using the primer designed in this study. Restriction digestion of the partial H gene products yielded 2 fragments, proving the CD viruses to be wild-type. Phylogenetic analysis of partial H and F gene nucleotide sequences revealed that the field CD viruses of this study were distinct and varied from the vaccine strain. None of the samples produced cytopathic effects evenafter 3 passages in 2 different cell lines. However, the presence of virus in 1 sample in both the cell lines after second passage was confirmed by RT-PCR.

Key words: Canine distemper virus, Phylogenetic analysis, RFLP, RT-PCR, Sequencing

Canine distemper (CD) is a highly contagious viral disease caused by canine distemper virus (CDV) affecting dogs and other wild carnivores throughout the world. Canine distemper virus belonging to genus *Morbillivirus*, family *Paramyxoviridae,* is a single stranded, negative sense RNA virus having a 15.7 kb genome. Canine distemper virus encodes seven viral proteins namely, phosphoprotein (P), large protein (L), nucleocapsid (N), matrix (M), haemagglutinin (H), fusion (F) proteins and one non-structural protein (C) (Von Messling *et al.* 2001). The main mode of transmission of the disease is through direct contact with the infected dogs (Greene and Appel 2006). The onset of the disease is accompanied by gastrointestinal, respiratory and nervous signs. Later, the infection progresses to central nervous system (CNS) and may result in death (Riley and Wilkes 2015). Based on the genetic variation of H gene, CDV genotypes have been classified geographically (Martella *et al.* 2006) into America 1, America 2, Asia1, Asia 2, Artic like, Europe and Europe-wildlife (Harder and Osterhaus 1997, Martella *et al.* 2006). Seroprevalence of CDV had been reported to be 70% from south India (Latha *et al.* 2007) but detailed study on molecular epidemiology is lacking till date (Swati *et al.* 2015). Earlier in Tamil Nadu, molecular characterization of CDV was done for H and N genes (Pawar *et al.* 2011). Therefore, phylogenetic analysis of CDV H and F genes will generate information on the prevalent genotypes circulating in this region. Further, isolation and adaptation of the field viruses in cell lines will be useful for the development of a new vaccine strain in future. Therefore, the present work focussed on detection, differentiation of CDV field virus from vaccine virus, phylogenetic analysis of H and F gene and isolation of CDV in MDCK/ vero cells expressing SLAM.

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MATERIALS AND METHODS

Collection of sample: Clinical samples were obtained from dogs suspected for canine distemper which were brought to clinics of Madras Veterinary College, Vepery and Blue Cross of India, Velachery. Ocular and nasal swabs were collected from suspected dogs. Sterile cotton swabs were used for the collection of samples. After sample collection, the swabs were suspended in 2 ml of sterile phosphate buffered saline and stored at –80°C until processing.

Extraction of total RNA: The total RNA was extracted from 250 µl of ocular and nasal swab sample using TRIzol reagent as per the manufacture's instruction with minor modifications at RNA pellet washing step wherein the pellet

was washed twice with 75% ethanol by centrifuging at 7,500 rpm for 5 min. The RNA pellet was air dried for 5 min and re-dissolved in nuclease free water (NFW). The concentration of RNA was estimated in spectrophotometer using nuclease free water as blank.

Synthesis of cDNA: Reverse transcription was performed with random hexamer using Verso cDNA synthesis kit as per manufacturer's instructions. The recipe was 2 µl of 5× cDNA synthesis buffer, 1 µl dNTP mix, 0.5 µl RTE enhancer, 0.5 µl Verso enzyme, 3 µl template RNA and 2.5 µl NFW and the mixture was subjected to 42ºC for 40 min, 95ºC for 2 min and 4ºC for ∞.

Reverse transcriptase polymerase chain reaction (RT-PCR): The CDV N gene was used as the target gene for identification of canine distemper virus. Two methods were used for N gene amplification (Table 1). The second method involved a second amplification with internal primers. The primers were used at the concentration of 10 µmol/µl.

