

Original Research Article

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Detection of Local Isolates of Canine Distemper Virus by Reverse-Transcription Polymerase Chain Reaction

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ABSTRACT

Keywords

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Distemper is a disease of several canids caused by canine distemper virus (CDV). The present study aimed to detect CDV directly from the swab samples of the suspected dogs by diagnostic RT-PCR. The positive samples were then subjected to virus isolation in MDCK cells after growing it in mitogen stimulated dog lymphocytes and the presence of the viral RNA again was confirmed by RT-PCR. Further, haemagglutinin (H) and large protein (L) genes were amplified and cloned into pGEMT Easy cloning vector, sequenced and phylogenetically characterized with the available reference CDV strains in GeneBank. Three samples were detected CDV positive by RT-PCR from a total of 50 samples and virus could be successfully isolated from one sample in MDCK cells after lymphocyte culture. After a number of serial passages, there were no consistent CPE observed but viral RNA could be detected from all the cell culture harvests of all passages which confirmed the presence of CDV. Mitogen stimulated dog lymphocyte culture proved to be an effective and economical method to isolate wild-type CDV from clinical samples. Partial haemagglutinin (H) and large protein (L) gene sequences of one of the CDV isolates made a distinct clade in phylogenetic tree which was clearly separated from commercial CDV vaccine groups (Lederle, Ondersteport, Convac and Synder Hill).

Introduction

Canine distemper virus (CDV) a Morbillivirus under Paramyxoviridae family is a contagious pathogen that causes canine distemper in carnivores. The disease is characterized with generalized infection with respiratory, nervous and gastrointestinal signs (Scagliarini *et al.*, 2003). CDV infects a broad range of animals such as domestic dogs, foxes, wolves, ferrets, minks and recently it has been

reported to infect seals, lions and tigers (Guo *et al.*, 2013). CDV is a single-stranded, RNA virus with genome size of 15.7 Kb which 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).

Initially, CDV isolation has been tried by different techniques and in different cells.

The attenuated form of CDV can readily be grown in epithelial and fibroblast cell lines but isolation of virulent CDV in these cells is difficult (Appel, 1978). CDV has been cultivated in canine alveolar macrophages (Appel and Jones, 1967); chorioallantoic membrane of embryonated chicken eggs (Ezeibe 2005; Haig 1956); co-cultivation of infected tissues with mitogen stimulated lymphocytes derived from healthy dogs (Appel *et al.*, 1992). But all of these methods are time consuming and multiple blind passages are needed before any proper cytopathic effects (CPE) of the virus become visible. CDV has been successfully isolated in cells like MDCK (canine epithelial kidney cells), MV1 LU (mink lung), Vero (African green monkey kidney cells) (Lednicky *et al.*, 2004), B95a (cells from marmosets) (Kai *et al.*, 1993), HmLu and BHK (hamster cell lines) (Sultan *et al.*, 2009). It is reported that CDV isolation in Vero cells expressing dSLAM receptor (dog-signalling lymphocyte activation molecule) is easy and simplest as CPE were observed from first day post infection (Seki *et al.*, 2003).

Although live attenuated vaccines have been widely used in most of the countries for controlling the disease yet CD has been reported in some vaccinated animals (Tatsuo *et al.*, 2001). It has been reported that genomic differences occur between the wild-type CDV and the vaccine strains (Li *et al.*, 2014). H gene is considered to be highly mutagenic (Martella *et al.*, 2006). The phylogenetic work performed based on H gene, showed six major genetic lineages i.e. America-1 (most vaccine strains), America-2, Europe, Artic-like, Asia-1, Asia-2 (Zhao *et al.*, 2010) of CDV from different geographical areas.

In India, limited reports are available on isolation of CDV from clinical samples. Pawar *et al.* (Pawar *et al.*, 2011) isolated

CDV from B95a cells which is a marmoset B lymphoblastoid cells and performed sequence analysis of partial N gene. Virus isolation is not only important for diagnosis purpose but also to provide further information about the genotype and origin of the virus based on direct sequence analysis of their genes. Therefore, this study aimed to isolate CDV from clinical samples collected from CDV suspected dogs, detect it by RT-PCR and further characterize it by cloning, sequencing and phylogenetic analysis of partial H and L gene sequences.

