

A phylogenetic study on the NP gene of detected canine distemper virus in (2008-2011) Iran

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Abstract:

BACKGROUND: Canine Distemper (CD) is a lethal systemic disease affecting a wide variety of terrestrial carnivores. **OBJECTIVES:** This study was performed to survey epidemiological and molecular characteristics of the canine distemper virus (CDV) strains circulating in Iran. **METHODS:** In this study, 19 CDV-suspected unvaccinated dogs from Northeast and center of Iran were analyzed for presence of CDV nucleoprotein (NP) gene using Nested-PCR during 2008-2011. Different biological samples of 14 dogs were positive. **RESULTS:** The phylogenetic analysis based on partial NP gene sequences indicated the presence of two major clusters that are clearly different from vaccine strains in Iran. One cluster belongs to the European group and the other one to the Arctic group. **CONCLUSIONS:** Due to a lack of phylogenetic analysis on CDV in countries bordering Iran, except Turkey, the ancestor of Iranian sequences specially Iranian Arctic sequences could not be definitely identified. This study is the first report on phylogenetic analysis of CDV from domestic dogs in Iran.

Introduction

Canine distemper virus (CDV) belongs to the genus Morbillivirus of the Paramyxoviridae family. CDV is a very contagious pathogen that causes canine distemper (CD) disease with high fatality rates in the Canidae family. Also, several studies have already reported on the CD infection of other terrestrial and aquatic carnivores (Appel et al., 1995; Guiserix et al., 2007).

CDV genome is composed of negative single-stranded RNA about 15690 nucleotides in length encoding six structural proteins (Nucleocapsid protein (NP), a single envelope-associated Matrix protein (M), the Phosphoprotein (P), and the Large protein (L), the Hemagglutinin protein (H) and the Fusion protein (F)) (Murphy et al., 1999).

The CDV NP gene is an important part of CDV, consisting of the conserved region, middle part, variable part, and N- and C-terminal (Yoshida et al., 1998).

Despite the fact that NP gene is a conserved region of the CDV genome, variations of the NP gene among CDV isolates have been shown (Parks et al., 1992). However, progresses in molecular studies reveal the usefulness of NP gene-based analysis for CDV detection and for molecular epidemiology studies (Jozwik et al., 2005; Keawcharoen et al., 2005; Scagliarini et al., 2003; Shin et al., 2004).

Although vaccination against CDV with attenuated virus is done to protect the majority of animals, there have been some evidences of CDV infection in vaccinated dogs worldwide (Blixenkroner-Moller et al., 1993; Iwatsuki et al., 1998).

According to these findings, it can be concluded

that there may be antigenic differences between some wild CDV and vaccine strain (Harder et al., 1997).

Categorization of the genetic diversity of CDV can lead us to a better understanding of its appearance and epidemiology, and also facilitates development of more effective vaccine to prevent CDV spread in the world.

CDV infections are very common disease of domestic dogs in Iran; however, to date, no molecular studies have been done on CDV.

The present study aims to analyze the molecular phylogenetic of Iranian CDV genome based on partial nucleotide sequences of the NP gene for the first time in Iran.

Materials and Methods

Clinical specimen: Samples taken from 19 unvaccinated rural dogs (16 living and 3 died dogs) showed representative symptoms of CD, such as fever, purulent ocular and nasal discharge, tonsillitis, bronchitis, and gastroenteritis. Neurological disturbances have not been diagnosed in the sample dogs.

The samples were submitted by veterinary medical teaching hospital of university of Tehran (Center of Iran) and local clinics of Golestan province (North- East of Iran) from 2008 to 2011. Clinical findings and histories, such as age, sex, vaccination, and breed have been recorded (data not shown)

Various biological specimens were screened for this study: whole blood (n= 18), Ocular swab (n= 19), serum (n= 18), and tissue (n= 3, lung, bladder, kidney, intestine and stomach).

Blood samples were poured into the EDTA tubes. 3 ml of blood were diluted 1:1 by phosphated buffered saline (PBS) and were gently mixed. 1 mg of homogenized internal tissue was kept in 1ml PBS (Ph 7.2), and Ocular discharges were wiped with sterilized cotton sticks and then eluted with 0.5 ml PBS (Ph 7.2). 2 ml of the serum were diluted 1:1 with PBS (Ph 7.2). The samples were kept in -70°C till they were used for the investigations.

RNA extraction & cDNA synthesis: Total RNA was extracted from all specimens by RNA extraction kit (Bioneer Co, South Korea) following the manufacturer's introductions. Vero cells were infected with Onderstepoort CDV (Ond-CDV). Vaccine

strain and ultra-pure water (Bioneer Co, South Korea) were used as positive and negative controls, receptively.

