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Identification of an additional N-glycosylation site and thermostable mutations within the hemagglutinin-neuraminidase gene of the Newcastle disease virus belonging to the VII.1.1 sub-genotype

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Abstract

Newcastle disease virus (NDV) is considered one of the most devastating avian viral pathogens affecting the avian population, and it causes a significant economic burden on the poultry industry worldwide. The study aimed to gain deeper understanding of the molecular and phylogenetic analyses of the complete hemagglutinin-neuraminidase (HN) coding region among NDV isolates. The samples were obtained from different parts of Iran from July 2017 to February 2020, were used for phylogenic analysis in this study. The results confirmed the predominance of sub-genotype VII.1.1, previously known as sub-genotype VIIL, which is circulating in commercial broiler farms of Iran. Identification of (a) an additional N-glycosylation site (NIS) at position 144; (b) mutations S315P and I369V which are related to increasing the viral thermostability; (C) cysteine residues at positions 123; (d) amino acid substitutions in the HN antigenic sites, especially the mutations I514V and E347Q, as well as the other mutant within HN binding sites of the VII.1.1 sub-genotype, suggests the idea that this new sub-genotype of NDV may possess a high level of pathogenicity and virulence compared to other NDV sub-genotypes. In conclusion, the results indicate the presence of an additional NIS at position 144, which may alter the virulence of the isolates. Furthermore, the presence of the thermostable mutations (S315P and I369V) and the other amino acid substitutions among the VII.1.1 sub-genotype isolates may have an impact on the vaccine immunity against this new NDV sub-genotype.

Introduction

Newcastle disease virus (NDV), also known as avian orthoavulavirus 1, is a highly fatal and devastating viral pathogen that affects a wide range of avian species. Newcastle disease (ND) is an extremely contagious viral disease caused by NDV.[1]

Newcastle disease virus belongs to the genus Orthoavulavirus in the family *Paramyxoviridae* and the order *Mononegavirales*. It is an enveloped, roughly spherical virus that contains a nonsegmented, negative-sense, single-stranded RNA [ssRNA (-)] genome with a length of approximately 15 kb.[2] The viral genome encodes six structural viral proteins in the following order: 30 - nucleo-capsid, phosphoprotein, matrix, fusion (F), hemagglutinin-neuraminidase (HN), and RNA large polymerase 50.[3]

Since the first identification of NDV in Iran in the early 1950s, outbreaks of the virus have been frequently reported in various geographical areas of the country.[4,5] Recently, the World Animal Health Information System (WAHIS) interface of the Office International des Epizooties (OIE) has identified ND as an endemic disease in Iran.[4]

The F protein and HN are two surface glycoproteins of the virus that contribute to the virus's virulence and are involved in the virus's entry into the host cell. These two proteins are responsible for stimulating protective neutralizing antibodies against NDV.[1] Although the F protein plays an important role in NDV pathogenicity, other

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parts of the genome, especially the HN protein, must be involved in the virus's virulence and immunostimulation activity. The HN protein is a surface glycoprotein that carries about 1998 nucleotides (nt) long with 571-577 amino acids in its coding region and is essential to the viral pathogenicity of NDV. The HN protein is responsible for neuraminidase activity, attachment, promoting fusion, and binding the virus particles to the surface cell receptors containing sialic acid. The globular head of HN protein can agglutinate the red blood cells (RBCs) by binding to the surface receptors on the RBCs.[6] Additionally, during the sialic acid endocytosis process of the virus, the HN protein can trigger a conformational change within the F protein, which is essential for promoting the fusion of NDV with the host cell membrane and allowing the ribonucleo-protein complex to enter the host cell cytoplasm.[7] In addition, the function of the HN protein and its ability to bind to a target could be influenced exclusively by the differentiation of amino acid sequences;[8] therefore, any sequence alteration within the *HN* gene should be accurately characterized by molecular studies.

The current study analyzed nine NDV strains isolated from Iranian commercial broilers flocks between 2017 and 2020 based on the complete nucleotide sequencing of the *HN* gene. This approach provides a more accurate and up-to-date understanding of the recent distribution of NDV in this region based on *HN* gene sequences.

