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Genotyping of Canine Distemper Virus Lineage in Clinically Infected Dogs in Puducherry, Southern India

Megavarnan Abirami[1], Mouttou Vivek Srinivas[1], Jayalakshmi Vasu[1], Prabhakar Xavier Antony[1], Jacob Thanislass[2], Muthuraj Muthaiah[3] and Hirak Kumar Mukhopadhyay[1*]

[1]Department of Veterinary Microbiology, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry - 605 009, India.
[2]Department of Veterinary Biochemistry, Rajiv Gandhi Institute of Veterinary Education & Research, Puducherry - 605 009, India.
[3]Government Hospital for Chest Disease, Puducherry, India.

Authors' contributions

This work was carried out in collaboration among all authors. Authors MA, MVS and HKM were involved in the design of this research work. Author MA performed the research. Authors MVS and HKM monitored all the activities being the supervisors. Authors MVS, HKM, PXA, JV, MM and JT assisted in this research work. Authors MVS, MA and HKM drafted and revised the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study aimed to determine the Canine Distemper Virus (CDV) lineage circulating in the Puducherry region (Southern India) and how they are genetically and antigenically related to the vaccine and other known CDV lineages around the world.
Study Design: The study involved genetic characterization of the canine distemper virus strains/lineages circulating in the clinically suspected dogs in the field.

**Corresponding author: E-mail: mhirak@rediffmail.com;*

Place and Duration of Study: Department of Veterinary Clinical Complex, Animal Husbandry Department, Private Veterinary Clinics, NGOs like Bark India, People for Animals organization, Puducherry between January 2018 and December 2019.

Methodology: The ocular and nasal swabs were collected from 40 dogs suspected for canine distemper from Puducherry state (Southern India). Following viral RNA Extraction and cDNA synthesis; the cDNAs were screened for virus by targeting the CDV Nucleocapsid (N) gene using Reverse Transcriptase PCR. Further, the N gene positive cDNAs were genetically characterized for sequence analysis of the CDV hemagglutinin (H) gene.

Results: A total of 15 (37.5%) out of 40 ocular/ nasal swabs were found to be CDV positive by RT-PCR targeting the N gene. The sequence analysis of the H gene revealed forty-nine non-synonymous and thirty-three synonymous mutations (out of 356 amino acids) in comparison with vaccine strain. The vaccine virus (Onderstepoort strain), which is related to the America 1 lineage, possessed high level of (30 – 35 i.e. more than 10%) amino acid divergence with the CDV sequences analysed in this study. The Phylogenetic analysis revealed that the present CDV sequences formed a separate monophyletic group with the CDV sequences of other Indian dogs and the Indian wildlife (Lion) and is clustered away from the vaccine strain. The CDV sequences were closer to the CDVs of Africa - 2 lineage than the other Asian lineages. The Recombination analysis revealed no potential breakpoints and recombination events.

Conclusion: Together, these findings highlighted the possible existence of the novel Indian CDV lineage/s distinct from the vaccine strain and from other known Asian lineages.

Keywords: Canine Distemper Virus (CDV); reverse transcriptase PCR; haemagglutine gene; sequencing; phylogenetic analysis.

1. INTRODUCTION

Canine distemper (CD) is a severe, life-threatening viral disease of domestic and wild Canids (dog, dingo, fox, coyote, jackal, and wolf) with a worldwide distribution [1]. Domestic dogs are the main reservoir of CDV and they pose a conservation threat to endangered species worldwide [2,3]. The causative agent Canine Distemper Virus (CDV) also infects a broad range of other families of animals, such as Mustelidae, Ursidae, Viverridae, Hyaenidae and Felidae [1,4-9].

The Canine Distemper Virus (CDV), an enveloped virus of about 150 to 300 nm in diameter, is classified under the genus Morbillivirus of the family Paramyxoviridae and has an un-segmented, negative-sense, single-stranded, ~15.7-kb RNA genome consisting of genes for two non-structural protein (C and V) and six structural proteins: large protein (L), haemagglutinin (H), phosphoprotein (P), nucleocapsid protein (N), fusion protein (F) and matrix protein (M). The nucleocapsid (N) gene is a part of the most conserved region of the CDV genome [10] and therefore it has been commonly employed for the detection of CDV in molecular diagnosis.

Of the six structural genes, Haemagglutinine (H) gene (1824 bp) which encodes an enveloped glycoprotein responsible for viral attachment and infection to the host cell [11], has the highest genetic diversity and is therefore a suitable target gene to understand or investigate the prevalent CDV lineage and also for performing the extensive molecular epidemiological studies [12,13]. At present, based on variation in the Haemagglutinine gene, seventeen geographically related CDV genetic lineages (genotypes) are reported worldwide namely America 1 to 5, Africa 1, Africa 2, Europe Wildlife, Arctic, Europe/South America 1, South America 2, South America 3, Rockborn-like and Asia 1 to 4 [14,15].

In India, all the commercially available vaccines use Onderstepoort strain as the candidate virus for the vaccination against CDV. Onderstepoort vaccine strain is genetically related to America 1 lineage and is used as vaccine in many countries since 1950s [16,17]. This vaccine preparations used for dogs are not recommended for felids (lion, jaguar, tiger, leopard, domestic cats, cheetah, puma, snow leopard, black panther etc).

