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ORIGINAL ARTICLE

Isolation and phylogenetic characterization of Canine distemper virus from India

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Abstract Canine distemper (CD), caused by canine distemper virus (CDV) is a highly contagious disease that infects a variety of carnivores. Sequence analysis of CDVs from different geographical areas has shown a lot of variation in the genome of the virus especially in haemagglutinin gene which might be one of the causes of vaccine failure. In this study, we isolated the virus (place: Ludhiana, Punjab; year: 2014) and further cloned, sequenced and analyzed partial haemagglutinin (H) gene and full length genes for fusion protein (F), phosphoprotein (P) and matrix protein (M) from an Indian wild-type CDV. Higher sequence homology was observed with the strains from Switzerland, Hungary, Germany; and lower with the vaccine strains like Ondersteport, CDV3, Convac for all the genes. The multiple sequence alignment showed more variation in partial H (45 nucleotide and 5 amino acid substitutions) and complete F (79 nucleotide and 30 amino acid substitutions) than in complete P (44 nucleotide and 22 amino acid substitutions) and complete M (22 nucleotide and 4 amino acid substitutions) gene/protein. Predicted potential N-linked glycosylation sites in H, F, M and P proteins were similar to the previously known wild-type CDVs but different from the vaccine strains. The Indian CDV formed a distinct clade in the phylogenetic tree clearly separated from the previously known wild-type and vaccine strains.

Keywords Canine distemper virus · Indian strain · Phylogenetic analysis · Genotype · N-Linked glycosylation

Introduction

Distemper is a disease of several canids caused by canine distemper virus (CDV), which is a member of the genus *Morbillivirus*, subfamily *Paramyxovirinae*, family *Paramyxoviridae*, in the order *Mononegavirales*. Other members in the Morbillivirus genus include measles virus, phocine distemper virus, peste-des-petit-ruminants virus and rinderpest virus [7]. CDV infects a broad range of animals such as Canidae (domestic dogs, foxes, wolves), Mustelidae (ferrets, minks, skunks, weasels, badgers), Procyonidae (raccoons), Ursidae (bears and pandas), Viverridae (civets, genets, and linsangs), Hyaenidae (hyenas) and Felidae (lions and tigers) [8]. It causes generalized infection with prominent respiratory, gastrointestinal and nervous signs [21]. Infected animals develop fever, cough, coryza and conjunctivitis [24].

CDV is a single-stranded, negative-sense, non-segmented, enveloped RNA virus with a diameter of about 150–300 nm [16]. The genome of CDV (approximately 15.7 Kb) consists of genes for one non-structural protein (C) and six structural proteins: large protein (L), haemagglutinin (H), phosphoprotein (P), nucleocapsid protein (N), fusion protein (F) and matrix protein (M). The non-structural protein (C) is produced by an alternative open reading frame in the P gene [12]. The haemagglutinin gene (H) of size 1824 bp, encodes an enveloped glycoprotein that helps in attachment of the virus to the host cell. The fusion protein gene (F) of size 1989 bp, encodes for the viral surface glycoprotein that mediates fusion between

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the virus and the infected cells or between the infected and the adjacent cells, plays an essential role in spreading the virus within the host [2]. Syncytia formation is due to F protein that enables CDV to fuse cells together. F protein is synthesized as an inactive precursor F0, which is further cleaved in its active form F1 and F2. The matrix protein gene (M) of size 1008 bp, encodes for a protein that is thought to play an important function during the virus maturation and also serves as a link between the nucleocapsid and the two surface glycoproteins, H and F [6]. Phosphoprotein gene (P) of size 1524 bp, encodes protein that is expected to play a function in RNA transcription and RNA replication [5, 17]. Several comparative studies have revealed that the H gene is subjected to a higher genetic variability than the other CDV genes, which makes it suitable for genetic analysis [9]. Based on nucleotide alignment of the H protein, six CDV genotypes have been classified i.e. America-1 (vaccine strains), America-2, Europe, Artic-like, Asia-1, Asia-2 [29]. Although vaccines have been widely used in most of the countries for controlling the disease yet CD still has been reported in some vaccinated animals. The hemagglutinin (H) protein is one of the most important antigens for inducing protective immunity against CD, and any variation of recent CDV strains may result in vaccination failure [14].

