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Identification of 23 specific nucleotide patterns in the HN gene of Newcastle disease viruses isolated from Iran

Majid ESMAELIZAD, Mehdi P. ASHTIANI, Saber JELOKHANI-NIARAKI, Khadije HASHEMNEJAD
Department of Biotechnology, Razi Vaccine and Serum Research Institute, Karaj - IRAN

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Abstract: Newcastle disease is considered the most contagious poultry disease and may cause severe economic loss in the poultry industry. The present study was undertaken to focus on molecular and phylogenetic analyses of the hemagglutinin-neuraminidase (HN) gene in the Iranian isolates. The nucleotide sequence of the HN (780-903 nt) coding region of 6 Newcastle disease viruses (NDVs), isolated from various outbreaks in Iran, was determined and aligned with previously published sequences of 26 NDV isolates in GenBank; the resulting alignment was then analyzed in terms of genetic variation. The number of nucleotide sequence variations presented by each of the isolates indicated that all 6 Iranian isolates contained 23 single nucleotide polymorphisms in the HN coding region. These polymorphisms can be considered signature nucleotides in this lineage. Specific positions are as follow: T795→C, T918→C, T1230→C, T1269→C, A819→G, A990→G, A1041→G, A1293→G, A1523→G, C834→T, C1299→T, C1383→T, C1386→T, C1545→T, C1560→T, G906→T, G1134→A, G1368→A, G1251→C, C1266→A, T1344→G, A1374→T, and T1401→A. Sequence analysis of the isolates showed that the 6 Iranian isolates examined share significant similarity with 2 Russian isolates, Sterna-Astr/2755/2001 and VOL95. Interestingly, both Russian isolates have the same nucleotide pattern as the Iranian isolates in all 23 nucleotide positions, except for isolate Sterna-Astr/2755/2001, which is only different at position 1560. The results may reflect that certain migratory birds, such as Sterna albifrons Pallas, might have contributed to the distribution of NDV in Russia and Iran. These best-known migratory birds travelling between Iran and Russia play a crucial role in the transmission of NDV.

Key words: RT-PCR, Newcastle disease virus, hemagglutinin-neuraminidase, single nucleotide polymorphism, Iran

Introduction

Newcastle disease (ND) is an extremely contagious viral disease of birds affecting domestic poultry, caged pet birds, and wild birds. ND has been shown to infect 250 species of birds in each age group. Since chickens are generally the most susceptible hosts, the disease has had a dramatic impact on the poultry industry, leading to tremendous economic losses. The World Organization for Animal Health considers ND to be one of the most important infectious diseases (1,2), and the Newcastle disease virus (NDV) continues to be regarded as one of the most important viral pathogens of poultry in the world (3). The etiological agent of ND, also known as fowl pest, is NDV, which is classified within the genus *Avulavirus* as a member of the family *Paramyxoviridae*. This virus has been divided into 2 classes and at least 9 genotypes on the basis of F gene phylogenetic analysis. Naturally occurring NDV isolates from outbreaks in different geographical areas exhibit a range of virulence variation among chickens (4-6). NDV outbreaks differ in clinical symptoms, disease severity, and virus transmissibility depending on the virus strain. ND is mainly characterized by respiratory distress,