CDV infection in vaccinated dogs have been reported in several occasions [18-21]. Since 1990s the question persists with the efficacy of the vaccine / vaccine strain on basis of these CDV infection in some vaccinated dogs as report in various countries [20,22-25]. Further, many

research studies have shown the antigenic differences between the CDV vaccine strains and the wild type isolates [20,26]. In one of the recent study, Bhatt et al., [27] have proposed a separate lineage existing in the Indian isolates, which is to be designated as Asia-5 /India-1 lineage. However, as of now, there are very limited extensive studies of molecular characterization of canine distemper virus have been reported in India.

Based on the above facts, it becomes prudent to determine the CDV strains/lineages circulating in various geographical regions of India and how they are genetically and antigenically related to the available vaccines and other known lineages of CDV around the world.

2. MATERIALS AND METHODS

2.1 Clinical Samples

The ocular and nasal swabs were collected from 40 dogs suspected for canine distemper from Puducherry & other state (Southern India) (Table 1) in sterile Phosphate buffered saline (PBS) of pH 7.4 and transported to the laboratory as soon as possible in ice. The samples were centrifuged at 5000 rpm for 5 min and the supernatants collected were stored at - 50°C until further use. The attenuated live vaccine Vanguard Plus (Pfizer) was used as a positive control, and ultrapure water and nasal swabs from healthy dogs were used as negative controls.

2.2 Extraction of Viral RNA and cDNA Synthesis

The viral RNA were extracted from 200 µl of ocular and nasal swab samples using Thermo Scientific Gene JET Viral RNA purification kit as per the manufacturer's instructions. The extracted RNA was used as the template for reverse transcription.

The RNA templates were subjected to first strand cDNA synthesis using Thermo Scientific RevertAid First Stand cDNA Synthesis kit with random hexamer primers, as per the manufacturer's instructions. Reverse transcription was performed with the 20 µl reaction mix prepared with random hexamer primers (1µl), RNA template (2µl), reaction buffer (4µl), Ribolock Rnase inhibitor (1µl), 10 mM dNTP mix (2µl), RevertAid M- MuLV RT (1µl) and nuclease free water (12µl). Reverse transcription was performed for 5 min at 25°C, followed by 60 min at 42°C in a thermal cycler (Eppendorf, Nexus gradient). The reaction was terminated by heating at 70°C for 5 min. The final products were then stored at -20°C until further use. The attenuated live vaccine Vanguard Plus (Pfizer) was used as a positive control, and ultrapure water and nasal swabs from healthy dogs were used as negative controls.

2.3 Screening of Samples for Canine Distemper Virus

Following reverse transcription; the synthesised cDNAs were screened for Canine distemper virus by PCR amplification targeting the CDV-N gene, which amplifies 242 bp-long fragments with the screening primer pair (Table 2). The reaction mixture (50 µl) consisted of 1X concentration of Taq DNA polymerase Ampliqon Master mix Red, 0.2 µM of each primer and 2 µl of template DNA. The thermo-cycling conditions were as follows: 5 min at 95°C (initial denaturation), 35 cycles of 30 sec at 95°C (denaturation), 30 sec at 56°C (annealing temperature), 1min at 72°C (extension), followed by final extension (72°C for 10min) and hold at 4°C. The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany). The PCR products were resolved 1.5% agarose gel electrophoresis in 1X Tris acetate EDTA (TAE) buffer.

2.4 PCR Amplification of H Gene and DNA Sequencing

The cDNAs which were positive for CDV-N gene were subjected to PCR amplification targeting the CDV-H gene with the specific primer pair designed in our laboratory (Table 2), which amplifies 1123 bp fragments (Partial H gene). The thermo-cycling conditions were as follows: 5min at 95°C (initial denaturation), 35 cycles of 30 sec at 95°C (denaturation), 30 sec at 56°C (annealing temperature), 1 min at 72°C (extension), followed by final extension (72°C for 10min) and hold at 4°C. The amplified PCR products were gel extracted and custom sequenced for both direction (5'-3' and 3'-5') using the automated sequencer, Applied Biosystem 3100.

Table 1. Details of sample collection and the results

Sample ID	Age	Sex	Breed	Vaccination status	Place of sample collected	PCR Result		GenBank Acc. ID H gene
						N gene	H gene	
Sample 1	9 year	F	Spitz	+	Puducherry, India	-		
Sample 2	7 year	F	Doberman	-	Puducherry, India	-		
Sample 3	3 year	F	ND	-	Puducherry, India	+	-	
Sample 4	2 year	M	ND	-	Puducherry, India	+	-	
Sample 5	1.5 year	M	ND	-	Puducherry, India	+	+	
Sample 6	2 year	M	ND	-	Puducherry, India	+	-	
Sample 7	2 year	M	ND	-	Puducherry, India	-		
Sample 8	5 month	F	ND	-	Puducherry, India	+	+	MN688781
Sample 9	5 month	F	ND	-	Puducherry, India	-		
Sample 10	6 month	F	Pug	+*	Puducherry, India	+	+	MN688782
Sample 11	2.5 year	F	Labrador	-	Puducherry, India	-		
Sample 12	2 year	M	Labrador	-	Puducherry, India	+	+	MN688783
Sample 13	1 year	M	ND	-	Puducherry, India	+	-	
Sample 14	9 year	M	ND	-	Puducherry, India	-		
Sample 15	1.5 year	M	ND	-	Puducherry, India	-		
Sample 16	1 year	M	ND	-	Puducherry, India	-		
Sample 17	7 month	M	Lab	-	Puducherry, India	-		
Sample 18	1.5 year	M	ND	-	Puducherry, India	+	-	
Sample 19	1.5 year	M	ND	-	Puducherry, India	+	+	
Sample 20	1 year	F	ND	-	Puducherry, India	+	+	MN688784
Sample 21	5 month	F	ND	-	Puducherry, India	+	+	MN688785
Sample 22	5 month	M	ND	-	Puducherry, India	+	+	MN688786
Sample 23	2 year	F	ND	-	Puducherry, India	+	+	MN688787
Sample 24	6 month	M	ND	-	Puducherry, India	-		
Sample 25	3 year	F	ND	-	Puducherry, India	-		
Sample 26	3 month	F	ND	-	Goa, India	+	+	MN688788
Sample 27	1 year	M	ND	-	Goa, India	-		

