Original Article

Molecular Identification and Phylogenetic Study Based on the Fusion Gene of Newcastle Disease Virus Isolated from Broiler Poultry Farms in Markazi Province, Iran

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ABSTRACT

Newcastle disease (ND) is an economically significant and extremely spreadable viral illness affecting a wide variety of avian species. ND can rapidly spread within poultry farms and result in considerable economic losses for the global poultry industry. This disease is endemic in Iran, and despite intensive vaccination efforts in the poultry industry, outbreaks of ND occur unexpectedly. This study aimed to isolate the Newcastle disease virus (NDV) from poultry farms with breathing problems in Markazi province, Iran, and investigate the evolutionary relationship and molecular characteristics of the isolates during 2017-2019. To this end, tissue samples (lung, brain, and trachea) were taken from 42 broiler farms exhibiting respiratory symptoms. The samples were inoculated into 9-11-day-old embryonated eggs, and the virus was isolated from 20 (47.6%) of the 42 farms. Subsequently, RT-PCR was used to amplify partial fusion gene sequences from the new isolates. The amplified products were sequenced and compared phylogenetically to the standard pilot dataset (125 selected sequences) generated by the NDV consortium. As determined by phylogenetic analysis, all nine isolates belonged to subgenotype VII.1.1 of genotype VII and were highly similar to isolates from other parts of Iran and China. Moreover, all isolates possessed a polybasic cleavage site motif (112RRQKRF117), characteristic of virulent strains. Furthermore, the present isolates shared a high nucleotide identity (96%) with viruses previously isolated from other provinces of Iran, as determined by BLAST searches and multiple alignments. In addition, they shared a high degree of sequence similarity but were distinct from the existing NDV vaccines. Therefore, the genetic dissimilarity between current vaccine strains and circulating NDVs must be considered in vaccination programs.

Keywords: Chicken, F gene, Genotype VII, NDV, Phylogenetic analysis

1. Introduction

Newcastle disease (ND) is a highly transmissible disease that affects various avian species, both domestic and migratory (1). It devastates the global poultry industry and causes significant economic losses. The disease is characterized by respiratory, neurological, and gastrointestinal hemorrhage lesions (2). The Newcastle disease virus (NDV) belongs to the avian orthoavulavirus 1 genus of the Paramyxoviridae family (3). NDV is a single-stranded, enveloped, and negative-sense RNA virus with six major structural proteins: phosphoprotein (P), nucleocapsid (N) protein, fusion (F) protein, matrix (M) protein, haemagglutinin-neuraminidase (HN), and large RNA-dependent RNA polymerase (L) (4). Pathogenicity is used to categorize NDV strains into five pathotypes: avirulent (asymptomatic enteric), mildly virulent (lentogenic), moderately virulent (mesogenic), and two types of exceedingly virulent (velogenic) strains with viscerotropic and neurotropic symptoms (5). This classification is mainly based on *in vivo* pathogenicity tests, including the intracerebral pathogenicity index and the mean death time (MDT) (6). In addition, virulence can be evaluated by analyzing the amino acid sequences of the F protein cleavage site (positions 112-117) (7). In virulent NDV strains, multiple basic amino acids (112R/K-R-Q-K/R-R116) are succeeded by phenylalanine at position 117. However, low-virulence viruses possess the amino acids 112G/E-K/R-Q-G/E-R116 and leucine at position 117 (8).

Multiple classification systems have been proposed based on F gene sequences for NDVs (4,10). Recent revisions classify NDV isolates into two divisions, I and II, based on phylogenetic analysis and genetic variation in the complete sequence of the F gene. Class I isolates are typically avirulent, less genetically diverse, and isolated from waterfowl and shorebirds around the globe. Class II genotypes, on the other hand, are typically isolated from wild and domestic animals and include various virulent and nonvirulent viruses. Furthermore, exhaustive analyses have identified at least 20 distinct genotypes in class II (1). Based on previous nomenclature, class II strains are further classified into 15 genotypes (I-XV). Genotypes VI and VII are genetically diverse and further divided into eight (A-H) and five (A-E) subgenotypes, respectively (11), which have been merged into subgenotype VII.1.1. in recent NDV classification systems (1).

