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Original Article

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 that can spread fast between poultry houses and cause a heavy economic burden on the poultry industry all around the world. Fusion and hemagglutinin-neuraminidase (HN) protein are important in the pathogenesis of the Newcastle disease virus (NDV). The HN protein is a critical viral protein with multiple functions and plays a key role in the formation of the virulence of NDV. Head of HN protein is responsible for receptor binding, neuraminidase activity. This study aimed to investigate the sequence homology of hemagglutinin-neuraminidase of two NDV isolates sampled from infected farms in Iran. The samples were collected from flocks that had been vaccinated by both types of live and killed vaccines for NDV. After isolation of NDV, the viruses were subjected to the polymerase chain reaction (PCR) amplification using two pairs of specific primers designed for the HN gene to amplify the complete HN gene (1730bp). Afterward, the PCR products were sequenced and analyzed by phylogenetic tree construction software. Based on the analysis, substantial sequence homology among Iranian isolates is within the range of 97.1-100%. Moreover, the sequence homology searching revealed a level of similarity between HN sequences of Iranian isolates and the HN sequences from other countries, particularly Asian ones. For instance, a high homology ratio (95.34%) was found between Iranian isolates and the sequences registered on online molecular databases from China. Based on phylogenetic analysis, the NDV isolates belong to the VIId genotype. Finally, it can be concluded that monitoring the circulation of NDVs among poultry and other birds can help to obtain an insight into the evolution of NDVs and control of panzootic viruses in the future.

Keywords: Hemagglutinin-neuraminidase, Iran, Newcastle Disease Virus, Phylogenetic tree

Séquence de L'hémagglutinine-neuraminidase et Analyse Phylogénétique de Deux Virus de la Maladie de Newcastle Isolés de Poulets en Iran

Résumé: La maladie de Newcastle est une infection virale très contagieuse affectant de nombreuses espèces d'oiseaux qui peut se propager rapidement entre les poulaillers et causer un lourd fardeau économique à l'industrie avicole du monde entier. La fusion et la protéine hémagglutinine-neuraminidase (HN) sont importantes dans la pathogenèse du virus de la maladie de Newcastle (NDV). La protéine HN est une protéine virale critique avec de multiples fonctions et joue un rôle clé dans la formation de la virulence du NDV. La tête de la protéine HN est responsable de la liaison aux récepteurs, de l'activité de la neuraminidase. Cette étude visait à étudier l'homologie de séquence de l'hémagglutinine-neuraminidase de deux isolats de NDV prélevés

dans des fermes infectées en Iran. Les échantillons ont été prélevés dans des troupeaux qui avaient été vaccinés par les deux types de vaccins vivants et tués contre le NDV. Après isolement du NDV, les virus ont été soumis à l'amplification par réaction en chaîne par polymérase (PCR) en utilisant deux paires d'amorces spécifiques conçues pour le gène HN afin d'amplifier le gène HN complet (1730pb). Ensuite, les produits de PCR ont été séquencés et analysés par un logiciel de construction d'arbres phylogénétiques. Sur la base de l'analyse, une homologie de séquence substantielle parmi les isolats iraniens est comprise entre 97.1 et 100%. De plus, la recherche d'homologie de séquence a révélé un niveau de similitude entre les séquences HN des isolats iraniens et les séquences HN d'autres pays, notamment asiatiques. Par exemple, un rapport d'homologie élevé (95.34%) a été trouvé entre les isolats iraniens et les séquences enregistrées sur les bases de données moléculaires en ligne de Chine. Sur la base d'une analyse phylogénétique, les isolats de NDV appartiennent au génotype VIId. Enfin, on peut conclure que la surveillance de la circulation des NDV parmi les volailles et autres oiseaux peut aider à obtenir un aperçu de l'évolution des NDV et du contrôle des virus panzootiques à l'avenir.
Mots-clés: Hémagglutinine-neuraminidase, Iran, virus de la maladie de Newcastle, arbre phylogénétique

Introduction

Newcastle disease (ND) is one of the most prevalent and devastating viral infections in poultry with a high mortality rate. It is also responsible for a significant part of the heavy economic losses in poultry houses around the world. From the taxonomic perspective, the ND virus (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 the avian paramyxovirus-1.