Sample ID	Age	Sex	Breed	Vaccination status	Place of sample collected	PCR Result		GenBank Acc. ID H gene
						N gene	H gene	
Sample 28	3 month	F	ND	-	Goa, India	-		
Sample 29	5 month	M	ND	-	Goa, India	-		
Sample 30	2 year	M	ND	-	Chennai, India	-		
Sample 31	4 month	F	ND	-	Chennai, India	-		
Sample 32	6 month	F	ND	-	Chennai, India	-		
Sample 33	2.5 year	F	ND	-	Puducherry, India	-		
Sample 34	3 year	M	Spitz	-	Puducherry, India	-		
Sample 35	2 month	F	ND	-	Puducherry, India	-		
Sample 36	5 month	F	ND	-	Chennai, India	-		
Sample 37	8 month	F	ND	-	Puducherry, India	-		
Sample 38	6 month	M	ND	-	Puducherry, India	-		
Sample 39	1 year	F	ND	-	Puducherry, India	-		
Sample 40	4 month	F	Kanni	-	Puducherry, India	-		

* *- Single dose*

Table 2. Oligonucleotide primer sequences used for screening of samples and sequence analysis (Haemagglutinine gene) in this study

Primer name		Sequence 5'–3' direction	Sense	Target	Amplicon size
Screening	CDV-NC-FP	CGGAAATCAACGGACCTAAAT	+	N gene	242 bp
Primer	CDV-NC-RP	TCCTTGAGCTTTCGACCCTT	-		
Sequencing	CDV-HA- FP	AACTTAGGGCTCAGGTAGTCCA	+	H gene	1123bp
primer	CDV-HA-RP	CAATGCAGGCACCATCCAGGT	-		

2.5 Sequence Analysis of H Gene

The sequencing results were evaluated for their specificity using nucleotide Basic Local Alignment Search Tool (BLAST) to determine the closest known relatives on the NCBI website (http://www.ncbi.nlm.nih.gov). The nucleotide sequences obtained were aligned with the corresponding sequences available in GenBank using Clustal Omega of MEGA 7 programme [28]. The sequences of H gene of CDV obtained from eight dogs in this study were submitted to the Genbank (www.ncbi.nlm.nih.gov/genebank) and the accession numbers were obtained. The deduced amino acid sequences of the H protein of the wild type CDV strains were aligned with multiple CDV amino acid sequences from different geographical regions using MEGA 7 to explore their amino acid profiles and potential differences between vaccine and wild type CDV strains.

2.6 Phylogenetic Analysis

The CDV-H gene sequences obtained from the eight samples were used for construction of a phylogenetic tree using Maximum Likelihood method implemented in MEGA 7 programme [28]. The confidence level of branching in the phylogenetic tree was evaluated with the bootstrap test based on 1000 re-samplings. The phylogenetic tree was constructed with the sequences from the vaccine virus, other known Indian CDVs, Indian wildlife CDVs and various CDV lineages around the world (GenBank).

2.7 Recombination Analysis

The genetic Algorithms for Recombination Detection were performed to detect the recombination breakpoints in the H gene alignment of the wild type Canine distemper virus obtained in this study by using GARD analysis in Datamonkey web server (http://www.datamonkey.org) and RDP4 analysis.

2.8 Selective Pressure Analysis

The rate of non synonymus (dN) to synonymus (dS) mutations ratios (dN/dS) with H gene of CDV was estimated in Datamonkey web server (http://www.datamonkey.org) using Single likelihood Ancestor counting (SLAC) method. The values of dN-dS<0, = and >0 indicate negative, neutral and positive selections respectively.

3. RESULTS AND DISCUSSION

3.1 Screening of the Clinical Samples

Of the forty dog samples screened in this study, 15 (37.50%) samples were found to be positive by RT- PCR assay targeting N gene (Fig. 1). The disease was predominantly noticed in dogs above one year of age (53.33%). Infection with CDV was widespread among unvaccinated dogs in this study, as most of the dogs detected positive for CDV were not vaccinated. Although in contrast, there are many reports of CDV positive cases in different geographical locations in vaccinated dogs also [29-33].

Of the fifteen N gene positive dog samples, ten samples (66.6%) yielded specific products in PCR assay targeting the H gene (Fig. 2). The less sensitivity of these Reverse Transcriptase PCR based detection on CDV Haemagglutinine gene may be due to the low level of this H gene expression over N gene in infected cells.

3.2 Sequence Analysis of H Gene of CDV

The BLAST analysis confirmed the amplified sequences and they were found to be highly specific to CDV as indicated by the maximum identity (94 - 97%) obtained with H gene sequences of other canine distemper virus strains available in the GenBank.