Since its first appearance in the mid-1920s in Java, Indonesia, Newcastle, and England (10), NDV has remained a persistent threat around the world. Iran reported its first instance of ND in the early 1950s (12). Despite the routine vaccination of industrial poultry farms using different active and inactive vaccines, outbreaks of NDV with high death rates and severe symptoms are reported in Iranian commercial poultry (13).

The majority of epidemics in the Middle East and Asia are currently caused by genotype VII strains (14), suggesting that subgenotype VII.1.1 may have recently become the most prevalent genotype in Iran's poultry farms. ND is endemic in Iran and causes considerable damage to the poultry industry every year (6,13-17). In this study, the NDV was isolated from poultry farms that exhibited respiratory symptoms in the Markazi province, Iran. The isolates' F genes were sequenced and phylogenetically characterized. In addition, the nucleotide and amino acid sequences of the F gene cleavage site were analyzed for each isolate.

2. Materials and Methods

2.1. Sample Collection and Virus Isolation

Tissue samples (lung, brain, and trachea) were collected from 42 vaccinated industrial broiler chicken farms in Markazi province, Iran, during 2017-2019. Sampled chickens exhibited respiratory symptoms, including sneezing, depression, coughing, wheezing, dyspnea, nasal discharges with or without diarrhea, central nervous system problems, and high mortality rates.

The samples were transferred on ice to the laboratory of the Arak branch of the Razi Vaccine and Serum Research Institute, labeled, and stored at -70°C until inoculation. Along with the samples, a form detailing the characteristics of the prepared samples was provided (including the characteristics of the injected vaccines, the name of the mother hen, clinical symptoms, and the potential diagnosis of the farm veterinarian).

To confirm the presence of NDV in infected chickens and the propagation and isolation of the virus, 0.2 mL of each homogenized tissue supernatant (mostly trachea and brain) was inoculated into the allantoic cavity of 9-11-day-old specific pathogen-free (SPF) embryonated chicken eggs, as described in the OIE Manual (18). The viability of the embryos was then monitored daily for five days while the eggs were being incubated at 37°C. All embryos that died after 24 h or survived until the incubation period were kept overnight at 4°C. After that, allantoic fluids were collected and subjected to a rapid hemagglutination (HA) assay (18). For rapid hemagglutination, 25µL of allantoic fluid was added to 25µL of 1% chicken RBC in PBS. This mixture was placed on a flat surface and allowed to produce hemagglutination for a few minutes. HA negative samples were serially passaged up to three times, and if the third harvested allantoic fluid was still negative for the HA test, it was considered negative for NDV. LaSota vaccine was used as the positive control in the HA test.

2.2. RNA Extraction and Reverse-Transcription PCR

Total RNA was extracted from the virus-infected allantoic fluids using RNX-plus TM solution (Cinnagen, Iran) in accordance with the protocol specified by the manufacturer. The quantity and quality of the extracted RNA were evaluated by nanodrop and stored at -70°C until Reverse-Transcription PCR (RT-PCR) was performed. RT-PCR was performed using a commercial cDNA synthesis kit (BioFact, South Korea) and a random hexamer.

The partial F gene containing the cleavage site sequence was amplified using Kant's primer sets (A: 5'- TTG ATG GCA GGC CTC TTG C -3', B: GGA GGA TGT TGG CAG CAT T -3') (19). The PCR conditions were 94°C for 5 min, followed by 35 cycles of 94°C for 45 sec, 58°C for 45 sec, and 72°C for 45 sec, as well as a 10-min final extension at 70°C. The RNA extracted from vaccine strains (B1 or Lasota) was a positive control sample in all PCR reactions. The products of PCR were electrophoresed on a 1.2% agarose gel and visualized using image capture in the Gel Documentation System (Biometra, Germany).

2.3 Sequence Analysis and Phylogenetic Study

The purification of nine positive PCR products was done using a PCR purification kit (Favorgen Co., Taiwan) and sent to the Pishgam Company for bidirectional sanger sequencing.

