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# Phylogenetic analysis of the Fusion (F) and Haemagglutinin (H) genes of Canine Distemper Virus from field isolates in Tamil Nadu

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## ABSTRACT

*Canine Distemper Virus (CDV) is a highly transmissible viral pathogen that causes the lethal canine distemper disease which has high mortality rates. In the present study, 15 clinical specimens, 8 ocular swab specimens, 6 blood specimens and 1 nasal swab specimen were collected from suspected dogs. The Fusion gene and Haemagglutinin gene regions of CDV were amplified using reverse transcriptase-PCR. Subsequently partial gene sequencing was performed to compare the genetic variation among the field isolate and vaccine strain. The obtained sequences were also compared with the reference CDV sequences available in the Gen Bank to establish the phylogenetic lineage. The results obtained suggested that CDV is prone to evolution and that a different CDV lineage may be present in Tamil Nadu. Variations in the amino acid site in the F and H gene regions indicated the need for the development of new candidate vaccines in Tamil Nadu.*

***Key words:*** *Canine distemper virus, Onderstepoort strain, Fusion gene, Haemagglutinin gene, Phylogenetic analysis*

## INTRODUCTION

Canine distemper virus is the causative agent of one of the most important viral diseases of wild and domestic *Canidae*. The family *Canidae* is a lineage of carnivorans that includes dogs, wolves, foxes and jackals. The onset of Canine distemper disease is accompanied by several indicators such as pyrexia, anorexia, nasal discharge, conjunctivitis and diarrhea. Some animals also show signs of hyperkeratosis, skin pustules and central nervous system disturbances[4]. CDV belongs to the genus Morbillivirus of the family Paramyxoviridaeand has a negative sense, single stranded ~15.7-kb RNA genome[5]. The genome of the CDV encodes the following virion proteins: matrix (M), fusion (F), hemagglutinin (H), nucleocapsid (N), polymerase (L), and phosphoprotein (P). The F and H gene proteins are glycoproteins and are highly susceptible to genetic variation. Analysis of the variations of the Fusion protein signal peptide (Fsp) sequence might be useful to conduct strain characterization studies[7]. The H gene protein is crucial for viral attachment to the cell host. Because of its variable nature, H gene is useful to analyse genetic changes between CDV isolates[1, 2]. Hence, the present study was undertaken with the object to compare the F and H gene of the field and reference strains of CDV and to differentiate them using sequence analysis.

## MATERIALS AND METHODS

**Collection of suspected samples:** A total of 15 clinical specimens, 8 ocular swab specimens, 6 blood specimens and 1 nasal swab specimen were obtained from suspected dogs admitted to the Madras Veterinary College. The suspect of CDV infection was initially found by the doctors on the basis of clinical signs (fever, respiratory, enteric and hyperkeratosis signs).

**RNA extraction and RT-PCR:** Total RNA was obtained from 250µl of ocular and nasal swabs using RNAiso Plus (Takara Bio Inc., Japan). Peripheral blood mononuclear cells (PBMC) were extracted from the blood samples and RNA was isolated from 250µl of the PBMC using RNAiso Plus. Reverse transcription was performed using the RevertAid RT kit (Thermo Scientific, USA) according to manufacturer's instructions.

**Cell line and culture:** The positive CDV samples were adapted on the MDCK (Madin-Darby canine kidney) cell line. The cells were grown routinely in 25 $cm^2$ polystyrene tissue culture flasks with filter caps at 37°C in a 5% humidified $CO_2$ atmosphere. The culture medium was composed of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) and 10% Fetal Bovine Serum (FBS)(Invitrogen, USA).