In sequence analysis, a total of 49 non-synonymous mutation sites were observed among 356 amino acids of H gene in the CDV sequence of dogs under this study (Fig. 3) in

comparison to prototype of the Onderstepoort vaccine CDV sequence. Among 49 sites; Singleton variations were observed at 17 sites (i.e. at amino acid position 4, 5, 25, 26, 30, 43, 155, 156, 162, 175, 191, 241, 245, 263, 332, 255 and 356) and Parsim informative variations were observed at 37 sites (i.e. at amino acid position 21, 30, 34, 47, 146, 155, 156, 160, 161, 162, 167, 180, 186, 197, 198, 217, 228, 238, 241, 243, 247, 262, 266, 277, 298, 301, 303, 311, 314, 315, 324, 327, 330, 331, 334, 342 and 346). In general there were 30 – 35 amino acid variation sites (~10%) between CDV field samples and the Onderstepoort vaccine strain. Therefore, high level of amino acid divergence between circulating field strains and the vaccine strain was observed. In addition, thirty-three synonymous mutations were also observed in the CDV sequences under this study.

Among the deduced amino acid residues for CDV (partial H gene) two glycosylation sites (N-X-S/T) were detected at 19-21 and 149 -151 (Table 3). Till date, nine potential glycosylation sites (19–21, 149–151, 309-311, 391–393, 422–424, 456–458, 584-586, 587–589 and 603–605) have been reported among the CDV strains worldwide. Number of studies has tried to address the role of glycosylation pattern of H protein in relation to virulence of CDV [11,34]. The glycosylation patterns of H protein may affect neutralization related sites leading to disruption of important epitopes.

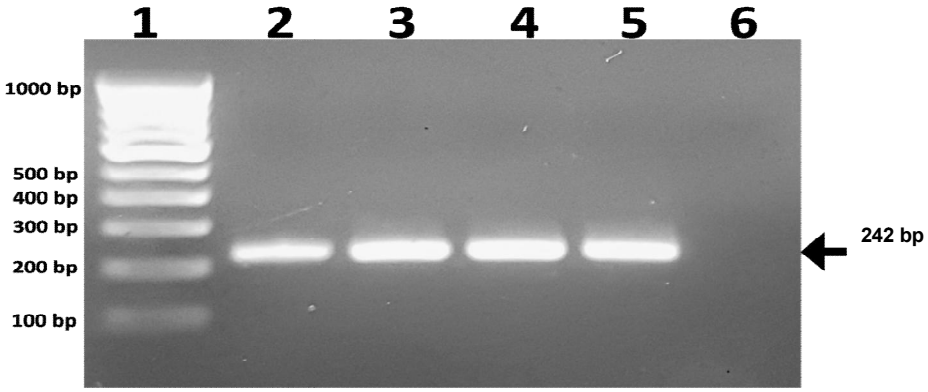

Fig. 1. Screening of clinical sample for canine distemper virus by PCR targeting Nucleocapsid (N) gene. Lane 1: 100bp DNA ladder; Lane 2 – 4: Clinical samples which are '+' by PCR; Lane 5: Positive control (Vaccine); Lane 6: Negative control (Sample from Healthy dog)

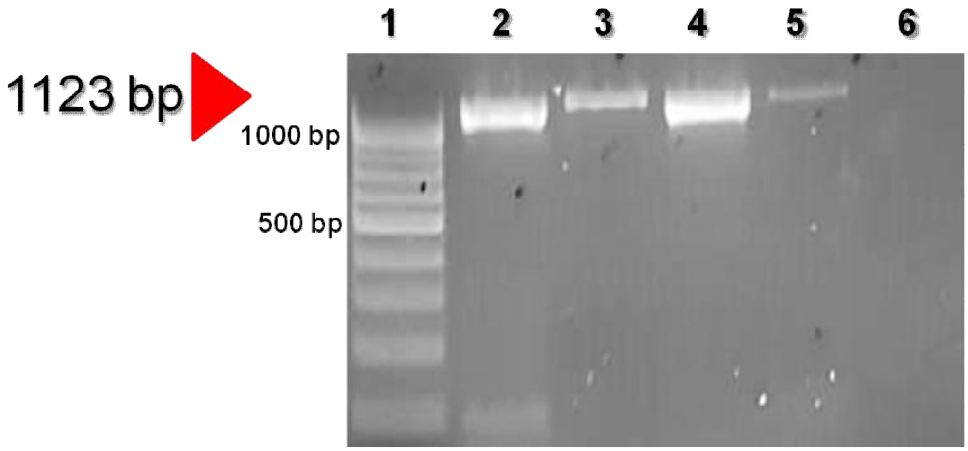

Fig. 2. Amplification of CDV gene by PCR targeting Haemagglutinine (H) gene. Lane 1: 100bp DNA ladder; Lane 2: Positive control (Vaccine); Lane 3 - 5: Clinical samples which are '+' by PCR; Lane 6: Negative control (Sample from Healthy dog)