diarrhea, circulatory disturbances, and central nervous system impairment (7). When disease outbreaks occur, disease-free regions suffer trade limitations and economic outcomes. Different strains of NDV can be categorized into 5 pathotypes based on their pathogenicity, depending on the disease intensity occurring in chickens: 1) viscerotropic velogenic, which produces a severe lethal infection along with hemorrhagic lesions seen in the intestine; 2) neurotropic velogenic, leading to a disease with high mortality resulting from respiratory and neurological symptoms; 3) mesogenic, causing a disease with low mortality, but with acute respiratory illness and neurological signs seen in some birds; 4) lentogenic, leading to a mild form of respiratory infection; and 5) asymptomatic enteric, an enteric infection with no visible signs of disease (1,8). Briefly, lentogenic strains do not cause disease and have been considered to be avirulent. Mesogenic viruses are characterized by intermediate virulence, and the virulent viruses resulting in high mortality are called velogenic. The viral particle has a nonsegmented, single-stranded negative RNA genome of about 15.2 kb nucleotides long. The RNA genome comprises 6 genes coding for RNA-directed RNA polymerase (L gene), hemagglutinin-neuraminidase (HN gene), fusion (F gene), matrix (M gene), phosphoprotein (P gene), and nucleocapsid (NC gene) proteins with the order of 5′ terminus to the 3′ terminus (5,9,10). The membrane of NDV contains 2 transmembrane glycoproteins: hemagglutinin-neuraminidase (HN) and fusion protein. In addition, a membrane-associated nonglycosylated M protein is located under the lipid bilayer, interacting with the HN protein and the nucleocapsid (11,12). The ability of NDV to agglutinate red blood cells (RBCs) is the result of binding the HN protein to receptors on the surface of the RBCs (13). The life cycle of NDV consists of RNA transcription by the viral RNA polymerase complex in order to generate the mRNAs that are needed for the creation of viral proteins. Viral RNA replication then begins to synthesize a full-length, positive-sense antigenome, which takes a turn as a template for the production of a full-length viral genome (6). The HN glycoprotein has different roles in attachment, fusion promotion activities, and the removal of sialic acid from progeny virions; it therefore acts as a neuraminidase. The HN protein is a main antigenic determinant in NDVs and has the responsibility of adsorbing the virus to sialic acid-containing receptors. In addition to this, HN contributes to the enzymatic cleavage of sialic acid (neuraminidase activity) from the surface of virions and infected cells (14). The HN gene is 1998 nt long, with a coding region involving 577 amino acids. The HN of some strains of NDV is produced as a biologically passive precursor (HN_0) and 90 residues are removed from the C-terminus in order to activate the molecule (15-17).

The main objectives of the present study were to provide some insight into the phylogenetic relationships among the NDVs circulating in Iran and other isolates from around the world, and to analyze the HN gene nucleotide sequences of sampled isolates from Iran.

Material and methods

Viruses and RNA extraction

Clinical samples of NDV were submitted to Razi Vaccine and Serum Research Institute. The serological test was carried out according to standard methods to confirm the presence of NDV. The laboratory of Poultry Disease Diagnosis collected a repository of NDV specimens, from which 27 field isolates, sampled from different states in Iran, were taken as viruses grown in specific pathogen-free embryonated chicken eggs. Viral RNA was extracted from the infected allantoic fluid using an RNX reagent (CinnaGen, Iran). In brief, allantoic fluid (1 mL) was centrifuged at 85,000 × *g* at 4 °C for 2 h. The pellet was resuspended in 200 µL of PBS and then in 400 µL of RNX reagent, mixed well, and left at room temperature for 5 min. Extraction was done with 0.2 mL of chloroform:isoamyl alcohol (24:1). The RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol. The mixture was centrifuged at 10,000 × *g* for 20 min. The pellet was washed with 75% ethanol and dissolved in 20 µL of RNase-free water.

Reverse transcription-polymerase chain reaction (RT-PCR)

In order to amplify the HN coding region, 2 forward oligonucleotide primers, F1 (5'-TTC ACA ACC TCC GTT CTA CC-3') and F2 (5'-AAG TCT TGC AGT

GTG AGT GC-3'), and 3 reverse oligonucleotide primers, R1 (5'-TTG CAC TCA CAC TGC AAG AC-3'), R2 (5'-TGA GAT GTC CCT ACT GTG AG-3'), and R3 (5'-TCA TCT TTG AGG ATC TCA AC-3'), were designed in this study using OLIGO software; this rendered amplification fragments from 780 to 903 bp. Reverse transcription of the viral RNAs was carried out in a 50-μL reaction mixture containing 10 μL of 5× reaction buffer, 4 μL of mixed dNTPs (2.5 mM each), 1 μL of AMV enzyme (Titan One Tube RT-PCR system kit, Roche Diagnostic, Germany), 1 μL of each primer (10 pmol each), 4 μL of RNA template, 2.5 μL of DTT, 3 μL of 25 mM $MgCl_2$, and 23.5 μL of H_2O. In vitro sequence amplification was done with a programmable thermocycler administering the following program: 42 °C for 30 min; 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 40 s; followed by a final extension at 72 °C for 5 min.