Seventy percent seroprevalence of CDV infection has been reported from South India [13]. In addition, isolation of Indian CDV from B95a cells and sequence analysis of partial N gene has also been reported [18], but detailed molecular epidemiology of the Indian CDV isolates is lacking till date. Therefore, the present study focused on the cloning, sequencing of H, F, P, M genes and the phylogenetic analysis of an Indian CDV strain as compared to the GeneBank available reference CDVs and the commercial CDV vaccine strains. To best of our knowledge this is the first report on sequencing and characterization of full length fusion protein (F), phosphoprotein (P) and matrix protein (M) genes of wild-type CDV from India.

Materials and methods

Clinical specimens

Ocular and Nasal swabs were collected in sterile 2 ml PBS from 25 dogs suspected of CDV infection. Description of the dogs from which the samples were collected is mentioned in the Table 1. The swabs were squeezed properly in PBS, centrifuged at 5000 rpm for 5 min and the supernatant was collected into a new sterile tube. Blood sample (2–4 ml) was taken aseptically in EDTA vial from cephalic vein of eight CDV suspected dogs and lymphocytes were isolated by density gradient method using HiSep1073 (HiMedia). The work was approved by the Institutional Animal Ethical Committee (IAEC).

Virus isolation

MDCK cell line procured from American Type Culture Collection (ATCC) was used for virus isolation. CDV suspected samples were inoculated at a volume of 500 µl into a 25 cm^2 tissue culture flask containing a sub-confluent monolayer of MDCK cells in serum free DMEM media. The flask was incubated at 37 °C for 1 h for virus adsorption. Further, the cells were washed with PBS and supplied with DMEM maintenance-media having 2 % serum. An uninoculated flask was used as negative control. The flasks were incubated at 37 °C and examined daily for cytopathic effects (CPE).

RNA extraction

Total RNA was isolated from 500 µl of the supernatant of the Ocular and Nasal discharge collected in 2 ml PBS and from the isolated lymphocytes, using 1 ml of Trizol reagent (Ambion, Life Technologies) as recommended. RNA quality and quantity were determined by spectrophotometric analysis with a Nanodrop 1000 (Thermo Scientific, USA) and then stored at −80 °C for further use.

cDNA synthesis

RNA templates having absorbance ratio (260/280) between 1.9 and 2.0 were subjected to first strand cDNA synthesis using First strand cDNA synthesis Kit (Thermo Scientific, USA) with Random hexamer primer, as per the manufacturer's instruction. An 11 µl reaction volume was prepared having 1 µl of Random hexamer primer, 5 µl of RNA template (103.8 ng/µl) and 5 µl of Nuclease free water. The mixture was subjected to denaturation at 65 °C for 5 min and then snap cooled on ice. Reverse Transcriptase (RT) mix solution consisting of 4 µl of 5× RT Buffer, 1 µl of Rnase Inhibitor, 2 µl of 10 mM dNTP and 2 µl of RT Enzyme was prepared. The RT mixture (9 µl) was added to the already denaturated template-primer mixture (11 µl) and the reverse transcription was performed in a total volume of 20 µl in a Thermal Cycler (Veriti, Applied Biosystems) for 5 min at 25 °C, followed by 60 min at 42 °C. The reaction was terminated by heating at 75 °C for 5 min. The final products were then stored at −20 °C.

