



N gene based detection and phylogenetic analysis of *canine morbillivirus* in dogs[#]

 K. M. Maneesh¹,  K. Sumod²,  Chintu Ravishankar³,  Koshy John⁴,  R. Rajasekhar²,

P. M. Arun¹, S. Shashank¹, G. S. Anjitha¹, P. Jishnu Haridas¹, K. V. Jayanth¹,

N. Madhan Raj¹ and L. Sri Ramya¹

Department of Veterinary Microbiology
College of Veterinary and Animal Sciences, Pookode, Wayanad-673 576
Kerala Veterinary and Animal Sciences University
Kerala, India

Citation: Maneesh, K.M., Sumod, K., Ravishankar, C., John, K., Rajasekhar, R., Arun, P.M., Shashank, S., Anjitha, G.S., Haridas, J.P., Jayanth, K.V., Raj, M.N. and Ramya, S.L. 2023. N gene based detection and phylogenetic analysis of *canine morbillivirus* in dogs. *J. Vet. Anim. Sci.* 54(2):486-491

DOI: <https://doi.org/10.51966/jvas.2023.54.2.486-491>

Received: 14.11.2022

Accepted: 06.01.2023

Published: 30.06.2023

Abstract

Canine distemper, a highly fatal systemic disease in domestic dogs and wild carnivores, has the second highest mortality rate after rabies and is responsible for a large number of animal deaths around the world. It is considered a major pathogen in the canine infectious respiratory disease complex. This paper reports the finding of a study conducted to detect and characterise canine distemper virus (Canine Morbillivirus) based on N gene. A total of 59 samples collected from cases of respiratory infections in dogs were subjected to N gene based reverse transcriptase polymerase chain reaction (RT-PCR) and eleven of them (18.64 per cent) were found positive. Sequencing and phylogenetic analysis revealed that all the canine distemper viruses obtained in the present study were related to Indian strains that were previously reported. However, the viruses from the same district were similar among themselves.

Keywords: *Canine distemper virus, phylogenetic analysis, reverse transcriptase-polymerase chain reaction, N gene*

Canine distemper (CD) is a highly contagious disease caused by *canine morbillivirus* (canine distemper virus) of the genus *Morbillivirus* in the family *Paramyxoviridae*. It can cause a highly fatal systemic disease in domestic dogs and wild carnivores (McCarthy *et al.*, 2007). Canine distemper virus (CDV) has the second highest mortality rate after rabies, and is responsible for

[#]Part of MVSc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

1. MVSc Scholar
2. Assistant Professor
3. Professor
4. Professor and Head

*Corresponding author: dmaneeshmohan95@gmail.com, Ph. 6238325092

Copyright: © 2023 Maneesh *et al.* This is an open access article distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

a large number of animal deaths around the world (Loots *et al.*, 2017). It is considered a major pathogen in the canine infectious respiratory disease complex (CIRDC) (Day *et al.*, 2020). The genetic material for *canine morbillivirus* is a single-stranded, negative sense and non-segmented RNA (Sidhu *et al.*, 1993). The complete viral genome contains 15,690 nucleotides and encodes six structural proteins, namely nucleocapsid protein (N), phospho protein (P), large protein (L), matrix protein (M), haemagglutinin protein (H), and fusion protein (F), and two accessory non-structural proteins (C and V). The viral particle has the glycoproteins H and F inserted on its surface; these proteins are crucial for the virion's adsorption and fusion with the host cell, respectively. The M protein covers the space between the envelope and the nucleoprotein, therefore contributing to viral shape and the packaging and budding process in the host cell membrane. The N protein stands out because it envelopes the genome and safeguards the genetic material. The transcription and replication of viral RNA are regulated by the L and P proteins. The P, C and V proteins contribute to RNA synthesis, help the viral genome move from primary transcription to replication and may have an impact on messenger RNA (mRNA) synthesis, respectively (Beineke *et al.*, 2015). The disease is characterised by multisystemic infection eliciting respiratory, gastrointestinal and nervous signs depending on the severity based on the age and immunity of the host animal. Canine distemper virus is endemic in Kerala but official reports and studies regarding molecular characterisation and phylogeny are scarce. This paper reports the findings of a study undertaken to detect and characterise the *canine morbillivirus* as a respiratory pathogen based on nucleotide sequence of N gene.