Materials and Methods

Clinical Samples

Ocular and nasal swabs were collected in sterile PBS from a total of 50 CD suspected dogs brought to Small Animal Veterinary Clinics, GADVASU, Ludhiana. The swabs were squeezed properly in PBS, centrifuged at 5000 rpm for 5 minutes and the supernatant was collected into a new sterile tube. Blood sample (2-4ml) was also taken aseptically in EDTA vial from cephalic vein of a healthy dog for lymphocyte isolation. The work has been approved by the Institutional Animal Ethical Committee (IAEC).

Detection of CDV in the Clinical Samples

Total RNA was isolated directly from suspected swab samples along with one CDV vaccine strain (Novibac-DHPPi) as positive control. 500ml of the supernatant of the ocular and nasal discharge were used to isolate RNA using 1ml 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. RNA templates having

absorbance ratio (260/280) between 1.9 and 2.0 were subjected to cDNA synthesis using First strand cDNA synthesis Kit (Thermo Scientific, USA) with random hexamer primer, as per the manufacturer's instruction.

CDV presence was confirmed by newly designed Large protein (L) gene based diagnostic primers. The sequences of the forward and reverse primers were 5'-CTGCAATCAACTGGGGCTTT-3' and 5'-GAAGGTCTAGGTAAATCATGTAACAGT-3' respectively. The PCR mixture was prepared in a final volume of 25ml containing 10ml cDNA template, 0.4 μ M each of forward primer and reverse primer, 1X of PCR reaction buffer with 15 mM of MgCl₂, 2 mM each dNTPs mix and 2 unit Taq DNA-Polymerase (Invitrogen). The reaction was carried out in Thermal Cycler (Veriti, Applied Biosystems) with the following conditions: initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds followed by a final extension at 72°C for 7 minutes. The PCR product was then subjected to agarose gel (2%) electrophoresis and then photographed.

Cell Culture and Media

Dog lymphocytes were isolated by density gradient method using HiSep1077 (HiMedia) as described by Boyum (Boyum, 1968). The cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% serum in 25cm² flask and kept at 37°C in a CO₂ incubator. The lymphocytes were stimulated by mitogen (PHA) phytohaemagglutinin A (Sigma) at a concentration of 15 μ g/ml for 48 hrs before inoculation with the CDV suspected samples. Madin Darby Canine

Kidney (MDCK) cell line was used for isolation of the virus. The cells were cultured routinely in polystyrene tissue culture 25cm² flask at 37°C in a 5% humidified CO₂ atmosphere in DMEM (Dulbecco's Minimum Essential Medium). The growth media was supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1X antibiotic- antimycotic solution (HiMedia).

Virus Isolation

RT-PCR positive ocular swab sample was subjected to virus isolation in primary lymphocytes and MDCK cells. The swab sample was vortexed, 2-3 drops of antibiotic (Antibiotic-Antimycotic Solution 100X Liquid Endotoxin Tested, HIMEDIA) was added and then incubated at 37°C for 1 hrs. The sample was then centrifuged at 8000 rpm for 10 minutes and the supernatant was collected into a new sterile tube which was used fresh or stored at -80°C till further use. 500 μ l of the inoculum was inoculated into a 25cm² tissue culture flask containing dog lymphocytes which was stimulated by mitogen PHA for 48 hrs. The flask was incubated at 37°C for 5-6 days in CO₂ incubator. Further, the cells were harvested and after three round of freezing and thawing, the content of the flask was centrifuged at 1500 rpm for 10 minutes in a 15 ml centrifuge tube and the supernatant was collected which served as the inoculum to infect a sub-confluent monolayer of MDCK cells in serum free DMEM media (maintenance media). The flask was incubated at 37°C for 1 hour for virus adsorption following inoculation with 500 μ l of the virus inoculum and then maintenance-media having 2% serum was added to make the volume 5ml. An uninoculated flask of MDCK cells was used as negative control. The flasks were incubated at 37°C in CO₂ incubator and examined daily for cytopathic

effects (CPE). Irrespective of the CPE observed, the samples were subjected to eight serial passages and the presence of CDV was confirmed by RT-PCR using the same set of diagnostic primers.