PCR amplification, cloning and sequencing

CDV presence was confirmed by L-gene (Largeprotein) based diagnostic primers (Table 2) along with the CDV vaccine (Novibac-Ondersteport strain) as a positive control. Further, the positive sample was used to amplify the other genes of CDV. New primers were designed to amplify H, F, M, P genes coding sequences of CDV using

Table 1 Description of the CDV suspected dogs (2013–2014)

S. no	Case no.	Sample type	Sex	Breed	Age	Place	RT-PCR
1	2888	O,N	F	Stray dog	1.5 y	Ludhiana	CD(+)
2	4927	O,N	M	Stray dog	9 m	Khanna	CD(−)
3	6763	O,N	F	Stray dog	6 m	Patiala	CD(−)
4	4894	O,N	F	Rottweiler	3 m	Patiala	CD(−)
5	4849	O,N	M	Stray dog	2.5 y	Ludhiana	CD(−)
6	5189	O,N	F	Stray dog	1.5 y	Ludhiana	CD(−)
7	12548	O,N	F	Pitt Bull	2.10 y	Ludhiana	CD(−)
8	13221	O,N	M	Stray dog	6 m	Ludhiana	CD(−)
9	7500	O,N,B	M	Labrador	4 y	Ludhiana	CD(−)
10	7738	O,N,B	M	Pomeranian	6 y	Kapurthala	CD(−)
11	7739	O,N,B	F	Pomeranian	2 y	Ludhiana	CD(−)
12	7736	O,N,B	M	Spitz	2.5 y	Moga	CD(−)
13	8334	O,N,B	M	Pug	8 m	Jagraon	CD(−)
14	1272	O,N,B	F	Pug	1 y	Ludhiana	CD(−)
15	8568	O,N,B	F	Pug	1.5 y	Ludhiana	CD(−)
16	5189	O,N,B	M	Pug	2 y	Amritsar	CD(−)
17	6069	O,N	F	Pomeranian	5 m	Barnala	CD(−)
18	6024	O,N	F	Pomeranian	1.4 y	Ludhiana	CD(−)
19	6025	O,N	M	Rottweiler	1.7 y	Ludhiana	CD(−)
20	962	O,N	M	German Shepherd	3 m	Ludhiana	CD(−)
21	2788	O,N	M	Stray dog	U	Ludhiana	CD(−)
22	4844	O,N	M	Stray dog	U	Ludhiana	CD(−)
23	8568	O,N	F	Pug	1.6 m	Ludhiana	CD(−)
24	8334	O,N	M	Pug	8 m	Jagraon	CD(−)
25	9875	O,N	M	German Shepherd	3 y	Ludhiana	CD(−)

O Ocular, *N* Nasal, *B* Blood, *F* Female, *M* Male, *U* Unknown, *m* months, *y* years

Table 2 Description of the primer pairs

Sl.No.	Sequence 5′–3′	Sense	Annealing temp (°C)	Target	Purpose	Amplicon size (bp)
1	CTGCAATCAACTGGGGCTTT	+	45	L gene	Diagnostic	268 bp
	GAAGGTCTAGGTAAATCATGTAACAGT	−				
2	AACTTAGGGCTCAGGTAGTCCA	+	54	H gene	Sequencing, phylogenetic analysis	1123 bp
	CAATGCAGGCACCATCCAGGT	−				
3	AACTTAGGACCCAGGTCCAACAA	+	53	P gene	Sequencing, phylogenetic analysis	1721 bp
	TGATCGAAGTCRTACACCTCAGTCA	−				
4	CAGACAAGCCCCATGCACAA	+	52	F gene	Sequencing, phylogenetic analysis	2153 bp
	TGGACTACCTGAGYCCTAAGT	−				
5	CCTTCCAAAGCTGACTTGCATCATTG	+	53	M gene	Sequencing, phylogenetic analysis	1268 bp
	GATTTAGAGAATTTTGAAAAGACCCTG	−				