The reaction mixture was composed of 5 µl master mix, 0.5 µl forward primer, 0.5 µl reverse primer, 2 µl template and 2 µl nuclease free water. The reaction was carried out under following conditions: initial denaturation at 95°C for 3 min, 29 cycles of denaturation at 95°C for 30 sec, annealing temperature at 57°C for 30 sec and extension at 72°C for 30 sec followed by final extension at 72°C. The PCR products were subjected to electrophoresis in 1.5% agarose gel. The positive PCR product was purified using gel purification kit as per manufacturer's protocol and sent for sequencing.

RT-PCR for H and F gene: The CDV N gene positive cDNA were then subjected to PCR for amplification of a part of H and F gene sequences. The primers were used at the concentration of 10 µmol/µl.

The primers customized in this study were designed using custom primers – oligo perfect designer (Thermoscientific online software) (Table 2). The reaction was carried out using following conditions for H gene: initial denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing temperature at 53°C for 30 sec and extension at 72°C for one min followed by final extension at 72°C for 5 min.

The thermal cycling profile for F gene consisted of initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing temperature at 54°C for 20 sec and extension at 72°C for 1 min followed by final extension at 72°C for 7 min. Gel electrophoresis was carried out using 1% agarose at 150 V for about 15–20 min. The gel was viewed under gel documentation system.

Sequencing and phylogenetic analysis: The purified PCR products were sequenced with forward and reverse primers using BDT v3 cycle sequencing kit on ABI 3730×1 genetic analyser (M/s Eurofins Genomics India Pvt.Ltd, Karnataka, India). Phylogenetic analysis of nucleotide sequence was computed and the phylogenetic tree was constructed by maximum likelihood method with a 1000 bootstrap repeats using the MEGA6.0 software.

Restriction fragment length polymorphism (RFLP): RFLP was carried out using *Nde*I with H gene positive PCR products as per manufacturer's instruction.

Viral isolation: MDCK cells were obtained from Translational Research Platform for Veterinary Biologicals (TRPVB), TANUVAS. Dog SLAM expressing Vero cell line was a gift from Dr Aravinth Babu, Assistant Professor, Centre for Animal Health Studies, Chennai. All samples were infected in vero cells expressing SLAM and MDCK cells and three passages carried out. After every passage, the presence of the virus in the cell line was checked using RT-PCR for N gene.

Table 1. Primers used for amplification of N gene

Target gene	Primer sequence	Annealing temp.	Product size	Reference
N	F: ACAGGATTGCTGAGGACCTAT R: CAAGATAACCATGTACGGTGC	57°C	287 bp	Frisk *et al*. (1999)
N	F: TAAGCTGGGTCAAAGTAAGACG R: GAATTGCTGAAATGATTTGTGAT	57°C	336 bp	Rosana *et al*. (2013)
	F: TTGGCATTGAAACTATGTATCC R: CGAAACCCAACCCTCCCATG	57°C	234 bp	

Table 2. Primers used for amplification of H and F gene

Target gene	Primer sequence	Annealing temp (°C)	Product size	Reference	Nucleotide position
H	F: TATCATGACRGYARTGGTTC R: AATYYTCRAYACTGGWTGTG	53°C	871 bp	Hashimoo *et al.* (2001)	7991-8010 8841-8861
H	F: TACTGAATGGAGACGGTATGGAT R: TCGATAGAATTGGTGACATCACA	53°C	449 bp	Present study	8379-8401 8827-8805
F	F: ACAGGTCAACCAGGTCCA R: GGGCCAAATATTGACAAC	54°C	1053 bp	Pardo *et al*. (2005)	4873-4890 5909-5926
F	F: ACCCTTCCACTACATCATGAACA\ R: TAGAGTCAACGCTTGGTTGATTG	54°C	451 bp	Present study	5113-5130 5549-5527