Cloning and Sequencing of CDV Partial H and L Gene

PCR was carried out for the bulk amplification of partial H and L genes using cDNA prepared from one positive sample. The forward and reverse primers used for H gene amplification were 5'-AACTTAGGGCTCAGGTAGTCCA-3' and 5'-CAATGCAGGCACCATCCAGGT-3' respectively, whereas the detail of primers used for L gene has already been mentioned. The bulk amplified PCR products were subjected to agarose gel (1%) electrophoresis and then purified by using GeneJET PCR Gel extraction Kit (Thermo Scientific, USA). The purified product was ligated into pGEMT Easy cloning vector (Promega, USA), transformed into Top10 competent cells and spread on agar plate containing Ampicillin (100mg/ml), X-gal (20µg/ml) and IPTG (100 mM). 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 of Sambrook and Russell (Sambrook and Russell, 2001). The recombinant plasmids carrying CDV genes were confirmed by EcoRI, restriction endonuclease digestion which released specific gene inserts. The positive recombinant plasmids were sequenced by commercial outsourcing.

Sequence Analysis of Partial H and L Gene

The obtained nucleotide sequences of CDV H and L genes were subjected to BLASTn (Altschul *et al.*, 1990) analysis for

comparing sequence identities and variations with other sequences of 22 CDV strains around the world present in NCBI database along with the CDV vaccine strains. Amino acid sequences were further subjected to multiple sequence alignment using Clustal-W method (Thompson *et al.*, 1990; Sievers *et al.*, 2011). The partial 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 (Tamura *et al.*, 2013). The topological accuracy of the tree was estimated by 1000 bootstrap replicates.

Results and Discussion

There are very limited reports on CDV isolation from the clinical samples from India and it is a well established fact that isolation of wild-type CDV is tedious and difficult. Out of 50 suspected samples, CDV could be detected in only three samples by RT-PCR as an amplicon of ~268 bp were observed (Fig. 1). Details of the positive samples obtained are mentioned in Table 1.

One of the RT-PCR positive ocular swab samples was subjected to CDV isolation in MDCK cells after growing the virus in mitogen stimulated dog lymphocytes. When the inoculum from the CDV infected lymphocyte flask was used to infect monolayer of MDCK cells, no consistent CPE in initial passages. However rounding, clumping and aggregation of the cells was visible in 3rd-4th passage onward. Existence of small syncytia in MDCK cells was also observed in the seventh passage (Fig. 3). In addition to the observed CPE, presence of CDV was confirmed by successful amplification of ~268 bp product by diagnostic RT-PCR from RNA extracted from cell culture harvests of all passages

(Fig. 4). Similarly, Tan et al (Tan *et al.*, 2011) isolated a virulent strain of CDV (ZJ7 isolate) in MDCK cell, which showed cytopathogenic effects like syncytia after six passages of the virus. Lednicky et al (Lednicky *et al.*, 2004) isolated wild-type CDV in different cells like MDCK, MV1 Lu, Vero cells and reported that CDV isolation was most effective in MDCK cells,

though CPE were most evident in Vero cells. Appel et al (Appel *et al.*, 1992) reported that unstimulated lymphocytes also support CDV growth, but to a lesser degree whereas optimal CDV growth was found in PHA (phytohemagglutinin) and (PWM) pokeweed mitogen stimulated peripheral blood lymphocytes.

Table.1 Description of CDV Positive Cases

Sl. No	Case No.	Sample type	Sex	Breed	Age	Place
1	2888	Ocular, Nasal swabs	Female	Stray dog	1.5yrs	Ludhiana
2	12411	Ocular, Nasal swabs	Male	Labrador	8yrs	Ludhiana
3	13529	Ocular, Nasal swabs	Female	German shepherd	4yrs	Sangrur

Fig.1 PCR Amplification of ~268bp Fragment of the L Gene of Cdv by Diagnostic Primer of Three Positive Samples: Lane 1: 2888 (Ocular), Lane 2: 2888 (Nasal), Lane 3: 12411 (Ocular), Lane 4: 12411 (Nasal), Lane 5: 13529 (Ocular), Lane 6: 13529 (Nasal), Lane 7: 1kb Plus Dna Ladder (Fermentas), Lane 8: Nobivac-Dhppi Vaccine, Lane 9: Ntc

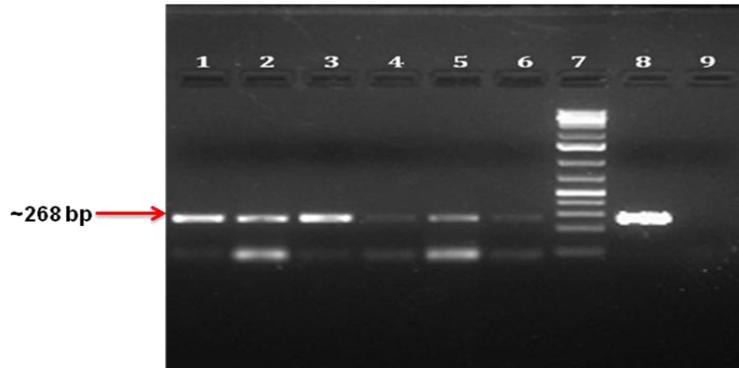


Fig.2 CPE of the Virus (a) cdv Infected Dog Lymphocytes (b) non infected mdck cells(c) cdv infected mdck cells (passage-7). Magnificaion 10x.