The RNA was reverse-transcribed into cDNA using two steps RT-PCR kit (Vivantis, Malaysia). The cDNA amplified was directly stored at -20°C until utilized for PCR.

PCR and nested-PCR: Two sets of primers specific for the CDV NP gene (with sequences obtained from GenBank) were elaborated and used in the study: CDV-1 (Forward) (5'-GGGTCGAAAGCTCAAGGAC -3') and CDV-2 (Reverse) (5'- CTGACACTAGCTGAGCCTCTTC -3'). This pair enabled amplification of 777-778bp region and the second pair of primers CDVa (Forward) (5'-CCTGCTCGCTAAAGCAGTG-3') and CDVb (Reverse) (5'-CCCTCCCATGGAGTTTTCA-3') enabled the amplification of 520 bp region.

Primer pair CDV-1 & CDV-2 were used for PCR as outer primers and pair CDVa & CDVb were used for Nested-PCR as inner primers

PCR with the use of outer primer (CDV-1 and CDV-2 primers) was performed in reaction mixture containing (25 ul): 1 µL (0.04 Unit UI-1) TaqDNA polymerase, 5 µL cDNA, 17 µL 10X PCR master mix (Vivantis, Cat No: RTPL12, Malaysia), 1 µL 10mM dNTP, 1 µL (10 mM) each primers.

The reaction mixture was incubated at 95°C for 5 min. Amplification was performed in 35 cycles with denaturation at 95°C for 1 min, annealing at 47°C for 1 min and elongation at 72°C for 1 min. The final elongation was also accomplished at 72°C for 5 min.

Additionally, Nested-PCR was carried out with the use of internal set of primers (CDVa, CDVb) and 1 µL of the first PCR product as template. Nested-PCR thermocycler program was equal to the first round. The positive and negative CDV controls were included in each run of both detections (NP gene) and genotyping. Products were investigated under ultraviolet (UV) light after electrophoresis on a 1% ethidium bromide agarose gel with 100 bp ladder (Fermentas, Canada) as a DNA size marker.

Four positive Nested-PCR results were purified using the Gel Purification kit (Bioneer, Cat No: K-3034, Korea). Purified DNA products were sequenced bi-directly using inner amplification primers in ABI 313 DNA sequencing devices (Seq Lab Co, Germany).

