Isolation and molecular characterization of virulent Newcastle disease virus isolates from commercial chickens

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Abstract

Introduction: Newcastle disease virus (NDV) sub-genotype VII.1.1 has been associated with severe outbreaks in vaccinated poultry flocks in several regions. This report describes the molecular characteristics, phylogenetic placement, and amino acid substitutions in key functional domains of the fusion (F) protein of two NDV isolates obtained from commercial flocks with neurological signs.

Materials and Methods: Tissue samples from a broiler breeder flock in Khorasan Razavi and a broiler flock in Mazandaran were screened using RT-PCR, and viruses were isolated. Sequence assembly, and phylogenetic reconstructions along with functional domain amino acid analyses were conducted using MEGA 12 and NDV Consortium reference datasets.

Results: The complete 1,662-bp F gene CDS (553 amino acids) was determined for both isolates. Both isolates harbored the multi-basic cleavage site motif ^{112}R-R-Q-K-R^{116}↓F^{117}, fulfilling the molecular criteria for virulent NDV. Phylogenetic analyses revealed that SNDV-1 and SNDV-2 clustered within class II, sub-genotype VII.1.1 (formerly VII j), sharing the highest nucleotide identities (>99.2%) with Egyptian and regional isolates rather than with historical Iranian isolates. Amino acid profiling of the transmembrane domain revealed distinct substitutions (I513 and A520) that matched the Egyptian lineage and differentiated these isolates from previously reported Iranian VII.1.1 strains (V513 and G520).

Conclusion: The results are consistent with the presence of virulent-type NDV sub-genotype VII.1.1 in the two sampled flocks. The close phylogenetic relationship with Egyptian isolates and the unique substitutions (I513 and A520) within the F protein transmembrane domain suggest the introduction of a distinct sublineage into Iran, differing from previously characterized local viruses.

Keywords: Newcastle disease virus, Sub-genotype VII.1.1, Fusion gene, Phylogenetic analysis, Poultry, Iran

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1- Introduction

Newcastle disease (ND), caused by virulent strains of Newcastle disease virus (NDV; species Avian orthoavulavirus 1, recently renamed Orthoavulavirus javaense), continues to impose significant economic losses on the global poultry industry through high mortality, reduced production, and the costs of vaccination and control programs. Based primarily on nucleotide sequence variation in the fusion (F) gene, AOAV-1 viruses are divided into two major phylogenetic classes, class I and class II, which are further organized into distinct genotypes and subgenotypes. Within class II, genotype VII, particularly sub-genotypes VII.1.1 and VII.2, has been increasingly associated with outbreaks in Asia, the Middle East, and Africa. Many viruses belonging to these subgenotypes exhibit velogenic pathotypes, highlighting their continued epidemiological importance in poultry populations [1-3].

In Iran, NDV has remained endemic for decades, with repeated outbreaks reported in commercial broiler flocks despite widespread vaccination. Molecular surveillance studies conducted on isolates from Iranian broiler farms between 2018 and 2019 demonstrated that the majority of field viruses clustered within genotype VII, exhibiting characteristic molecular features of velogenic strains and distinct phylogenetic positioning relative to vaccine strains [2]. Complementary work in Markazi Province confirmed the circulation of genotype VII viruses in broiler poultry, with *fusion* gene phylogenies supporting their classification as virulent field isolates [3]. Analysis of the *haemagglutinin-neuraminidase* (HN) gene from NDV isolates recovered from Iranian broilers further revealed genetic signatures typical of genotype VII, including specific amino acid substitutions that may influence antigenicity, receptor binding, and thermostability [4].

A pivotal development in Iranian NDV research has been the isolation and standardization of a sub-genotype VII.1.1 strain as a local challenge virus for vaccine efficacy trials. This isolate, derived from field material in Iran, has been molecularly characterized and is now utilized to better reflect contemporary circulating strains in potency testing [5]. More recently, in 2024, genotype VII.2 NDV was detected and pathotypically characterized from imported cockatiels in Iran, demonstrating the virus's expanding host range beyond commercial poultry and highlighting the risk of introduction or dissemination through the pet bird trade [6]. These Iranian findings align with broader regional patterns: genotype VII.2 has also been identified in wild birds in neighboring Turkey, while dominant G-VII strains continue to drive outbreaks in commercial chickens in Bangladesh [7, 8]. Parallel surveillance in other endemic regions, including whole-genome sequencing efforts in Ethiopia and genomic analyses in Pakistan and Tanzania, underscores the ongoing global diversification and evolutionary pressure on AOAV-1 within genotype VII [6, 9].