The sequences were edited, aligned, and analyzed utilizing the BioEdit software (version 7.2.5) and compared to other NDV sequences in the GenBank utilizing the Basic Local Alignment Search Tool (BLAST, NCBI). The pathogenicity of NDVs was studied by analyzing the amino acid sequence corresponding to the cleavage site of protein F (positions 112 to 117) (7,8). Multiple sequence alignments of the NDV partial F gene were conducted using the ClustalW method in the MEGA X software (20).

The phylogenetic tree was constructed according to the NDV consortium's most recent classification system (1). The F gene sequences from the current study (nine isolates) were compared to the pilot dataset, containing 125 isolates covering all class II NDV genotypes (1), totaling 136 sequences. The maximum-likelihood method was used to generate a phylogenetic tree using the MEGA X software (20) and 1,000 bootstrap replicates based on the optimal nucleotide method (K2+G+I). In addition, the evolutionary distances between the isolates in this investigation were determined using the maximum composite likelihood method with the gamma

distribution model (shape parameter=1) and 1,000 bootstrap replicates in the MEGA X software. The first, second, third, and noncoding codon positions were included in the analysis, and all positions with missing or incomplete information were eliminated.

3. Results

3.1. RT-PCR, Sequencing, and Homology Analysis

Of the 42 samples collected, 25 were successfully isolated from SPF eggs and had rapid hemagglutination activities. Their harvested allantoic fluid was used for subsequent experiments. Negative samples were stored at -70°C for future studies on other viruses involved in the respiratory syndrome. As expected, RT-PCR using AB primers amplified a 362 bp fragment of the F gene (Figure 1). Altogether, 20 of 42 (47.6%) samples were evaluated as positive for NDV. Positive isolates from different cities in the Markazi province, Iran, were selected for sequencing, and finally, nine isolates were sequenced.

Comparing the nucleotide sequences of the nine NDV isolates demonstrated a high degree of sequence similarity (97.74% to 99.72% nucleotide-level identity). The partial F gene analysis of nine NDV isolates revealed nucleotide sequence identities ranging from 97.42% to 99.72%, similar to the previously characterized Ck/IR/MAM68/2017 NDV isolates from Iran. Comparison of the F gene sequences of the current NDV isolates to GenBank reference strains revealed a significant similarity between the isolated NDVs and genotype VII. All nine sequenced isolates corresponded to subgenotype VII.1.1, and no other genotype or subgenotype was detected. The isolated NDVs displayed minimal nucleotide sequence similarity to the commonly used NDV vaccines in Iranian poultry farms (81.7% to 83.4% nucleotide sequence identity to the LaSota vaccine and 81.3 to 83.4% nucleotide sequence identity to the B1 vaccine).

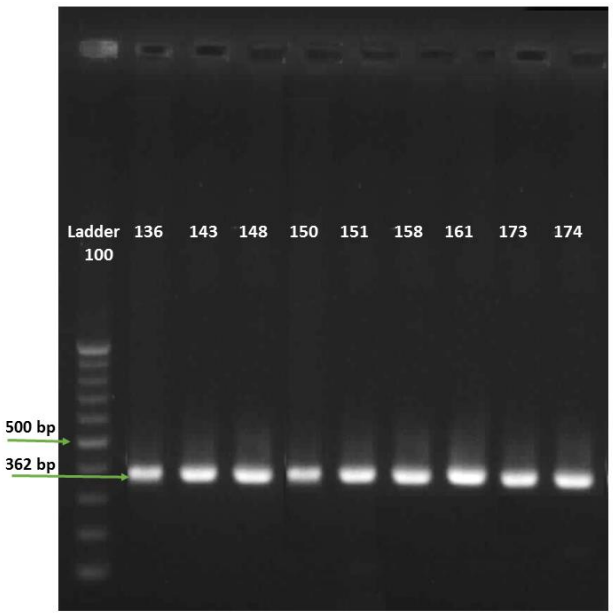

Figure 1. PCR product electrophoresis of some NDV samples on TAE 1.2% gel. The 362 bp bands indicate NDV detection

Moreover, the predicted amino acid sequences of NDV isolates were compared. All NDV isolates had the amino acid sequence 112RRQKRF117 at the F0 cleavage site, characteristic of highly virulent strains (7,8) and indicates that all new isolates from Markazi province were virulent. However, NDV virulence is believed to be a polygenetic factor (14).