The NDV contains an RNA genome with about 15 kb length coding six viral proteins in the following order: 5-NP-P-M-F-HN-L–3 (Cao et al., 2013). The NDV strains are grouped in two separate classes (i.e., I and II) within a single serotype. Class I contains nine genotypes (1–9) and class II includes 18 genetic groups, 10 of which (i.e., I–IX, and XI) have been previously established while five of them (i.e., X, XII, XIII, XIV, and XV) are new genotypes (Diel et al., 2012). Virulence of all NDV isolates all around the world is monitored by veterinary organizations. 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 World Organisation for Animal Health (OIE, 2018).

Virulence of NDVs is related to fusion (F) amino acid sequences at the cleavage site. However, hemagglutinin-neuraminidase (HN) protein, as a critical viral protein with multiple functions, plays a key role in forming the virulence of NDV. 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 good binding property, the HN protein can agglutinate red blood cells (RBCs).

The mechanism by which the HN protein can agglutinate the RBCs consists of binding 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., 2010).

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, highly virulent NDV infections have posed challenges to a considerable number of industrial farms (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 VIId (Hosseini et al., 2014).

Most publications analyzed NDVs based on F protein sequences. Nevertheless, there is little information about HN protein sequences and diversities (Esmaelizad et al., 2012).

The present study aimed to distinguish the genetic variation of two Iranian NDV isolates from current outbreaks by phylogenetic analysis of the HN sequences and compare them to HN sequences of NDV strains from other countries.

Material and Methods

Specimen Collection and Virus Culture. Two NDV-infected broiler flocks located in Gilan and Isfahan provinces were selected for specimen collection. Sampled chickens were displaying NDV-typical nervous symptoms and high mortality rates (more than 25%). The broiler flocks had been previously vaccinated with a routine vaccination program which includes both live and killed NDV vaccines.

Heads of the affected chickens were submitted to the Laboratory. To confirm the presence of NDV in infected chickens, the brains were cultured into the embryonated eggs to isolate the viruses and 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 evaluated using the hemagglutination assay method. Subsequently, allantoic fluids specimens with hemagglutination activity were subjected to the hemagglutination inhibition test according to 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 the allantoic fluid using RNeasy Mini Kit (Qiagen, Germany) according to the protocol provided by the manufacturer. Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Canada) was used to convert the viral RNA to the complementary DNA (cDNA) based on the recommendations of the manufacturer.

Afterward, the cDNAs produced during the synthesizing step were incorporated into PCR procedures along with HN-specific primers. Two pairs of primers, specific for the HN region were used for the amplification of the complete HN gene (1730 bp). Sequences of forward primers F1 and F2 were 5'-TTC ACA ACC TCC GTT CTA CC-3' and 5'-AAG TCT TGC AGT GTG AGT GC-3', respectively. Moreover, the sequences of reverse primers R1 and R3 were 5'-TTG CAC TCA CAC TGC AAG AC-3' and 5'-TCA TCT TTG AGG ATC TCA AC-3', respectively.

Each pair of primers, capable of amplifying a part of the HN gene sequence, was incorporated into a separate PCR assay; accordingly, 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 which included denaturation (94 °C for 30 sec), annealing (51 °C for 30s), and extension (72 °C for 40s). All these three steps were repeated for 35 cycles; subsequently, a prolonged extension period at 72 °C for 10 min was also set as a final step after completion of the 35 cycles.

Sequences and construction of a phylogenetic tree. The PCR products were visualized in two steps; first, they were run on a 1.5% gel electrophoresis, and afterward, they were stained with ethidium bromide. A PCR purification kit (AccuPrep® PCR Purification Kit, Bioneer Co., Korea) was used to purify the amplified DNA fragments from the PCR product. Afterward, 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 were configured according to the instructions described by the manufacturer. The samples were

sequenced using an ABI sequencing machine (Prism 310 Genetic Analyzer, Applied Biosystems, USA).

Subsequently, the raw results produced by the ABI machine, which were computer-produced nucleotide sequences, were checked for any errors or wrong data and edited before exporting to a digital file with FASTA format with the help of Chromas Lite software (version 2). The erroneous and ambiguous parts of the sequences were also omitted using the Mega software (version 7).