R-A/G, Y-C/T

Primer3 programme following whole genome sequence alignment of 45 different CDV strains available in NCBI. The complete details of the primers have been listed in Table 2. The PCR mixture was prepared in a final volume of 50 µl containing 5 µl cDNA template, 0.4 µM each of forward primer and reverse primers, 1× of PCR reaction buffer with 15 mM of $MgCl_2$, 2 mM each dNTPs mix and 2 Unit High Fidelity PCR Enzyme Mix (Thermo Scientific, USA). The reaction was carried out in Thermal Cycler (Veriti, Applied Biosystems) with the following conditions: initial denaturation at 94 °C for 10 min, 35 cycles of denaturation at 94 °C for 1 min, annealing temperature

listed in the Table 2 for 30 s and extension at 72 °C for 1.5 min followed by a final extension at 72 °C for 10 min. The PCR product was then subjected to agarose gel (1 %) electrophoresis and then purified by using GeneJET PCR Purification Kit (Thermo Scientific, USA). The eluted product was checked for quality and quantity in horizontal agarose gel electrophoresis and Nanodrop reading respectively. The purified product was ligated into pJET 1.2/blunt vector (Thermo Scientific, USA) in 1:3 vector insert molar ratio, transformed into Top10 competent cells and spread on agar plate containing Ampicillin (100 mg/ml). Recombinant white colonies were picked, grown overnight in LB broth at 37 °C on a shaker incubator and subjected to plasmid isolation by alkaline lysis method as per the protocol [19]. The recombinant plasmids carrying CDV genes were confirmed by *Bgl* II, restriction endonuclease digestion which released specific gene inserts. The positive recombinant plasmids were sequenced by commercial outsourcing.

Sequence analysis

The obtained nucleotide sequences (forward and reverse) of CDV H, F, M and P genes were subjected to BLASTn [1] to compare for sequence identities/variations with other sequences of CDV strains around the world present in NCBI database (http://blast.ncbi.nlm.nih.gov/). The sequences of CDV vaccine strains available were also considered. Further, in silico translated amino acid sequences were subjected to multiple sequence alignment using Clustal-W method [23, 26]. The deduced amino acid sequences of the genes along with the other CDV strains from different geographical areas were used to construct the phylogenetic tree using maximum likelihood (ML) method in MEGA 6.06 software [25]. The topological accuracy of the tree was estimated by 1000 bootstrap replicates. Potential N-linked glycosylation sites in H, F, M and P proteins were predicted with NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc) [15].

Results

Virus isolation, RT-PCR detection and amplification of CDV genes

Only one, out of 25 samples showed cytopathic effect in form of syncytia at 5 days post-infection in MDCK cells which was named as Indian (LDH) isolate. The presence of the virus was further confirmed by RT-PCR, an amplicon size of 268 bp was detected by L gene based diagnostic primer (Fig. 1a). RT-PCR carried out using gene specific primers, could successfully amplify CDV H, F, M and P genes with amplicons of 1123, 2153, 1268 and 1721 bp respectively.