Materials and Methods

Sample collection and virus isolation. A total of 540 clinical samples, suspected of being infected by NDV, were collected from 36 commercial broiler farms located in 18 different provinces of Iran between July 2017 and February 2020. Selection of the farms was based on typical clinical signs of ND such as neck or wing paralysis, green diarrhea, and respiratory symptoms with high mortality rates of 10.00 - 80.00%. All farms were vaccinated against ND. Nine NDV isolates were utilized for sequencing and phylogenetic analysis of the complete HN coding region. Sampling was carried out in each geographical direction, especially in the provinces that shared borders with neighboring countries, including North provinces (Ardabil, Gilan, Mazandaran, and Golestan), East province (Khorasan Razavi), South province (Hormozgan), and West provinces (West Azerbaijan and Kurdistan). The minimum number of farms required for the sample size was calculated to estimate a prevalence of 36.50%.[9]

The NDV specimens were isolated from positive samples according to standard methods as previously described.[4] Briefly, the supernatants of homogenized tissues (lungs, brain, and trachea) were prepared to obtain 10.00% (w/v) suspension in Phosphate buffered saline (PBS) containing antibiotics streptomycin and penicillin and then centrifuged at 2,800 rpm for 10 min. Afterward, 200 µL of each supernatant were propagated via the allantoic sac route inoculation into a total of 10 nine-day-old specific-pathogen-free (SPF) embryonated chicken eggs. The incubated (HLYX-16986; Zhengzhou Hongle Machinery Equipment Co., Henan, China) eggs were observed every 12 hr for 4 days, and the minimum lethal doses to kill all the inoculated embryos, known as mean death time (MDT), were calculated. After harvesting, the allantoic fluid from eggs was subjected to the rapid hemagglutination (HA) test to confirm the presence of the virus. Briefly, 25.00 µL of allantoic fluid and 25.00 µL of 1.00% chicken RBC in PBS were mixed slowly on a flat surface for 2 min, and the hemagglutination reaction was allowed to develop. Positive allantoic fluids were stored at – 80.00 ˚C for further experiments. Finally, serological tests such as HA and hemagglutination inhibition (HI) were carried out based on the standard methods described earlier in the OIE guidelines.[10] All experimental procedures and chicken handling were carried out according to standard animal experimentation protocols and this study was approved by the Veterinary Ethnics Committee of Urmia University (Reference No.: IR-UU-AEC 2381/DP/3).

Primers. The specific Primers used for amplification and sequencing of *HN* gene in this study were; *HN1*-Forward 1: AGTCACCCTTCAATCGGAAAT, position (6347-6367), temperature (55.92 ˚C), Guanine-Cytosine (42.86%), *HN1*-Reverse 1: GGTGTCGTCTTCCTAACCATC, position (8167-8187), temperature (61.01 ˚C), Guanine-Cytosine (45.38%), *HN2*-Forward 2: GATCACTCGCACTCACATCAATAC, position (7009-7032), temperature (61.01 ˚C), Guanine-Cytosine (45.38%), *HN2*-Reverse 2: AGTGTGATTGTGTTGGGTGGAATA, position (7584-7607), temperature (59.30 ˚C), Guanine-Cytosine (41.67%).

RNA extraction and polymerase chain reaction (PCR). According to the manufacturer's instructions, viral RNA was extracted from all HA positive allantoic fluids using High Pure Viral RNA Isolation Kit (Roche, Mannheim, Germany). In the next step of the process, the samples containing the entire coding region of the *HN* gene were subjected to the Reverse transcription-polymerase chain reaction (RT-PCR) using Biotechrabbit™ one-step RT-PCR kit (Berlin, Germany). In brief, 50.00 µL master mixtures containing 25.00 µL one step mix, 2.50 µL reverse transcriptase-ribonuclease inhibitor (RT-RI) Blend, 2.00 µL forward primer, 2.00 µL reverse primer, 1.00 µL RNA template, and the variable PCR grade, RNase-Free water were PCR amplified. A programmable thermocycler (49.00 ˚C for 30 min; 95.00 ˚C for 2 min; 35 cycles of 95.00 ˚C for 40 sec, 50.00 ˚C for 40 sec, and 72.00 ˚C for 5 min; followed by an additional final extension at 72.00 ˚C for 10 min) has been used in the presented study. Two pairs of specific oligonucleotide primers were designed based on the available sequences from National Center for Biotechnology Information (NCBI) database to bind regions flanking the *HN* genes of predominantly virulent NDV strains.

Sequence and phylogenetic analysis. The sequences were aligned and edited by DNAstar Laser gene package (version 8.0; DNAStar, Madison, USA) and compared with other 46 published NDV sequences of each genotype from various parts of the world, including Iran and its neighboring countries. The rendered NDV strains were retrieved from NCBI to construct the phylogenetic tree. The representative sequences were used to conduct the phylogenetic analysis in MEGA Software (Biodesign Institute, Tempe, USA) using the maximum likelihood method based on the optimum nucleotide method (GTR + G + I),[11,12] and a 1,000 bootstrap replicates, as described by Diel *et al*.[13] At least four sequences must be assessed in a phylogenetic analysis to identify a new sub-genotype of NDV strains, as reported in a previous study[13] Thus, the complete *HN* gene sequence of nine isolates was utilized in our study to confirm validity. The mean inter-populational evolutionary and homology distances between nucleotide sequences of the isolates and other strains belonging to class II NDV were calculated using the neighbor-joining method with the maximum composite likelihood and the gamma distribution models.[14] Besides, the prediction of amino acid (AA) sequences and their alignments was carried out using CLC sequence viewer (version 8.0; Qiagen, Hilden, Germany) to compare the AA sequences between the isolates and other common NDV vaccine strains.