Next, the final purged sequences were used as 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 for smalls fragments of the template sequences in an extensive 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 the identity of sequences, they were submitted 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 a phylogenic tree, we retrieved several HN sequences registered on GenBank which were submitted by other research teams from different countries.

The strains names and accession numbers 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), ZHJ-3/97 (FJ766529), YZ-23-07-Pi (GQ281092), NDV05-029 (FJ766528), QH1 (FJ751918).

A phylogenetic tree was constructed by importing the retrieved HN sequences along with our two sequences into the MEGA software (version 7.0) (Kumar et al., 2016) and running the phylogenetic tree analysis by setting the specific algorithms. The algorithm which was used in the software for the construction of the tree was the Neighbor-Joining (NJ) (Offeddu et al.) method (1000 replicates for bootstrap).

Furthermore, 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 the 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 the NJ method. The values above 70 were supposed to be well-supported by the sequence alignments, while the lower values were not.

Results

The RT-PCR amplification of viral RNA using HN-specific primers resulted in the amplification of two viral RNA fragments with 1000 bp and 730 bp lengths. Each of the amplicons is in fact an amplified part of the original viral HN sequences. Afterward, the PCR-amplified products (HN gene) were sequenced. Molecular phylogeny of the Iranian NDV strain isolates as well as their similarities and possible relationships to other representative NDV strains from other countries were assessed based on genetic variations on the HN gene sequence.

It was found that the HN gene of the Iranian NDV isolates has high sequence similarity. The search for homology between the HN gene sequences of our isolates and those of other Iranian isolates showed a high level of similarity within the range of 98.9-100%. Moreover, the constructed phylogenetic tree revealed a close molecular relationship among our two Iranian NDV isolates (95.34%) and HN sequences which are from China, SGM01 (DQ234592).

Despite the fact that there were close phylogenetic relationships between Iranian NDV isolates and several genotypes of NDV, such as V, VI, and VIII, the closest genetic association was found with the VIId genotype. Isolates of this study were clustered in one monophyletic clade with a common internal node with

several genotypes of NDV (Figure 1). The difference between the La Sota vaccine strain and these two NDVs in antigenic sites are summarized in Table 1.