Considering that virulent NDV constantly circulates regardless of the implemented preventive measures, there is an acute need to determine the pathogenicity and molecular characterization of contemporary field strains to assess the suitability of currently used vaccines. Here, we report the isolation and full-length fusion gene characterization of two contemporary NDV field isolates from commercial chicken outbreaks in Iran. Accordingly, this study was undertaken to isolate and molecularly characterize NDV from recent outbreaks in Iranian commercial chicken flocks, and to determine the phylogenetic placement of the obtained isolates in relation to historical Iranian, regional, and global reference sequences. A further aim was to analyze the

amino acid profile of key functional domains within the F protein, with particular emphasis on the fusion peptide and transmembrane regions, in order to identify molecular signatures that may reflect viral evolution and have implications for vaccine matching.

2- Materials and Methods

2-1- Collection of samples

This study focused on commercial chicken flocks with a history of neurological signs. Fresh carcasses of two chickens from two separate flocks were submitted to the Diagnostic Laboratory of the SANA Institute for Avian Health and Diseases Research (Tehran, Iran) under cold-chain conditions. The first case was a broiler breeder from Khorasan Razavi Province, submitted in October 2024, and the resulting isolate was designated Ck/IR/SNDV-1/2024 (hereafter SNDV-1). The second case was a broiler from Mazandaran Province, submitted in December 2024, and the isolate was designated Ck/IR/SNDV-2/2024 (hereafter SNDV-2). Both birds exhibited Newcastle disease-like neurological signs, including torticollis, opisthotonus, and limb paresis. Following necropsy, brain, trachea, lung, spleen, and cecal tonsil samples were collected aseptically and stored at −70 °C until further processing.

2-2- RNA Extraction and Preliminary Molecular Detection

Viral genomic RNA was extracted from clinical samples using the Sambio™ RNA Extraction Kit according to the manufacturer's instructions and stored at −70 °C until reverse transcription PCR (RT-PCR) was performed. Complementary DNA (cDNA) was synthesized using a commercial cDNA synthesis kit (Yekta Tajhiz, Iran) according to the manufacturer's instructions. For initial detection, a 363-bp fragment of the F gene was amplified using the primer set and PCR conditions described by Kant et al. [10] (Table 1).

2-3- Virus isolation

For virus isolation, clinical and post-mortem samples were homogenized to make a 10% w/v suspension in phosphate buffer saline (PBS) pH 7.0−7.2 containing 10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 250 IU amphotericin-B/mL. The supernatant fluid was inoculated at 0.2 mL into 9- 11-day-old SPF embryonated chicken eggs (Razi Serum and Vaccine Research Institute, Iran) via the chorioallantoic cavity. The inoculated eggs were incubated at 37 °C, and samples were serially passaged up to three times. Embryos that died within 24 hours post-inoculation were discarded. Allantoic fluids were harvested from dead or live embryos during 6 days post-inoculation, after which allantoic fluids were collected and subjected to a rapid hemagglutination (HA) assay [11].

2-4- PCR and Sequencing of fusion gene

Total viral RNA was extracted from the allantoic fluid infected with NDV using a Sambio™ RNA Extraction Kit, according to the manufacturer's instructions. cDNA was subsequently synthesized using random hexamers and M-MLV reverse transcriptase (cDNA Synthesis Kit, Yektatajhiz, Tehran, Iran). Subsequently, for detection of virulent NDV with a partial F gene containing the cleavage site sequence was amplified using primer set A & C as previously described by Kant et al. (Table 1). An 1,871-bp fragment encompassing the entire F gene was then amplified using Super PCR Master Mix (Yektatajhiz, Tehran, Iran) with the F-Fwd and F-Rev primers (Table 1), which target conserved regions flanking the F gene of virulent NDV strains with a genome length of 15,192 nt, as previously described by Molouki et al. [12]. The cycling conditions comprised an initial denaturation at 95 °C for 3 min, followed by 35 cycles

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of 95 °C for 30 s, 52 °C for 30 s, and 72 °C for 4 min, with a final extension at 72 °C for 10 min. PCR products were sequenced using dye-terminator Sanger sequencing (Gen Fanavaran, Iran) using the same PCR primers together with two additional internal primers designed for sequencing only (Table 1).