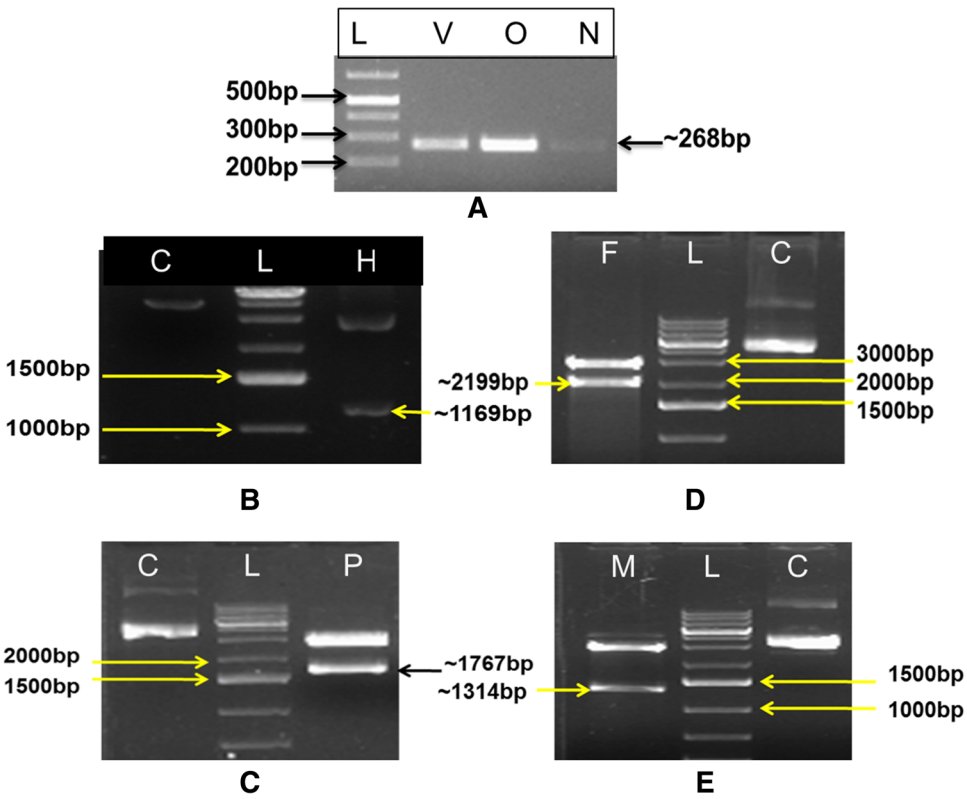

Fig. 1 Diagnostic PCR for CDV and Restriction digestion of recombinant plasmids by *Bgl* II enzyme. **a** PCR amplification of ~268 bp fragment of the L gene of CDV by diagnostic primer. *Lane V* Nobivac vaccine, *Lane O* ocular sample, *Lane N* nasal sample. Restriction digestion of recombinant plasmids for the release of insert of sizes. *Lane H* ~1169 bp of H-gene (**b**); *Lane P* ~1767 bp of P gene (**c**); *Lane F* ~2199 bp of F gene (**d**); *Lane M* ~1314 bp of M gene (**e**); *Lane C* undigested controls, *Lane L* 1 kb plus DNA Ladder (Fermentas)

Cloning and sequencing of CDV genes

The amplified CDV genes were successfully cloned into pJET 1.2/blunt vector and the recombinant plasmids prepared from the selected clones were screened for the presence of desired inserts by *Bgl* II restriction endonuclease digestion. *Bgl* II restriction enzyme could successfully release the cloned H, P, F and M genes from the recombinant pJET 1.2/blunt vector backbone with the product size of 1169, 1767, 2199 and 1314 bp respectively (Fig. 1b–e). The plasmid carrying individual CDV genes were sequenced by outsourcing, analyzed by BLASTn and then manually edited and assembled to get partial sequence of H gene (1118 bp) and full length cds of F gene (1989 bp), P gene (1524 bp) and M gene (1008 bp). The nucleotide sequences were submitted to DDBJ and the accession numbers obtained were LC011102.1, LC011103.1, LC011104.1 and LC011105.1 for F, H, M and P genes, respectively.

Sequence analysis of CDV haemagglutinin (H) gene/protein

Comparison of Indian CDV H gene nucleotide sequence with that of the other 45 CDV strains across the world, revealed 91.9–96.9 % nucleotide identity. In-silico translated amino acid (aa) sequence alignment of different CDVs revealed that the haemagglutinin (H) aa sequences of Indian (LDH) strain showed a higher degree of identity with Switzerland (97 %), Germany (96.3 %) and a lower with that of the vaccine Convac (92.1 %), Ondersteport (92.9 %) and CDV3 (94.8 %) strains (Supplementary file 1). A total of five aa substitutions were found within the 372 aa (partial cds) sequences of Indian (LDH) CDV strain at positions 156 (Threonine → Isoleucine), 160 (Arginine → Lysine), 161 (Lysine → Glutamic acid), 241 (Glycine → Glutamic acid) and 262 (Aspartic acid → Asparagine). The variation at position 161 i.e. Glutamic acid in place of Lysine was unique in Indian (LDH) strain.