Materials and methods

A total of 59 samples from dogs having respiratory infections were collected in virus transport medium from hospitals in Wayanad, Kannur, Kozhikode, Malappuram and Ernakulam districts of Kerala during the period 2021-2022 for the study. The samples consisted of nasal swabs and tissue samples including lungs, spleen, liver and kidney. The

samples were transported on ice and were stored at -80°C after proper labelling, till further processing.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was isolated from the tissue samples using the Gene JET RNA Purification Kit from ThermoScientific (USA) as per the manufacturer's protocol. The total RNA from nasal swabs was extracted by the TRIzol reagent method. Briefly, the nasal swabs were soaked in 500 µL of NFW and vortexed. Then the swab was squeezed against the wall of the tube and discarded. A volume of 250 µL of the fluid was mixed with 750 µL TRIzol reagent and incubated for 5 min. In the next step 200 µL chloroform per mL of TRIzol reagent was added and the tube was shaken vigorously for 15 s. The aqueous phase was carefully pipetted and transferred to another tube and 0.7 volume of isopropanol was added, followed by incubation at room temperature for 5 min. The tube was centrifuged at 14000 x g for 10 min. The supernatant was decanted, and the pellet was washed in 500 µL of 70 per cent ethanol and centrifuged at 14000 x g for 5 min. The alcohol was decanted, and the RNA dried at room temperature or in a dry bath at 37°C. The dried RNA was reconstituted in 30 µL of nuclease free water (NFW) and stored at -80°C.

Complementary DNA (cDNA) was synthesised using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per the manufacturer's protocol. To a PCR tube kept on ice, 2 µL RNA, 1 µL (0.2 µg/µL) of random hexamer primer and 9 µL of nuclease-free water (NFW) were added to make the volume up to 12 µL. The mixture was incubated at 65°C for 5 minutes. The above mixture was cooled down to 25°C and 4 µL of 5X reaction buffer, 1 µL (20 U/µL) Ribolock RNase inhibitor, 2 µL of 10 mM dNTP mix, and 1 µL (200U/µL) RevertAid H minus M-MuLV reverse transcriptase were added and thereafter the mixture was incubated at 25°C for 5 min followed by 60 min at 42°C. The reaction was terminated by heating at 70°C for 5 min. The cDNA was stored at -80°C for further use.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of *canine morbillivirus* was performed employing N gene specific primers as described by Frisk *et al.* (1999) with slight modifications. Briefly, the reaction was carried out in a volume of 12.5 μ L EmeraldAmp GT PCR master mix (2X) (Takara, Japan), 1 μ L each of respective primers (10 pmol), 2 μ L of cDNA, and 8.5 μ L of NFW. The PCR condition was standardized as 94°C for 1min (initial denaturation), 35 cycles of 94°C for 1 min (denaturation), 55°C for 2 min (annealing), and 72°C for 1 min(extension). Final extension was carried out at 72°C for 5 min. Complementary DNA prepared from Nobivac® DHPPi (MSD Animal health, Mumbai, India) was kept as the positive control. No template control (NTC) which does not contain template was also kept for each run.

The PCR products were resolved electrophoretically on 1.25 per cent agarose gels containing ethidium bromide and were visualised in a gel documentation system under UV illumination to identify the size of the amplicon.

Phylogenetic analysis

The PCR products of representative positive samples were purified using Gene JET Gel Extraction Kit (Thermo Fisher Scientific, Massachusetts, USA) and sent to AgriGenome Lab Private Limited, Cochin, India for sequencing. With the help of Chromas Lite v2.01 software (<http://www.technelysium.com.au>) the chromatograms of the sequences were analysed for their proper assembly. BLAST analysis was conducted (<http://www.ncbi.nlm.nih.gov/BLAST>) to confirm the presence of gene specific to canine distemper virus.