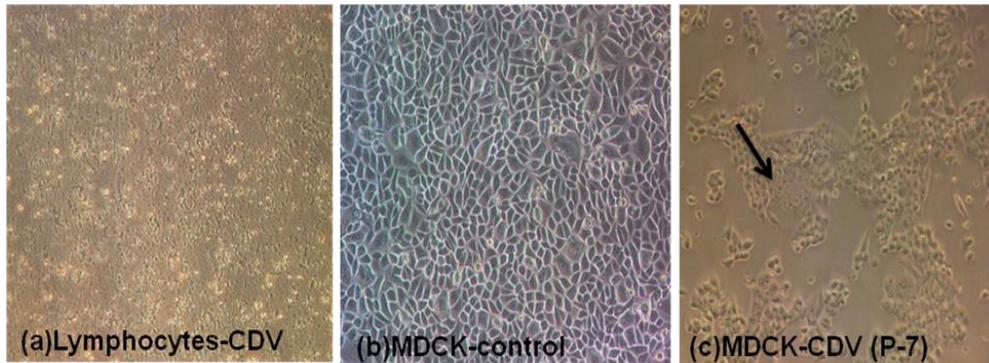


Fig.3 PCR Amplification of Different Viral Passages by Diagnostic Primer: Lane 1-8: Passage 1-8, Lane 9: Nobivac-Dhppi Vaccine, Lane 10: Ntc, Lane 11: 1kb Plus Dna Ladder (Fermentas)

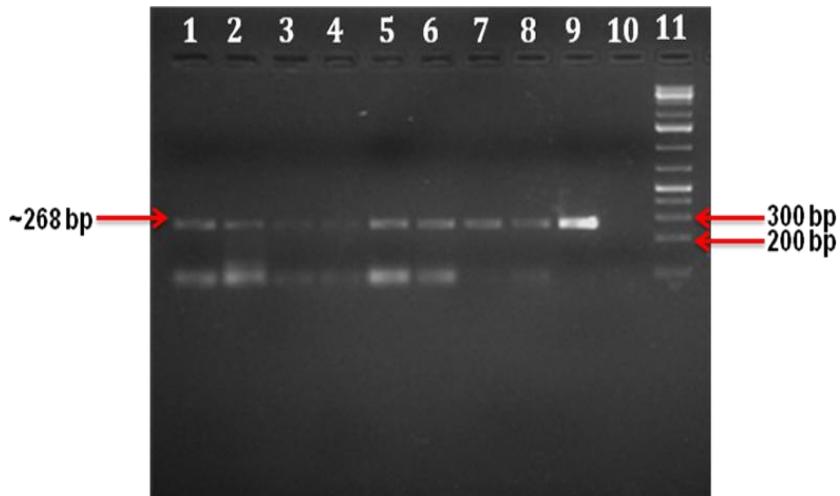


Fig.4 RE Digestion of Cdv Genes by Ecori Enzyme: Lane M: 1 Kb Plus Dna Ladder (Fermentas), Lane H: Insert Release Of ~1169bp Size Of H Gene, Lane L: Insert Release of ~268bp Size of L Gene