Results

All isolates were classified into the velogenic VII.1.1 (VIIL) sub-genotype. The consensus nucleotide sequences of the HN coding region were submitted to the GenBank database under the accession numbers presented in Table 1. The MDT of isolates showed a range from 40 to 72 hr (Table 1), suggesting that they belong to the velogenic Newcastle strains. As shown in Fig. 1, the phylogenetic tree was constructed using the entire sequences of the HN hypervariable region of the isolates and other 46 NDV strains from genotypes class II retrieved from GenBank. Based on the Phylogenetic tree, all the isolates analyzed in the present study were classified into genotype VII within class II and were closely related to the sub-genotype VII.1.1 (previously VIIL).

The *HN* gene of sub-genotype VII.1.1 has the lowest percentage of sequence homology with the V4 and LaSota NDV vaccine strains. The nucleotide sequence differentiation of the *HN* gene among the isolates and five vaccine strains (including V4, LaSota, VG/GA, B1, and PHY-LMV42) was found to be 22.92%, 22.65%, 22.36%, 22.34%, and 19.81%, respectively. Sequence distance analysis revealed the lowest percentage of sequence homology with the V4 and LaSota strains.

The HN protein of sub-genotype VII.1.1 contains 571 amino acid residues. While there are different lengths of the HN protein among NDV strains, all the isolates contain the same length of 571 AA residues within their HN proteins. Nucleotide sequence determination of the HN coding region can help us to understand the evolution of RNA viruses. This continuous and complex process can lead to genetic variations during the replication and create new genetic groups. All nine sequences were analyzed and compared with the other 29 VII.1.1 NDV sequences retrieved from Iran to understand such kind of phenomenon. A careful analysis between the isolates and the most similar isolate to our viruses (Chicken/Iran/2015 with accession number MF417546.1) demonstrated at least 27 single nucleotide polymorphisms (SNPs), including A→G at positions 56, 774, 840, 972, 1,245, and 1584; T→C at positions 369, 462, 555, 732, 831, 1,326, and 1,677; C→T at positions 24, 113, 552, 582, 846, 927, and 1,566; T→G at positions 216, and 378; G→A at positions 169, 492, and 1,491; and T→A at positions 108, and 1,141. These SNP markers will be valuable for future genetic investigations.

An additional N-linked glycosylation site (NIS) has been found at residue 144 among the HN protein of sub-genotype VII.1.1. The isolates of the present study contain a total of four conserved potential glycosylation sites at positions 119 (asparagine-asparagine-serine), 341 (asparagine-asparagine-threonine), 433 (asparagine-lysine-threonine), and 481 (asparagine-histidine-threonine) as seen in five commonly used vaccine strains. However, the N-linked glycosylation sites at residues 508 and 538 are absent in the VII.1.1 sub-genotype. In addition, we have found the N-glycan Asparagine-Isoleucine-Serine as a new N-linked glycosylation site at residue 144 of the VII.1.1 HN protein. This suggests that the VII.1.1 sub-genotype contains the N-linked glycosylation site at position 144 which is different from typical vaccine strains such as V4, Lasota, VG/GA, B1, and PHY-LMV42.

The mutations S315P, I369V, and V329A may influence the thermostability of the sub-genotype VII.1.1. The HN Sequence alignment of the VII.1.1 isolates showed the AA substitutions S315P, I369V, and V329A compared with the LaSota strain (Fig. 2). These mutations, especially S315P and I369V, seem to suggest that the VII.1.1 isolates may influence the level of thermostability features.

There are three AA substitutions at the sialic acid-binding sites and antigenic sites of the sub-genotype VII.1.1 compared with the five commonly used vaccine strains. Based on multiple alignments of the HN sialic acid-binding sites, the results indicated three substitutions at F156→Y, Y203→H, and T522→I (Fig. 2) that differs between the isolates and five commonly used vaccine strains, respectively. In addition, the results demonstrated three mutations at positions 347 (glutamic acid to glutamine), 494 (glycine to aspartic acid), and 514 (isoleucine to valine) on the antigenic sites of the VII.1.1 sub-genotype. These AA residue substitutions may influence the antigenicity of HN glycoprotein.

Table 1. Information for isolates used in this study and preliminary test results. All nine isolates were very virulent.