Phylogenetic analysis of the sequences obtained in this study was carried out using MEGA X software. For phylogenetic analysis, sequences of CDV isolates from other parts of India and from other countries were downloaded from GenBank (www.ncbi.nlm.nih.gov/genbank/). Using ClustalW program of MEGA 10.2, alignment of the downloaded

sequences was done followed by trimming of the same to match sequence lengths obtained in this study. The evolutionary history was inferred by using Maximum Likelihood tree method (Yang, 1997). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein, 1985) was taken to represent the evolutionary history of the analysed sequences. Determination of evolutionary distances were done by Tamura-3-parameter method (Tamura, 1992) for canine distemper virus they had the lowest Bayesian Information Criterion scores (BIC).

Results and discussion

Of the 59 tested samples, eleven were found positive for canine distemper virus nucleic acid by PCR targeting the N gene (Fig.1.). Percentage positivity was calculated to be 18.64 percent in the present study. Specific amplicons of 287 bp generated by PCR reaction were visualized on agarose gel electrophoresis. No band was observed in the NTC.

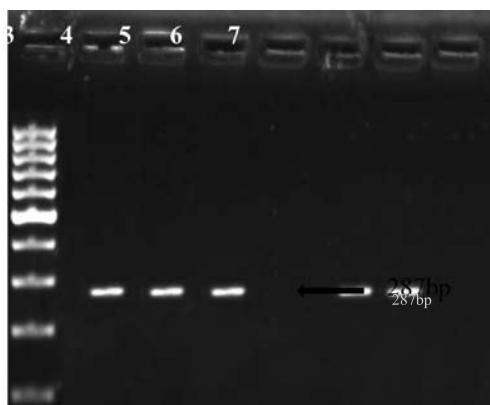


Fig. 1. Detection of N gene of canine distemper virus of dogs

Ashmi *et al.* (2017) reported a CD outbreak in Tamil Nadu. In their study, 21 per cent were positive for CD by RT-PCR targeting the N gene. In another study conducted by Shin *et al.* (2004), N gene of *canine morbillivirus* was selected for amplification from nasal swabs. Twenty-two per cent of the samples were positive.

On BLAST analysis of N gene sequences, the isolates obtained in this study showed similarity to other Indian sequences.

The samples from Kannur showed 98.91 per cent identity with a sequence from a palm civet (MF964188) from Uttar Pradesh, India. One sample from Malappuram showed the greatest similarity with the sequence from Hisar (MN128876), India with a 98.14 per cent identity. One sample from Ernakulam showed 97.45 per cent identity with a sequence from Orathanadu (MH536200), Tamil Nadu. Two sequences from Wayanad showed identities of 99.10 per cent and 99.11 per cent with a Hyderabad sequence (MT905031).

On phylogenetic analysis, all the sequences in the present study clustered together with Indian sequences. These sequences also clustered separately from the Asia-1 and Asia-2 lineages. It was observed that strains from particular district clustered together in the phylogenetic tree as observed in the case of strains from Wayanad and Kannur. Strains from Ernakulam and Malappuram though genetically related branched separately in the phylogenetic tree (Fig. 2.). Based on the