2-5- Sequence assembly, alignment, and phylogenetic analysis

Four overlapping sequencing reads were edited and assembled using Unipro UGENE v53, yielding a 1,662-bp consensus covering the complete coding sequence of the F gene. The identity of the assembled sequences as NDV was confirmed by BLAST analysis against the National Center for Biotechnology Information (NCBI) database. The sequences of both isolates (SNDV-1 and SNDV-2) were deposited in GenBank under the accession numbers listed in Table 2.

Phylogenetic trees were reconstructed using maximum likelihood (ML) as implemented in MEGA 12, with branch support evaluated using 1,000 bootstrap replicates. The most recent FASTA files available from the Newcastle disease virus (NDV) Consortium were downloaded from the Consortium's GitHub repository (https://github.com/NDVconsortium/NDV_Sequence_Datasets), and the F gene sequences of our isolates were added to the files for analysis. The sequences were aligned using the ClustalW algorithm implemented in MEGA 12, and the resulting alignments were subsequently used for phylogenetic analysis. Two independent ML analyses were performed using the best-fitting substitution models selected by the Bayesian Information Criterion (BIC). The first tree, constructed under the GTR+G+I model, was used to assign the isolates to their respective genotypes. The second tree, inferred under the T92+G+I model, was based on representative Iranian isolates from the NDV Consortium dataset, recent Iranian sequences, sequences from regional and neighboring countries, and the sequences showing the highest nucleotide identity to the isolates analyzed in this study. This second phylogenetic analysis was performed to further assess the genetic relationships and evolutionary placement of the study isolates in relation to closely related and geographically relevant NDV strains.

Table 1. Specific primers used for RT-PCR amplification and full-length fusion (F) gene sequencing of Newcastle disease virus isolates in this study

Primer Name	Sequence (5′→3′)	Application	Amplicon Size (bp)	Reference
A	TTGATGGCAGGCCTCTTGC	Initial Detection	363 (with B) / 252 (with C)	Kant et al. [10]
B	GGAGGATGTTGGCAGCATT	Initial Detection	363	
C	AGCGTYTCTGTCTCCT	Virulent F Cleavage Site Detection	252	
F-Fwd	YTGCTTATAGTTAGTTYACCTGTC	Full F gene flanking	1,871	Molouki et al. [12]
F-Rev	ACCCGTGTATTGCTYTTYGG	Full F gene flanking	1,871	
F-Fwd-middle	GCAACCAATGAAGCTGTGCATGA	Internal sequencing	Internal (770)	
F-Rev-middle	ACAGCTTCTCCATAATTTTGCGA	Internal sequencing	Internal (770)	

Finally, deduced amino acid sequences were compared with those of previously reported NDV genotypes circulating in Iran and homologous sequences from other countries to identify residue substitutions within functionally critical domains of the F protein: the fusion cleavage site (residues 112–117), the fusion peptide (residues 117–141), and the transmembrane domain (residues 501–521).

3- Results

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3-1- Molecular Identification, Virus isolation, and Full-length *Fusion* Gene Sequencing

Two samples tested positive in the initial RT-PCR screening and were subsequently selected for virus isolation in SPF-embryonated chicken eggs. Following inoculation, both isolates produced characteristic hemorrhagic and congestive lesions in embryos. The isolates were further examined using hemagglutination (HA), hemagglutination inhibition (HI), and RT-PCR assays, which confirmed the presence of Newcastle disease virus (NDV) in both samples. A 1,871-bp genomic region encompassing the complete fusion (F) gene coding sequence (CDS) and its flanking regions was successfully amplified and sequenced for both isolates. The complete F gene CDS obtained from each isolate was submitted to GenBank, and the corresponding nucleotide and protein accession numbers, along with relevant isolate characteristics, are presented in Table 2.

Table 2. Characteristics and GenBank accession numbers of Newcastle disease virus isolates analyzed in this study

Isolate name	Nucleotide accession No.	Protein accession No.	Tissue origin	Date of isolation	Province
Ck/IR/SNDV-1/2024	PX452495	YBG53389	Brain	Oct 2024	Khorasan Razavi
Ck/IR/SNDV-2/2024	PX452496	YBG53390	Lung and Trachea	Dec 2024	Mazandaran

3-2- Sequence similarity and phylogenetic analysis

The complete F gene coding sequence of both isolates was successfully determined, each comprising 1,662 nucleotides encoding a 553-amino-acid fusion protein, and deposited in GenBank under accession numbers PX452495 (SNDV-1) and PX452496 (SNDV-2), with corresponding protein accessions YBG53389 and YBG53390(Table 2).