Name	GenBank accession Number	Date of isolation	Province	MDT (hr)	HI (4HA)
CK/IR/SBWA18/HN/2017	MZ605758	Jul-2017	West Azerbaijan	<48 hr	6
CK/IR/SBMA128/HN/2018	MZ605759	Jun-2018	Mazandaran	<48 hr	7
CK/IR/SBGI132/HN/2018	MZ605761	Dec-2018	Gilan	<48 hr	8
CK/IR/SBKE133/HN/2018	MZ605762	Jun-2018	Kerman	<72 hr	8
CK/IR/SBWA140/HN/2018	MZ605763	Nov-2018	West Azerbaijan	<48 hr	7
CK/IR/SBGI148/HN/2019	MZ605764	Jan-2019	Gilan	<48 hr	6
CK/IR/SBEA151/HN/2019	MZ605765	Feb-2019	East Azerbaijan	<48 hr	8
CK/IR/SBGO152/HN/2019	MZ605766	Feb-2019	Golestan	<48 hr	9
CK/IR/SBWA179/HN/2020	MZ605767	Feb-2020	West Azerbaijan	<48 hr	9

MDT: mean death time, and HI: hemagglutination inhibition**.**

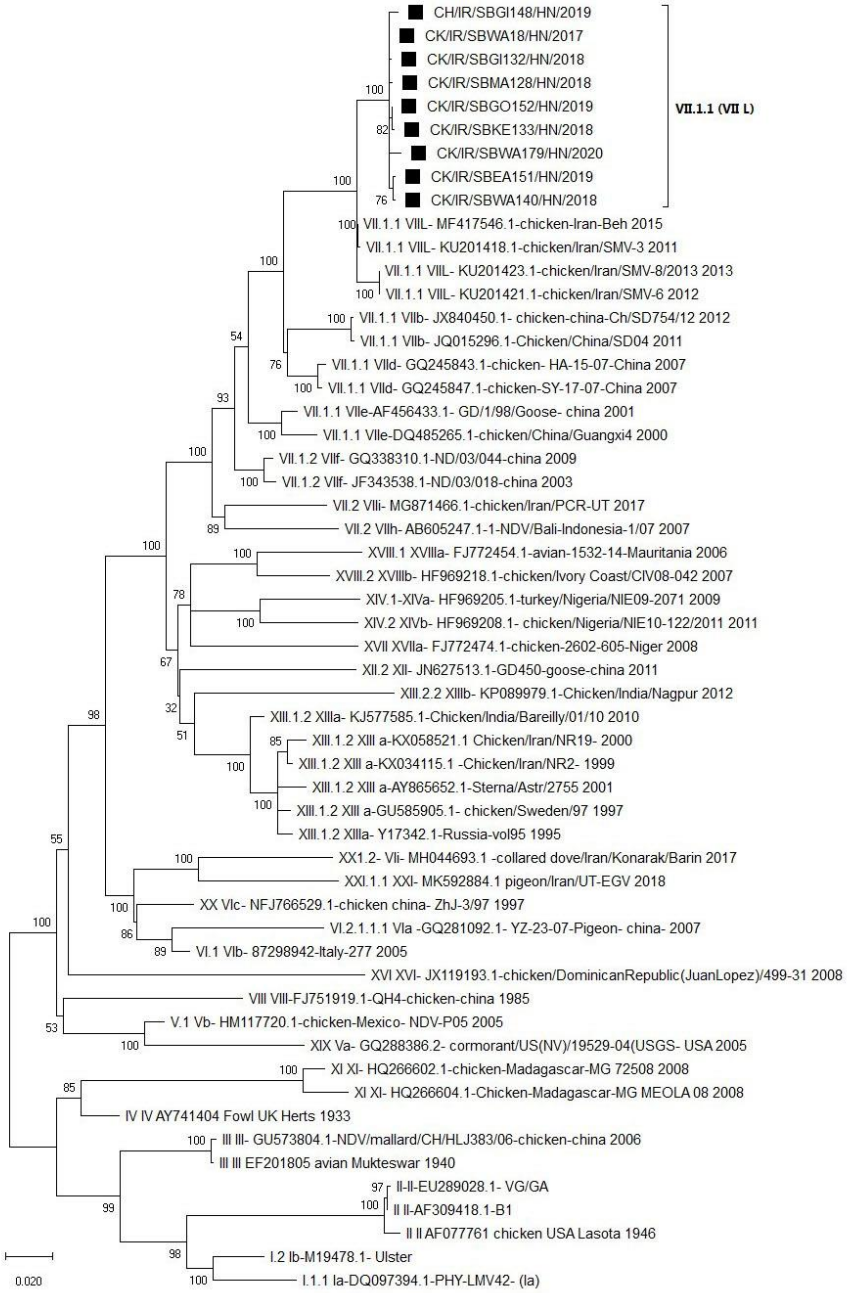

Fig. 1. Phylogenetic analysis tree belongs to the full length of *HN* gene ORF of 9 NDV isolates by maximum likelihood method based on the optimum nucleotide method (GTR + G + I) and a 1,000 bootstrap replicates.

