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Molecular characterization of haemagglutinin-neuraminidase gene among virulent Newcastle disease viruses isolated in Iran

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Summary

Background: Virulent Newcastle disease virus (vNDV) causes great economic losses to the poultry industry throughout the world. Despite the endemicity of Newcastle disease (ND) and occurrence of recurrent outbreaks, the nature and genetic features of circulating NDV strains in Iran are largely unknown. **Aims:** This study was conducted to characterize 13 NDV isolates obtained from different outbreaks in various regions of Iran during 1999-2000 by sequencing and phylogenetic analysis of complete coding sequences of haemagglutinin-neuraminidase (HN) gene. **Methods:** All isolates were analyzed based on the previously determined *in vivo* pathogenicity indices and amino acid (aa) sequences of fusion (F) protein cleavage site (FPCS). **Results:** Phylogenetic analysis based on the HN gene coding region revealed a very close relationship of these viruses with the recently defined genotype XIII, and more specifically, subgenotype XIIIa viruses. Analysis of HN gene nucleotide (nt) sequences revealed that all studied isolates encode for a protein length of 571 aa and there is no C-terminal extension on HN aa sequences. Sequence analysis revealed multiple aa residue substitutions at antigenic sites or neutralizing epitopes on the HN glycoprotein of studied viruses compared with commonly used vaccinal strains. **Conclusion:** In this study, molecular characterization of vNDV isolates, obtained from commercial poultry farms in Iran, were conducted through complete sequencing and analysis of HN gene. Isolation and molecular characterization of further NDV isolates from other parts of Iran and from neighboring countries in the region will be helpful to identify the nature and origin of indigenous viruses.

Key words: Hemagglutinin-Neuraminidase gene, Iran, Newcastle disease virus, Phylogenetic analysis, Poultry

Introduction

Newcastle disease virus (NDV) or avian paramyxovirus 1 (APMV-1) is the causative agent of a highly contagious fatal disease of a broad range of avian species (Alexander *et al*., 2012; Miller and Koch, 2013). Newcastle disease virus encodes six structural proteins, among which only fusion (F), haemagglutinin-neuraminidase (HN), and matrix (M) proteins interact with viral envelope and are responsible for the main pathogenic and antigenic features of NDV strains (Kai *et al*., 2015). The HN glycoprotein is multifunctional and has various activities such as haemagglutination (HA), neuraminidase (NA), and F promotion activities during the process of viral infectivity and pathogenicity and is the main antigenic determinant of the paramyxoviruses (Lamb and Kolakofsky, 1996). According to recent updates, NDV strains are grouped into two classes, I and II, according to genetic variation and phylogenetic analysis of complete coding region of F gene. Class I strains contain only one genotype including non-virulent strains, while class II strains are composed of 18 genotypes from I to XVIII including both non-virulent and virulent strains (Diel *et al*., 2012; De Almeida *et al*., 2013; Snoeck *et al*., 2013).

Newcastle disease viruses have been classified into four major pathotypes including avirulent (asymptomatic enteric), mildly virulent (lentogenic), moderately virulent (mesogenic), and virulent (velogenic) based on *in vivo* pathogenicity tests such as mean death time (MDT) and intracerebral pathogenicity index (ICPI) (OIE, 2004). Virulence of NDVs may also be determined by prediction of amino acid (aa) sequences of F glycoprotein cleavage site (FGCS) at molecular level (OIE, 2004) but not in all NDV strains (Dortmans *et al*., 2009). Apparently, the FGCS may not be the only factor involved in pathogenicity of NDVs. Therefore, other parts of the NDV genome, mainly HN gene, have to be involved in full expression of viral pathogenicity. In spite of the crucial role that HN plays in virulence and immunostimulation of NDVs, there is limited data in the literature on molecular characteristics of this gene and there is a paucity of information on HN gene among Iranian NDVs (Esmaelizad *et al*., 2012; Esmaelizad and Ashtiani, 2015).

