Research Article

Phylogenetic Analysis of Canine Distemper Virus in a Domestic Dog in Pakistan

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Abstract | Canine distemper (CD), caused by canine distemper virus (CDV), is highly contagious disease of carnivores and is quite prevalent in Pakistan. The CDV is known to exist as 11 distinct clades around the globe; however, as to what prevail in Pakistan to devise appropriate diagnostics and control strategies is yet to be known. Despite the use of modified live vaccine to dogs, occurrence of disease is not uncommon. Here, we sequenced and phylogenetically analysed the complete hemagglutinin gene of CDV from a male dog (14 months old) exhibiting clinically suggestive symptoms. With a characteristic amino acid residue for dog at Y549, sequence analysis showed geographically distinct pattern clustering the study strain to clade Asia-2 but clearly distinct from vaccine strains. Though limited and preliminary data, the findings enhance our knowledge towards CDVs circulating in Pakistan. Further studies are much necessary to ascertain genetic characteristics of CDVs over a wide geographical area of the country together exploring the potential reasons of vaccine failure.

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Introduction

Canine distemper (CD) is a highly contagious disease of carnivores caused by canine distemper virus (CDV). It occurs as acute, sub-acute or febrile illness and has been expanded to non-human primates (Appel and Summers, 1995; Sun et al., 2010). The causative agent (CDV) belongs to the genus *Morbillivirus* within the family *Paramyxoviridae*. It exists as negative sense single stranded RNA virus that possesses a helical nucleocapsid surrounded by envelop. With an approximate length of 15Kb, the genome encodes six proteins named the nucleocapsid (N), the phosphoprotein (P), the matrix (M), the fusion (F), the hemagglutinin (H) and the polymerase (L) (Sidhu et al., 1993). Among these, the H protein is of much importance as it determines viral tropism and an effective immune response raised in host against it prevent from infection (Sidhu et al., 1993; Martella et al., 2008; Woma et al., 2010). Since it is considered the least conserved gene in the CDV genome with up to 10% variability among different strains giving a distinct pattern of geographical spread, exploring its sequence, the viruses around the globe are phylogenetically classified into eleven different lineages known as America-1 (vaccine strains), America-2, Europe-1/South America-1, Europe wildlife, Africa, Rockborn-like CDVs, Arctic, Asia-1, Asia-2, Asia-3 and Asia-4 (Iwatsuki et al., 2000; Woma et al., 2010; Zhao et al., 2010; Panzera et al., 2012; Bi et al., 2015).

A prevalence of 11% of CD has been reported in Pakistan based upon clinical diagnosis (Jafri and Rabbani, 1999). In another study, while comparing suitable clinical samples for early detection of CDV from naturally infected dogs through nucleoprotein gene based RT-polymerase chain reaction, Shabbir et al. (2010) revealed prevalence of CDV as 22.22%. Though vaccine schedule is being practiced as per manufacturer's guidelines for modified live vaccine, occurrence of disease even in vaccinates is not uncommon. Given the pronounced genetic diversity for CDVs worldwide in the recent years, understanding toward genetic profile of circulating variants than vaccine strains thus become much important for a particular geographical area to articulate suitable diagnostics and control strategy. Since the spread and occurrence of CDVs across the globe have consistent evolutionary relevance and there is an absolute lack of information from Pakistan pertaining to circulating lineage and its nucleotide and amino acid profile to reference and vaccine strains, here we sequenced H gene from a dog clinically suspected of CDV samples collected from Lahore. The obtained sequence was processed for phylogeny of prevalent strain and amino acid substitution analysis giving an insight towards genetic diversity of indigenous strain and their comparative evolution to those reported earlier so far.