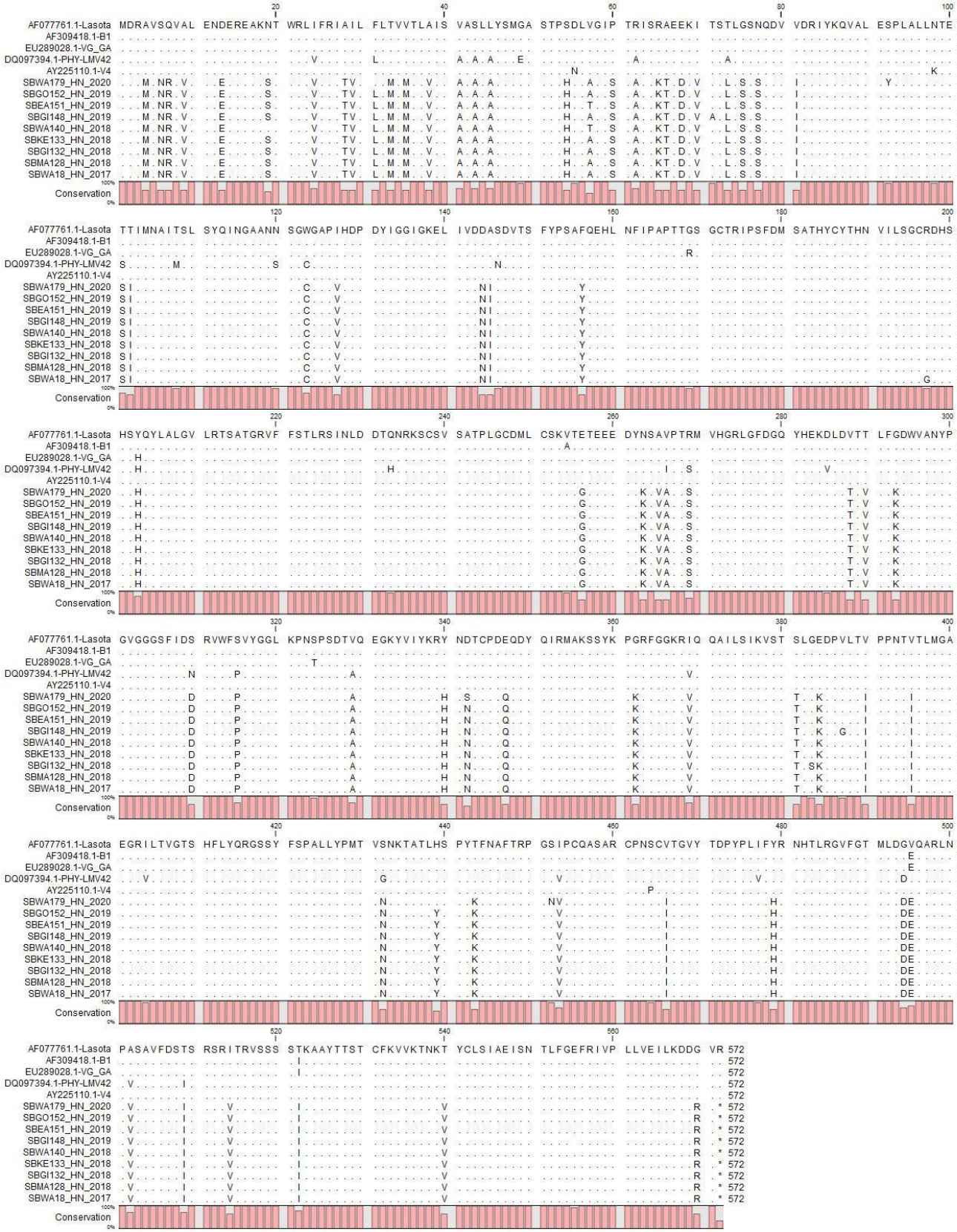

Fig. 2. The alignment of the predicted amino acid sequences belonging to the HN proteins of 9 Iranian NDV isolates and five commonly used vaccine strains was performed using CLC sequence viewer 8.00.

Discussion

Considering the capability of the high evolutionary rate in NDV strains, new genotypes and sub-genotypes have been described globally in recent decades, and it is possible that many more will be recognized by further investigations in the future. RNA replication is a complicated process in nature. During the recombination process, these viruses can generate new variants.[15] Therefore, genome sequence data will be one of the best tools for tracking the evolution of the viruses and monitoring the field isolates in the regions where such diseases are endemic.[15]

Considering the crucial roles of the HN protein in protective immunity and escape of vaccine immunity,[6] this study was conducted to analyze the complete *HN* gene of nine ND isolates by detecting the possible genetic variations in antigenic epitopes of HN protein among Iranian field isolates and five commonly used vaccine strains (LaSota, B1, VG/GA, V4, and PHY-LMV42).