BLAST analysis confirmed the NDV nature of both sequences, with the highest nucleotide identity observed against Egyptian strains. SNDV-1 shared 99.46% identity with NM09/2019 (OP219681) and NM97/2019 (OP219679), 99.34% with CFGS1/2021 (OP611416), and 99.28% with Egy-Beh-NR726/2016 (MH899933) and MN51/2019 (MN519684); SNDV-2 showed a comparable profile (99.40% with NM09/2019 and NM97/2019, 99.28% with CFGS1/2021). Notably, no previously reported Iranian NDV sequence appeared among the closest matches for either isolate.

Consistent with these results, both isolates clustered within class II genotype VII, sub-genotype VII.1.1 (formerly VII j), in figure 1 (Prototype Tree), forming a monophyletic cluster with prototype strains of Egyptian and Kyrgyz origin and remaining distinct from the previously circulating Iranian VII.1.1 (former VII l) strains MN615882.1/chicken/Iran/ABD/2018 and MF417546/chicken/Iran/Beh/2012. The figure 2 (regional tree) resolved this relationship further: SNDV-1 and SNDV-2 grouped with the Egyptian isolates NM09/2019, NM97/2019, CFGS1/2021, MN51/2019, ME6/2017 (MN481245), Ismailia32/2016 (KY075895), and Egy-Beh-NR726/2016, clearly separated from both the well-established Iranian VII.1.1 (VII l) clade and the Iranian VII.2 (VII i) strains, while Iranian pigeon-derived genotype XXI and genotype XIII isolates occupied distant clades. Together, these findings indicate that SNDV-1 and SNDV-2 belong to the VII.1.1 sub-lineage, phylogenetically distinct from NDV strains previously reported in Iranian poultry and most closely related to strains circulating in Egypt and the wider region.

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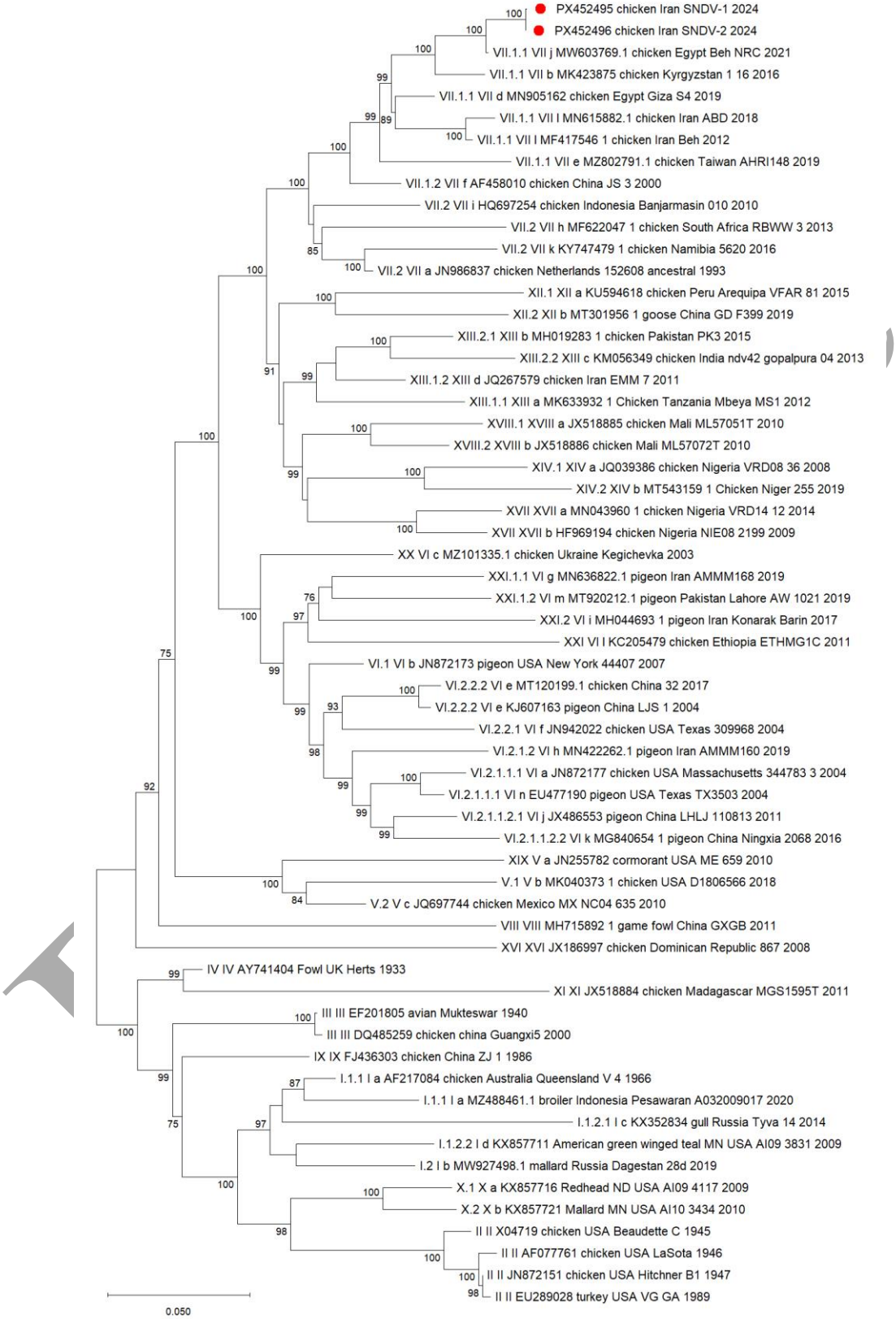