3.2. Phylogenetic Evaluation

A phylogenetic tree was created based on the most recent NDV classification system published by Dimitriove et al. (1) by applying the partial F gene nucleotide sequences of the current study (nine sequences) isolates and the standard pilot dataset (125 selected reference strains representative of each genotype and subgenotype sequences) recommended by the NDV consortium (1). In the phylogenetic tree, our isolates clustered with genotype VII and subgenotype VII.1.1 strains in a cluster that was well-supported (89% bootstrap value) (Figure 2). The isolates used in this research are marked with the • symbol. Due to the large number of sequences and the size of the obtained tree, a part related to the isolates of the current study is shown as a subtree in figure 3. Additionally, the calculated evolutionary distance among the nine new NDV isolates was between 0.00

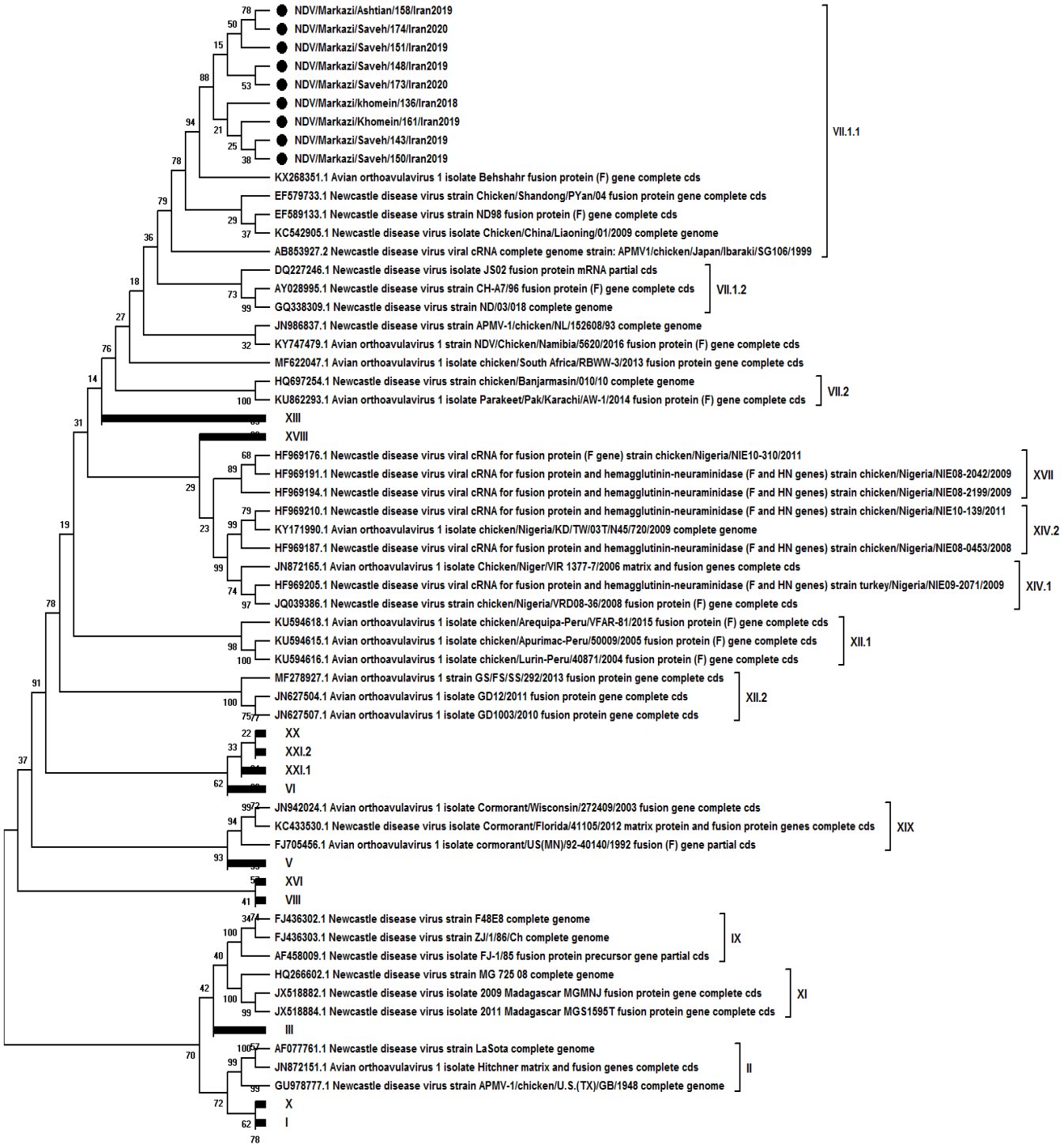

Figure 2. Phylogenetic tree of NDV strains based on the nucleotide sequence of the F gene. The maximum-likelihood method was used to generate a phylogenetic tree using the MEGA X software (19) and 1000 bootstrap replicates based on the optimal nucleotide method (K2 + G + I). The analysis involved nine isolates from this study and standard pilot dataset (125 selected sequences) generated by the NDV consortium (1). Black circle indicates the current study isolates.