In this study, 13 NDV isolates partly characterized previously by partial sequencing of the F gene (not published) and *in vivo* pathogenicity indices in Razi

Vaccine and Serum Research Institute (RVSRI) in Karaj, Iran, were further studied by complete nucleotide (nt) sequencing of the HN gene.

Materials and Methods

Viruses

During a national surveillance plan conducted by RVSRI, from January 1999 to February 2000, samples suspected of Newcastle disease (ND) were collected from commercial chicken farms showing high mortality rate ranging from 10 to 80%. All applicable international, national, and institutional guidelines for the use and care of animals were followed. Approximate locations of sampled farms are shown on the map in Fig. 1. From NDV collection obtained during this national surveillance, 13 velogenic isolates were chosen (OIE, 2004; Abdoshah *et al*., 2012; Hemmatzadeh and Kazemimanesh, 2017). The main characteristics of 13 NDV isolates of this study including GenBank accession numbers are shown in Table 1.

Reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNAs were extracted from all HA-positive and HI-confirmed allantoic fluids using High Pure Viral RNA Isolation kit (Roche, Germany) as described by the manufacturer. cDNA synthesis was carried out by using random hexamers and with the RevertAid® Reverse Transcriptase kit (Fermentas-Thermo Fisher Scientific, Canada) in a 20 µL reaction volume containing 1 ng viral RNA, 60 pM final concentration of primer, 20 mM each dNTPs, 80 U Mmulv enzyme Revertaid®, 5 U Riboluck® RNase inhibitor, 4 µL 5X reaction buffer, and up to 20 µL DEPC treated water. The reaction mixture was incubated for 60 min at 45°C followed by 10 min at 70°C.

Overlapping PCR products covering complete coding region of HN gene were accomplished using three pairs of specific primers. Three primer pairs were designed using available sequences from the National Center for Biotechnology Information (NCBI) database and synthesized by Cinnagen (Iran) (Table 2).

The PCR reaction mixture for each sample consisted of 50 mM $MgCl_2$, 2.5 µL 10X PCR buffer, 10 mM each

Fig. 1: Sites of sample collection in different provinces of Iran. Approximate locations of sample collection are indicated by black squares. Numbers on the map correspond to the following provinces: 1: Tehran, 2: Ghom, 3: Ghazvin, 4: Isfahan, 5: Kordestan, 6: Ardabil, 7: East Azarbaijan, 8: West Azarbaijan, 9: Khorasan, and 10: Fars

Table 1: Characteristics of 13 Iranian NDV field isolates used in this study

NDV isolate	Strain	Province	MDT (h)	ICPI	F accession No.	HN accession No.
NR2	NDV/Chicken/Iran/NR2/1999	West Azarbaijan	52.8	1.76	JN001184	KX034115
NR9	NDV/Chicken/Iran/NR9/1999	East Azarbaijan	48	1.78	JN001185	KX034116
NR10	NDV/Chicken/Iran/NR10/1999	East Azarbaijan	49.6	1.79	JN001186	KX034117
NR13	NDV/Chicken/Iran/NR13/1999	Ardabil	54.4	1.86	JN001187	KX034118
NR14	NDV/Chicken/Iran/NR14/1999	Isfahan	52.8	1.91	JN001188	KX034119
NR19	NDV/Chicken/Iran/NR19/2000	Tehran	41.6	1.88	JN001190	KX058521
NR24	NDV/Chicken/Iran/NR24/1999	Khorasan Razavi	48.8	1.85	JN001191	KX058522
NR31	NDV/Chicken/Iran/NR31/2000	Khorasan Razavi	ND	1.87	KC161991	KX058523
NR35	NDV/Chicken/Iran/NR35/2000	Fars	ND	1.86	-	KX058524
NR36	NDV/Chicken/Iran/NR36/1999	Ghazvin	60	1.81	GU228501	KX058525
NR41	NDV/Chicken/Iran/NR41/1999	Ghom	49.3	1.81	JN001193	KX058526
NR45	NDV/Chicken/Iran/NR45/1999	Ghom	46.4	1.86	JN001194	KX058527
NR46	NDV/Chicken/Iran/NR46/1999	Kurdistan	51.2	1.86	GU228503	KX058528