Figure 1. Maximum-likelihood phylogenetic classification of Newcastle disease virus (NDV) isolates based on complete coding sequences of the fusion (F) gene (1,662 bp). The phylogenetic tree was reconstructed in MEGA 12 using the General Time Reversible model with gamma-distributed rate variation among sites and invariant sites ("GTR"+"G"+"I"), identified as the best-fitting nucleotide substitution model according to the Bayesian Information Criterion (BIC). The dataset includes curated reference strains representing established class II genotypes obtained from the NDV Consortium repository. Isolates characterized in this study (SNDV-1 and SNDV-2) are indicated by solid red circles. Bootstrap support values based on 1,000 replicates are shown at major nodes (values ≥70%are presented). Genotype designations follow the unified phylogenetic classification system [1].

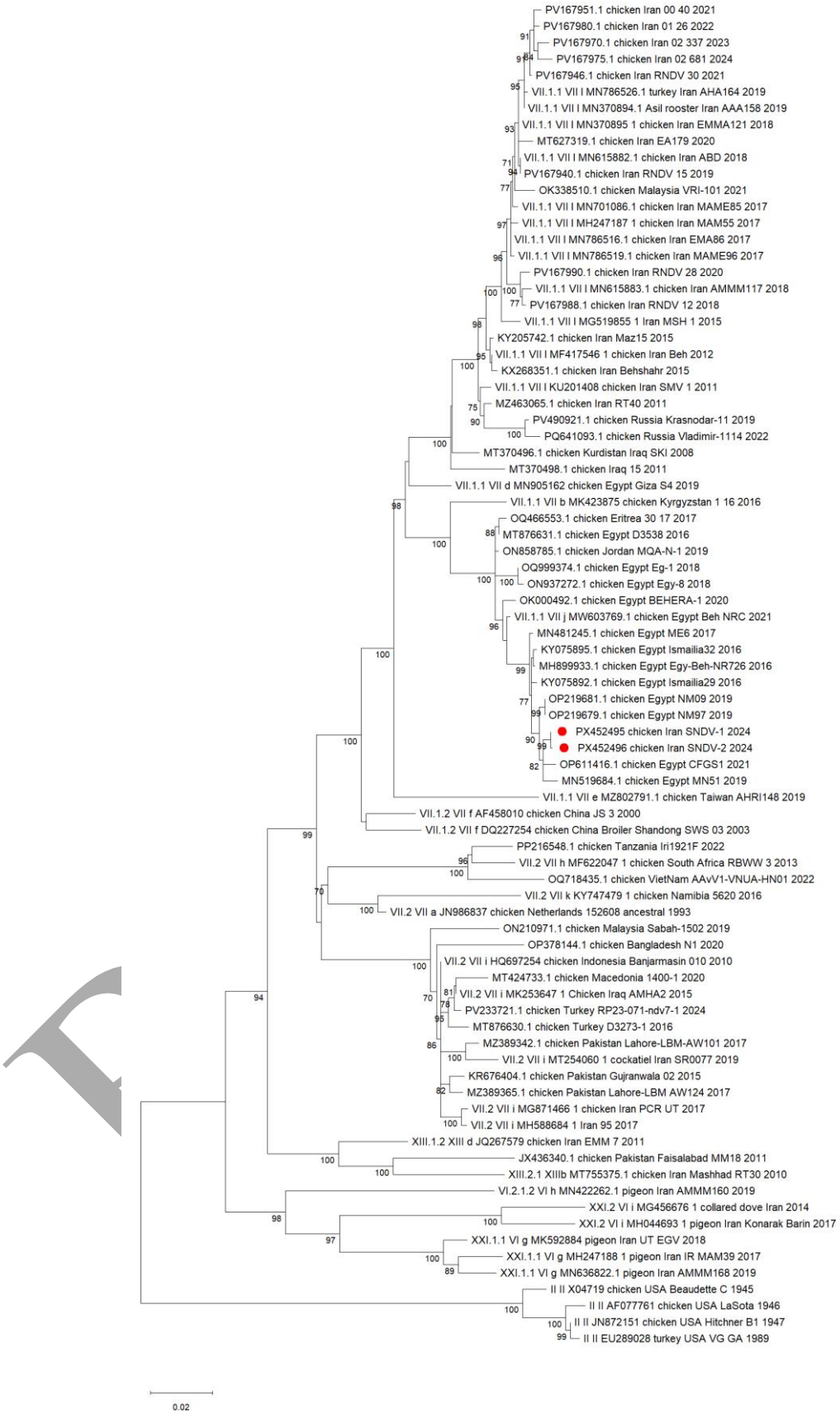

Figure 2. Phylogenetic placement of Iranian NDV isolates relative to local, regional, and closely related sequences. Maximum-likelihood tree inferred from the complete F gene coding region (1,662 bp) using the Tamura 3-parameter model with gamma distribution and invariant sites ("T92"+"G"+"I") in MEGA 12. The analysis incorporates the study isolates (indicated by solid red circles), historical and recent Iranian reference sequences from the NDV Consortium, isolates from regional and neighboring countries, and top BLAST nucleotide matches. Node robustness was assessed using 1,000 bootstrap replicates, with values ≥70%indicated at relevant branches.

3-3- Amino acid analysis of the F protein

