Hemagglutinin-neuraminidase sequence and phylogenetic analysis of two Newcastle disease virus isolated from chickens in Iran

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Abstract:
Newcastle disease is a highly contagious viral infection affecting many species of birds which can spread fast between poultry houses and cause heavy economic burden on the poultry industry all around world. Fusion and hemagglutinin-neuraminidase (HN) protein are important in pathogenesis of newcastle disease virus. HN protein is critical viral protein with multiple functions plays a key role to form the NDV’s virulence. The head of HN protein is responsible for receptor binding, neuraminidase activity. The aim of this study was to investigate the sequence homology of hemagglutinin-neuraminidase of two Newcastle disease virus isolates sampled over the infected farms in Iran. Samples were collected from flocks that had been vaccinated by both types of live and killed vaccines for NDV. After isolation NDV, the viruses were subjected to the polymerase chain reaction (PCR) amplifications using two pairs of specific primers designed for HN gene to amplify complete HN gene (1730bp). Next, the PCR products were sequenced and analyzed by
phylogenetic tree constructing software. The analysis showed substantial sequence homology among Iranian isolates which is ranged between 97.1 to 100%. Moreover, the sequence homology searching revealed a level of similarity between HN sequences of Iranian isolates and the HN sequences from other countries particularly those in Asia. For instance, we found high homology ratio (95.34%) between Iranian isolates and the sequences registered on online molecular databases from China. Based on phylogenetic analysis the NDV isolates belong to the VIIId genotype. Monitoring of circulation NDVs from poultry and other birds would help to obtain an insight into the evolution of NDVs and control of panzootic viruses in future.

**Key words:** Newcastle Disease Virus, Hemagglutinin-neuraminidase, Phylogenetic tree, Iran.

**INTRODUCTION**

Newcastle disease (ND) is one of the most prevalent and devastating viral infections in poultry with high mortality and is responsible for the significant part of the heavy economic losses in poultry houses around the world. From the taxonomic perspective, the NDV is a member of the genus *Avulavirus* within the family *Paramyxoviridae*, in the order *Mononegavirales*. Based on the taxonomy, the infectious agent causing ND is named as the avian paramyxovirus-1 (APMV-1). The NDV contains a RNA genome with about 15 kb length, coding six viral proteins in the order as following: 5-NP-P-M-F-HN-L-3(Cao et al., 2013). NDV strains grouped in the two separate classes (I and II) within a single serotype. Class I contains nine genotypes (1–9) and class II includes 18 genetic groups which 10 of them have been previously established (I–IX, and XI) and five are new genotypes (X, XII, XIII, XIV and XV)(Diel et al., 2012). The virulence of all NDV isolates all around world monitored by veterinary organizations and according to the instructions, Upon isolation of a virulent NDV strain, with ICPI of 0.7 and IVPI of 1.40 a report should be sent to OIE (OIE, 2018).
Virulence of NDVs are related to fusion (F) amino acid sequences at cleavage site. However, hemagglutinin-neuraminidase (HN) protein as a critical viral protein with multiple functions plays a key role to form the NDV’s virulence. The protein is a surface glycoprotein with several subunits including a cytoplasmic domain, a transmembrane region, a stalk region and a globular head. The head is the part that is responsible for receptor binding, neuraminidase activity, and all the antigenic properties of HN protein. As a virulence factor with a good binding property, the HN protein can agglutinate red blood cells (RBCs). The mechanism by which the HN protein can agglutinate the RBCs is handled by binding of the HN protein to the surface receptors on the RBCs. However the HN protein is multifunctional therefore several other virulence-associated activities such as the attachment, fusion promotion, deletion of the sialic acid from progeny virion particle and neuraminidase activity have been attributed to the HN protein (Hu et al., 2010a).

In Iran, ND has been described as an endemic disease due to its frequent incidence in various geographical areas and different industrial poultry houses. In recent years, considerable numbers of industrial farms get challenged by highly virulent NDV infections (Samadi et al., 2014; Ghalyanchilangeroudi et al., 2018; Sabouri et al., 2018). In most cases, the predominant NDV genotype that is circulating in industrial farms in Iran was VIIId (Hosseini et al., 2014). Most of publications were analyzed NDVs based on F protein sequences. Nevertheless, there is a little information about HN protein sequences and diversities (Esmailizad et al., 2012).