The phylogenetic tree clustered the sequences of a 1716-bp fragment of the *HN* gene coding region from each of the given NDV isolates into the representative strains of genotype VII and sub-genotype VII.1.1. Although some of the poultry farms used for sampling are located more than 1,000 km apart, we did not identify any sub-genotype other than VII.1.1 in commercial broiler farms. According to the procedure of Malirat *et al*., the genotype variation of 2.00 - 5.00% in the phylogenetic interpretations is consistent with the fact that the viruses can originate from a similar geographical zone.[16] The evolutionary distance between nucleotide sequences of the isolates and the other sequences deposited by neighboring countries belonging to the class II genotype was found to be more than 5.00% (Table 2). Furthermore, a previous study by Dimitrov *et al*. noted that group distances less than 5.00% represented the same sub-genotype.[2] The evolutionary distance analysis among all isolates presented in this study as a group showed a range between 99.00 - 100% (Table 2). These results suggest that the novel sub-genotype VIIL is unique to the area. However, Dimitrov *et al*. proposed a new classification system by representing the entire VII (b+d+e+J+L) sub-genotypes as one group named VII.1.1.[2] This idea strongly argues that VII.1.1 is the most predominant sub-genotype of NDV in this part of the Middle East.

According to the different lengths of the HN protein,[17] most of those NDV isolates belong to class I genotype, and class II sub-genotype I possess 616 AA. In addition, class II genotype II viruses have 577 AA, whereas genotypes III, IV, V, VI, VII, VIII, and IX contain 571 AA. As expected, all nine isolates belonging to genotype VII indicated HN proteins containing only 571 AA residues.

The most NDV strains cannot tolerate the heat and will lose their infectious ability when exposed to 50.00 ˚C for 30 min. However, several thermostable NDV strains, which usually belong to genotype I with low pathogenicity to chickens, have been identified that are able to keep their infectivity and HA titer at 56.00 ˚C for at least 30 min.[18]

Thus far, the molecular mechanism of HN protein thermostability is still unknown.[19] As shown in Fig. 2, the results demonstrated mutations S315P, I369V, and V369A among the VII.1.1 isolates. These results are consistent with a recent study,[19] which revealed that residues 315, 329, and 369, especially mutations S315P and I369V, could significantly enhance the viral thermostability, HA, and NA activities.

Table 2. Estimates of evolutionary divergence between nine Iranian NDV field isolates and other genotypes of class II NDV, especially virulent genotypes in Iran and neighboring countries (genotypes VII and XIII).

No.	Isolates	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	I_(I)(n= 2)																			
2	II_(II)(n = 3)	0.11																		
3	XIII.2.2_(XIIIb)(n = 1)	0.19	0.23																	
4	XIII.1.2_(XIIIa)(n = 6)	0.18	0.21	0.11																
5	VII.2_(VIIh)(n = 1)	0.19	0.23	0.15	0.11															
6	VII.2_(VIIi)(n = 1)	0.18	0.22	0.14	0.10	0.08														
7	VII.1.2_(VIIf)(n = 2)	0.17	0.21	0.12	0.09	0.07	0.06													
8	VII.1.1_(VIIe)(n = 2)	0.18	0.22	0.13	0.10	0.09	0.08	0.05												
9	VII.1.1_(VIIb)(n = 2)	0.19	0.23	0.14	0.11	0.09	0.10	0.06	0.06											
10	VII.1.1_(VIId)(n = 2)	0.18	0.22	0.14	0.11	0.09	0.09	0.05	0.05	0.04										
11	VII.1.1_(VIIL)(n = 4)	0.19	0.22	0.14	0.11	0.10	0.10	0.07	0.07	0.06	0.05									
12	SBWA179	0.20	0.23	0.15	0.12	0.11	0.11	0.08	0.08	0.07	0.06	0.02								
13	SBGO152	0.20	0.23	0.15	0.12	0.10	0.11	0.07	0.08	0.07	0.06	0.02	0.01							
14	SBEA151	0.20	0.23	0.15	0.12	0.10	0.11	0.07	0.08	0.07	0.06	0.02	0.01	0.00						
15	SBGI148	0.20	0.23	0.16	0.13	0.11	0.11	0.08	0.08	0.07	0.06	0.02	0.01	0.01	0.01					
16	SBWA140	0.20	0.22	0.15	0.12	0.10	0.10	0.07	0.08	0.07	0.06	0.02	0.01	0.00	0.00	0.01				
17	SBKE133	0.20	0.23	0.15	0.12	0.11	0.11	0.07	0.08	0.06	0.07	0.02	0.01	0.00	0.01	0.01	0.01			
18	SBGI132	0.20	0.23	0.15	0.12	0.10	0.11	0.07	0.08	0.07	0.06	0.02	0.01	0.00	0.00	0.01	0.00	0.00		
19	SBMA128	0.20	0.23	0.15	0.12	0.10	0.11	0.07	0.08	0.07	0.06	0.02	0.01	0.00	0.00	0.01	0.00	0.00	0.00	
20	SBWA18	0.20	0.23	0.15	0.12	0.10	0.11	0.07	0.08	0.07	0.06	0.02	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00