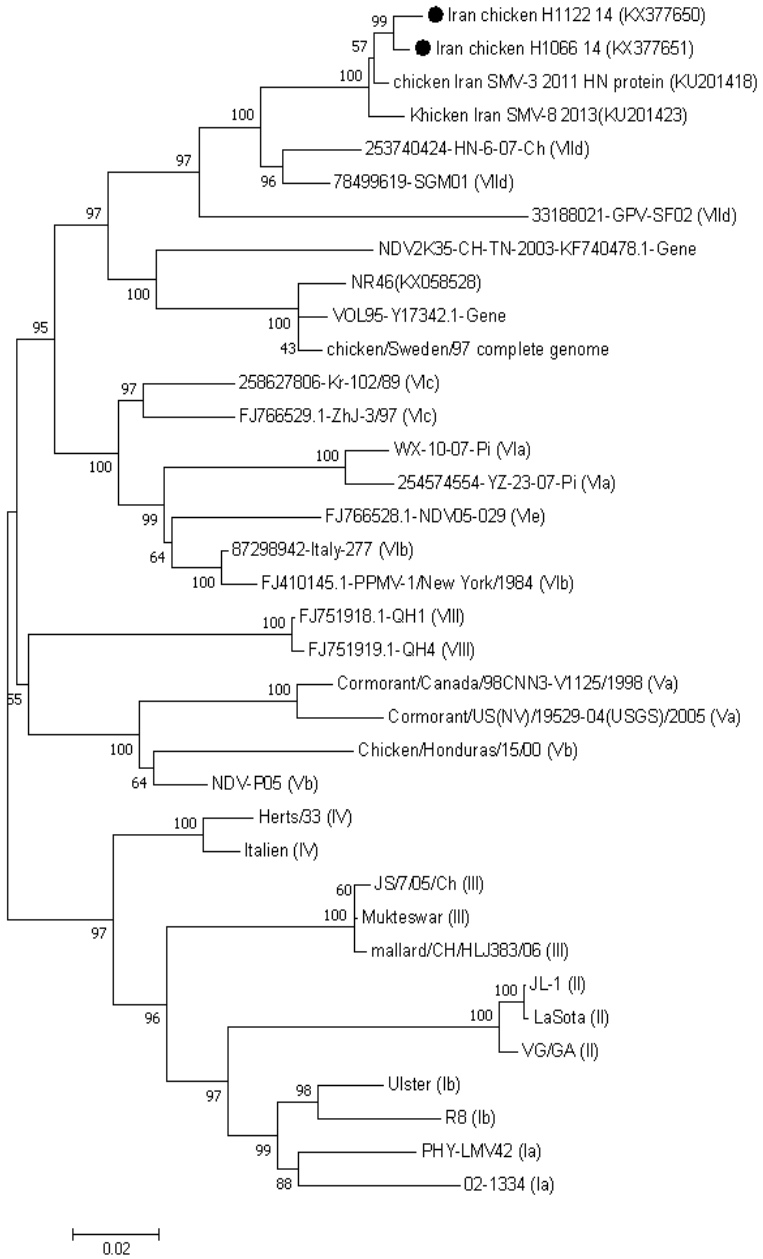

Figure 1. This figure represents a phylogenetic tree that was constructed using various sequences of hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) and also two sequences of Iranian isolates. The tree was constructed based on a specific algorithm (Neighbor-Joining) in MEGA software (version 7.0). Numbers on the tree represents the bootstrap value in percent (from 1000 replicates) supporting the reliability of each branch of the tree. Lengths of the lines indicate the phylogenetic distances between 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 are characterized with black circles on the tree. The sequences were obtained from Gene Bank.

Table 1. Amino acid sequence of haemagglutinin-neuraminidase protein: comparison of Lasota vaccine strain with two Iranian Newcastle disease viruses

Virus	Hemagglutinin-neuraminidase protein									
	25-45	**193-201**	**263**	**287**	**321**	**332-333**	**346-353**	**356**	**494**	**513-521**
LaSota/Vaccine (KJ563939)	FRIAILFLTVVTLAISVASLL	LSGCRDHSH	**N**	**D**	**K**	GK	DEQDYQIR	**K**	**G**	RITRVSSSS
Iran_Chicken_H1066_14	A28T I29V F31L T33M V35M V41A S43A L45A	-	K	-	-	-	E347Q	-	D	I514V
Iran_Chicken_H1122_14	A28T I29V F31L T33M V35M V41A S43A L45A	-	E	-	-	-	E347Q	-	D	I514V

Discussion

In the present study, two NDVs were isolated from vaccinated broilers in two different geographical locations. Based on the sequence and phylogenetic analysis of the F gene of these two viruses, they were close to VIId genotypes that had been previously reported in Iran (Kiani et al., 2016). However, limited information is available about the HN gene sequence of Iranian NDVs (Esmaelizad et al., 2012).

In this study, amplification of the complete HN gene was performed to find variations in the HN gene of the NDVs circulating in Iran. The ND is one of the most prevalent and economically important viral diseases of poultry in Iran. In order to clear the molecular footprint and study the genetics of the NDV deeply, the phylogenetic research on isolated strains of NDV in Iran seems to be of great importance.

Results of genetic studies can present us with a clear structure of the genetic contents of the virus which, in turn, provide us with critical information about the most important genes (virulence genes) as well as the taxonomy and even geographical origin of the virus. Viral particles of NDV, similar to most of the other viruses, have an envelope consisting of specific glycoproteins that act as an attachment facilitator, protection, and virulence agent for the virus.

The NDV, as a highly infectious virus, benefits from its structural virulent glycoprotein called HN which is named based on its hemagglutination and neuraminidase activity. The protein helps the viral particles to attach to host cells and attack the protective proteins on target cells. However, it was generally accepted that the two glycoproteins of NDV, i.e., HN and F, are the major viral agents responsible for its virulence (Diel et al., 2012; Cao et al., 2013).