At the present study we concentrate on distinguishing the genetic variation on two Iranian NDVs isolates from current outbreaks by phylogenetic analysis on the HN sequences and comparing them to HN sequences NDV strains from other countries.
MATERIALS AND METHODS

Specimen collection and virus culture. Two of NDV-infected broiler flocks located in Gilan and Isfahan provinces were selected for specimen collection. Sampled chickens were showing NDV-typical nervous symptoms and also high mortality rates (more than 25%). The broiler flocks had been previously vaccinated with a routine vaccination program which also includes live and killed NDV vaccines. The heads of the affected chickens submitted to the Laboratory. To confirm the presence of NDV in infected chickens, we cultured the brains into the embryonated eggs to isolate the viruses and then confirm them using antigen-testing. To culture the viruses, 0.2-ml aliquots of suspensions from the infected tissues were inoculated into the allantoic cavity of embryonated eggs (9-day-old). After incubation at 37°C for three days, the allantoic fluid was harvested and then evaluated using HA assay method. Then, allantoic fluids specimens with hemagglutination activity were subjected to the HI test according the recommended procedures to further confirm the presence of NDVs (Manual, 2018).

RNA Extraction and Reverse-transcription PCR (RT-PCR). Total viral RNA was extracted from allantoic fluid using RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Reverse Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Canada) was used to convert the viral RNA to the complementary DNA (cDNA) based on the manufacturer’s recommendations. Next, the cDNAs produced from synthesizing step, were incorporated into PCR procedures along with HN-specific primers. Two pair of primers, specific for HN region were used to amplification of the complete HN gene (1730 bp). The sequences of forward primers (F1 and F2) were as followings: F1 (5’-TTC ACA ACC TCC GTT CTA CC-3’) and F2 (5’-AAG TCT TGC AGT GTG AGT GC-3’), and the sequences of reverse primers were as followings: R1 (5’-TTG CAC TCA CAC TGC AAG AC-3’) and R3 (5’-TCA TCT TTG AGG
ATC TCA AC-3’). Each pair of primers, capable of amplifying a part of HN gene sequence, were incorporated into a separate PCR assay which in that way the whole HN gene sequence was amplified (Esmaelizad et al., 2012). Therefore, the primers amplified DNA fragments with different lengths. A PCR program was set in the thermal cycler for amplification of templates as followings: denaturation (94°C for 30s), annealing (51°C for 30s), and extension (72°C for 40s) which all three steps were repeated for 35 cycles, then a prolonged extension period at 72°C for 10 min was also set as a final step after completion of 35 cycles.

**Study of the sequences and construction of phylogenetic tree.** The PCR products were visualized in two steps; first they were run on a 1.5% gel electrophoresis and then were stained with ethidium bromide (EB). A PCR purification kit (AccuPrep® PCR Purification Kit, Bioneer Co., Korea) was used to purify the amplified DNA fragments from the PCR product. Then the purified PCR products were sequenced using both forward and reverse primers. An enhanced sequencing package (BigDye Terminator Cycle Sequencing Kit) including the required materials from ABI (Applied Biosystems, USA) was used to run the sequencing reactions. The reactions configured according to the instructions described by the manufacturer. Samples were sequenced using an ABI sequencing machine (Prism 310 Genetic Analyzer, Applied Biosystems, USA). Afterwards, the raw results produced by the ABI machine which are computer-produced nucleotide sequences, were checked for any error or wrong data and edited for length before exporting to a digital file with FASTA format with the help of Chromas Lite software. The erroneous and ambiguous parts of the sequences were also omitted using appropriate software. Next, the final purged sequences, were used as the templates for homology searching in a nucleotide online database (NCBI GenBank) with the help of its online software; BLAST (The Basic Local Alignment Search Tool). The software as its name implies, searches smalls fragments
of the template sequences against a huge database of the nucleotides sequences of all genes from all species based on specific algorithms. Our HN sequences were incorporated in BLAST analysis to confirm their identity. After validation of identity of sequences, we submitted them into the GenBank database and received their accession numbers after evaluation by NCBI. We registered two HN sequences of Iranian NDV in GenBank with accession numbers: KX377650 and KX377651. Moreover, to construct phylogenic tree we retrieved several HN sequences registered on GenBank which were submitted by other research teams from different countries. The strains name and accession number of obtained sequences were as followings: HN-6-07-Ch (GQ245845), JS/2/05/Go (EU044810), JS-1-05 (DQ469830), SSX03 (DQ234581), JS/2/98/Go (AF456430), SL03 (DQ234579), SGM01 (DQ234592), SWS03 (DQ234588), HN-7-06-Ch (GQ245854), XZ-9-08-Ch (GQ245867), GD/1/98/Go (AF456433), GPV-SF02 (AY325796), KR-102/89 (GQ507802), ZHI-3/97 (FJ766529), YZ-23-07-P1 (GQ281092), NDV05-029 (FJ766528), QH1 (FJ751918). A phylogenetic tree was constructed by importing the retrieved HN sequences along with our two sequences into the MEGA7.0 software (Kumar et al., 2016) and then running the phylogenetic tree analysis by setting the specific algorithms. The algorithm in which was used in the software for construction of tree was the Neighbor-Joining (Offeddu et al.) method (1000 replicates for bootstrap). Also, a matrix table containing evolutionary distances was computed by the Pairwise Distance method. According to the configurations which were set in the MEGA software, simulated trees with lowest possibility (Bootstrap values lower than 50) were omitted from the final results. The constructed tree was interpreted based on the values which were calculated according to NJ method. The values above 70 were supposed to be well-supported by the sequence alignments but the lower values were in fact failed to be supported by sequences.
RESULTS