RESULTS AND DISCUSSION

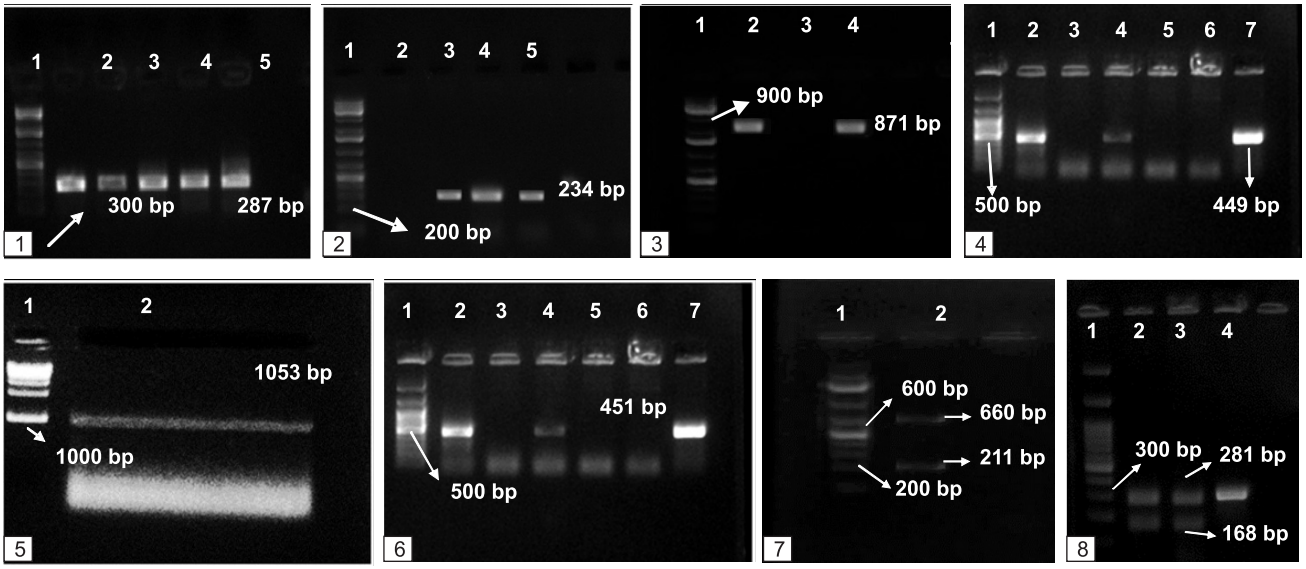
RT-PCR for N gene: Out of the 90 samples tested, 19 samples (21%) were found positive by RT-PCR method producing 287 bp product (Fig. 1) and 21 samples (23%) were found positive by the second method involving a nested PCR step producing 234 bp product (Fig. 2). As N gene is highly conserved, it has been commonly employed for the detection of CDV. RT-PCR was a highly sensitive and specific method for the ante-mortem diagnosis of canine distemper virus (Frisk *et al.*1999). This increased sensitivity in the second method could be due to additional nested PCR step.

RT-PCR for H gene and F gene: Out of 19 N gene positive samples, only 1 sample (sample Id: 70) yielded positive H gene amplification using published primer (Fig. 3) and 2 samples (sample Id: 29 and sample Id: 36) were amplified by primers designed in this study (Fig. 4). Out of 19 N gene positives, only 1 sample (sample Id: 63) got amplified for F gene using published primer (Fig. 5) and 2 samples (sample Id: 29, sample Id: 36) got amplified by primers designed in this study (Fig. 6). Compared to N gene, H and F gene are transcribed at lower levels in morbilli viruses. The lower rate of RT-PCR detection of partial H and F genes may be due to low amount of the virus in the clinical samples and low expression of H and F genes when compared to N gene in infected cells.

Restriction fragment length polymorphism: The H gene positive PCR product of 871 bp yielded fragments of 660 bp and 211 bp when digested with restriction enzyme (Fig. 7). The H gene positive PCR product of 449 bp when digested yielded fragments of 281 bp and 168 bp (Fig. 8). This revealed that the CDV detected in this study were wild type viruses but not vaccine strains. Restriction digestion of H gene PCR product is useful for ruling out the re-isolation of vaccine virus and hence can be adopted as a first step during molecular characterization and virus isolation studies. Calderon *et al.* (2007) reported that field CDV on restriction digestion with *Nde*I yielded 2 restriction fragments, where vaccine remained uncut.