**PCR amplification:** The cDNA synthesized from the extracted RNA was subjected to PCR amplification using oligonucleotide primers designed to amplify the F gene and H gene of the CDV. The partial length F gene primers (CDVF1: 5'-ACAGGTCAACCAGGTCCA-3', CDVF2: 5'-GGGCCAAATATTGACAAC-3') used in this study were designed by Pardo *et al* (2005). After initial denaturation at 95°C for 5 min, the amplification was carried out for 35 cycles each consisting of 30s at 95°C, 20s at 54°C and 90s at 72°C with a final extension of 7 min at 72°C. The partial length H gene primers (CDVH1: 5'-TCGAAATCCTATGTGAGATCACT-3', CDVH2: 5'-ACACTCCGTCTGAGATAGC-3') were designed by Lan *et al* (2006) and Pardo *et al* (2005). The temperature profile includes initial denaturation at 95° for 5 min, the amplification was carried out for 40 cycles each consisting of 1 min at 95°C, 1 min at 48°C and 2 min at 72°C; with a final extension of 7 min at 72°C[10]. Negative control reactions (no template added) were added to check for contamination of the PCR reaction.

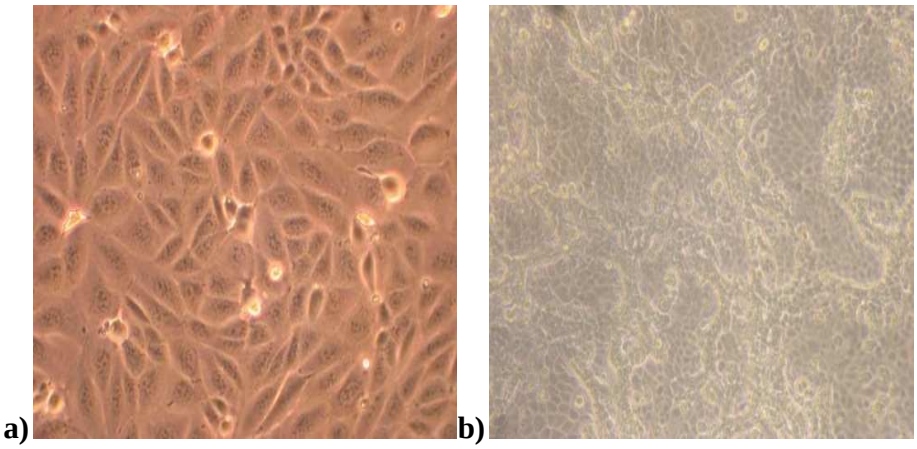
**Sequencing and Phylogenetic analysis:** The amplified PCR products were purified using EZ-10 Spin Column DNA Gel Extraction Kit (BioBasicInc., Canada) according to the manufacturer's instructions. The purified products were subjected to sequencing. The obtained nucleotide sequences of F and H gene were compared with the Onderstepoort vaccine strain sequence available in GenBank. The alignment command available in MEGA 4.0 software was used to create multiple sequence alignment of the field, vaccine and other strains of CDV. Phylogenetic tree was then constructed using the neighbour joining method[9].

## RESULTS AND DISCUSSION

The cell line MDCK was successfully used for CDV isolation from the third passage by the observation of CDV cytopathic effect (CPE) **(Fig. 1)**. The presence of viral RNA detection through RT-PCR was carried out for the cell culture samples by adding RNAiso Plus directly into the tissue culture flask. The infected culture fluid showed PCR amplification using the CDV primers specific for F and H gene.Virus isolation is a standard method of confirming the etiological agent. Hence the virus was passaged in MDCK cell line and based on the characteristic cytopathic effect and molecular method, the presence of virus in the infected culture fluid was confirmed.