NDV: Newcastle disease virus, MDT: Mean death time, ICPI: Intracerebral pathogenicity index, ND: Not determined, F: Fusion protein gene, and HN: Haemagglutinin-neuraminidase gene

Table 2: The sequences of primers used for amplification of haemagglutinin-neuraminidase gene in this study

Primer	Sequence	Position[a]	TM	GC%	PCR product size (bp)
HN1-F	GCACAGCAAAAGACCTTACTATG	6140-6162	58.1	43.4	899
HN1-R	TGCTAAGTATTGATGTGAATGTG	7016-7038	55.5	34.7	
HN2-F	ACGAATAATAGCGGGTGTGG	6769-6788	59.8	50.0	900
HN2-R	GCCTCGTTGGTACAAGAAGTG	7648-7668	59.7	52.3	
HN3-F	TAATAACACATGCCCCGATG	7437-7456	59.2	45.0	877
HN3-R	GCGACTAAAGAAGGGACTCAG	8293-8313	58.2	52.3	

[a] Newcastle disease virus isolate Bareilly complete genome (accession No. KF727980) was used to design primers. TM: Temperature, and GC: Guanine-Cytosine

Table 3: Comparison of amino acid substitutions at different positions of the deduced haemagglutinin-neuraminidase protein sequences between 13 Iranian field NDV isolates and three NDV vaccine strains[a]

Virus	Amino acid substitutions at residues																																						
	Antigenic or neutralizing sites																													FPR		Sialic acid binding site							
	Site 23											Sites 1 and 14										Sites 2 and 12																	
	Residues 193-201											Residues 345-353										Residues 494, 513-521, and 569																			Residues 234-239					
	L	S	G	C	R	D	H	S	H	P	D	E	Q	D	Y	Q	I	R	R	I	T	R	V	S	S	S	S	D	D	I	A	N	R	K	S	C	S
PHY-LMV42 (DQ097394)																																							
B1 (AF309418)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
Lasota (AY845400)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.
NR2 (KX034115)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR9 (KX034116)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR10 (KX034117)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR13 (KX034118)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	-	T	.	.	.	.	.	.	.	.
NR14 (KX034119)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR19 (KX058521)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR24 (KX058522)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR31 (KX058523)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR35 (KX058524)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR36 (KX058525)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR41 (KX058526)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	V	T	.	.	.	.	.	.	.	.
NR45 (KX058527)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	.	T	.	.	.	.	.	.	.	.
NR46 (KX058528)	.	.	.	.	.	.	.	.	.	.	.	K	.	.	.	.	.	.	.	V	.	.	.	.	.	.	N	.	V	.	T	.	.	.	.	.	.	.	.

[a] Amino acid sequences of Iranian NDV field isolates and three NDV vaccine strains have been compared. Dots indicate sequences identical to those of the NDV strain PHY-LMV42. Abbreviations used for amino acids are as follows: FPR: Fusion promotion region, L: Leucine, S: Serine, G: Glycine, C: Cysteine, R: Arginine, D: Aspartic acid, H: Histidine, P: Proline, E: Glutamic acid, Q: Glutamine, Y: Tyrosine, I: Isoleucine, T: Threonine, V: Valine, A: Alanine, N: Asparagine, and K: Lysine

dNTPs, 2 pM concentration of each primer, 1.5 U *Taq* DNA polymerase, 2 µL cDNA in a final volume of 25 µL. Amplification was programmed in a thermocycler (Peqstar 2X®, Peqlab) as follows: 94°C for 4 min followed by 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The amplified products were detected on SYBR® Green-stained (Invitrogen, USA) 1% agarose gel after electrophoresis and ultraviolet illumination. All chemicals, unless differently stated, were provided from Cinnagen.