Amino Acid Variation at Haemagglutinin (HA) of Canine Distemper Virus

Site	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
Amino Acid position at HA	4	5	21	25	26	30	34	43	47	78	146	155	156	160	161	162	167	175	180	186	191	197	198	217	228	238	241	243	245	246	247	262	263	266	277	298	301	303	311	314	315	324	327	330	331	332	334	342	346	355	356
Onderstepoort Vaccine AF378705	Y	Q	T	L	V	H	R	L	V	M	V	E	S	R	K	A	A	L	S	H	A	K	V	I	Y	D	R	F	T	Q	E	D	M	L	N	E	T	L	S	G	I	W	P	H	I	E	V	M	H	K	D
America-1 HQ403645	.	.	S	.	.	.	.	.	.	.	.	.	T	.	.	S	.	.	G	Y	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	V	.	.	.	.	.	.
Europe/South America-1 DQ889177	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	T	.	Y	G	.	.	.	K	N	.	F	.	D	.	.	.	.	V	G	.	Q	V	.	M	V	.	.	.
European Wildlife DQ889188	.	.	S	.	.	.	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	.	.	.	.	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Rockborn-like GU810819	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	F	.	.	Y	G	.	.	.	K	.	.	.	.	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
South America-2 JN215475	.	.	S	.	.	Q	.	.	.	.	.	D	T	.	.	S	.	.	G	Y	.	R	.	T	.	Y	G	.	.	.	K	N	.	.	.	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	N
South America-3 KF835412	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	.	.	.	.	D	.	.	.	S	V	G	.	Q	V	.	.	V	.	.	.
America-2 AF112189	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	.	.	.	.	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Arctic-like DQ226088	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	.	S	.	.	Y	G	.	.	.	K	.	.	F	.	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Asia-1 EF445051	.	P	S	.	.	Q	.	.	I	.	I	D	T	K	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	S	.	K	.	.	.	T	D	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Asia-2 EU716073	.	.	S	.	.	Q	.	.	.	L	I	D	T	K	.	S	.	.	G	Y	.	R	.	.	.	Y	.	.	.	.	K	.	.	.	.	D	.	.	.	S	.	G	.	Q	V	.	.	V	.	.	.
Asia-3 EU743934	.	.	S	.	.	Q	.	.	.	L	I	D	A	.	.	S	.	.	G	Y	.	R	.	.	.	Y	K	.	.	R	.	.	.	.	.	D	.	.	.	S	.	E	.	Q	V	.	.	V	.	I	.
Asia-4 KJ437596	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	.	.	.	.	A	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Africa-1 FJ461707	.	.	S	.	.	Q	.	.	.	.	I	D	T	.	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	.	.	.	.	G	.	.	.	.	.	G	.	Q	V	.	.	V	.	.	.
Africa-2 KC916717	H	.	S	.	M	R	.	.	.	.	I	D	T	K	.	S	.	.	G	Y	.	R	.	.	.	Y	G	.	.	.	K	N	.	.	.	D	.	.	.	.	.	G	L	Q	V	.	.	V	.	.	.
Lion Gujarat India 2018 MK037468	.	.	S	.	.	Q	.	.	I	I	I	D	I	K	.	S	.	.	G	Y	S	R	.	.	.	Y	E	.	.	P	K	N	.	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	V	.	.	.
MF964183/CDV/H gene/India/2018	.	.	S	.	.	Q	.	.	.	.	I	D	I	K	E	S	.	.	G	Y	.	.	.	.	.	Y	E	.	.	.	K	N	.	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	I	.	.	.
MF964180/CDV/H gene/India/2018	.	.	S	.	.	Q	.	.	I	.	I	D	I	K	E	S	.	.	G	Y	.	.	.	.	.	Y	E	.	.	.	K	N	.	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	I	.	.	.
MF964185/CDV/H gene/India/2018	.	.	S	.	.	Q	.	.	.	.	I	D	M	K	E	S	.	.	G	Y	.	R	.	.	.	Y	E	.	.	.	K	N	.	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	V	.	.	.
MF964186/CDV/H gene/India/2018	.	.	S	.	.	Q	.	.	.	.	I	D	I	K	E	S	.	.	G	Y	.	R	.	.	.	Y	E	.	.	.	K	N	.	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	V	.	.	.
Sample 8HA MN688781	.	.	S	.	.	Q	G	.	.	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	P	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 10HA MN688782	.	.	S	.	.	Q	.	.	G	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	.	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 12HA MN688783	.	.	S	.	.	Q	G	.	.	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	P	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 20HA MN688784	.	.	S	.	.	Q	G	.	.	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	P	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 21HA MN688785	.	.	S	.	.	Q	.	.	G	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	.	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 22HA MN688786	.	.	S	.	.	Q	.	.	.	.	I	D	I	K	E	S	.	R	G	Y	.	R	.	.	.	Y	D	.	M	.	K	N	.	.	S	D	.	V	P	S	.	G	.	Q	V	D	.	V	L	R	G
Sample 23HA MN688787	F	P	S	F	G	Q	G	.	G	.	I	D	T	K	.	S	V	.	G	Y	.	.	I	V	F	Y	G	L	.	.	K	.	.	F	.	D	.	F	.	.	F	G	L	Q	V	.	L	V	L	.	.
Sample 26HA MN688788	.	.	S	.	.	P	.	V	.	.	I	N	V	K	E	F	.	.	G	Y	G	R	.	.	.	Y	E	.	.	.	K	N	T	.	S	D	.	V	P	S	.	G	L	Q	V	.	.	V	.	.	.

Fig. 3. Amino acid residues and substitutions in the H gene of the CDV study sequences in comparison with the other Indian dog and wildlife CDV sequences, CDV lineages around the world and CDV vaccine