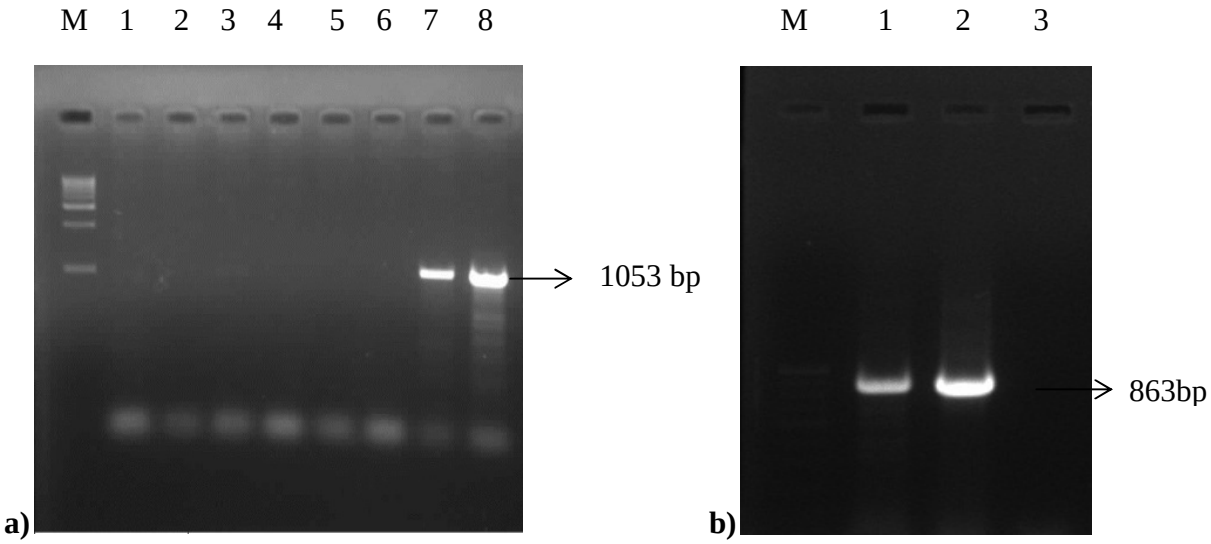
RNA extraction followed by PCR was done for the 15 clinical samples using the pair of primers for the partial length F and H genes respectively. The expected amplicon size of 1053 bp for the F gene was observed when tested in 1% agarose gel with 1 kb DNA molecular weight marker **(Fig. 2a)** as reported by Pardo *et al* (2005). The expected amplicon size of 863 bp for the H gene was observed when tested in 1.2% agarose gel with 100bp DNA molecular weight marker **(Fig. 2b)**. This clearly indicates the specificity of H gene primers as reported by Lan *et al* (2006) and Pardo *et al* (2005). No amplification was noticed in the negative control samples.

**Fig. 1: Virus isolation in MDCK cell line**



**a)** MDCK cell control. **b)** CDV infected sample (100X)

**Fig. 2: RT-PCR amplified products**




**a)**1053bp partial F gene amplicon (M-1 kb DNA ladder, lane 1 to 5-negative samples, lane 6- negative control, lane 7 and 8- positive samples) **b)** 863bp partial H gene amplicon (M-100 bp DNA ladder, lane 1 and 2- positive samples, lane 3- negative control)

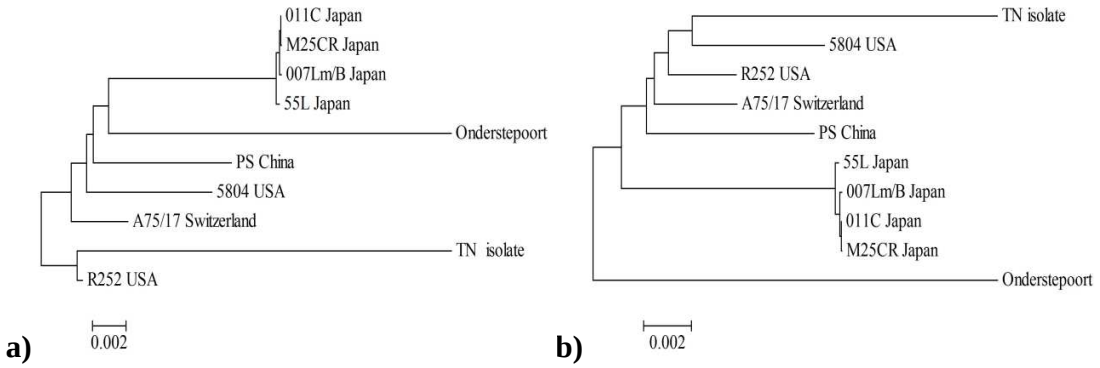
The F and H gene sequences of the field isolate were compared with the vaccine and foreign strains using the National centre for biotechnology information basic alignment search tool (NCBI BLAST) program and the results were furnished in **Table 1**. The Onderstepoort vaccine strain (AF378705.1) showed 91% homology with the H gene of the Tamil Nadu field isolate and 89% homology with the F gene of the field isolate. There was only a homology of 95% between the field isolate and the R252, USA strain. All the strains from Japan showed similar homology with the Tamil Nadu field isolate. The H gene of local strain shows more homology with the reference strains than the F gene. Hence it can be inferred that the CDV strain is prone to genetic variation in the F and H gene regions.