Sequence and phylogenetic analysis

PCR products were purified using the AccuPrep® DNA Gel Purification kit (Bioneer, Korea) according to the manufacturer's instruction and submitted for automated sequencing in both directions at the Bioneer Company using PCR primers as sequencing primers. The sequences were aligned and analyzed by BioEdit software ver. 7.0.9.0 (Hall, 1999) and DNASIS MAX 3.0 (Hitachi Solutions America), and compared with selected sequences available in GenBank. Phylogenetic tree was constructed using maximum likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993) with MEGA 7 software (Kumar *et al*., 2016). The evolutionary distance and homology of the respective coding region were also estimated using Pairwise Sequence Comparison in MEGA 7 software (Kumar *et al*., 2016) and analyzed in excel. Prediction of aa sequences and their alignments were also performed by MEGA 7 (Kumar *et al*., 2016). The sequence data from the present study were submitted to the GenBank database with the accession numbers shown in Table 1.

Results

Genomic features

Approximately, 1987 bases from HN gene of each isolate were amplified. Transcriptional start signal or gene start (nt 1-10) of HN gene was found to be completely conserved among all isolates and determined as ACGGGTAGAA. Translation initiation (ATG, nt 92-94) and termination (TAA, nt 1805-1807) codons were placed in identical positions in all 13 sequenced HN genes.

All 13 isolates demonstrated HN proteins containing only 571 aa residues which was consistent with virulent Newcastle disease viruses (vNDVs) and contained the suggested sialic acid binding site (NRKSCS) at position 234-239 (Jorgensen *et al*., 1987). Three aa residues at positions 401 (E), 416 (R), and 526 (Y) that have been reported to be key residues for receptor recognition were also conserved in all 13 NDVs (Connaris *et al*., 2002) as seen in the commonly used vaccine strains. Examination of antigenic sites on the HN glycoprotein revealed that there were a total of five aa residue substitutions at positions 347 (E to K), 494 (G to D), 514 (I to V), 521 (S to N), and 569 (D to V) in comparison with common vaccine strains (Iorio *et al*., 1991) (Table 3). These mutations may affect the antigenicity of HN protein. It was also shown that all HN glycoproteins of 13 isolates possessed 13 cysteine residues at positions 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, and 542. There were four conserved potential glycosylation sites at position 119 (NNS), 341 (NNT), 433 (NKT), and 481 (NHT) among all 13 isolates. However, one site at position 508, considered non-conserved among