Materials and Methods

Blood sample (3 mL) was collected from a recently 14 months old mortile German shepherd dog (male) that had a history of vaccination. Clinical picture revealed typical symptoms of CD such as chewing gum fits together with increased body temperature (108 °F), muco-purulent oculo-nasal discharge, occasional coughing, diarrhoea and infrequent vomiting followed by severe dehydration since last 3 days. The necessary supportive therapy was given by the local veterinarian including antibiotics and multivitamin in fluids. Viral genome was extracted from blood using QIAamp Viral RNA extraction Mini Kit, Qiagen, Valencia city, CA, USA). according to the manufacturer instructions. For genome amplification, cDNA was synthesized from the extracted RNA using cDNA synthesis kit (Thermoscientific® ReertAid First Strand Kit) as per manufacturer instructions. The prepared cDNA was stored at -20 °C until further used in genome amplification using polymerase chain reaction assay (PCR). The complete hemagglutinin coding genome region was amplified using previously reported primers (Chen et al., 2018). The amplified amplicons were visualized using gel electrophoresis system for separation of bands under the influence of electricity.

The PCR product amplicons were purified using Wizard® SV Gel and PCR Clean-Up System (Promega, Co. Madison, WI, USA) according to the manufacturer's guidelines. The H genome was sequenced in both directions with same primers used for amplification through BigDye Terminator method and detected in ABI PRISM Genetic Analyzer 3130x1 version (Applied Biosystems, Foster City, CA, USA) in accordance to manufacturer's instructions. The obtained sequences were compared to GenBank database using BLAST tool at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The Hgenome sequences representing so-far reported distinct CDV strains were retrieved from the public database. Assembled sequence of each isolate was aligned with strains representing distinct strains (GenBank) using ClustalW methods in BioEdit® version 5.0.6 (Hall, 1999) for subsequent H gene-based phylogenetic and comparative residue analysis. To determine phylogenetic clustering and residue differences of study sequence, H gene sequence was analysed in comparison with previously reported distinct CDV strains around the globe (http://www.ncbi.nlm.nih.gov/) using distance-based neighbour-joining (1000 replication bootstrap values) method in MEGA® version 6.0 software (Tamura et al., 2013).

Results and Discussion

We performed H gene based nucleotide and amino acids sequence phylogeny for the study isolate to determine its evolutionary relationship between CDV isolates together determining genetic variations among wild-type and vaccine strains. Since the said gene is more variable, plays an important role in host immune reaction and a large number of complete sequence of H gene are available in public database than others of CDV, this gene has been widely used for comparative evolutionary studies so far giving a robust and definitive results of nucleotide and amino acid divergence (Bolt et al., 1997; Pardo et al., 2005). The Neighbour-Joining tree generated using complete H gene sequence retrieved from GenBank evidenced geographic-related patterns of segregation as reported previously for CDVs. In the phylogenetic tree, study strain sequence clustered together with strains corresponding to the clade Asia-2 reported previously from Asian countries (Figure 1).