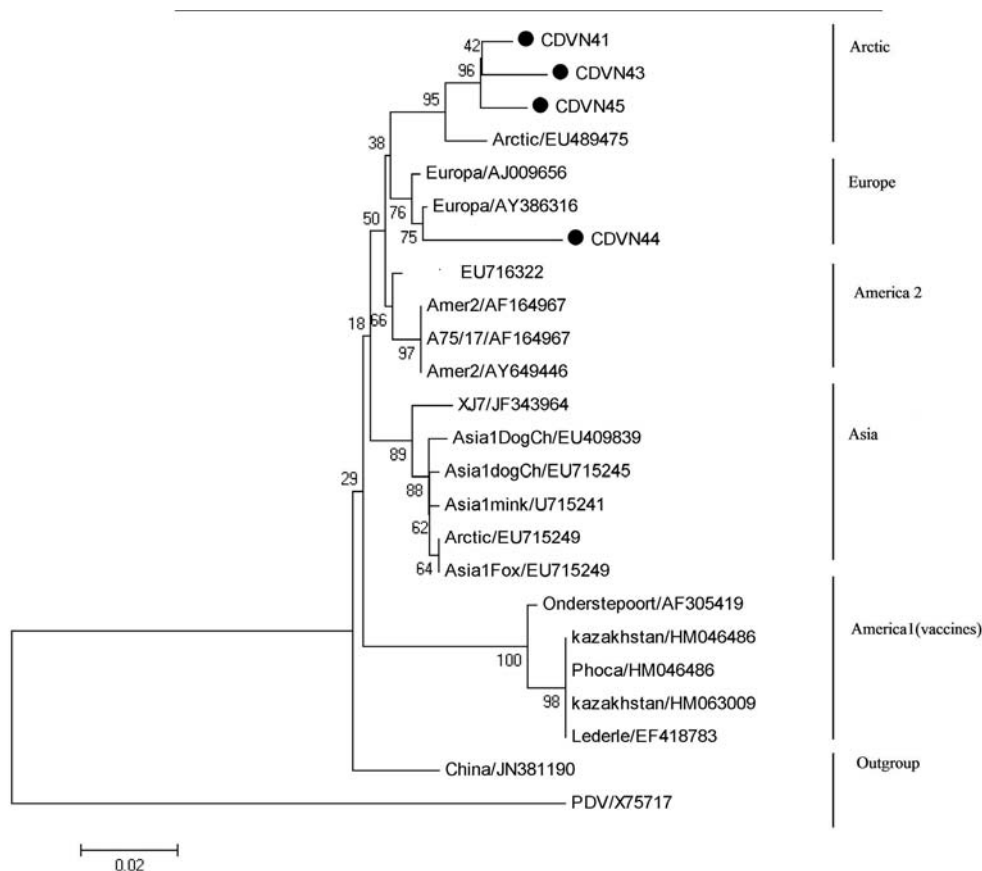


Figure 1. RT-Nested PCR results on agarose gel (1%) electrophoresis of different samples by outer and inner primer of *NP* gene: Lanes 1: molecular marker (100-bp ladder); lanes 2: CDV Onderstepoort strain; lane 3: Suspected sample; lanes 4: Ultrapure water. Numbers on the left are molecular sizes (in base pairs).

Phylogenetic analysis: The partial nucleotide sequences of the CDV *NP* gene were compared with submitted sequences in GenBank. Initially, the CDV sequences were checked over by the MEGA 4.0 software. The alignments were subsequently used as an input for phylogenetic analysis using MEGA.4.0 software. The phylogenetic tree was designed by the neighbor-joining technique and Distances values with a 1000 bootstrap replicates with MEGA 4.0 software (Thompson et al., 1994).

Gene bank submission: Four CDV strains identified in this study have been presented to GenBank and are available in the GenBank data base under accession numbers: JN941240-43.

Results

Nested-PCR: 14 samples out of 19 CDV expected dogs from Northeast and center of Iran were positive for canine distemper virus based on Nested-

PCR assay (Figure 1).

Sequence and phylogenetic analysis: Figure 2 displays the Iranian *NP* gene sequences in the distance Neighbor-Joining tree among CDV strains obtained from Gene Bank database. Iranian *NP* gene sequences from dogs with the numbers CDV N41, CDV N43, CDV N45, and CDV N44 demonstrated %93.46 to %98.46 homology to each other. In phylogenetic analysis, four identified Iranian sequences were divided into two known clusters: cluster A, dogs with the numbers CDV N41, CDV N43, CDV N45 and cluster B, the dog number CDV N44. Cluster A is closely related to the Arctic group, and cluster B is related to the European group. The highest genetic connection from %94.42 to %97.88 for the Iranian Arctic *NP* gene was found with *NP* gene sequences of a CDV strain identified in a breeding fox by Zeng et al. in China (Zeng et al., 2000).

The nucleotide similarity of Iranian *NP* sequences

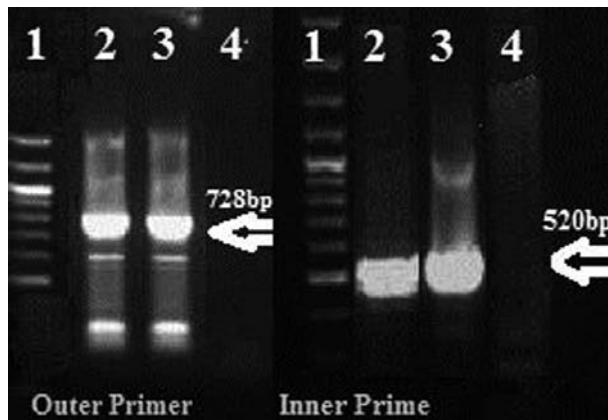


Figure 2. Phylogenetic analysis of NP gene of CDV from local and worldwide strains. GenBank Accession numbers are indicated in each strain.

reduces to %92.50 to %93.27 compared with that of the Ond-CDV strain (data not shown) (Gassen et al., 2000).

Discussion

Canine distemper virus (CDV) generates a lethal systemic infection disease in dogs and wildlife carnivores throughout the world (Greene et al., 2006). In this study, for the first time in Iran, partial sequences of the CDV NP gene (728bp and 520bp) were successfully identified and compared with other CDV lineages.

Sequence analysis of CDV strains is generally performed using different genetic targets, such as H, NP, and P genes (Castilho et al., 2007; Mochizuki et al., 1999; Pardo et al., 2005). Although the use of genetic targets has produced a large data set of CDV sequences, it has hindered uniform comparison of various CDV strains.