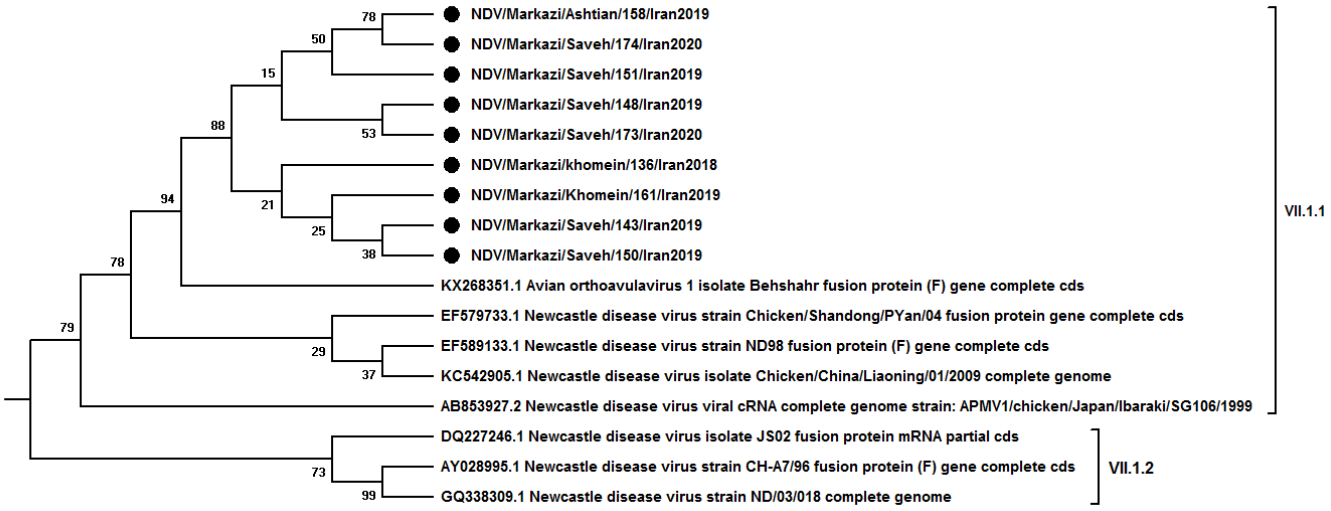

Figure 3. Genotype VII branch derived from phylogenetic tree shown in fig2. The nine isolates of this study marked with ●

Table 1. Estimates of Evolutionary Divergence among current study isolates based on partial F gene coding sequences

	136	**143**	**148**	**150**	**151**	**158**	**161**	**173**	**174**
136									
143	0.002								
148	0.006	0.004							
150	0.002	0.000	0.004						
151	0.006	0.004	0.008	0.004					
158	0.012	0.009	0.009	0.009	0.009				
161	0.004	0.002	0.006	0.002	0.006	0.012			
173	0.010	0.009	0.008	0.008	0.011	0.014	0.011		
174	0.014	0.011	0.011	0.011	0.011	0.002	0.014	0.014	

The number of base substitutions per site from between sequences are shown. Standard error estimate(s) are shown above the diagonal and were obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Maximum Composite Likelihood model (20). This analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 368 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (19).

and 0.014 (Table 1), revealing a high degree of similarity between nucleotide sequences and that the isolates are closely related.