**Table 1: Comparison of nucleotide sequence homology between field and reference strains**

| GenBank Accession no. | Strain description | Nucleotide identity of field isolate | |
|---|---|---|---|
| | | F gene | H gene |
| KF640687.1 | R252, USA | 95% | 95% |
| AF164967.1 | A75/17, Switzerland | 93% | 95% |
| AY386315.1 | 5804, USA | 92% | 95% |
| AB490680.1 | 007Lm/B, Japan | 92% | 94% |
| AB476401.1 | 011C, Japan | 92% | 94% |
| AB475097.1 | M25CR, Japan | 92% | 94% |
| AB475099.1 | 55L, Japan | 92% | 94% |
| JN896331.1 | PS, China | 91% | 94% |
| AF378705.1 | Onderstepoort strain | 89% | 91% |

Representative sequences from top hits obtained from nucleotide BLAST were chosen for phylogenetic analysis. The neighbour joining tree with relatively higher bootstrap values provided clear resolution of all the nodes. The Onderstepoort strain which is considered to be completely apathogenic and used in vaccines was used as a reference strain for phylogenetic analysis[8] .The current field isolates did not cluster with the Onderstepoort strain but was seen as a separate clade in the phylogenetic tree.The amino acid sites in F and H gene of field and reference strains were presented in **Table 2.** Variation in the amino acid is seen between the field isolate and Onderstepoort vaccine strain.

**Fig. 3: Phylogenetic tree construction using neighbour joining method**




**a)** Phylogenetic tree for the F gene sequences of representative CDVs and Tamil Nadu field isolate. **b)** Phylogenetic tree for the H gene sequences of representative CDVs and Tamil Nadu field isolate.

**Table 2: The amino acid site variation in the field isolate and reference strains of F and H genes of CDV (Amino acids are in single letter code)**

| Reference strains | Amino acid position in F gene | | | | Amino acid position in H gene | | | |
|---|---|---|---|---|---|---|---|---|
| | 1648 | 1730 | 1867 | 1946 | 2341 | 2391 | 2436 | 2503 |
| TN isolate | T | V | R | L | V | E | G | T |
| Onderstepoort strain | R | L | K | P | E | G | R | A |
| R252, USA | T | V | K | L | E | G | R | T |
| A75/17, Switzerland | T | A | K | L | E | G | R | T |
| 5804, USA | T | V | K | L | E | G | R | T |
| 007Lm/B, Japan | R | V | R | L | E | G | G | A |
| 011C, Japan | R | V | R | L | E | G | G | A |
| M25CR, Japan | R | V | R | L | E | G | G | A |
| 55L, Japan | R | V | R | L | E | G | G | A |
| PS, China | T | V | K | L | A | G | R | T |

## CONCLUSION

Gene mutation, deletion and arrangement of some key nucleotides or amino acids were responsible for the reduced efficiency of the CDV vaccine which is widely used in Tamil Nadu. At present in India, imported vaccines are available in the market; hence an ideal cell culture adapted candidate vaccine strain would be useful for proper control of the canine distemper disease.

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