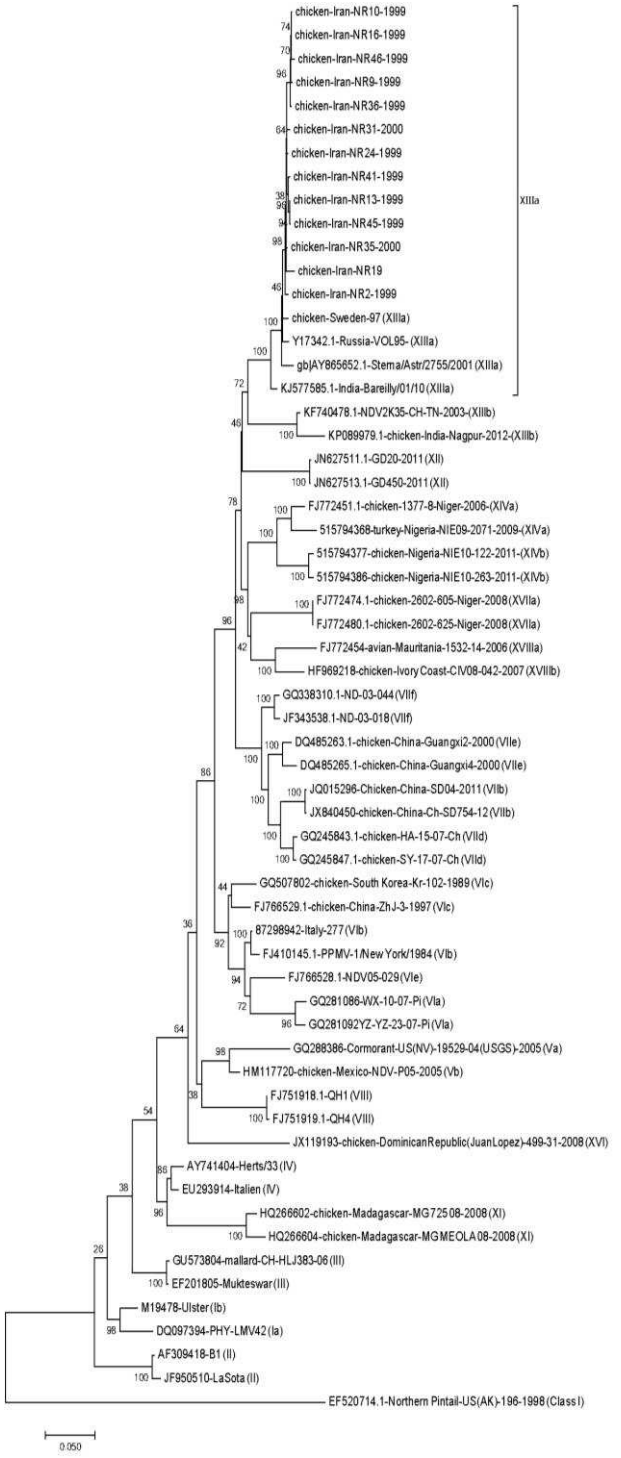

Fig. 2: Phylogenetic analysis based on the full length of HN gene ORF. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The tree with the highest log likelihood (-18047.1983) is shown. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1708 positions in the final dataset. Previously identified HN gene sequences of NDV strains representing different genotypes have been provided from the GenBank with their accession numbers. Numbers indicate the bootstrap values (1000 replicates). Horizontal distances are proportional to sequence distances. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016)

paramyxoviruses, was absent in all 13 NDVs (McGinnes and Morrison, 1995). F glycoprotein precursor cleavage site was determined as 112RRQRRF117 in 11 NDV isolates of this study (not determined for NR31 and NR35) (not published) which was similar to a virulent cleavage site and consistent with *in vivo* pathogenicity indices (Abdoshah *et al*., 2012).

Phylogenetic analysis and distance estimation

Based on the complete coding region of HN gene (1716 bp), all 13 isolates analyzed in the present study were closely related to genotype XIII and more specifically subgenotype XIIIa viruses. Among available sequences of HN complete coding region, Sweden/97 (GenBank: GU585905) was found as the most similar isolate to our isolates with 99% homology. After construction of phylogenetic tree by using complete sequence of HN gene ORF, representative viruses of each genotype were clearly differentiated based on branching patterns and tree topology (Fig. 2). The analysis showed that all 13 NDVs isolated from different parts of Iran clustered with genotype XIII viruses along with other previously characterized viruses from Russia, Sweden, Burundi, and India. The nt (1716 bp), and aa (571 aa) similarities of HN gene and protein sequences among the 13 studied NDV isolates ranged from 98.5-99.9% and 98.7% to 100%, respectively. The percentage of nt and aa sequence identity of HN gene and glycoprotein between our 13 NDVs and three vaccine strains (Lasota, B1 and PHY-LMV42) were in the 78.4% to 82.1% and 84.7% to 88.1%, range, respectively. Sequence analysis showed that 13 Iranian NDV isolates had the lowest percentage of sequence homology with Lasota strain. Estimates of evolutionary distances between viruses of the present study and genotypes of class II NDV are presented in Table 4.

Discussion

In this study, the HN gene of 13 ND viruses previously isolated from various parts of Iran were fully sequenced to observe possible changes in antigenic epitopes of indigenous NDV and the genetic relatedness between commonly used vaccine strains (Lasota, B1 and PHY-LMV42) and Iranian field isolates. Identity of the HN protein aa sequences among these 13 isolates varied from 98.7% to 100%, while the corresponding range between the Iranian field isolates and the vaccine strains were from 84.7% to 88.1%. This suggests that the circulating strains in distinct parts of Iran were considerably different from the vaccine strains in use and, therefore, the role of antigenic differences in weak vaccine-induced protection can be presumed. Furthermore, aa substitutions in some positions such as 347 (E to K), 514 (I to V), 521 (S to N), 494 (G to D) and 569 (D to V) were detected at neutralizing epitopes of the HN glycoproteins in our field isolates when compared with commonly used vaccine strains in Iran. These aa substitutions may result in the disruption of antibody recognition and neutralization capability. Our