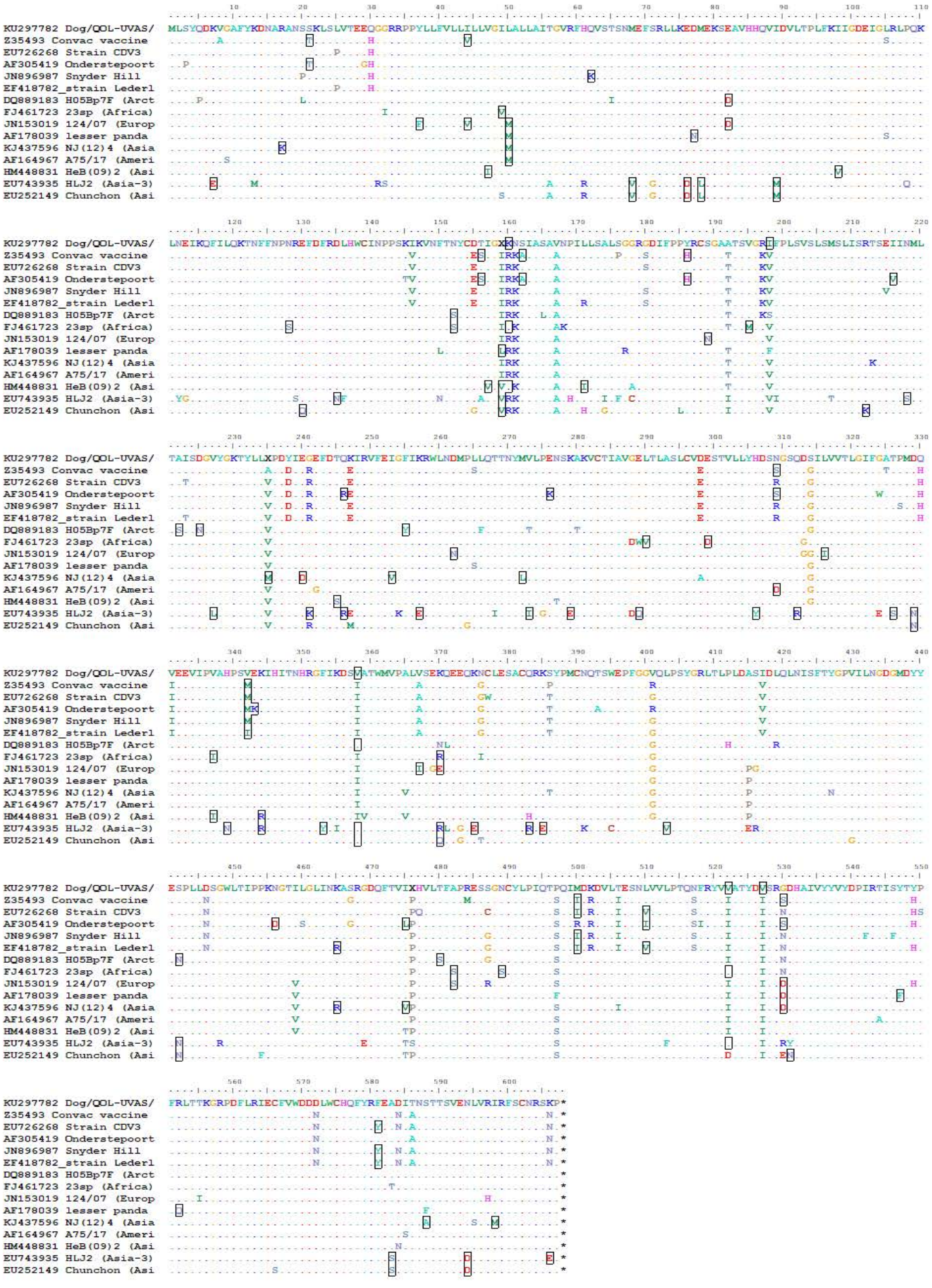

Figure 1: *Comparative residue analysis for substitutions and localization of biologically, functionally and structurally important motifs/domains.*