Sequence analysis of CDV fusion (F) gene/protein

F gene of Indian CDV showed 91.4-96.1 % nucleotide similarity with that of the other 32 known CDV strains. Similar to that of H protein, Indian LDH strain's fusion protein amino acid (aa) sequences showed a higher percent identity with that of Germany (95.6 %) and Switzerland (93.7 %); and a lower with that of vaccine Ondersteport (90.2 %) and CDV3 (90.6 %) strains (Supplementary file 2). A higher rate of aa sequence variation were found in the fusion protein of all the CDVs analyzed. The Indian (LDH) strain showed 30 aa substitutions within the fusion protein coding 671 aa, as compared to the consensus sequences. Interestingly, out of 30, 25 aa changes were found at position 1-120, which is the part of signal peptide region (1–135 aa) of F protein. In F2 region (136–224 aa) only a single change at 192nd position (Alanine → Serine) was observed. The region 226–416 was found to be highly conserved. In F1 region (225–671), four aa substitutions at positions 225 (Valine → Isoleucine), 417 (Valine → Isoleucine), 493 (Serine → Cystein) and 599 (Aspartic acid → Glutamic acid) were found.

Sequence analysis of CDV Phosphoprotein (P) gene/protein

Nucleotide similarity of 93.7–96.9 % was observed for P gene of Indian CDV with that of the other 22 known strains. For phosphoprotein amino acid (aa) sequences, a higher percent identity was observed with the strains from Hungary (95.3 %) and Switzerland (95.1 %) and a lower with the vaccine CDV3 (90.7 %) and Ondersteport (90.9 %) strains (Supplementary file 3). The Indian (LDH) strain CDV phosphoprotein of 507 aa possessed 22 aa change from the consensus, with maximum substitution at N-terminal within the region 1-169 and a single aa substitutions in 425th position (Isoleucine → Leucine) at C-terminal end of the protein.

Sequence analysis of CDV Matrix protein (M) gene/protein

The M gene nucleotide sequence of Indian CDV was 93.6–97.1 % identical to that of the other 23 known strains. Matrixprotein amino acid (aa) sequence showed a higher percent identity with Switzerland (98.8 %) and Hungary (95.7 %) and a lower with vaccine CDV3 (96.1 %) and Ondersteport (96.4 %) strains (Supplementary file 4). The Indian (LDH) strain CDV matrixprotein of 335 aa residue, showed only four aa substitutions at positions 131 (Serine → Asparagine), 269 (Alanine → Threonine), 301 (Lysine → Arginine), 319 (Isoleucine → Valine), and was found to be more conserved. It is clearly noted that, H and F proteins aa sequences are more variable than M and P proteins. Overall, it was observed that Indian field CDV strain was more identical with the CDV strains of Switzerland, Hungary and Germany; and was less identical with the vaccine strains Ondersteport, CDV3 and Convac for H, F, P and M proteins.

Phylogenetic analysis of CDV

The present study reports for the first time that the field CDV strain from North India based on M, P, F and H amino acids (aa) sequences, formed a distinct clade in the phylogenetic tree, clearly separated from the previously known wild-type and vaccine strains (Fig. 2a–d,