4. Discussion

Newcastle disease is an economically significant viral illness that can spread quickly across poultry farms and result in substantial economic losses that affect the global poultry business. NDV is one of the most severe threats to the poultry industry in developing nations, such as Iran, and plays a significant role in the occurrence of respiratory syndromes in broiler farms (10). The broad circulation of NDV in poultry populations has led to the significant genetic diversity of the virus and the constant emergence of NDV variants. Given the clinical and economical relevance of NDV to the poultry industry and the broad use of live ND vaccines worldwide, sequencing and phylogenetic analysis have become the methods of choice for the characterization of NDV strains circulating in the field. The identification of various factors involved in the endemicity of NDV is essential for controlling the disease.

In the current study, NDV was isolated from 20 (47.6%) of 42 vaccinated broiler farms with

respiratory syndromes in Markazi province, Iran, during 2017-2019. Nine isolates were selected from different cities in the Markazi province for nucleotide sequencing and genotyping. The current isolates shared extensive nucleotide homology (96%) with previously isolated NDVs from other provinces of Iran, as determined by BLAST searches and multiple alignments.

According to the findings of the phylogenetic study, our isolates were highly related to each other. Moreover, phylogenetic analysis confirmed that all nine examined isolates corresponded to subgenotype VII.1.1 (VIIl) of genotype VII. Consequently, our data obtained from sequencing and bioinformatics evaluations verified the existence of VII.1.1 strains in the Markazi province. Genotype VII (class II of the NDV) is a large group with extensive genetic variation involved in many NDV infection outbreaks around the world. In the 1980s, genotype VII viruses developed initially in the Far East and expanded to Europe, South Africa, the Middle East, and Asia in the 1990s, forming the fourth pandemic of ND (20,21). Genomic studies have demonstrated that genotype VII is the most widely spread NDV genotype circulating in Asia (4,6,10,13,15).

Molecular and epidemiological investigations of NDV in Iran (1995-2016) by Mayahi and Esmaelizad (4) demonstrated that subgenotypes XIIId, XIIIa, VIg, VIj, VIIj, and VIId have circulated in Iranian industrial chicken farms for over 17 years. Furthermore, Molouki et al. (2019) (14) recently recognized subgenotype VII.1.1 as a probable dominant genotype in Iranian poultry flocks. Numerous studies have also previously disclosed the existence of subgenotypes VIIb, VIId, VIIg, VIIi, VIIj, and VIIl in Iran, which have been merged into subgenotype VII.1.1. in the recent NDV classification system (6,13,16,17,23-26). Some previous phylogenetic analyses based on P, M, and F gene partial sequencing proposed that there was a close relationship between Iranian NDV isolates and those in Russia (AY865652), Pakistan (JN682210), and India (27-29). Nevertheless, our phylogenetic analysis, using the partial sequencing of the hypervariable region of the F gene following several recent studies (14,16), indicated that Iranian NDV isolates are most closely related to Chinese VII.1.1 strains. Based on BLAST analysis, our isolates shared high levels of nucleotide homologies with recent isolates (subgenotype VIIl) from Isfahan and Mazandaran (Ck/IR/MAM68/2017 and Ck/IR/MAM52/2017, respectively) (14). Moreover, the evolutionary analyses showed a 0.00 to 0.014 distance between the isolates of the current study (Table 2), demonstrating that the latest dominant NDV strains prevalent in chicken farms in Markazi province are identical to VII.1.1 and favorably similar.