Table 4: Estimates of evolutionary divergence over sequence pairs of haemagglutinin-neuraminidase gene between 13 Iranian NDV field isolates and genotypes of class II NDV

	Genotype (No. of sequences)	No. of base substitutions per site, SE[a]																	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	Genotype I (n: 6)		0.008	0.007	0.008	0.010	0.011	0.011	0.010	0.011	0.007	0.010	0.011	0.011	0.011	0.013	0.012	0.012	0.011
2	Genotype II (n: 5)	0.096		0.009	0.010	0.012	0.012	0.013	0.012	0.012	0.009	0.012	0.012	0.013	0.013	0.014	0.013	0.014	0.013
3	Genotype III (n: 4)	0.082	0.133		0.007	0.010	0.010	0.010	0.010	0.010	0.009	0.010	0.011	0.011	0.010	0.011	0.012	0.012	0.011
4	Genotype IV (n: 1)	0.091	0.138	0.083		0.009	0.009	0.010	0.009	0.010	0.009	0.009	0.011	0.009	0.009	0.011	0.011	0.011	0.010
5	Genotype V (n: 4)	0.159	0.195	0.154	0.134		0.009	0.010	0.009	0.010	0.012	0.013	0.010	0.009	0.010	0.011	0.011	0.010	0.009
6	Genotype VI (n: 9)	0.134	0.185	0.126	0.103	0.115		0.007	0.008	0.008	0.011	0.012	0.008	0.007	0.008	0.011	0.008	0.009	0.008
7	Genotype VII (n: 8)	0.161	0.209	0.162	0.136	0.139	0.086		0.010	0.003	0.011	0.012	0.008	0.008	0.008	0.012	0.009	0.009	0.008
8	Genotype VIII (n: 1)	0.145	0.190	0.146	0.122	0.126	0.111	0.142		0.010	0.011	0.012	0.010	0.009	0.010	0.011	0.011	0.011	0.009
9	Genotype IX (n: 1)	0.165	0.211	0.167	0.144	0.142	0.090	0.015	0.147		0.011	0.012	0.009	0.008	0.009	0.012	0.010	0.009	0.008
10	Genotype X (n: 1)	0.073	0.109	0.111	0.117	0.182	0.160	0.178	0.160	0.183		0.012	0.012	0.012	0.012	0.013	0.013	0.013	0.012
11	Genotype XI (n: 1)	0.151	0.200	0.147	0.106	0.188	0.166	0.194	0.187	0.201	0.187		0.012	0.012	0.012	0.014	0.013	0.013	0.012
12	Genotype XII (n: 1)	0.175	0.216	0.180	0.156	0.151	0.115	0.111	0.153	0.120	0.196	0.213		0.008	0.009	0.012	0.010	0.009	0.008
13	Genotype XIII (n: 4)	0.164	0.207	0.161	0.134	0.136	0.090	0.086	0.138	0.093	0.187	0.183	0.102		0.008	0.012	0.009	0.009	0.002
14	Genotype XIV (n: 4)	0.168	0.209	0.164	0.139	0.147	0.100	0.101	0.147	0.109	0.184	0.201	0.114	0.088		0.011	0.009	0.009	0.009
15	Genotype XVI (n: 1)	0.177	0.220	0.178	0.153	0.162	0.150	0.166	0.158	0.175	0.200	0.210	0.185	0.174	0.175		0.012	0.011	0.012
16	Genotype XVII (n: 4)	0.180	0.223	0.184	0.150	0.163	0.119	0.118	0.157	0.125	0.204	0.212	0.135	0.102	0.107	0.187		0.010	0.009
17	Genotype XVIII (n: 1)	0.179	0.229	0.185	0.160	0.161	0.121	0.115	0.159	0.118	0.207	0.213	0.131	0.108	0.105	0.187	0.119		0.009
18	Iran-NDV isolates (n: 13)	0.166	0.209	0.165	0.135	0.136	0.093	0.089	0.139	0.095	0.187	0.185	0.104	0.006	0.092	0.176	0.107	0.112	