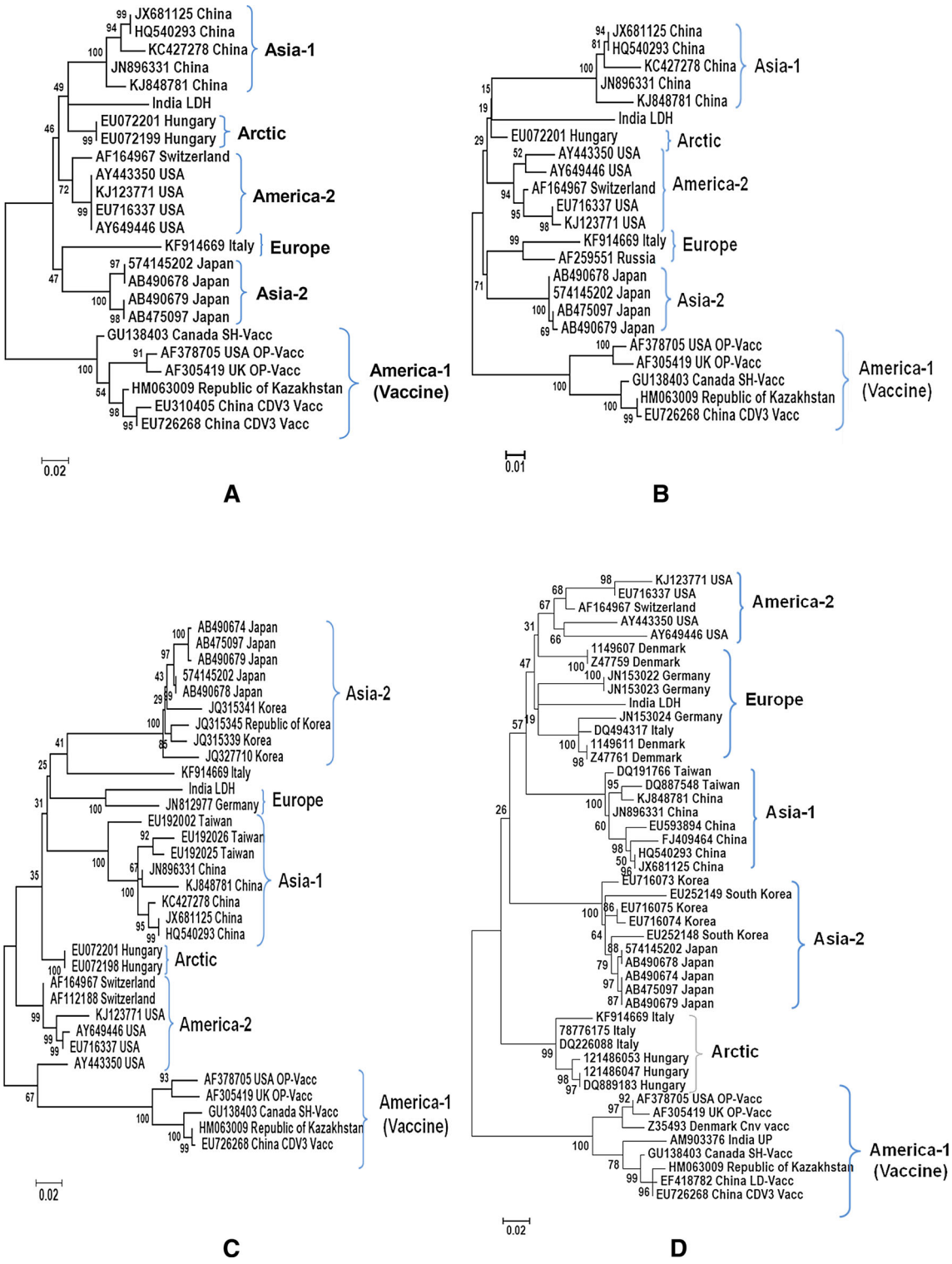

Fig. 2 Phylogenetic tree of CDV strains, on the basis of the amino acid alignment (**a**) complete M protein (**b**) complete P protein (**c**) complete F protein (**d**) partial H protein of India LDH strain, constructed using maximum likelihood method. The *scale bar* indicates the branch length equivalent to 0.01/0.02 amino acid substitutions per site; the numbers on branches are percentages of bootstrap values determined for 1000 iterations

respectively). Indian CDV cluster was most distant from all the available commercial vaccines sequences (Onderstepport, CDV3 and Convac) that come under America-1 group of CDVs.