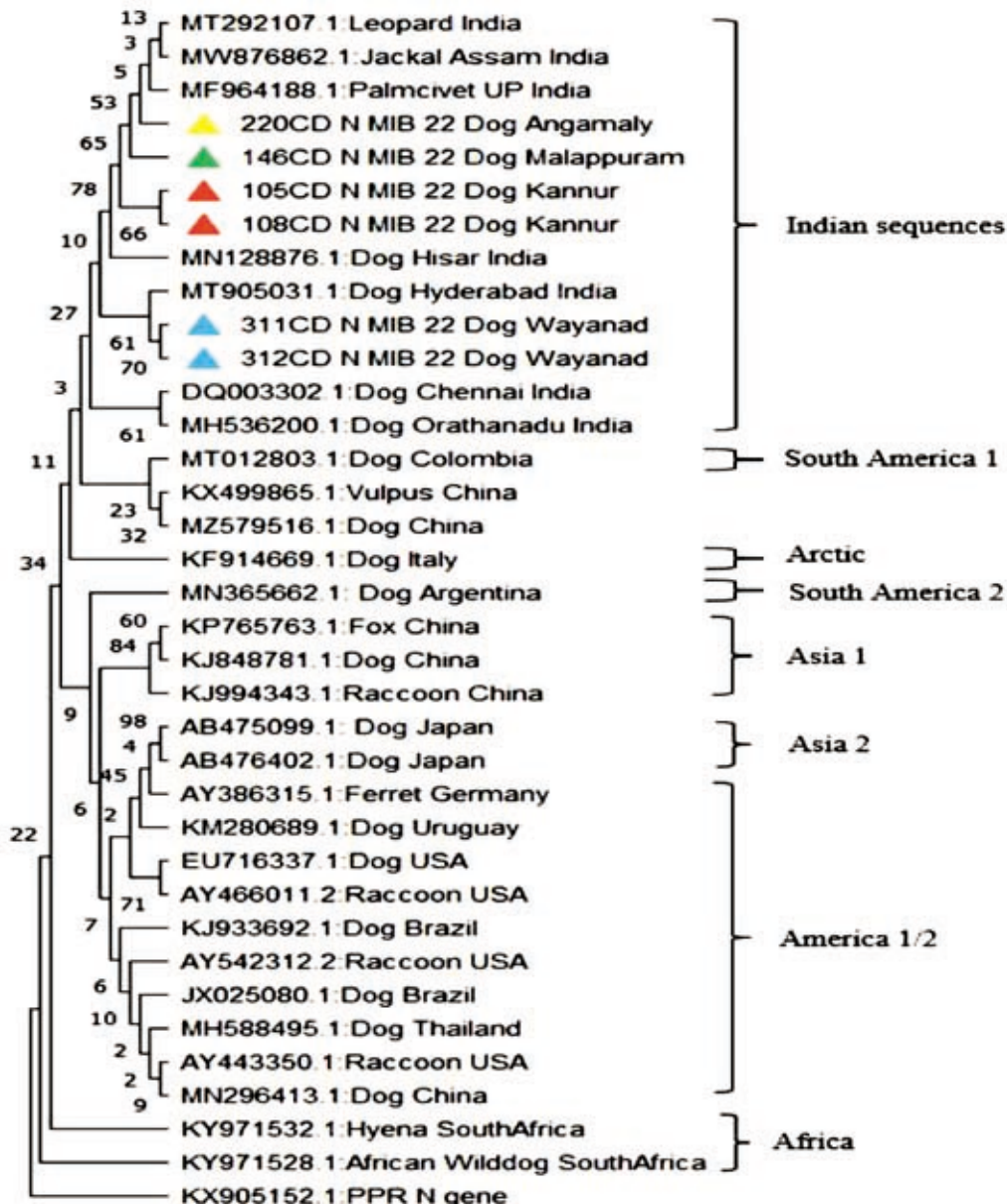


Fig. 2. Maximum likelihood tree constructed using nucleotide sequences of N gene of CDV

partial N gene sequences of two Indian CDV from dogs, Pawar *et al.* (2011) reported similar findings. Based on the sequences of the H, F, M, and P proteins, Deka *et al.* (2015) found that the Indian canine distemper viruses formed a separate clade in the phylogenetic tree. Bhatt *et al.* (2019) studied the H gene of CDV and reported that the Indian CDV were grouped together by phylogenetic reconstruction into a discrete monophyletic group which was clearly distinct from the known CDV lineages. They also reported that a novel genetic variant ("Lineage India- 1/Asia- 5") is present and circulating among Indian dog populations and that in comparison to other Asiatic lineages, the Indian CDV displayed a significant level of divergence, indicating that the Indian CDV and other CDV strains have distinct origins. These studies are in agreement with our finding that the Indian sequences cluster together and separately from Asia-1 and Asia-2 lineages.