SE: Standard error, estimates are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). [a] The number of base substitutions per site from consensus sequences between groups are shown. Analyses were conducted using the maximum composite likelihood model. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1715 positions in the final data set

results were comparable to the findings of a recent Iranian study (Esmaelizad and Ashtiani, 2015) that investigated aa substitutions of partially-sequenced HN glycoprotein in vNDVs obtained from different parts of Iran in comparison with known lentogenic strains. Most of the aa substitutions were identical in both studies but there were some differences. At residue 523, substitution of R to G occurred in one of our isolates and there was no aa substitution at residue 550 in comparison with virulent isolates examined in that study (Esmaelizad and Ashtiani, 2015). Both residues 523 and 550 are located close to antigenic, catalytic and glycosylation sites on HN glycoprotein and the potential role of these residues in antigenic structure and immunogenic potential of NDV should be further investigated. Investigators have already shown that very small changes in the nt sequence of an NDV strain may lead to prominent effects on the pathogenic specifications of the virus (Mohamed *et al*., 2009).

The HN gene in different NDV strains may have various lengths of open reading frame (ORF) that are able to encode for various protein lengths between 571-616 aa due to appearance of translation termination codons at different locations. On the other hand, the relation between the total length of HN protein and strain virulence has already been observed (Sakaguchi *et al*., 1989; Zhao *et al*., 2013). The HN gene of standard avirulent or slightly virulent strains has extended ORF and may encode a longer protein containing up to 616 aa residues (Sakaguchi *et al*., 1989). In the present study, C-terminal extension length analysis of HN glycoprotein gene indicated the lack of aa extension length. So, the total length of 571 aa could be predicted for all 13 isolates indicating high virulence in all viruses. The molecular analysis of 13 NDV isolates reinforced our previous data on *in vivo* pathogenicity tests of 13 isolates performed by MDT and ICPI assays (Abdoshah *et al*., 2012). The values obtained for MDT revealed virulent or velogenic nature of all the studied viruses and were further verified by the ICPI assay and characteristic multibasic pattern of FPCS (112-RRQRRF-117). It has been previously shown that genotypes III-VIII strains which contain the smallest aa length (571 aa) of HN glycoprotein, exclusively, consist of viscerotropic velogenic strains (Czeglédi *et al*., 2006).

As a result of the high evolutionary rate of NDV strains, new genotypes have been reported in the last few decades and it is possible that many more will be identified in the future (Miller *et al*., 2015). Phylogenetic analyses, based on the complete nt sequence of the HN gene coding region of the 13 Iranian isolates, revealed that all were closely related to the representative strains of genotype XIII and subgenotype XIIIa. Genotype XIII strains, previously considered as genotype VII in Iran (Ebrahimi *et al*., 2012; Samadi *et al*., 2014), include vNDVs that have been isolated in Pakistan, Russia, Burundi, India, and Sweden between 1997 and 2014 (Usachev *et al*., 2006; Cattoli *et al*., 2010; Linde *et al*., 2010; Munir *et al*., 2012; Morla *et al*., 2014; Kumar and Kumar, 2015; Jakhesara *et al*., 2016). Phylogenetic relatedness of Iranian and Russian NDVs was previously determined through partial sequencing of HN gene in 6 NDV isolates obtained from Iran (Esmaelizad *et al*., 2012). It appears that genotype XIII viruses along with XII and XIV genotypes have the same ancestor with genotype VII that generates distinct lineages in the course of its evolution (De Almeida *et al*., 2013). Strain cockatoo/India/7847/1982 (GenBank: JN942041) has been previously reported as the ancestor strain and is the most closely related strain to the NDVs of XIIIa subgenotype isolated in Iran and Pakistan, but due to the unavailability of the relevant HN gene sequence, its comparison with our studied viruses was not possible (Miller *et al*., 2015). Isolation and characterization of viruses from this lineage, XIIIa or previously defined VIIb from 1995 to 2011 in Iran may indicate that NDV genome in our country has mainly been under negative or purifying selection pressure with low evolution rate (Ebrahimi *et al*., 2012; Samadi *et al*., 2014). Overall, according to the results of some studies, to date, the circulation of the genotypes XIIIa, XIIId, VIIj, and VIId NDV strains in commercial poultry (Ebrahimi *et al*., 2012; Hosseini *et al*., 2014; Samadi *et al*., 2014; Esmaelizad *et al*., 2017; Mayahi and Esmaelizad, 2017), the genotype VI in domestic pigeons (Rezaei Far *et al*., 2017) and most recently new subgenotype of the