Phylogenetic analysis: The CDV nucleotide sequences that were obtained in this study were deposited in the National Centre for Biotechnology Information Database and assigned GenBank accession numbers viz. KY011297 (TNCDV_29H), KY011298 (TNCDV_36H), KY011299 (TNCDV_70H), KY011300 (TNCDV_29F), KY011301 (TNCDV_36F), KY011302 (TNCDV_63F).

The phylogenetic tree constructed with H gene nucleotide sequences is depicted in Fig. 9 and for F gene nucleotide sequences in Fig. 10. The possibility of point mutations at the attachment site of the primers is relatively high for H gene. It was reported that in addition to H gene, F gene also had highest genetic/antigenic variation in CDV (Mochizuki *et al.* 1999). Therefore, phylogenetic analysis



Figs 1–8. **1.** RT-PCR for detection of CDV N gene. Lane 1,100 bp ladder; lane 2, positive control (vaccine); lane 3,4,5,6, positive samples (287 bp); lane 7, negative control. **2.** Nested PCR for detection of CDV N gene. Lane 1, 100 bp ladder; lane 2, non-template control; lane 3, positive control (vaccine); lane 4, positive sample (Id: 1); lane 5, positive sample (Id 12). **3**. RT-PCR detection of H gene. Lane 1,100 bp ladder; lane 2, positive control (vaccine); lane 3, non-template control; lane 4, positive sample (Id: 70). **4.** RT-PCR amplification of partial CDV H gene (449 bp). Lane 1, 100 bp ladder; lane 2, positive control (vaccine); lane 3, non-template control; lane 4, positive sample (Id: 29); lanes 5,6, negative sample; lane 7, positive sample (Id: 36). **5.** RT-PCR detection of F gene. Lane 1, 1 kb ladder; lane 2, positive sample (1053 bp). **6.** RT-PCR detection of F gene. Lane 1, 100 bp ladder; lane 2, positive control (vaccine); lane 3, positive sample (Id: 1); lane 4, positive sample (Id: 29); lane 5, positive sample (Id: 12); lane 6, positive sample (Id: 16); lane 7, positive sample (Id: 36). **7.** RFLP of 871bp H gene PCR product. Lane 1, 100 bp ladder; lane 2, RFLP positive product (wild type virus sample yielding 2 fragments of 211 and 660 bp after digestion with *Nde*I). **8.** RFLP of 449 bp H gene PCR product. Lane 1,100 bp ladder; lane 2,3, positive samples (Id: 29,36) (RFLP of 449 bp PCR product yielding two fragments of 281 and 168 bp); lane 4, vaccine–449 bp (remains uncut).