RT-PCR amplification of viral RNA using HN-specific primers resulted in amplification of two viral RNA fragments with lengths as following: 1000 bp and 730 bp. Each of the amplicons is in fact an amplified part from the original viral HN sequences. Afterwards, the PCR-amplified products (HN gene) were sequenced. The molecular phylogeny of the Iranian NDV strains isolates, and their similarity and possible relationship to other representative NDV strains from other countries was assessed based on genetic variations on HN gene sequence. We found that HN gene of the Iranian NDV isolates have high sequence similarity. The homology searching between the HN gene sequences of our isolates and the HN sequences of other Iranian isolates showed a high level of similarity which is ranged between 98.9% and 100%. Moreover, the constructed phylogenetic tree revealed close molecular relationship between our two Iranian NDV isolates (95.34%) with HN sequences which are belonged to China; SGM01 (DQ234592). Although there were close phylogenetic relationships between Iranian NDV isolates and several genotypes of NDV such as V, VI and VIII but most close genetic association was found with VIIa genotype. Our isolates were clustered in one monophyletic clade with common internal node with several genotypes of NDV (Fig. 1). Difference between La Sota vaccine strain and these two NDVs in antigenic sits presented in table 1.

DISCUSSION

In this present study two NDV were isolated from vaccinated broiler in two different geographical locations. The sequence and phylogenetic analysis of F gene of these two viruses showed that they are close to VIIa genotypes previously reported from Iran (Kiani et al., 2016). Limited information is available about HN gene sequence of Iranian NDVs (Esmaeilzad et al., 2012). In this study amplification of complete HN gene was done to find variation in HN gene of circulating NDVs in
Iran. ND is one of the most prevalent and economically important viral diseases of the poultry in Iran. In order to clear the molecular footprint and to go more in depth of the genetics of the NDV, the phylogenetic studies on isolated strains of NDV in Iran seems to be of great importance. The results of genetic studies can draw us a clear structure of the genetic contents of the virus which as a consequence also provide us the critical information about the most important genes (virulence genes) and the taxonomy and even geographical origin of the virus. Viral particles of NDV, similar to most of the other viruses have an envelope consisted from specific glycoproteins, acting as an attachment-facilitating, protection and virulence agent for the virus. NDV as the highly infectious virus favors from its structural virulent glycoprotein called HN which is named for its hemagglutination and neuraminidase activity. The protein helps the viral particles to attach on host cells and attack the protective proteins on target cells. However, it was generally accepted that the two glycoproteins of NDV, HN and F are the major viral agents responsible for its virulence (Diel et al., 2012; Cao et al., 2013). Evolutionary studies (e.g. phylogeny) on various strains of NDV in different areas (Asia, Europe) have cleared that velogenic genotypes have been spread extensively in these areas and are responsible for the outbreaks. Recently, the most prevalent genotype which is involved in many of the NDV infection outbreaks all around Europe, South Africa and Far East, emerged in the 1990s and later named genotype VII of NDV. The VII genotype of NDV is in fact a family of subgenotypes named from VII-a to VII-h. The first members of the family consisting VII-a to VII-e are largely spread over China, Malaysia, Kazakhstan and Kyrgyzstan. The remaining genotypes including VII-f to VII-h were repeatedly isolated within African countries (Ebrahimi et al., 2012).