According to evolutionary studies (e.g. phylogeny) on various strains of NDV in different areas (i.e., Asia and Europe), velogenic genotypes have spread extensively

in these areas and are responsible for the outbreaks. Recently, the most prevalent genotype which was involved in many of the NDV infection outbreaks all around Europe, South Africa, and the Far East during the 1990s was 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, namely VII-a to VII-e, are largely spread over China, Malaysia, Kazakhstan, and Kyrgyzstan. The remaining genotypes, from VII-f to VII-h, were repeatedly isolated within African countries (Ebrahimi et al., 2012).

Esmaelizad et al. (2012) studied phylogenetic analysis for the first time in Iran to find the origin of the HN gene of NDV isolates in 2012. They found an obvious sequence relationship in phylogenetic analysis among six Iranian isolates and NDV isolates from a country near Iran, Russia. Two Russian isolates, including Sterna-Astr/2755/2001 (VIIb) and VOL95 showed significant similarity with Iranian isolates. Surprisingly, both Russian NDV isolates had 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).

The HN plays an important role in pathogenesis and immunity; therefore, Shahriari et al. (2015) used the HN gene to produce a recombinant vaccine. Firouzamandi et al. (2016), have used the HN gene to improve immunity against NDV. Sometimes, the studies performed on genetic footprints lead the researchers to characterize new sub-genotypes or sublineages of viruses.

For instance, a group of researchers in one of the neighboring countries of Iran, Pakistan, found a particular difference in the phylogeny of NDV isolates when they were evaluating 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 the emergence of a novel divergent group of NDV under lineage 5 which are slightly different from NDV isolates previously obtained in the area (Munir et al., 2012).

In another study performed in 2010 on virulent and velogenic strains of NDV which were isolated from a backyard and healthy poultry, the researchers found a Y526Q substitution in the HN protein. The aminoacids on that position play a crucial role in determining the binding activity of the neuraminidase receptor and the fusion activity of NDV (Munir et al., 2012).

Moreover, Otim et al. (2004) reported variations in the HN sequence of freshly isolated ND samples from poultry farms in Uganda as well as 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 (Otim et al., 2004). According to the results of another study, three local ND isolates could not be identified by the specific monoclonal antibodies. These local isolates also had different variations in their HN sequence in comparison to the reference ND isolates (Hu et al., 2010).

Results of the present study are in line with those of the above-mentioned studies. It was also observed that the Iranian ND isolates were different from those of other countries in terms of their HN sequence. In this study, two NDV strains that were isolated from different farms in Iran were placed within a clade with a common internal node and as a monophyletic group next to other genotypes of NDV from different countries.

However, there was a high similarity rate (94.5-94.9%) between the HN sequences of isolates in this study and an isolate from China (SGM01; DQ234592, VII). Therefore, it can be said that the Iranian isolates could also be grouped within the VIId genotype. Based on the findings of most studies conducted in Iran, NDV isolates belong to the VIId genotype (Ahmadi et al., 2014; Mehrabanpour et al., 2014).

In the present study, it was found that the NDV isolates belong to the VIId genotype regarding the

results from HN phylogenetic analysis. These findings are consistent with genotyping based on fusion gene. In addition, the collected data helps us to design a recombinant vaccine based on HN sequences of Iranian NDV isolates.

Authors' Contribution

Sampling and technical procedures: M. H. K.
Data analysis: M. H. B.
Bioinformatics and technical procedures: H. H.
Paper discussions: S. Ch.
Bioinformatics and data analysis: A. Gh.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that there was no conflict of interest in this research.

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References

Ahmadi, E., Pourbakhsh, S.A., Ahmadi, M., Talebi, A., 2014. Pathotypic characterization of the Newcastle disease virus isolated from commercial poultry in northwest Iran. Turk J Vet Anim Sci 38, 383-387.

Cao, Y., Gu, M., Zhang, X., Liu, W., Liu, X., 2013. Complete Genome Sequences of Two Newcastle Disease Virus Strains of Genotype VIII. Genome Announc 1.

Diel, D.G., da Silva, L.H., Liu, H., Wang, Z., Miller, P.J., Afonso, C.L., 2012. Genetic diversity of avian paramyxovirus type 1: proposal for a unified nomenclature and classification system of Newcastle disease virus genotypes. MEEGID 12, 1770-1779.