geneotype VII in domestic poultry (Sabouri *et al*., 2016) has been proven in our country, and the total data may be indicative of the continuous circulation of strains causing third (VI) and fourth (VII) global panzootics of ND in Iran. These data show that VIIj and XIIIa are among the most predominant NDV subgenotypes circulating in Iran between 1995 and 2016. Additionally, the subgenotype XIIId has recently been introduced through reclassifying some of the previously characterized isolates (Mayahi and Esmaelizad, 2017). Isolation of a virus belonging to subgenotype XIIIa from a wild little tern (*Sterna albifrons*) in Russia in 2001 implies the probability of NDV spillover from poultry into wild birds and the potential role of these birds in the spread of the virus (Usachev *et al*., 2006). The low degree of genetic diversity between NDVs from Iran and those isolated in Russia, Sweden, and India indicates clear intra and inter-continental transmission of genotype XIII strains like other genotypes throughout the named countries. Human interventions related to poultry farming and pet-bird trades (Seal, 1995; Munir *et al*., 2012), in many cases, have been considered as the most probable cause of vNDV distribution around the world, however, involvement of migratory birds in NDV spread to poultry cannot be ruled out (Ramey *et al*., 2013).

The identical pattern of aa substitutions at important sites of HN glycoprotein epitopes among our 13 isolates may be an indication of their common origin in spite of being obtained from different provinces of Iran. Extensive commercial relations between various provinces of Iran may be the reason for this prominent similarity among isolates from different regions of the country. The comparison of our 13 isolates with other NDV strains was not possible because the full HN gene sequences of previously reported isolates from Iran and most of the neighboring countries were not available. Since, separate analysis of NDVs based on F or HN genes has led to similar classification of NDV genotypes (Munir *et al*., 2012; Snoeck *et al*., 2013), the results presented in this study can be reliably appreciated.

Due to lack of sufficient knowledge on molecular epidemiology and biological characteristics of Iranian vNDVs, it seems necessary to conduct extensive studies of NDV isolates from poultry, free-living and companion birds in Iran. It should also be noted that the identification of VIId and VIb subgenotypes was carried out through partial sequencing of F gene in NDV isolates obtained from Iran and, therefore, this data may not be fully reliable until complete sequencing of F gene coding region in these viruses is released.

This was one of the first studies to analyze complete HN gene sequence of NDVs in Iran. In conclusion, the findings of the present study showed variations in important antigenic sites of studied NDVs isolated from different provinces of Iran that may be responsible for vaccine failure during previous years and, hence, the occurrence of NDV outbreaks. To determine the biologic impact of aa sequence differences in important antigenic sites of the HN in NDVs of the present study with those of vaccine strains, site directed mutagenesis can be employed. Data obtained in our study reinforces the need for examination of commonly used vaccines by systematic protection assays to assess immunization capability against currently circulating indigenous viruses (Asl Najjari *et al*., 2017). Further isolation, molecular and biological characterization of NDVs from other provinces of Iran as well as the neighboring countries will be beneficial to ascertain the nature of the indigenous viruses and enhance our knowledge about the evolution of vNDV strains in the region. Moreover, the present results may also indicate the need for designing and producing new efficient NDV vaccines in Iran.

Conflict of interest

The authors declare that they have no conflict of interest.

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