Sequencing and sequence analysis

After the amplification of DNA fragments, the fragments were purified using a PCR product purification kit (Roche Diagnostic) following the recommendations of the suppliers. PCR products of 6 isolates from the 27 samples were selected and applied to low melting point agarose, and the distinct bands were purified from gel for direct sequencing (MWG Co., Germany). Purified fragments were sequenced from both directions. The published sequences of 26 NDV isolates recovered from different parts of the world were included in this analysis and compared with the corresponding sequences from Iran. Reference NDV sequences were obtained from the National Center for Biotechnology Information (NCBI). To determine the degree to which genetic diversity is reflected in the HN genes, multiple alignments and comparisons of the nucleotide sequences of isolates were carried out (Figure 1). The nucleotide sequence homology/divergence was calculated using the MegAlign tool of DNAStar software package version 5.1 (DNAStar, USA). Descriptions of the viruses examined in this study along with the NDV sequences taken from GenBank are given in the Table. The nucleotide sequences of the HN coding region of the Iranian isolates were submitted to the GenBank database under the accession numbers AF548619, AF548620, AY370771, AY372671, AY372672, and AY372673.

Results and discussion

The evolution of RNA viruses has been broadly investigated and findings show that virus evolution is a complicated process in nature. During RNA replication, these viruses rapidly generate genetically different variants (18). According to earlier observations, it has been revealed that various strains of NDV may evolve via synonymous mutations, and there is no evidence to support the idea that the recombination process can cause evolution (19). Viral genome sequence data are undoubtedly appropriate tools for tracking the origin of ND outbreaks and monitoring the field isolates in areas in which the disease is endemic. Poultry industries in Iran are affected by the velogenic and viscerotropic strains of NDV. As in many other countries, ND is one of the most important restrictions on the profitability of poultry farms in Iran's villages (20). NDV includes 2 glycoproteins that are considered to be connected with the viral envelope. The HN glycoprotein is included in hemagglutinating and neuraminidase actions that have the responsibility of binding the virus to host cell receptors and destroying receptors, respectively. The fusion (F gene) is the protein responsible for fusion with the host cell membrane. It has long been thought that the 2 glycoproteins mentioned above can affect the virulence of NDV strains (13,15). In the family *Paramyxovirinae*, NDV is a unique member that infects birds but cannot be spread to mammals (10). A nucleotide sequence determination of the HN protein from Iranian isolates would help to clarify the origin of the disease. The nucleotide sequence of the HN coding region was determined for 6 isolates obtained from 27 clinical samples collected from different provinces of Iran, including Ghom, Isfahan, Kerman, Ghazvin, Azarbaijan, and Khorasan. These sequences were aligned and compared with 26 published reference sequences deposited in the GenBank database. All NDV isolates studied from the outbreaks in Iran showed a fairly limited degree of nucleotide sequence variation in the HN gene, with values of more than 98% genetic similarity found among the isolates (Figure 1). A careful analysis of the 32 isolates allowed for the identification of single nucleotide polymorphisms (SNPs). Surprisingly, all 6 Iranian isolates indicated 23 SNPs in the HN coding region; this finding has been considered a diagnostic SNP marker in this lineage. The 23 SNP markers are:

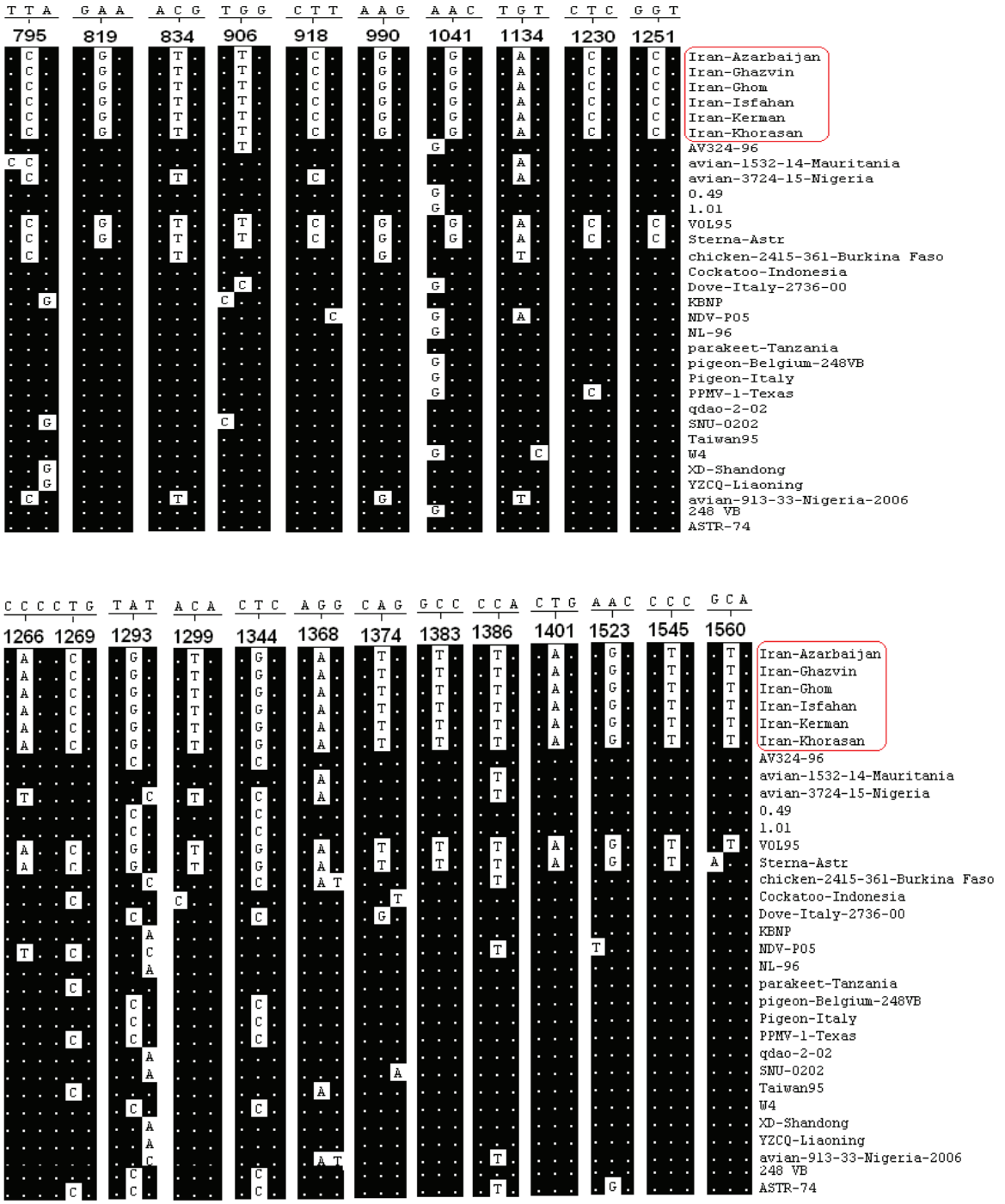

Figure 1. Numbers 795 to 1560 indicate the 23 polymorphic positions in the HN gene nucleotide sequence of 6 Iranian isolates. A dot (.) indicates sequence identity in relation to the Iranian isolates (Iran-Ghom, Iran-Khorasan, Iran-Isfahan, Iran-Kerman, Iran-Ghazvin, and Iran-Azarbaijan). Sequence variation is presented with a single-letter code. Accession numbers are as detailed in the Table.

Table. Descriptions and origins of the Newcastle disease viruses studied.