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	Onderstepoort_Vaccine_AF378705	100.0																													
2	Rockborn-Candur_Vaccine_GU266280	93.5	100.0																												
3	America-1_HQ403645	96.9	94.7	100.0																											
4	Europe/South_America-1_DQ889177	91.9	96.1	93.0	100.0																										
5	European_Wildlife_DQ889188	93.0	97.2	94.1	94.9	100.0																									
6	Rockborn-like_GU810819	93.5	100.0	94.7	96.1	97.2	100.0																								
7	South_America-2_JN215475	93.0	96.6	94.1	97.2	95.5	96.6	100.0																							
8	South_America-3_KF835412	91.3	95.5	92.4	94.9	95.2	95.5	94.9	100.0																						
9	America-2_AF112189	93.8	97.8	94.9	96.1	96.6	97.8	96.6	95.5	100.0																					
10	Arctic-like_DQ226088	92.4	95.8	93.5	94.4	94.9	95.8	94.4	93.3	95.5	100.0																				
11	Asia-1_EF445051	91.6	95.5	92.7	93.5	94.1	95.5	94.1	93.5	95.2	93.5	100.0																			
12	Asia-2_EU716073	91.3	94.7	92.7	93.3	93.3	94.7	93.3	92.7	94.4	92.7	94.1	100.0																		
13	Asia-3_EU743934	83.1	85.7	84.0	83.7	85.1	85.7	84.3	84.0	85.4	84.3	84.6	88.8	100.0																	
14	Asia-4_KJ437596	93.0	96.9	94.1	95.2	95.8	96.9	95.8	94.9	96.9	94.7	94.4	93.5	84.6	100.0																
15	Africa-1_FJ461707	92.1	95.5	93.5	93.8	95.5	95.5	94.4	93.8	95.5	94.9	94.1	92.7	84.0	94.9	100.0															
16	Africa-2_KC916717	92.1	95.5	93.5	94.4	94.9	95.5	94.9	93.3	95.5	93.8	94.1	93.3	84.0	94.7	94.1	100.0														
17	MF964183/CDV/H_gene/India/2018	91.9	93.5	92.4	93.0	92.4	93.5	93.5	92.4	93.8	92.4	92.4	92.1	83.1	92.7	91.9	93.5	100.0													
18	MF964185/CDV/H_gene/India/2018	91.3	93.8	92.1	92.7	93.0	93.8	93.3	92.1	94.1	92.1	92.7	92.4	83.7	93.0	92.4	94.7	96.6	100.0												
19	MF964186/CDV/H_gene/India/2018	92.1	94.7	92.7	93.5	93.5	94.7	94.1	93.0	94.9	93.0	93.5	93.3	84.0	93.8	93.0	94.7	97.8	97.8	100.0											
20	Lion_Gujarat_India_2018_MK037468	92.1	94.7	92.7	93.5	93.5	94.7	94.1	93.0	94.9	93.0	94.1	93.5	84.6	93.8	93.0	94.7	96.6	96.6	97.8	100.0										
21	Lion_Gujarat_India_2018_MK037467	92.1	94.7	92.7	93.5	93.5	94.7	94.1	93.0	94.9	93.0	94.1	93.5	84.6	93.8	93.0	94.7	96.6	96.6	97.8	100.0	100.0									
22	Lion_Gujarat_India_2018_MK037469	92.1	94.7	92.7	93.5	93.5	94.7	94.1	93.0	94.9	93.0	94.1	93.5	84.6	93.8	93.0	94.7	96.6	96.6	97.8	100.0	100.0	100.0								
23	Sample_8HA_MN688781	91.6	94.4	92.4	93.8	93.0	94.4	93.3	92.1	94.4	93.8	92.7	91.9	82.3	93.3	92.4	93.5	93.0	92.7	93.5	93.5	93.5	93.5	100.0							
24	Sample_10HA_MN688781	91.9	94.7	92.7	94.1	93.3	94.7	93.5	92.4	94.7	94.1	93.3	92.1	82.6	93.5	92.7	93.8	93.3	93.0	93.8	94.1	94.1	94.1	99.2	100.0						
25	Sample_12HA_MN688781	91.6	94.4	92.4	93.8	93.0	94.4	93.3	92.1	94.4	93.8	92.7	91.9	82.3	93.3	92.4	93.5	93.0	92.7	93.5	93.5	93.5	93.5	100.0	99.2	100.0					
26	Sample_20HA_MN688781	91.6	94.4	92.4	93.8	93.0	94.4	93.3	92.1	94.4	93.8	92.7	91.9	82.3	93.3	92.4	93.5	93.0	92.7	93.5	93.5	93.5	93.5	100.0	99.2	100.0	100.0				
27	Sample_21HA_MN688781	91.9	94.7	92.7	94.1	93.3	94.7	93.5	92.4	94.7	94.1	93.3	92.1	82.6	93.5	92.7	93.8	93.3	93.0	93.8	94.1	94.1	94.1	99.2	100.0	99.2	99.2	100.0			
28	Sample_22HA_MN688781	91.6	94.1	92.1	93.0	93.0	94.1	93.8	92.4	94.4	92.4	93.3	92.7	83.7	93.3	92.4	93.5	95.8	95.8	96.9	96.3	96.3	96.3	93.0	93.3	93.0	93.0	93.3	100.0		
29	Sample_23HA_MN688781	90.4	93.3	91.3	92.7	91.9	93.3	92.1	91.0	93.3	92.7	92.4	90.7	81.2	92.1	91.3	93.0	91.9	91.6	92.4	92.7	92.7	92.7	98.3	98.6	98.3	98.3	98.6	91.9	100.0	
30	Sample_26HA_MN688781	92.1	93.8	92.4	92.7	93.0	93.8	93.3	92.1	94.1	92.1	92.7	92.4	83.1	93.0	92.1	94.1	96.1	96.3	97.2	96.9	96.9	96.9	92.7	93.0	92.7	92.7	93.0	95.8	91.6	100.0

% amino acid similarity between Onderstepoort Vaccine & Study sequences

% amino acid similarity between Asian Lineages & Study sequences

% amino acid similarity between Africa-2 Lineage & Study sequences

% amino acid similarity between Indian sequences & Study sequences

% amino acid similarity between India-WildLife & Study sequences

Fig. 4. Estimates of Evolutionary Divergence between Sequences. The percentage amino acid similarity per sequence from between sequences are shown. The analysis involved 30 amino acid sequences covering 356 amino acid positions of H gene