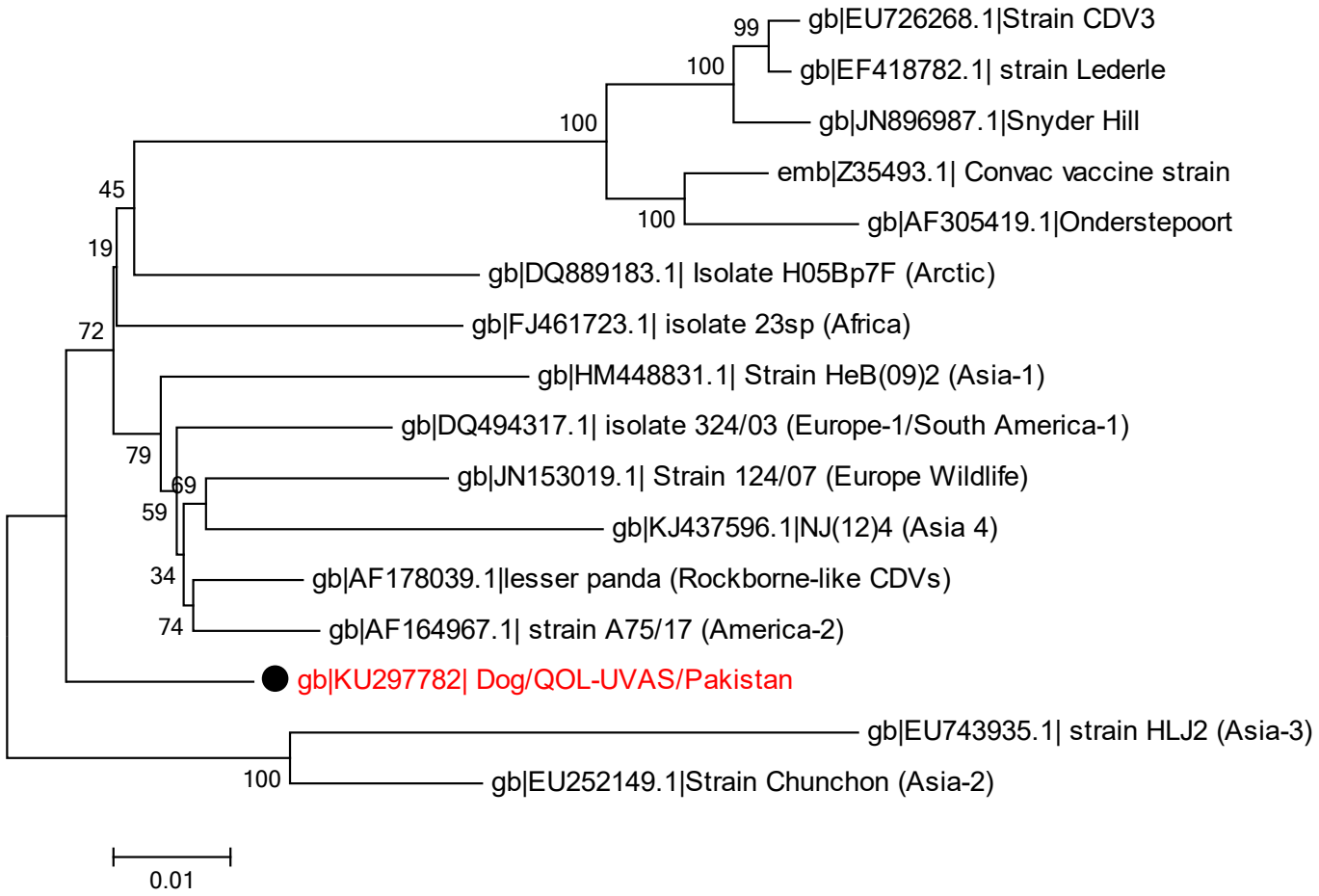

Figure 2: *A complete hemagglutinin (H) gene based phylogenetic tree of Pakistan originated CDV.*

From the predicted amino acid sequence of H gene of study isolate, a total of nine potential N-linked glycosylation sites (N-X-S/T) were identified for the conserved sites at aa position 19-21, 149-151, 309-311, 391-393, 422-424, 456-458, 584-586, 587-589 and 603-605 (Figure 2). The N-glycosylation has been described important for protein folding, transport and functions of fusion and attachment glycoproteins (Hu et al., 1994). Differences in glycosylation sites, thus may result in subsequent differences neutralization efficacy, replication and virulence of corresponding CD strain (Lan et al., 2007). Since reduced glycosylation is considered an important attenuating factor for vaccine strains, a significant molecular difference between wild-type and vaccine could result in potential vaccine failure. Of the vaccine strains, the strains Onderstepoort has only four N-glycosylation sites while the strains CDV3, Lederle and Convac displayed seven glycosylation sites. Nevertheless, so far, seven or nine sites have been detected in all wild CDVs where 309-311 connected amide asparagine glycosylation site are specific to CDV field strains (Guo et al., 2013). Further, when compared by amino acid alignment to other CDVs particularly vaccine strains, 12 of 12 cysteine residues, 33 of 35 proline residues and 33 of 35 glycine residues were conserved that are considered important for the secondary structure of the protein.