In line with standard approaches, such as the MDT, sequence evaluation of the F protein cleavage site may be applied to determine the probable pathogenicity of NDV (7,8). Similar to previously published Iranian sequences (13,30), the amino acid sequence of the F0 cleavage site was discovered to be 112RRQKRF117 in all isolates from the present investigation, characteristic of virulent strains. Hosseini et al. (13) also reported that all nine virulent NDV isolates from Iranian industrial farms in their study followed the pattern 112RRQKR/F117 at the cleavage site of the F protein. According to the phylogenetic data, these isolates belong to subgenotype VIId (13). In the study conducted by Makki et al. (31) in four provinces of Iran, 10 out of 16 NDV isolates matched subgenotype VII.1.1. It was also found that most isolates had the 112RRQKR/F117 motif at the F protein cleavage site. Recently, NDV subgenotype VII.1.1 was isolated from chicken farms in Northern Iran. All isolates carried the pattern 112RRQKR/F117 at the cleavage site of the F protein (25). Furthermore, in a previous study on NDV isolates from 18 Iranian provinces, all isolates were classified into the VII.1.1 group. The isolates possessed the amino acid sequence 112RRQKRF117 for the F0 cleavage site, as determined by sequence analysis (10). Following the findings of several studies on velogenic NDV isolates, subgenotype VIIb (29), subgenotype VIId (27), and subgenotype VIIg (17) have the 112RRQKR/F117

motif in the cleavage site. Ghalyanchi et al. (6) demonstrated that acute subgenotype VIIi also carried the 112-RRQKRF-117 motif.

Findings of the present research and the above-mentioned studies prove that remarkably similar fourth panzootic NDV subgenotype VII.1.1 variants continue circulating in Iranian poultry farms. Previously, Allahyari et al. (10) hypothesized that the unique Iranian VII.1.1, which is not reported from other countries, primarily spreads across the country due to human factors, including transportation and commerce. Although Iran uses a combination of B1, LaSota, and V4 vaccine strains as live or inactivated vaccines, ND remains a major threat to Iranian poultry flocks. The vaccines listed above are classified as genotype II and are distinct from the dominant genotype VII. Furthermore, they have low nucleotide sequence similarity to the isolates of the current study (81% to 83% identities), which could clarify the diminished efficacy of vaccination programs. Consequently, the frequent occurrence of ND in different parts of the country may be related to this issue.

In summary, evidence from the present study, as well as some similar recent works, shows that the extremely comparable fourth panzootic NDV subgenotype VII.1.1 variants are still circulating in Iran (10,13-16,26,27). It was also observed that vaccine isolates widely employed in Iran differ from the prevalent genotype VII. The emergence and identification of new sublineages provide insight into the high rate of genetic drift occurring in NDV strains in Iran and raise many concerns about the efficacy of current ND control measures in the country.

Regarding the vaccination against ND at the provincial and national levels, unfortunately, some cases of the disease occur in industrial poultry farms, which can be considered a serious threat to the poultry industry. Therefore, according to the obtained results, it is necessary to continue epidemiological and genetic surveys in the country. Furthermore, the challenge of vaccinated chickens should be carried out using circulating isolates to check the efficacy of existing vaccines. Finally, in addition to the current vaccination program, it is recommended to produce and administer a lethal vaccine derived from the commonly identified genotype VII strains.

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Authors' Contribution

Study concept and design: Ebrahimi, S.; Akbari, N.;
Acquisition of data: Ebrahimi, S.; Akbari, N.; Jafari, P.; Vahidi, V.;
Analysis and interpretation of data: Ebrahimi, S.; Akbari, N.; Jafari, P.; Vahidi, V.;
Drafting of the manuscript: Vahidi, V.; Ebrahimi, S.;
Critical revision of the manuscript for important intellectual content: Ebrahimi, S.; Akbari, N.;
Statistical analysis: Vahidi, V.; Ebrahimi, S.;
Administrative, technical, and material support: Ebrahimi, S.; Akbari, N.;
Study supervision: Ebrahimi, S.; Akbari, N.;

Ethics

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

Conflict of Interest

The authors declare that they have no conflict of interest.

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