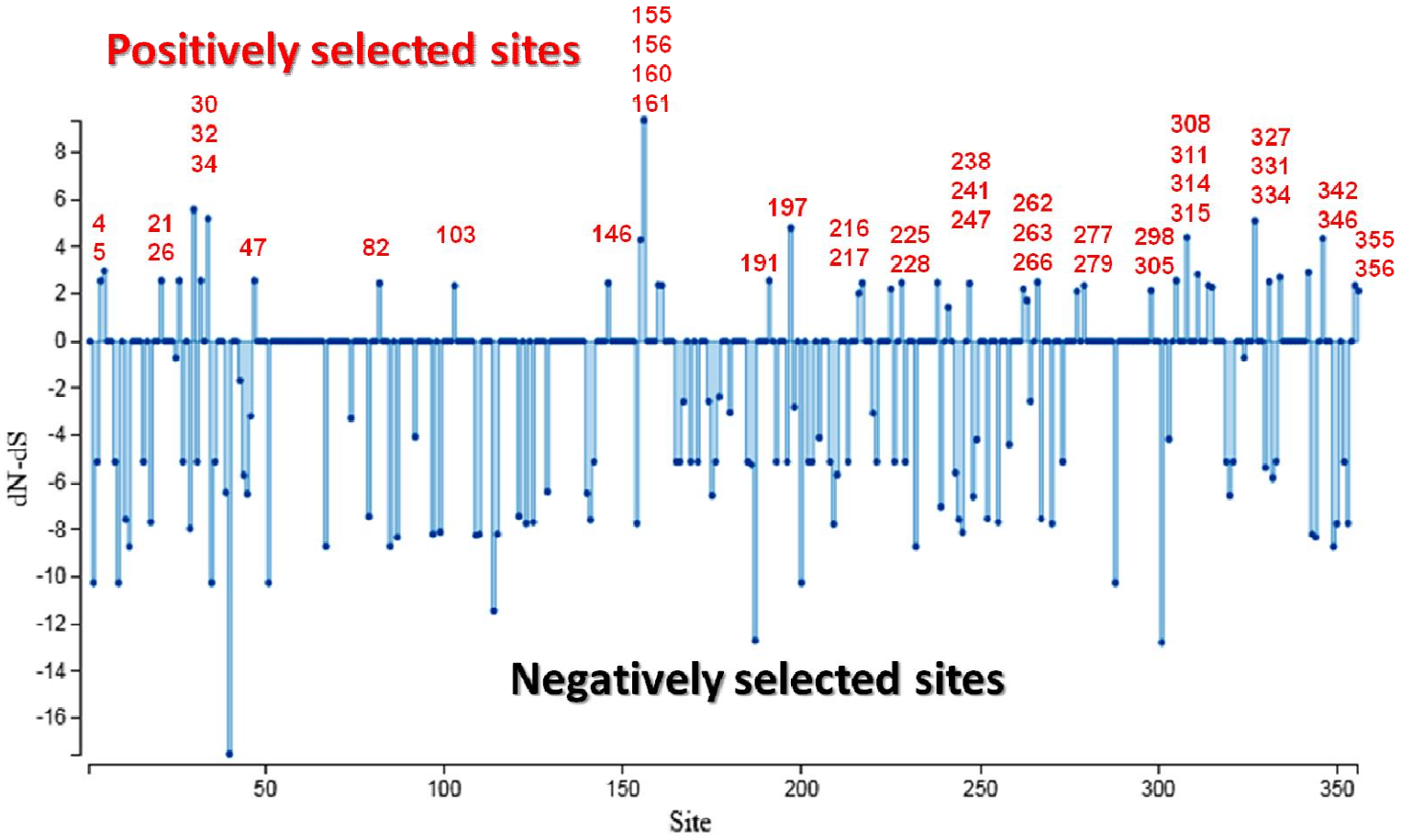

Fig. 5. Estimation of Synonymous (dS) & Non-synonymous (dN) substitution rates per site to determine the selective pressures on individual sites of codon alignments using Single Likelihood Ancester Counting (SLAC) method