Both isolates possessed the ^{112}R-R-Q-K-R^{116}↓F^{117} motif at the fusion protein cleavage site, a polybasic sequence with phenylalanine at position 117 that is characteristic of virulent (mesogenic/velogenic) NDV strains according to WOAH criteria, and consistent with the neurological presentation observed in the affected flocks. This motif was identical to that observed in the closely related Egyptian genotype VII.1.1 strains. In comparison, the lentogenic reference strains LaSota and Hitchner B1 possessed the corresponding cleavage site configuration associated with lentogenic viruses. The Iranian pigeon-derived genotype XXI isolates (MH247188, MK592884, and MN636822) exhibited a K^{112}RQKR^{116}↓F^{117} variant, differing from the motif identified in the two isolates examined in this study (Table 3).

Within the fusion peptide (residues 117–141), both isolates displayed F117, V121, S124, and V125, a profile shared with the majority of virulent genotype VII strains analyzed, in contrast to the L117/I121/G124 configuration of LaSota and Hitchner B1. Analysis of the transmembrane domain (residues 501–521) revealed the pattern V506, V509, I510, I513, A516, L517, and A520 in both isolates. This combination was identical to that of the Egyptian strains NM09/2019, CFGS1/2021, Ismailia32/2016, Egy-Beh-NR726/2016, MN51/2019, and ME6/2017. Importantly, it differed from previously reported Iranian VII.1.1 (VII l) isolates, including SMV-1/2011 (KU201408), Beh/2012 (MF417546), MAM55/2017 (MH247187), and ABD/2018 (MN615882), which uniformly carried V513 and G520. The I513 and A520 residues therefore distinguish the present isolates from the Iranian VII.1.1 strains characterized to date and support their placement within the Egyptian-related sub-lineage identified by phylogenetic analysis.

Table 3. Amino acid sequence alignment and substitutions across functionally critical domains of the fusion (F) protein in Iranian NDV isolates compared with national and international reference strains.