N-linked glycosylation sites of the H, F, M and P proteins

As H gene was partially sequenced, the deduced amino acid sequence of length 372 residues showed N-linked glycosylation sites (N-X-S/T) only at two positions 19–21, 149–151. However, N-linked glycosylation site 309–311 was absent, in our strain and in wild type CDV strain of Switzerland when compared to other known CDVs. In case of F protein, a total of seven N-linked glycosylation sites were found at aa positions 11–13, 62–64, 108–110, 141–143, 173–175, 179–181 and 517–519. One of these seven glycosylation sites, at position ^{11}NQT, was unique in Indian CDV strains. In M protein, four N-linked glycosylation sites were found at aa positions 115–117, 131–133, 206–208 and 223–225. ^{131}NGS site was unique to our sequence. In P protein, only two N-linked glycosylation sites were found at aa positions 158–160 and 163–165.

Discussions

The genotyping or phylogenetic analyses of CDV studied so far are mostly based on H gene/protein, as H gene has the highest rates of mutation. It has been reported that the CDV isolates from foxes, raccoon dogs and minks, when compared with other CDVs from different geographical areas cluster into six major genetic lineages—America-1 (most vaccine strains), America-2, Europe, Artic-like, Asia-1, Asia-2 [29] based on H protein. In the present study, similar genetic lineages were found not only based on H protein but also on M, P, F proteins. Based on sequences of the proteins (H, F, M and P) studied, Indian wild-type CDV showed a distinct clade in the phylogenetic tree. Similar finding has been reported earlier [18] based on partial N gene nucleotide sequences of two Indian CDV isolates.

Maximum genetic/antigenic variation was observed for H and F proteins compared to M and P proteins of Indian CDV. Similar pattern of high antigenic variation for H and F proteins has also been reported from previously known CDVs [22]. In F protein, maximum sequence variation was found in the signal peptide region (1–135 aa). Signal peptide region of F protein has the lowest amino acid homology and is reported to be geographically distinct [14].

N-linked glycosylation in case of viruses has a potential role in its virulence and immune interaction properties [27]. N-linked glycosylation site at aa positions 309–311 in H protein, is reported to be specific for virulent strains of CDV with the exception of (AF164967) Switzerland [4, 10, 14]. This glycosylation site was also missing in Indian strain. Phylogenetic analysis of Indian CDV strain based on H amino acid sequences showed a highest degree of identity with strain from Switzerland and both lack this 309–311 N-linked glycosylation site. In case of F protein, our strain showed four out of seven N-linked glycosylation sites in F1 region at aa positions 141–143, 173–175, 179–181, 517–519, as this region is thought to be highly conserved among all CDV strains [3, 11, 28]. N-linked glycosylation site at position 108–110 was present in Indian CDV which is specific for wild-type CDV strains. Asia-1 group of CDV possess two N-linked glycosylation sites in F2 region of F protein at specific position and sequences, i.e. ^{62}NRT and ^{108}NAT and was different from Indian CDV ^{62}NKT and ^{108}NGS; which clearly shows that our strain is different from the Asian groups of CDV. Also, an extra N-linked glycosylation site ^{11}NQT of F protein and ^{131}NGS of M protein was observed, which was unique to Indian strain. It has been hypothesized earlier that variation or an increase in N glycosylation could affect the immune response to the virus and may result in vaccine failure [20].

The present study clearly shows the distinct genetic relationships of Indian wild-type virus from vaccine strains and other known lineages of CDV around the world. H gene of a new CDV isolate TM-CC showed low identity (90.4 % nt and 88.9 % aa) with the H gene of the classical Onderstepoort vaccine strain and this could be one of the reason for host's inability to mount a protective immune response against CDV [14]. The observed diversity between the vaccine strains and the wild-type CDVs may be due to antigenic escape, genetic recombination between wild-type strains, adaptation to new host species that drive the evolution of the virus [8]. Wild- type Indian CDV is phylogenetically distinct from other known Asian types (Asia-1 and Asia-2) and vaccine types but more similar to viruses from Switzerland, Hungary and Germany. Further, an elaborative study on CDV isolates from different parts of India is required to provide an extensive understanding on the origin and the evolution of Indian CDVs.

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