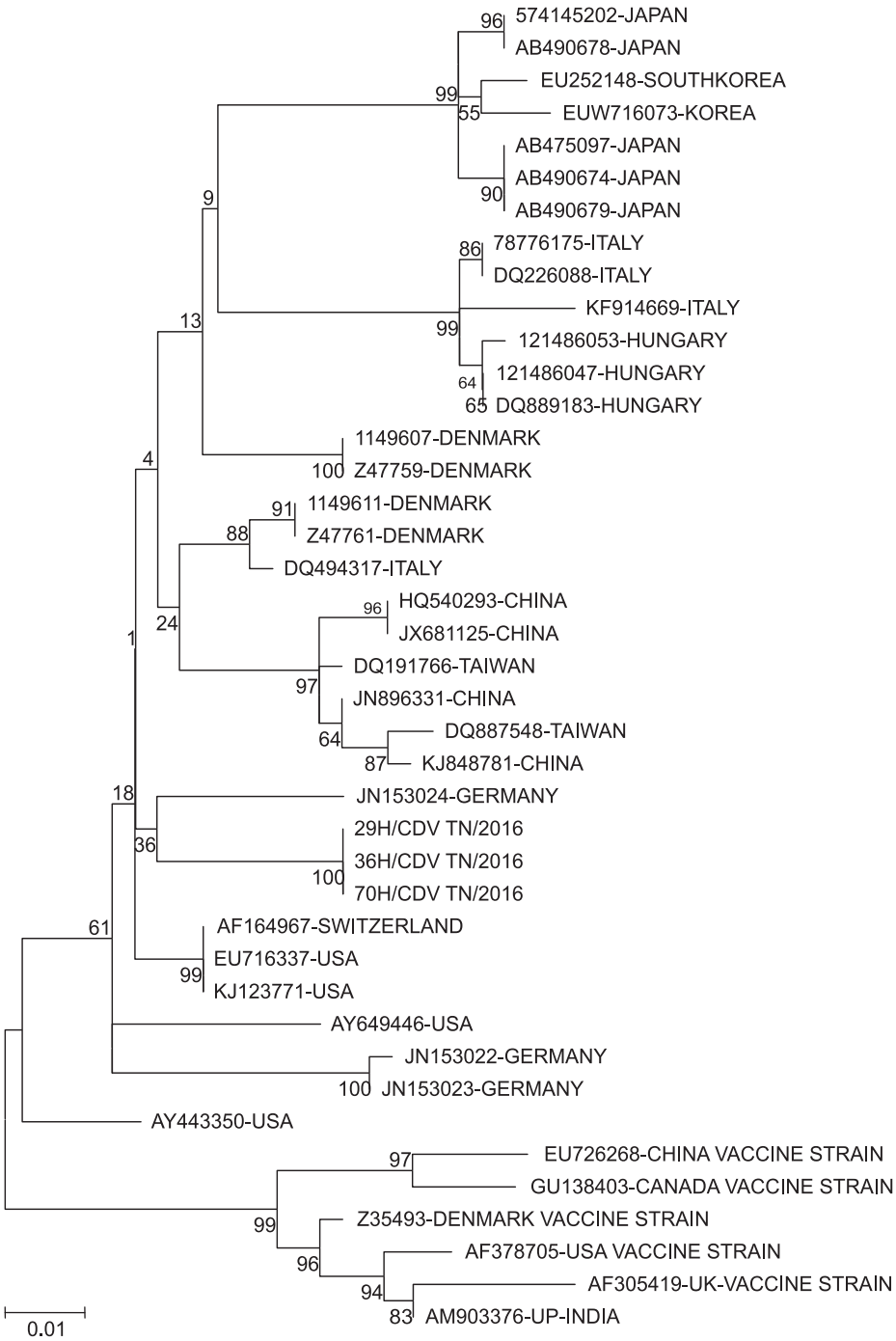


Fig. 9. Phylogenetic tree of CDV strains on the basis of nucleotide alignment of H gene constructed using maximum likelihood method. The scale bar indicates the branch length equivalent to 0.01 nucleotide substitution per site with bootstrap value determined for 1000 iterations.

of H and F gene nucleotide sequences were carried out and it was found that H and F gene sequences did not cluster with the vaccine strain but were seen as separate clade in the phylogenetic tree and fell under Europe lineage. Earlier studies on the phylogenetic analysis of CDV H and F gene in India also reported that the prevalent CDV did not cluster with the vaccine strain (Aarthi *et al.* 2015, Swati *et al.* 2015).

Viral isolation: Until third passage, the cytopathic effect was not observed. One sample (Id:70) was found positive by RT-PCR from second passage in both the cell lines. The poor isolation rate could be due to low titres of viable virus in the clinical samples collected in this study. It has been reported that cell lines such as vero cells does not allow propagation of field isolates which could also be another reason for the failure of isolation of CDV virus (Appel and Jones 1967). Other cell lines such as marmoset lymphoid cell line (B95a) is said to be a good host for CDV isolation (Kai *et al.* 1993). It has been reported that co-cultivation of