Names	Fusion Peptide (117-141)				Transmembrane domain (501-521)							Cleavage Site (112-117)
	117	121	124	125	506	509	510	513	516	517	520	112-117
PX452495_chicken_Iran_SNDV-1_2024	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
PX452496_chicken_Iran_SNDV-2_2024	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
OP219681.1_chicken_Egypt_NM09_2019	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
OP611416.1_chicken_Egypt_CFGS1_2021	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
KY075895.1_chicken_Egypt_Ismailia32_2016	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
MH899933.1_chicken_Egypt_Egy-Beh-NR726_2016	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
MN519684.1_chicken_Egypt_MN51_2019	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
MN481245.1_chicken_Egypt_ME6_2017	F	V	S	V	V	V	I	I	A	L	A	RRQKRF
VII.1.1_VII_l_KU201408_chicken_Iran_SMV_1_2011	F	V	S	V	V	V	I	V	A	L	G	RRQKRF
XXI.2_VI_i_MG456676_1_collared_dove_Iran__2014	F	I	S	I	I	V	I	V	A	L	A	RRQKRF
VII.2_VII_i_MG871466_1_chicken_Iran_PCR_UT_2017	F	V	S	I	A	V	I	F	V	L	G	RRQKRF
XXI.2_VI_i_MH044693_1_pigeon_Iran_Konarak_Barin_2017	F	I	S	I	I	V	I	V	A	L	V	RRQKRF
VII.1.1_VII_l_MG519855_1__Iran__MSH_1_2015	F	V	S	V	V	I	I	V	A	L	G	RRQKRF
VII.1.1_VII_l_MF417546_1_chicken_Iran_Beh_2012	F	V	S	V	V	V	I	V	A	L	G	RRQKRF
VII.1.1_VII_l_MH247187_1_chicken_Iran_MAM55_2017	F	V	S	V	V	V	I	V	A	L	G	RRQKRF
XXI.1.1_VI_g_MH247188_1_pigeon_Iran_IR_MAM39_2017	F	I	S	V	V	V	A	V	A	L	I	KRQKRF
XXI.1.1_VI_g_MK592884_pigeon_Iran_UT_EGV_2018	F	I	S	V	I	V	I	V	A	L	I	KRQKRF
VII.2_VII_i_MT254060_1_cockatiel_Iran_SR0077_2019	F	V	S	V	A	V	I	F	V	L	G	RRQKRF
VII.1.1_VII_l_MN615882.1_chicken_Iran_ABD_2018	F	V	S	V	V	V	I	V	A	L	G	RRQKRF
VI.2.1.2_VI_h_MN422262.1_pigeon_Iran_AMMM160_2019	F	I	S	V	V	I	I	V	A	L	V	RRQKRF
XXI.1.1_VI_g_MN636822.1_pigeon_Iran_AMMM168_2019	F	I	S	V	V	V	I	V	A	L	I	KRQKRF
XIII.2.1_XIIIb_MT755375.1_chicken_Iran_Mashhad_RT30_2010	F	V	S	V	V	A	I	I	A	L	V	RRRKRF
XIII.1.2_XIII_d_JQ267579_chicken_Iran_EMM_7_2011	F	V	S	V	V	V	I	V	A	L	V	RRRKRF
VII.2_VII_k_KY747479_1__chicken_Namibia_5620_2016	F	V	G	V	V	V	I	V	A	L	V	GRQGRL
VII.2_VII_a_JN986837_chicken_Netherlands_152608_ancestral_1993	F	V	S	V	V	V	I	V	A	L	V	GRQGRL
VII.2_VII_h_MF622047_1_chicken_South_Africa_RBWW_3_2013	F	I	S	V	V	V	I	V	A	L	I	RRQKRF
VII.1.2_VII_f_AF458010_chicken_China_JS_3_2000	F	V	S	V	V	V	T	V	A	L	V	RRRKRF
VII.1.1_VII_e_MZ802791.1_chicken_Taiwan_AHRI148_2019	F	V	S	I	V	V	I	V	A	L	V	RRQKRF
VII.1.1_VII_b_MK423875_chicken_Kyrgyzstan_1_16_2016	F	V	S	V	V	V	I	I	A	L	A	KRQKRF
VII.1.1_VII_j_MW603769.1_chicken_Egypt_Beh_NRC_2021	F	V	S	V	V	V	I	I	A	L	A	RRKKRF
VII.1.1_VII_d_MN905162_chicken_Egypt_Giza_S4_2019	F	V	S	V	V	V	I	V	A	L	G	RRQKRF
VII.2_VII_i_MK253647_1_Chicken_Iraq_AMHA2__2015	F	V	S	V	A	V	I	F	V	L	G	RRQKRF
II_II_JN872151_chicken_USA_Hitchner_B1_1947	L	I	G	V	V	I	I	V	I	L	I	RRQKRF
II_II_AF077761_chicken_USA_LaSota_1946	L	I	G	V	V	I	I	V	I	L	I	RRQKRF