Conclusion

The strains of canine distemper virus obtained from different geographic regions of Kerala are related to other viruses reported from India. However, among the Kerala strains, subtle variations exist based on the geographical location.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- McCarthy, A.J., Shaw, M.A. and Goodman, S.J. 2007. Pathogen evolution and disease emergence in carnivores. *Proc. R. Soc. B: Biol. Sci.* **274**: 3165-3174.
- Loots, A.K., Mitchell, E., Dalton, D.L., Kotzé, A. and Venter, E.H. 2017. Advances in canine distemper virus pathogenesis research: a wildlife perspective. *J. Gen. Virol.* **98**: 311-321.
- Day, M.J., Carey, S., Clercx, C., Kohn, B., Marsillo, F., Thiry, E., Freyburger, L., Schulz, B. and Walker, D.J. 2020. Aetiology of canine infectious respiratory disease complex and prevalence of its pathogens in Europe. *J. Comp. Path.* **176**: 86-108.
- Sidhu, M.S., Husar, W., Cook, S.D., Dowling, P.C. and Udem, S.A. 1993. Canine distemper terminal and intergenic non-protein coding nucleotide sequences: completion of the entire CDV genome sequence. *Virology*. **193**: 66-72.
- Beineke, A., Baumgärtner, W. and Wohlsein, P. 2015. Cross-species transmission of canine distemper virus—an update. *One Health*. **1**: 49-59.
- Deem, S.L., Spelman, L.H., Yates, R.A. and Montali, R.J. 2000. Canine distemper in terrestrial carnivores: a review. *J. Zoo Wildlife Med.* **31**: 441-451.
- Frisk, A.L., König, M., Moritz, A. and Baumgartner, W. 1999. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. *J. Clin. Microbiol.* **37**: 3634-3643.
- Yang, Z. 1997. PAML: a program package for phylogenetic analysis by maximum likelihood. *Comp. Appl. Biosci.* **13**: 555-556.
- Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*. **39**: 783-791.
- Tamura, K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases. *Mol. Biol. Evol.* **9**: 678-687.
- Bhatt, M., Rajak, K.K., Chakravarti, S., Yadav, A.K., Kumar, A., Gupta, V., Chander, V., Mathesh, K., Chandramohan, S., Sharma, A.K. and Mahendran, K. 2019. Phylogenetic analysis of haemagglutinin gene deciphering a new genetically distinct lineage of canine distemper virus circulating among domestic dogs in India. *Transbound. Emerg. Dis.* **66**: 1252-1267.

- Deka, D., Uppal, S.K. and Verma, R. 2015. Isolation and phylogenetic characterization of Canine distemper virus from India. *Virus Dis.* **26**: 133-140.
- Ashmi, J.M., Thangavelu, A., Senthilkumar, T.M.A. and Manimaran, K. 2017. Molecular characterization of Canine distemper virus from Tamil Nadu, India. *Indian J. Anim. Sci.* **87**: 1062–1067.
- Pawar, R. M., Raj, G. D., Gopinath, V. P., Ashok, A. and Raja, A. 2011. Isolation and molecular characterization of Canine distemper virus from India. *Trop. Anim. Hlth Prod.* **43**: 1617–1622.
- Shin, Y.J., Cho, K.O., Cho, H.S., Kang, S.K., Kim, H.J., Kim, Y.H., Park, H.S. and Park, N.Y. 2004. Comparison of one-step RT-PCR and a nested PCR for the detection of canine distemper virus in clinical samples. *Aust. Vet. J.* **82**: 83-86. ■