Serial No.	Isolate	Location	Date of collection	Accession No.
1	Iran-Ghom	Iran	2002	AF548619
2	Iran-Khorasan	Iran	2002	AF548620
3	Iran-Isfahan	Iran	2002	AY370771
4	Iran-Kerman	Iran	2002	AY372671
5	Iran-Ghazvin	Iran	2002	AY372672
6	Iran-Azarbaijan	Iran	2002	AY372673
7	NL-96	China	1996	AF204872
8	qdao-2-02	China	2002	DQ023152
9	XD-Shandong/08	China	2008	FJ608365
10	YZCQ-Liaoning/08	China	2008	FJ608369
11	W4	China	2005	HM063423
12	0.49	UK	unknown	EF026580
13	1.01	UK	unknown	EF026582
14	248 VB	Belgium	1998	EF026584
15	Pigeon-Belgium-248VB/1998	Belgium	1998	EF026579
16	SNU-0202	South Korea	2000	EU140951
17	KBNP	South Korea	2004	EU140954
18	Parakeet-Tanzania, Belgium, China/28710/93	USA	1993	AY288988
19	Pigeon-Italy/1166/00	USA	2000	AY288996
20	Cockatoo-Indonesia/14698/90	USA	1990	AY288998
21	PPMV-1-Texas-1998	USA	1998	FJ410148
22	Avian-3724-15-Nigeria-2008	Nigeria	2008	FJ772488
23	Avian-913-33-Nigeria-2006	Nigeria	2006	FJ772448
24	Avian-1532-14-Mauritania-2006	Mauritania	2006	FJ772454
25	Chicken-2415-361-Burkina Faso-2008	Burkina Faso	2008	FJ772457
26	AV324-96	Ireland	1996	GQ429292
27	Dove-Italy-2736-00	Italy	2000	GQ429293
28	NDV-P05	Mexico	2005	HM117720
29	Taiwan95	Taiwan	1995	NDU62620
30	Sterna-Astr/2755/2001	Russia	2001	AY865652
31	VOL95	Russia	1995	Y17342
32	ASTR-74	Russia	1974	Y18725