Ebrahimi, M.M., Shahsavandi, S., Moazenijula, G., Shamsara, M., 2012. Phylogeny and evolution of Newcastle disease virus genotypes isolated in Asia during 2008–2011. Virus Genes 45, 63-68.

Esmaelizad, M., Ashtiani, M.P., NIARAKI, S.J., Hashemnejad, K., 2012. Identification of 23 specific nucleotide patterns in the HN gene of Newcastle disease viruses isolated from Iran. Turk J Biol 36, 135-142.

Firouzamandi, M., Moeini, H., Hosseini, D., Bejo, M.H., Omar, A.R., Mehrbod, P., *et al.*, 2016. Improved immunogenicity of Newcastle disease virus inactivated vaccine following DNA vaccination using Newcastle disease virus hemagglutinin-neuraminidase and fusion protein genes. J Vet Sci 17, 21-26.

Ghalyanchilangeroudi, A., Hosseini, H., Jabbarifakhr, M., Fallah Mehrabadi, M.H., Najafi, H., Ghafouri, S.A., *et al.*, 2018. Emergence of a virulent genotype VIIi of Newcastle disease virus in Iran. Avian Pathol 47, 509-519.

Hosseini, H., Ghalyanchi-Langeroudi, A., Torabi, R., 2014. Molecular Characterization and Phylogenetic Study of Newcastle Disease Viruses Isolated in Iran, 2010-2012. Avian Dis 3, 373-376.

Hu, S., Wang, T., Liu, Y., Meng, C., Wang, X., Wu, Y., *et al.*, 2010. Identification of a variable epitope on the Newcastle disease virus hemagglutinin-neuraminidase protein. Vet Microbiol 140, 92-97.

Kiani, M.H., Bozorgmehrifard, M.H., Hosseini, H., Charkhkar, S., Ghalyanchi, A., 2016. Molecular Characterization and Phylogenetic Study of Newcastle Disease Viruses Isolated in Iran, 2014–2015. Iran J Virol 10, 53-57.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33, 1870-1874.

Manual, O.T., 2018. Newcaslte Disease.

Mehrabanpour, M.J., Khoobyar, S., Rahimian, A., Nazari, M.B., Keshtkar, M.R., 2014. Phylogenetic characterization of the fusion genes of the Newcastle disease viruses isolated in Fars province poultry farms during 2009-2011. Veterinary research forum: an international quarterly journal. Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, p. 187.

Munir, M., Cortey, M., Abbas, M., Afzal, F., Shabbir, M.Z., Khan, M.T., *et al.*, 2012. Biological characterization and phylogenetic analysis of a novel genetic group of Newcastle disease virus isolated from outbreaks in commercial poultry and from backyard poultry flocks in

Pakistan. Infect Genet Evol 12, 1010-1019.

Offeddu, V., Cowling, B.J., Peiris, J.M., 2016. Interventions in live poultry markets for the control of avian influenza: a systematic review. One Health 2, 55-64.

OIE, M., 2018. Manual of diagnostic tests and vaccines for terrestrial animals. Paris: Office International Des Epizooties.

Otim, M.O., Christensen, H., Jørgensen, P.H., Handberg, K.J., Bisgaard, M., 2004. Molecular characterization and phylogenetic study of Newcastle disease virus isolates from recent outbreaks in eastern Uganda. J Clin Microbiol 42, 2802-2805.

Sabouri, F., Vasfi Marandi, M., Bashashati, M., 2018. Characterization of a novel VIIl sub-genotype of Newcastle disease virus circulating in Iran. Avian Pathol 47, 90-99.

Samadi, S., Kianizadeh, M., Najafi, M.F., Nasab, S.D.M., Davatgar, A.M.H., Royaee, A., *et al.*, 2014. Molecular characterization and phylogenetic study of velogenic Newcastle disease virus isolates in Iran. Virus Genes 48, 290-295.

Shahriari, A.G., Bagheri, A., Bassami, M.R., Malekzadeh Shafaroudi, S., Afsharifar, A.R., 2015. Cloning and expression of fusion (f) and haemagglutinin-neuraminidase (hn) epitopes in hairy roots of tobacco (nicotiana tabaccum) as a step toward developing a candidate recombinant vaccine against newcastle disease. J Cell Mol Res 7, 11-18.