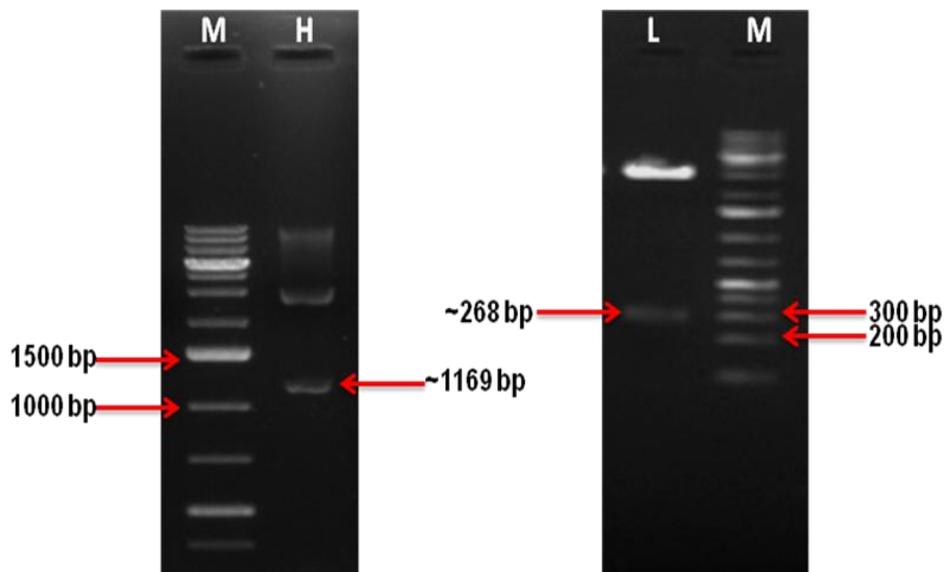
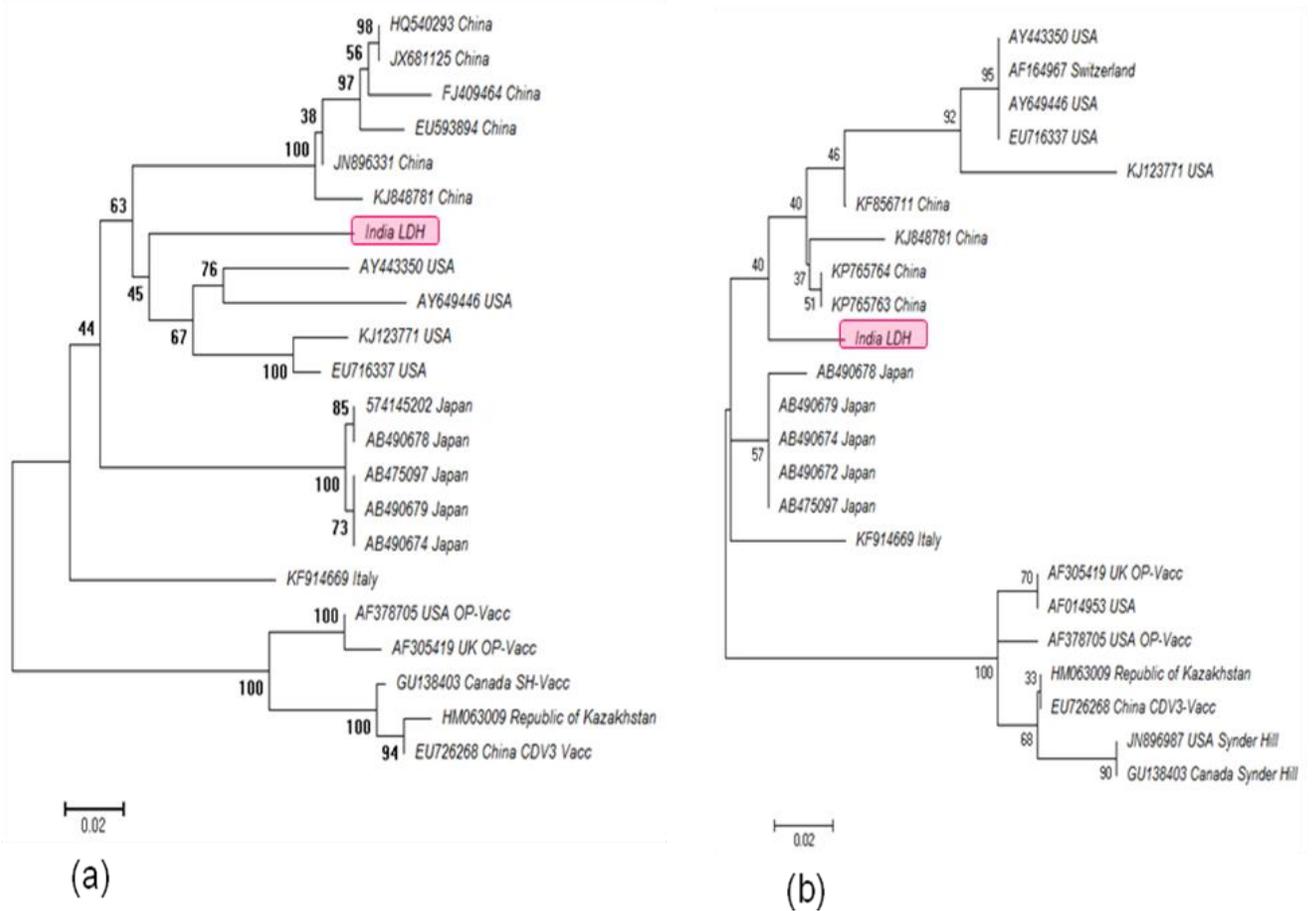