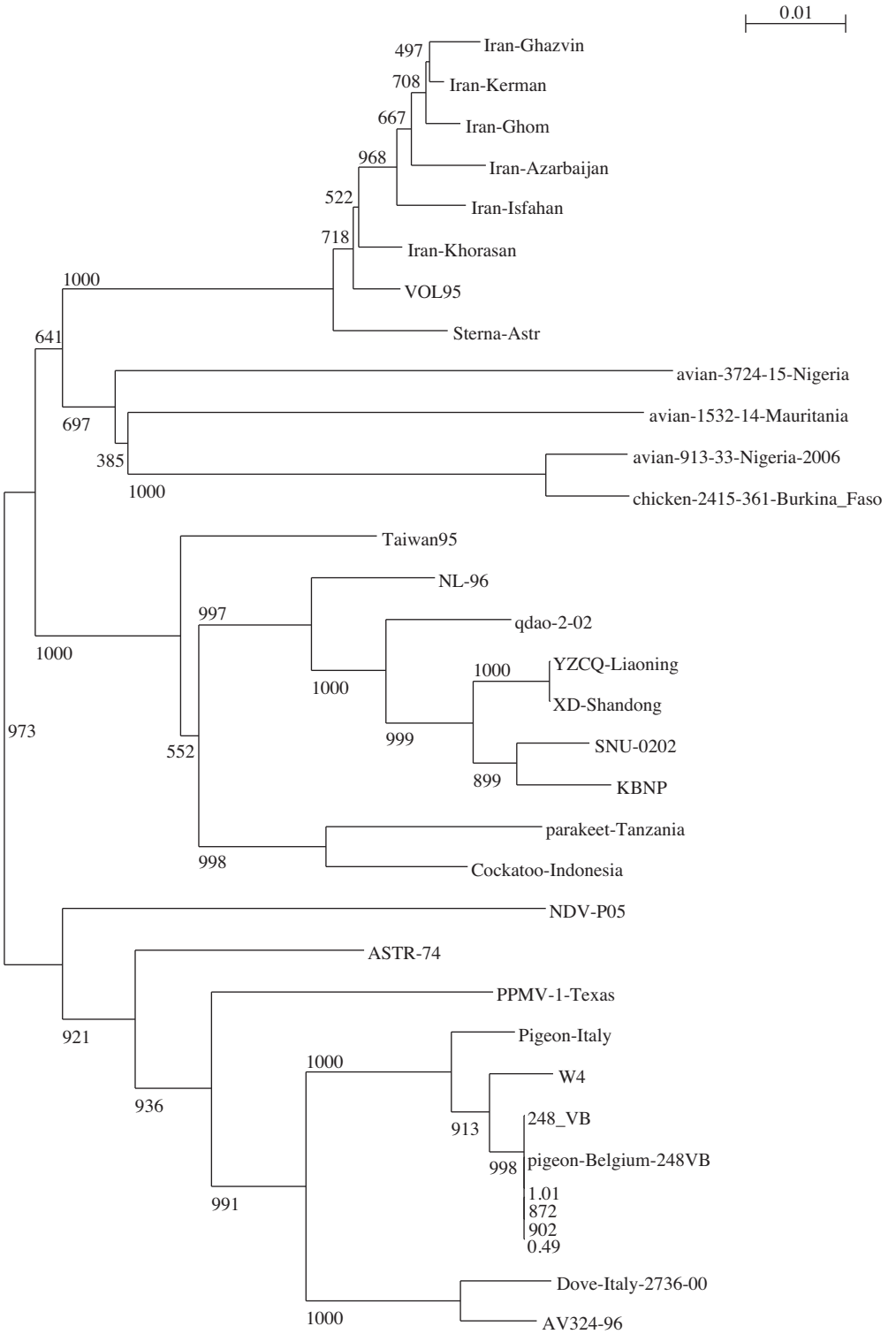

Figure 2. The phylogenetic relationship among 6 of the Iranian isolates examined and 26 sequences of NDV obtained from the GenBank database. The lineages and bootstrap values are shown in the tree.

T→C at positions 795, 918, 1230, and 1269; A→G at positions 819, 990, 1041, 1293, and 1523; C→T at positions 834, 1299, 1383, 1386, 1545, and 1560; G→T at position 906; G→A at positions 1134 and 1368; G→C at position 1251; C→A at position 1266; T→G at position 1344; A→T at position 1374; and T→A at position 1401. These signature nucleotides in the HN proteins are of value for genetic studies.

In order to conduct a phylogenetic analysis, a 800-base pair (bp) fragment of the HN gene of 6 field isolates obtained from RT-PCR and a set of 26 sequences of the homologous region of NDV isolates registered in GenBank were used. Figure 2 shows a phylogenetic tree generated based on the sequence alignment of 32 genomes that are distinctly clustered into 5 lineages. The Iranian isolates (Iran-Ghom, Iran-Khorasan, Iran-Isfahan, Iran-Kerman, Iran-Ghazvin, and Iran-Azarbaijan) clustered with 2 Russian isolates into a separate branch from the other NDV isolates (lineage A). Sequence distance analysis indicates that the Iranian isolates grouped in lineage A share a similarity of over 97% with isolates Sterna-Astr/2755/2001 and VOL95. A minimum homology of 89.5%-90% in the nucleotide sequence was noted between the Iranian isolates and the other 29 remaining isolates (data not shown). The topology of the phylogenetic tree revealed that the other remaining isolates of the present study originated from different geographical locations and formed 4 genetically different lineages (lineages B, C, D, and E).

According to phylogenetic interpretations of RNA viruses, virus genotypes showing 2%-5% difference from each other are mostly believed to originate from the same geographical region (21). As can be seen in Figure 1, both of the Russian isolates (Sterna-Astr/2755/2001 and VOL95) have genomic patterns that are identical to the viruses isolated in Iran in all 23 nucleotide positions, except for isolate Sterna-Astr/2755/2001, which differs only at position 1560. The phylogenetic analysis based on the HN gene is in complete agreement with an earlier study documenting the possible role of migratory birds in the spread of ND (1). There might be some carrier birds immigrating to the different geographical regions in question. Migratory birds such as *Sterna albifrons* Pallas might have contributed to the distribution of NDV in Russia and Iran. These birds travelling between Iran and Russia play a remarkable role in the transmission of NDV.

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Corresponding author:

Majid ESMAELIZAD

Department of Biotechnology,

Razi Vaccine and Serum Research Institute,

Karaj - IRAN

E-mail: m.esmaelizad@rvsri.ir

References

1. Linde AM, Munir M, Zohari S et al. Complete genome characterization of a Newcastle disease virus isolated during an outbreak in Sweden in 1997. Virus Genes 41: 165-173, 2010.
2. Singh K, Jindal N, Gupta SL et al. Detection of Newcastle disease virus genome from the field outbreaks in poultry by reverse transcription - polymerase chain reaction. Int J Poult Sci 7: 472-475, 2005.
3. Huang Y, Wan HQ, Liu HQ et al. Genomic sequence of an isolate of Newcastle disease virus isolated from an outbreak in geese: a novel six nucleotide insertion in the non-coding region of the nucleoprotein gene. Arch Virol 149: 1445-1457, 2004.
4. de Leeuw OS, Koch G, Hartog L et al. Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemagglutinin-neuraminidase protein. J Gen Virol 86: 1759-1769, 2005.
5. Huang Z, Krishnamurthy S, Panda A et al. Newcastle disease virus V protein is associated with viral pathogenesis and functions as an alpha interferon antagonist. J Virol 77: 8676-8685, 2003.