It was previously suggested that the proline residues at specific sites, especially in b-turns or loop regions, play a crucial role in protein stabilization.[20] The pyrrolidine rings of these residues could affect the protein thermostability by restricting the backbone bond rotation of protein, thereby reducing the conformational entropy throughout the protein unfolding process and consequently increasing the protein thermostability features.[21] Moreover, Omony *et al*. showed that a higher proportion of amino acids Val, Leu, and Ile might be identified within the HN protein of heat-resistant NDV strains.[22] The results showed 44, 47, and 34 amino acid residuals of Val, Leu, and Ile at the HN stalk and globular regions of the VII.1.1 sub-genotype compared with 40, 47, and 33 amino acid residuals from the LaSota strain, respectively. Overall, these data suggested that the VII.1.1 isolates might contribute to possess higher thermostability features than the other NDV sub-genotypes across this area of the Middle East. However, whether these mutations in amino acid sequences of the HN protein affect the thermostability of the VII.1.1 sub-genotype or not remains to be further studied.

The N-linked glycosylation sites are found to be necessary for some biological activity of proteins, such as proper conformation, disulfide bond formation, correct folding, and oligomerization. Several studies have previously suggested that any changes in the glycosylation sites of NDV might affect its biological functions.[23] Examination of N-linked glycosylation sites of the HN glycoprotein of NDV revealed that there are a total of six predicted sites for the addition of N-linked carbohydrates at residues 119, 341, 433, 481, 508, and 538.[23] Four of these sites (residues 119, 341, 433, and 481) have been found functional in NDV strains with the consensus sequence motif Asparagine-X-Serine (NXS) or Asparagine-X-Threonine (NXT), where X can be any amino acid except proline or aspartic acid.[23] Earlier studies have illustrated that losing a glycosylation site may lead to either decreasing or increasing the virulence of a virus, depending on its location.[24-26] For example, some researchers have mentioned that the elimination of N-glycans in NDV HN (especially at position 481) could have reduced the pathogenicity of the virus.[26] These data have suggested that the VII.1.1 sub-genotype might possess higher virulence than those parental viruses. Whether the new N-glycosylation site (NIS) can influence the virulence of the virus or not requires further investigation.

Functional antigenic sites studies on HN glycoprotein indicated that a total of seven overlapping antigenic sites (sites 1, 2, 3, 4, 12, 14, and 23) could influence the attachment of the virion to the cellular receptor and neutralize viral infectivity.[27] Five of these sites (residues 193 to 201 as site 23; 345 to 353 as sites 1 and 14; and a domain composed of residues 494, 513 to 521, and 569 as sites 2 and 12) have been identified as the principal glycoprotein antigenic sites of HN protein, which could affect the attachment of virion to a cellular receptor and consequently neutralize viral infectivity.[27] As shown in Fig. 2, the AA substitutions G494→D and I514→V (antigenic sites 12 and 2), as well as the mutation E347→Q (sites 1 and 14), have been detected (Fig. 2).

Previously, functional inhibition studies on antigenic sites 12, 2, and 23 have demonstrated that the monoclonal antibodies against these sites could influence the biological activities of NDVs by either preventing viral attachment to chick cell monolayers or decreasing HA and NA of the virus.[27] The mutant I514V (sites 12 and 2) is reported in this study and previously among several virulent isolates of NDV strains.[28]

Based on the previous studies, the AA substitution in HN residue 347 is contributed to the lower antigenic relationship between field isolates and vaccine strains.[29] The mutation E347K has previously been reported in genotype VII viruses by several researchers from Asia, especially in Korea[30] and China.[31] Moreover, the recent study by Zhu *et al*. reported the failure of vaccination (by LaSota strain) of chicken infected with a variant strain carrying mutation E347K.[29] In addition, the effect of an AA substitution in residue 347 on NDV HI titers was reported earlier.[31] Overall, this suggests that the mutation of E347Q may lead the VII.1.1 sub-genotype to the new antigenic variation and novel amino acid substitutions in the HN linear epitope. These AA residue substitutions may influence the antigenicity of HN glycoprotein.