Fig.5 Phylogenetic tree of CDV strains, on the basis of the amino acid alignment (a) partial H protein (b) partial L protein of India LDH strain, constructed using maximum likelihood method. The scale bar indicates the branch length equivalent to 0.02 amino acid substitutions per site; the numbers on branches are percentages of bootstrap values determined for 1000 iterations.



CDV have been successfully isolated in a number of different cells earlier. Vero cells expressing dog SLAM receptor is not only useful for CDV isolation but also passaging the virus without any alteration of its genome (Lan *et al.*, 2006). B95a cells which intrinsically expresses mammalian SLAM receptor is also considered good for CDV isolation. But due to unavailability of such cells in many laboratories, lymphocytes from healthy dogs would be a simple option for the multiplication and isolation of virulent CDV from clinical samples. Stimulated dog lymphocytes or any canine lymphoid cell-line can be an easy option to

isolate and propagate virulent CDV as SLAM receptor is expressed on cells of the immune system. Therefore, stimulated dog lymphocyte method as described in the present study for growing, multiplying and further isolating virulent CDV from clinical samples is both useful and economical for many laboratories.

The PCR amplified CDV partial H and L genes of local isolate in this study were successfully cloned into pGEMT Easy vector and the recombinant plasmids prepared from the selected clones when screened for the presence of desired inserts

released an 1169 bp and 268 bp H and L genes specific products by EcoRI restriction endonuclease digestion respectively (Fig. 5). The plasmids carrying CDV H and L genes were sequenced by outsourcing, analyzed by BLASTn and then manually edited and assembled to get partial sequences of H gene (1118bp) and L gene (268bp). The nucleotide sequences were submitted to DDBJ and the accession numbers obtained were LC011103.1 and KM868711.1 for H and L genes respectively.

Comparison of local isolate of CDV's partial H gene with that of the other 21 CDV strains showed 91.9-95.9% nucleotide and 92.9-95.5% amino acid sequence identity. In case of partial L gene, there was 94.8-98.1% nucleotide and 87.6-95.5% amino acid sequence identity. Based on multiple sequence alignment, for both H and L amino acid sequences, LDH strain had the highest degree of identity with that of China and the lowest with that of the vaccine strains i.e. Onderstepoort and Synder Hill. Percentage identity and divergence matrix for H and L gene of different CDV strains based on both nucleotide and amino acid alignment have been shown in (Fig 6). A total of five aa substitutions were found within the 372 aa in partial cds of H protein and four aa substitutions within the 89 aa in partial cds of L protein were observed.

The genotyping or phylogenetic analyses of CDV studied so far are mostly based on H protein, as it has the highest rates of mutation (Iwatsuki *et al.*, 1997). Most of the vaccine strains (Lederle, Onderstepoort, Convac and Synder Hill) are observed to cluster in America-1 type (Maria *et al.*, 2014; Bi *et al.*, 2015). In the present study, the phylogenetic tree based on the partial sequences of H and L genes showed a distinct clade for local CDV isolate which was clearly separated from the known

vaccine group of CDVs (Fig. 7). The similar pattern has also been reported earlier based on partial N gene nucleotide sequences of two Indian CDV isolates (Pawar *et al.*, 2011). Recently, another Indian CDV isolate was reported to make a separate branch compared to vaccine group of CDVs in phylogenetic tree not only based on H protein but also for F, M and P proteins (Swati *et al.*, 2015). An elaborative study is required for genotyping CDV from different places of India to focus on their evolutionary origin.

In conclusion, Growing CDV from clinical samples in mitogen stimulated dog lymphocytes was an effective and economical method to isolate wild-type CDV. Though the method requires a number of blind passages for appearance of proper CPE, the presence of the viral RNA was easily detectable from the first passage. Molecular characterization of the partial H and L genes revealed that the isolated CDV was least identical to vaccine strains being used currently.

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