6. Phillips RJ, Samson AC, Emmerson PT. Nucleotide sequence of the 5'-terminus of Newcastle disease virus and assembly of the complete genomic sequence: agreement with the "rule of six". Arch Virol 143: 1993-2002, 1998.

7. Lin MY, Liu HJ, Ke GM. Genetic and antigenic analysis of Newcastle disease viruses from recent outbreaks in Taiwan. Avian Pathology 32: 345-350, 2003.

8. Momayez R, Gharahkhani P, Pourbakhsh SA et al. Isolation and pathogenicity identification of avian paramyxovirus serotype 1 (Newcastle disease) virus from a Japanese quail flock in Iran. Arch Razi Ins 62: 39-44, 2007.

9. Gould AR, Kattenbelt JA, Selleck P et al. Virulent Newcastle disease in Australia: molecular epidemiological analysis of viruses isolated prior to and during the outbreaks of 1998-2000. Virus Res 77: 51-60, 2001.

10. de Leeuw O, Peeters B. Complete nucleotide sequence of Newcastle disease virus: evidence for the existence of a new genus within the subfamily Paramyxovirinae. J Gen Virol 80: 131-136, 1999.

11. Cobaleda C, Munoz-Barroso I, Sagrera A et al. Fusogenic activity of reconstituted newcastle disease virus envelopes: a role for the hemagglutinin-neuraminidase protein in the fusion process. Int J Biochem Cell B 34: 403-413, 2002.

12. Munoz-Barroso I, Cobaleda C, Zhadan G et al. Dynamic properties of Newcastle disease virus envelope and their relations with viral hemagglutinin-neuraminidase membrane glycoprotein. Biochim Biophys Acta 1327: 17-31, 1997.

13. Kianizadeh M, Ideris A, Shahrabadi MS et al. Biological and molecular characterization of Newcastle disease virus isolated from Iran. Arch Razi Ins 50: 1-10, 1999.

14. Lamb RA, Kolakofsky D. Paramyxoviridae. In: Fields BN, Knipe DM and Howley PM. eds. The Viruses and Their Replication. Lippincott-Raven Publishers; 1996: pp. 477-522.

15. Millar NS, Chambers P, Emmerson PT. Nucleotide sequence of the fusion and haemagglutinin-neuraminidase glycoprotein genes of Newcastle disease virus, strain Ulster: molecular basis for variations in pathogenicity between strains. J Gen Virol 69: 6132-6120, 1988.

16. Millar NS, Chambers P, Emmerson PT. Nucleotide sequence analysis of the hemagglutinin-neuraminidase gene of Newcastle disease virus. J Gen Virol 67: 1917-1927, 1986.

17. Nagai Y, Klenk HD, Rott R. Proteolytic cleavage of the viral glycoproteins and its significance for the virulence of Newcastle disease virus. Virology 72: 494-508, 1976.

18. Dopazo J, Sobrino F, Palma EL et al. Gene encoding capsid protein VP1 of foot and mouth disease virus: a quasispecies model of molecular evolution. Proc Natl Acad Sci USA 85: 6811-6815, 1988.

19. Gould AR, Hansson E, Selleck K et al. Newcastle disease virus fusion and haemagglutinin-neuraminidase gene motifs as markers for viral lineage. Avian Pathol 32: 361-373, 2003.

20. Hassanzadeh M, Bozorgmeri Fard MH. A serological study of Newcastle disease in pre- and post-vaccinated village chickens in north of Iran. Int J Poult Sci 10: 658-661, 2004.

21. Malirat V, de Barros JJ, Bergmann IE et al. Phylogenetic analysis of foot-and-mouth disease virus type O re-emerging in free areas of South America. Virus Res 124: 22-28, 2007.