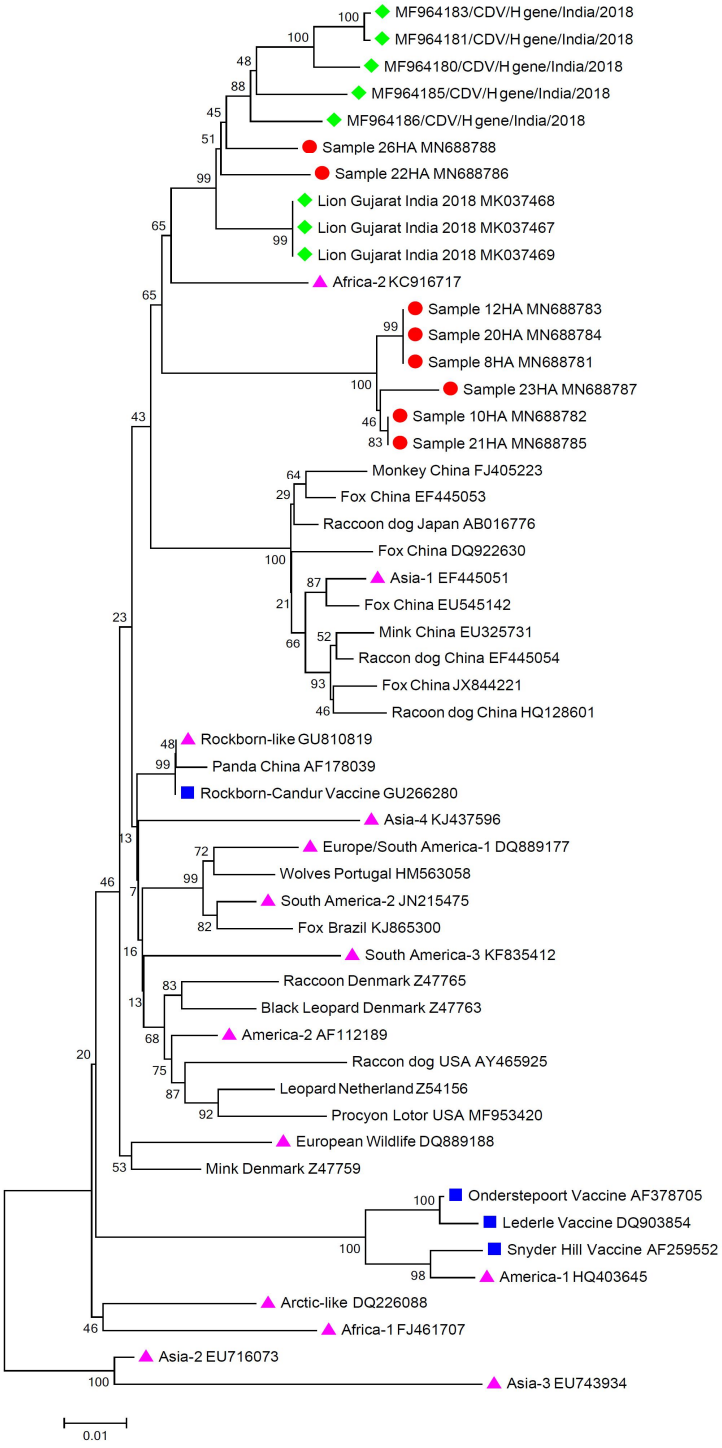

Fig. 6. Maximum likelihood tree depicting phylogenetic relationship among canine distemper virus. Canine distemper virus obtained from dogs sequenced in this study are shown with solid circles respectively, the vaccine CDV are shown with solid square, the CDVgenetic lineage around the world are shown with solid triangle and the other Indian dog/ wildlife CDVs are shown with solid star. Bootstrap values are shown next to the branches in the phylogenetic tree

Table 3. Glycosylation sites (N-X-S/T) at the Amino acid residues of CDV (partial H gene)

Amino Acid	19 - 21	149 -151	309 - 311
CDV8_MN688781	N – S – S	N – F – T	S – G – S
CDV10_MN688782	N – S – S	N – F – T	S – G – S
CDV12_MN688783	N – S – S	N – F – T	S – G – S
CDV20_MN688784	N – S – S	N – F – T	S – G – S
CDV21_MN688785	N – S – S	N – F – T	S – G – S
CDV22_MN688786	N – S – S	N – F – T	S – G – S
CDV23_MN688787	N – S – S	N – F – T	S – G – S
CDV26_MN688788	N – S – S	N – F – T	S – G – S
GLYCOSYLATION SITE	Present	Present	Absent

3.3 Amino Acid Divergence

The estimate of evolutionary divergence between sequences shows the study sequence had 90-92% amino acid similarity with Onderstepoort CDV vaccine (Fig. 4). Based on the criteria to define a genotype, at least 95% identity is required [35]. A high degree of identity (93-94%) was observed with Africa-2, Rockborn-like and America-2 lineage. Comparatively lower amino acid similarity (81-83%) was observed with the Asia-3 lineage. Therefore, none of the known lineages present worldwide had identity of ≥ 95% with the study sequences. A broad range of 91-100% amino acid similarity was observed within the study sequences. In comparison, 91-97% and 92- 96% amino acid similarity was observed with the Indian dog CDV sequences and with Indian wildlife CDV sequences respectively.

3.4 Recombination Analysis

No potential breakpoint and recombination events were identified in the study CDV sequences using RDP4 analysis.

3.5 Selection Pressure Analysis

The selection pressure estimated by SLAC method using Datamonkey web-server revealed dN/dS ratio of 0.249 indicating Negative / Purifying selection (Fig. 5). Site by site selective pressure analysis for the CDV sequences, executed in FEL, IFEL and FUBAR, indicated that majority of the sites were under negative selection. Thus, the CDV strains under this study were proved to be under rapid adaptation and mutation.

3.6 Phylogenetic Analysis

The phylogenetic tree constructed in this study revealed that all the CDV sequences from Puducherry State, Southern India formed separate monophlytic group with other CDV sequences from India as well as from Indian wildlife (Lion) and was clustered away from the vaccine strains (Fig. 6). The sequence under this study was also found closer to the Africa - 2 lineage than the other known Asian lineages. This is possibly the first report of the novel Indian CDV strains showing closer relationship with Africa 2 lineage.

4. CONCLUSION

The present study depicts the prevalence of CDV infection among dogs in Puducherry state (Southern India). Our findings suggest that there may be an existence of novel Indian CDV genetic lineage circulating in India, which can be designated as Asia-5 /India-1 lineage, distinct from vaccine strains and from other known Asian lineages. As the CDV strains were found to be under immense selection pressure and realizing the constant mutations happening in the field strains, detection of strain/lineage circulating in wider geographical regions of India would be of utmost importance. This will help devising suitable strategies for preventive and therapeutic management of canine distemper virus infection. In addition, there is an urgent need for assessing the level of protection elicited by vaccine strains against those novel variant strains through well controlled antigenic studies.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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