The study strain contained glycine (G) at 530 and tyrosine (Y) at 549 in the H protein. Though varying in observations, both positions are considered much important in determining the variations within lineage and affecting or switching host species. McCathy et al. (2007) have identified both amino acid residue associated with host specificity; glycine (G) or glutamic acid (E) at position 530 in canine samples to aspartic acid (D) or asparagine (N) or arginine (R) in non-dog host while the substitution of tyrosine (Y) at position 549 in canine to histidine (H) in non-canid hosts. Beside characteristic residues at both positions for canids and wildlife samples, Benetka et al. (2011) reported G/E530D in canids attributing it to previous interspecies transmission between wildlife and dogs accompanied by viral evolution. While comparing complete H gene sequence from domestic dog and non-dog species, Guo et al. (2013) suggested positive selection for residues at 530 and 549 assuming that

presence of specific residue at these positions may result in disease to novel host species. However, Nikolin et al. (2010) and Bi et al. (2015) revealed residue at 530 as conserved within CDV lineages irrespective of host species. Residue at 549 (Y) has shown significant bias for wild-canids whereas non-canids are independent of either H or Y at this specific site (Nikolin et al., 2010), whereas in another study, tyrosine (Y) residue has been identified in both canids and non-canids at this site (Guo et al., 2013). In another study involving characterization of CDV in raccoons in the USA, glycine (G) and tyrosine (Y) residues were identified at position 530 and 549 in some of the samples than expected aspartic acid/asparagine/arginine (D/N/R) and histidine (H), respectively (Lednicky et al., 2004).

The study dog was 14 months old and had the history of vaccination with modified live vaccine. However, the immune response to vaccine was not determined so that appropriate intervention could be applied in terms of re-vaccination etc. Further, the strain detected was much distinct from known vaccine strains as has been reported previously for other field CDVs strains (Bolt et al., 1997; Maes et al., 2003; Pardo et al., 2005). In dogs particularly that are pets, despite high rate of vaccinations against canine diseases including CDV, clinical cases do occur sporadically (Bi et al., 2015; Pardo et al., 2005).

Varying speculation are presented to explain potential vaccine failure but most often includes variations in the pups genetics to develop immunity, potential interference of maternal antibodies against active immune response, genetic distance and subsequent alterations in the antigenic profile of H gene at sites important for neutralization (Iwatsuki et al., 2000; Maretall et al., 2008; Pardo et al., 2015) and inappropriate handling or vaccine administration. Interference of maternal antibodies is well known fact in developing enough immune response towards vaccine; nevertheless, this could not be the case in this dog as it was 14 months old. The strain identified differed in sequence from the vaccine strains, exposure of dog to wild-CDV is suggested than revertant of vaccine strains much similar to recent report from China (Bi et al., 2015). Since potential mutation enough to escape from neutralization titter produced by the vaccine has been reported for wild strains and the current isolate analysis has reported mutations too, whether the vaccine has produced enough antibody titre or not is also a question of concern. Vaccines are normally administered but a question always remains whether they have produced antibodies enough to combat field challenge. Serum analysis in this regard is much important and this practise is totally lacking in Pakistan that must be introduced. The vaccines are used in the recent past against surface proteins. In Pakistan DHPPI+L, and DHPPi2-L as combination vaccine against canine parvo, canine parainfluenza, canine hepatitis and leptospira. Recently recombinant vaccine against CDV planned to use but it is under trail. However, in recent years, genetic and subsequent antigenic diversity has been reported in field strains disrupting neutralization-related epitope that are considered important for immune protection (Blixenkrone-Moller et al., 1993; Iwatsuki et al., 2000; Zhao et al., 2010).

Conclusions and Recommendations

This is the first report revealing genetic characterization of canine distemper in dogs in Pakistan. Phylogenetic analysis of H gene implied an evolutionary relationship of prevalent strain to Asia-2 but genetically distinct from vaccine strains. Further studies with much larger datasets are necessary to ascertain a wide-geographic divergence of CDVs in the country together exploring potential reasons to vaccine failure either due to divergence from vaccine strains or presence of maternal antibodies or lack of evaluation of immune response to intervene accordingly.

Author's Contribution

All authors were involved in sample collection, laboratory and data analysis. All authors read and approved the final draft of the manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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