Esmaeilzad et al. studied phylogenetic analysis for the first time in Iran to clear the origin of HN gene of NDV isolates on 2012 (Esmaeilzad et al., 2012). They found obvious sequence relationship
in phylogenetic analysis between six Iranian isolates and NDV isolates from a near country to Iran; Russia. Two Russian isolated including Sterna-Astr/2755/2001 (VIIb) and VOL95 showed significant similarity with Iranian isolates. Surprisingly, both Russian NDV isolates showed high homology and sequence similarity with Iranian isolates at all nucleotide positions except only one position on Sterna-Astr/2755/2001 (Esmaelizad et al., 2012). HN plays important role in pathogenesis and immunity so, Shahriari et al., have applied HN gene to produce recombinant vaccine (Shahriari et al., 2015). Firouzamandi et al., have used HN gene to improve immunity against NDV (Firouzamandi et al., 2016). Sometimes, the studies on genetic footprints lead the researches to characterize new sub-genotypes or sublineages of viruses. For instance, a group of researchers in an Iran’s neighbor country; Pakistan could reveal a particular difference in phylogeny of NDV isolates when they evaluate the sequences of HN, F and matrix (M) genes of the NDV isolates. The isolates were obtained from outbreaks among commercial poultry and also backyard poultry in various areas in Pakistan. The phylogenetic evaluation showed emergence of novel divergent group of NDV under the lineage 5, which are slightly different from NDV isolates previously obtained in the area (Munir et al., 2012). In another study in 2010 on virulent and velogenic strains of NDV which was isolated from a backyard and healthy poultry, the authors could reveal a Y526Q substitution in the HN protein. The aminoacids on that position play crucial role on determining the binding activity of the neuraminidase receptor and also in fusion activity of NDV(Munir et al., 2012). Also, Maxwell et al. reported variations in the HN sequence of freshly isolated ND samples from poultry farms in Uganda and the previously obtained sequences from ND isolates. The phylogenetic evaluations demonstrated that some of the new isolates differ in their genotype from the previously obtained isolates in Uganda (Otím et al., 2004). Another study showed that three local ND isolates could not be identified by the monoclonal antibodies specific
against known reference isolates of ND. These local isolates also had different variations in their HN sequence in comparison to the reference ND isolates (Hu et al., 2010b).

The results of the present study are also in agreement with the mentioned studies. We also observed that the Iranian ND isolates showed some differences in their HN sequence in comparison to isolates from other countries. In our hands, two NDV strains which were isolated from different farms in Iran, both were placed within a clade with common internal node and as a monophyletic group next to other genotypes of NDV from different countries. However, since there was a high similarity rate (94.5 to 94.9%) between the HN sequences of our isolates and an isolate from China (SGM01; DQ234592, VII), we might also say the Iranian isolated could be grouped also within VIId genotype. Most studies in Iran, reported NDV isolates belonging to the VIId genotype (Ahmadi et al., 2014; Mehrabanpour et al., 2014).

At present study we demonstrated that the NDV isolates belong to the VIId genotype regarding the results from HN phylogenetic analysis. The findings are in agreement with genotyping based on Fusion gene. In addition, the data helps us to design recombinant vaccine based on HN sequences of Iranian NDV isolates.

References:


Figure 1: The figure represents a phylogenetic tree constructed using various sequences of HN gene of NDV and also two sequences of Iranian isolates. The tree was constructed based on a specific algorithm (Neighbor-Joining) in MEGA software (version 7.0). Numbers on the tree represents the bootstrap value in percent (from 1000 replicates) supporting the reliability of the each branches of the tree. The lengths of the lines indicate the phylogenetic distances between each of the divergent sequences. The vertical lines were used to just place a space between names and numbers. There is a scale bar on the bottom of the tree which is a guide to measure the phylogenetic distance between sequences. Iranian isolates of NDV were characterized with Black Circles on tree. The sequences were obtained from Gene Bank.
Table 1: Amino acid sequence of haemagglutinin-neuraminidase protein: Comparison between Lasota vaccine strain and two Iranian NDVs.

<table>
<thead>
<tr>
<th>Virus</th>
<th>HN Protein</th>
<th>LaSota/Vaccine (KJ563939)</th>
<th>Iran_Chicken_H106 6_14</th>
<th>Iran_Chicken_H112 2_14</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25-45</td>
<td>193-201</td>
<td>26 3</td>
<td>28 7</td>
</tr>
<tr>
<td></td>
<td>26 3</td>
<td>28 7</td>
<td>32 1</td>
<td>33 2-33 3</td>
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<td>33 2-33 3</td>
<td>346-353</td>
<td>35 6</td>
<td>49 4</td>
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<tr>
<td></td>
<td>346-353</td>
<td>35 6</td>
<td>49 4</td>
<td>513-521</td>
</tr>
<tr>
<td>LaSota/Vaccine (KJ563939)</td>
<td>FRIAILFLT VTVTALISV ASLL</td>
<td>LSGCRDH SH N D K GK DEQDYQ IR K G RITRVSS SS</td>
<td>A28T I29V F31L T33M V35M V41A S43A L45A</td>
<td>A28T I29V F31L T33M V35M V41A S43A L45A</td>
</tr>
<tr>
<td>Iran_Chicken_H106 6_14</td>
<td></td>
<td></td>
<td>K</td>
<td>E347Q E514V</td>
</tr>
<tr>
<td>Iran_Chicken_H112 2_14</td>
<td></td>
<td></td>
<td>E</td>
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