In general, CDV can be clustered into 6 major genetic lineages designated as America-1 (including several vaccine strains), America-2, Asia-1, Asia-2, Europe and Arctic, according to their geographical distributions (Haas et al., 1997).

The nucleocapsid protein (NP) gene producing the main structural viral protein is the most conserved gene and seems to be a suitable target for the detection of all CDV strains (Calderon et al., 2007; Harder et al., 1997; Kim et al., 2001).

A phylogenetic distance analysis based on Neighbor-Joining method revealed that the four

Iranian NP gene sequences created two clusters: dog number CDVN44 (JN941243) from the center of Iran (Tehran province) belongs to European genotype and the dogs numbered CDV N41 (JN941240), CDV N43 (JN941241), and CDV N45 (JN941242) are from North-East (Golestan province) belonging to the Arctic genotype.

A Similar finding was achieved about nucleotide sequence analysis of nucleoprotein gene in Thailand (Keawcharoen et al., 2005). Arctic CDVs had been identified since the 1980s and then spread in many countries globally (Martella et al., 2006).

In the present study, the Iranian Arctic NP sequences, identified from Golestan province, showed the highest affinity to EU489475 strain isolated from a breeding-fox in China which belonged to arctic genotype (Zeng et al., 2008).

So far, no molecular analysis has been conducted on CDV in the countries neighboring Iran, except Turkey. Turkish CDV lineages are related to European sequences; therefore, the Turkish-Europe sequences can function as one of the origins of the European sequence in Iran (Ozkul et al., 2004).

The Iranian European sequence was most closely related to AY386316 which has been assigned to the European cluster by Von et al. (Von et al., 1999).

The four CDV sequences identified in Iran showed homologies of % 93.46 to %98.46 to each other. Keawcharoen et al. also found %90.75 to %97.61 similarity among NP gene sequences of canine distemper virus isolated in Thailand (Keawcharoen et al., 2005).

Besides, similar to some previous studies, NP genes sequenced in this study were genetically distant from the known vaccine strains (Castilho et al., 2007; Tan et al., 2011).

Due to a lack of information about molecular features of CDV in countries neighboring Iran, the ancestor of the Arctic sequences in Iran could not be identified.

This study merely analyzed CDV genome in central and northeastern parts of Iran; however, more surveys are needed before drawing any conclusion in a country as wide as Iran, which is reservoir of many susceptible species of carnivores to CDV.

We recommend more molecular and phylogenetic analyses in other geographic areas and also molecular studies on complete sequences of CDV genes such as

P, H, and NP genes in Iran.

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مطالعه فیلوژنتیکی ویروس دیستمپر شناسایی شده در ایران، براساس ژن نوکلئوپروتئین (۱۳۸۷-۱۳۹۰)

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چکیده

زمینه مطالعه: بیماری دیستمپرسگ ها یک بیماری بسیار کشنده که بسیاری از گوشتخواران را مبتلا می کند، می باشد. **هدف:** این مطالعه با هدف بررسی وضعیت اپیدمیولوژیک ویروس دیستمپرسگ ها و ساختمان ژنی آن در ایران صورت گرفت. **روش کار:** در این مطالعه با استفاده از تست واکنش زنجیره ای پلیمرز داخلی، حضور ژن نوکلئوپروتئین ویروس دیستمپر در نمونه های تهیه شده از ۱۹ سگ دارای علائم بیماری دیستمپر، مورد مطالعه قرار گرفت (۱۳۸۷-۱۳۹۰). **نتایج:** در نمونه های مختلفی از ۱۴ سگ، ژن نوکلئوپروتئین مورد شناسایی قرار گرفت. مطالعات فیلوژنتیکی انجام شده براساس بخشی از ژن نوکلئوکپسید، موجب شناسایی ۲ شاخه ویروس دیستمپر که بطور واضح با سویه واکسن متفاوتند، در ایران شد. یک شاخه، متعلق به دسته ویروس های دیستمپراوپایبی و یک شاخه متعلق به دسته آرکتیک می باشد. **نتیجه گیری نهایی:** با توجه به مطالعات فیلوژنتیک محدود موجود، بر روی وضعیت ژنتیکی ویروس دیستمپر در کشورهای همسایه ایران، نمی توان به راحتی اجداد ویروس دیستمپر در ایران، بخصوص شاخه آرکتیک را ریشه یابی کرد. این مطالعه اولین مطالعه فیلوژنتیکی انجام شده بر روی ویروس دیستمپرسگ ها در ایران می باشد.

واژه های کلیدی: ویروس دیستمپرسگ ها، ژن نوکلئوپروتئین، مطالعه فیلوژنتیک

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