Furthermore, sites 1 and 14 were found effective in the HA activity of NDV strains with no detectable influence on NA function.[27] The data obtained from our HI assay demonstrated the HI titers of 7-9 (Table 1). These results suggest that the high level of HI titers might be closely related to the AA substitutions of VII.1.1 antigenic sites.

Regarding the mechanism of interaction between the HN and F proteins, there are two sialic acid binding sites in the stalk and globular head of the HN protein: first, the residues located in the sialic acid-binding site I at positions 234-239;[32] second, the residues are composed of four loops at the dimer interface of HN protein in the sialic acid-binding site II: residues 156 to 174, 191 to 203, 515 to 527, and 547 to 556.[33] It has been previously shown that the HN protein is responsible for both the entire fusion procedure and the initial triggering of the F protein. While site I triggers the interaction with F protein and modulates neuraminidase activity, site II operates to maintain contact with the target cell during the fusion process.[34] According to the multiple alignments shown in Fig. 2, the results showed a total of three AA substitutions F156→Y, Y203→H, and T522→I in the sialic acid-binding site II among the VII.1.1 isolates.

As the HN protein of NDV probably exists in virions in both nondisulfide-linked and disulfide-linked forms, and due to the crucial role of cysteine residues in the disulfide linkage of the HN homodimers, the number of these

residues and their positions have been accurately studied in NDV strains.[35,36] The result showed a total of thirteen cysteine residues at positions 123, 172, 186, 196, 238, 247, 251, 344, 455, 461, 465, 531, and 542 in the VII.1.1 sub-genotype. Although twelve of these residuals have been identified conserved among all nine isolates, the cysteine at residue 123 was observed to be variable in comparison with the LaSota strain. The residue 123 is noticeable by its deficiency in the major part of the NDV isolates analyzed so far. The previous study by McGinnes and Morrison has revealed the higher fusogenicity of a mutant virus than that of the ancestral virus. These authors have suggested that the mutation at residue 119, located near the Cys-123 residue in the stalk region of HN protein, might implicate better covalent linkage by enhancing the efficiency of Cys-123 in an intermolecular covalent bond. Consequently, it can increase the fusogenicity of the virus.[23] By analyzing the sequences of VII.1.1 sub-genotype isolates, we found a substitution at Trp123→Cys in the stalk region of the HN protein (Fig. 2). This suggests that mutation of residue 123 might cause higher fusion promotion among the VII.1.1 sub-genotype isolates.

Despite acceptable biosecurity and vaccination programs within the Iranian poultry flocks using various kinds of commercial vaccines such as the LaSota strain, outbreaks of ND is still a massive problem in this region of the Middle East.[4] As mentioned earlier, the sequence distances analysis among nucleotide and AA sequences of the isolates under study has illustrated the lowest percentage of the sequence homology with the LaSota and V4 strains.

This study also suggests that the vaccination programs of broiler farms infected by mutant strains may be affected by these AA substitutions. This is because the current commercial vaccines, which comprise F or HN proteins of other types of viruses from another country, may not provide promising results against newly emerging genotypes.[37] Thus it is recommended that further studies on molecular epidemiology of the circulating NDVs should be carried out within the region. This can help us to design a new neutralization map of the sub-genotype VII.1.1 by constructing a functional profile of each antigenic site. The results of this comprehensive study can be used for future investigations to develop more effective and protective recombinant NDV vaccines that carry VII.1.1 surface HN protein.

In conclusion, the phylogenetic analysis of the isolates retrieved from the new ND outbreak during 2017 - 2020 has confirmed the novel VII.1.1 sub-genotype, previously known as sub-genotype VIIL, as the predominant sub-genotype circulating in poultry farms of Iran. Sequence analysis between sub-genotype VII.1.1 isolates and commonly used vaccine strains revealed multiple AA residue substitutions on the HN glycoprotein of the VII.1.1 sub-genotype. This study identified two AA substitutions (S315P, I369V), previously found responsible for enhancing the viral thermostability of NDV strains. Furthermore, an additional N-glycosylation site at position 144 (NIS), as well as the cysteine residue at position 123, was identified in the investigation. Each of these has been previously found essential for NDV fusogenicity and pathogenicity. Identification of AA substitutions in the HN antigenic sites, especially the mutations I514V and E347Q, as well as the other mutant within HN binding sites of the VII.1.1 sub-genotype, suggest that these mutations may influence the HA and NA activities of the isolates. These multiple AA residue substitutions may increase the virulence activity of the field isolates and can be responsible for vaccination failure in commercial poultry farms.

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Conflict of interest

The authors declare they have no conflict of interest.

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