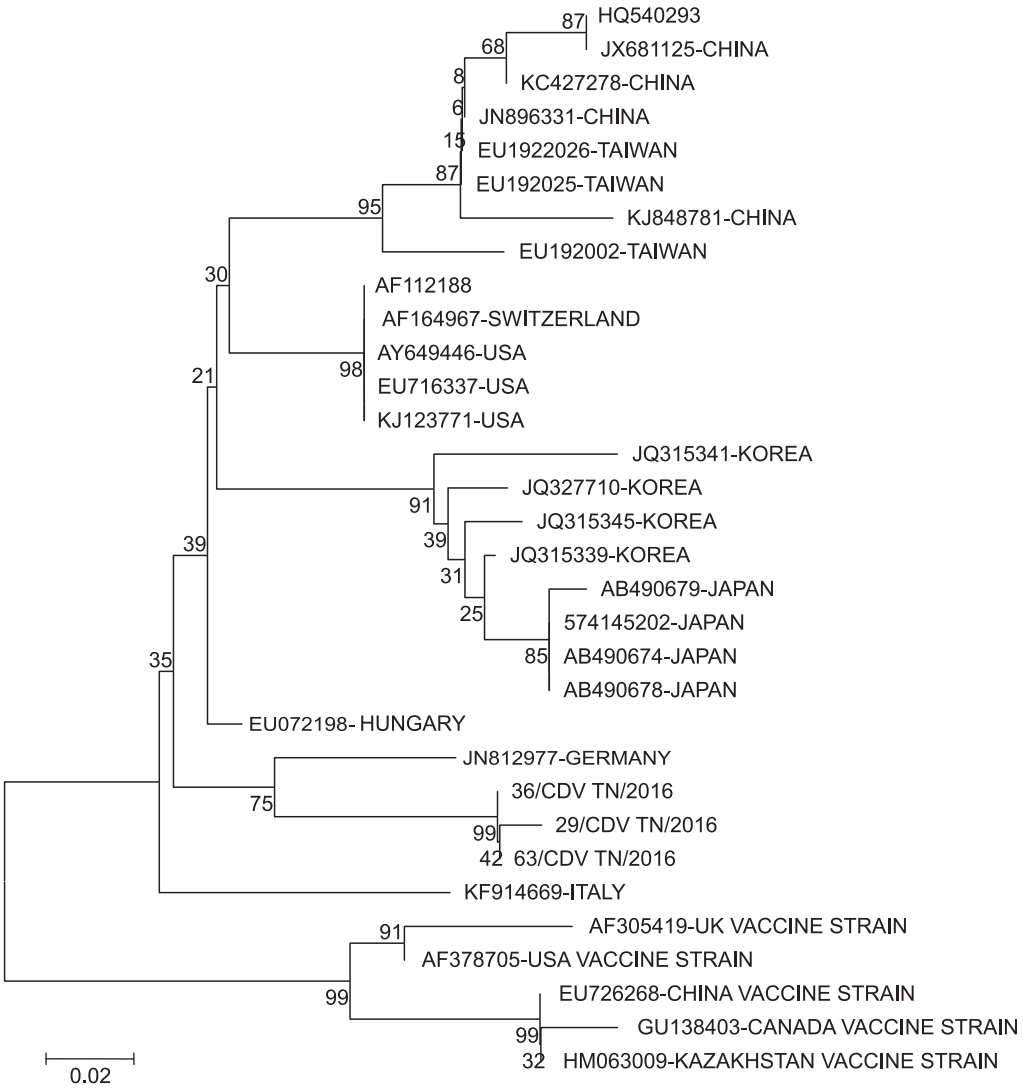


Fig. 10. Phylogenetic analysis of CDV strains on the basis of nucleotide alignment of F gene constructed using maximum likelihood method. The scale bar indicates the branch length equivalent to 0.02 nucleotide substitution per site bootstrap value determined for 1000 iterations.

lymphocytes from suspected dogs with mitogen stimulated dog lymphocytes are useful in CDV isolation (Appel *et al.* 1992).

In developing countries, particularly in India, dog populations in rural areas are large and free-ranging. As free ranging dogs are generally not vaccinated against the prevailing infectious diseases, they act as a major source of infection. Canine distemper is characterized by varied symptoms,which may resemble other diseases thus complicating the clinical diagnosis. Therefore it is essential to have a rapid and sensitive diagnostic technique for the confirmation of the disease so that necessary measures can be taken to prevent the spread of infection. Therefore nested PCR can be employed for the detection of canine distemper virus. In the present study, the incidence of the disease is high among non-vaccinated dogs thereby stressing the need for proper vaccination in dogs.Further an extensive study on complete H and F genes is required to find out the viral strains presently circulating in our country, which would in turn aid in the development of candidate vaccines.

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