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4- Discussion

In this study, two NDV isolates recovered from commercial chicken flocks in Khorasan Razavi and Mazandaran provinces were characterized at the molecular level. Both isolates belonged to sub-genotype VII.1.1, carried a virulent F protein cleavage site motif, and shared a combination of residues (I513 and A520) in the transmembrane domain that differed from previously reported Iranian VII.1.1 strains. Phylogenetically, the isolates grouped with Egyptian strains rather than with the Iranian sequences available for comparison. The polybasic cleavage site is the main molecular determinant of virulence and is compatible with the neurological signs seen in both flocks; however, pathogenicity indices were not determined, and other causes of neurological disease were not excluded. These findings add limited molecular data on NDV in Iran and raise questions about the origin and evolution of the viruses involved, which these two isolates cannot resolve. These findings are consistent with earlier reports that genotype VII viruses, including sub-genotype VII.1.1, continue to be detected in poultry in Iran despite vaccination programs [2, 3, 12]. Notably, phylogenetic analysis placed these isolates in close proximity to Egyptian strains rather than historical Iranian sequences. These findings provide new insights into the molecular epidemiology of NDV in Iran and raise important questions regarding the origin, evolution, and control of circulating virulent strains.

The two isolates characterized here (SNDV-1 and SNDV-2) clustered within subgenotype VII.1.1 and shared high nucleotide identity with Egyptian strains, a pattern that mirrors recent regional reports. For instance, surveillance in Egypt between 2019 and 2024 identified a high prevalence of VII.1.1 strains in domestic poultry, with full F gene sequencing placing representative isolates within the same subgenotype [13]. Similarly, whole-genome sequencing of Ethiopian isolates revealed virulent VII.1.1 strains in vaccinated flocks, a finding that may reflect antigenic differences between field viruses and commonly used vaccine strains [8]. These parallels are consistent with VII.1.1 viruses circulating more widely in the region, although two isolates cannot show that the lineage detected here is part of a broader pattern. In contrast, earlier reports from Iranian broiler farms described VII.1.1 viruses of the VII.1.1 lineage [12], whereas the isolates in this study carried different transmembrane residues. Whether this difference reflects a recent introduction, limited availability of Iranian sequences in public databases, or local sequence variation cannot be determined from these data.

A key finding of this study is the presence of distinct amino acid substitutions (I513 and A520) within the transmembrane domain of the F protein, which differentiated our isolates from previously reported Iranian VII.1.1 strains (e.g., SMV-1/2011, Beh/2012, MAM55/2017, and ABD/2018) that uniformly carry V513 and G520. Although the functional consequences of these specific substitutions remain to be experimentally validated, the transmembrane domain is known to play a critical role in the structural stability and fusogenic activity of the F protein [14]. Changes in this region could potentially influence membrane fusion, but no data from this study address that possibility. The presence of the same residues in Egyptian strains is compatible with a shared origin or viral exchange between the two regions. Other explanations, such as independent change or incomplete sampling of Iranian viruses, cannot be excluded without further data.

From a control perspective, the detection of virulent VII.1.1 viruses in vaccinated commercial flocks, as also documented in Ethiopia [8] and Iraq [15], highlights the limitations of current vaccination strategies. The genetic and antigenic divergence between circulating field strains and vaccine strains (genotypes I and II) is well established [14]. The present study did not include antigenic or vaccine-challenge data, so it cannot show whether these isolates differ in

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their susceptibility to vaccine-induced immunity. The findings may nevertheless be relevant to ongoing discussions of genotype-matched vaccines and to continued biosecurity and molecular surveillance, which would need to be evaluated in dedicated studies. Backyard and free-range flocks have been reported as possible reservoirs of virulent NDV in Slovenia [16] and northern Iran [17, 18]. Their role was not examined here, and the sequences from those reports may be useful comparators in future analyses.

Limitation of this study is that it is based on two isolates, one from each of two provinces, obtained through diagnostic submissions rather than systematic sampling. The findings therefore describe these two viruses and cannot be generalized to NDV in Iran or to the provinces of origin. In addition, the absence of closely related Iranian sequences among the BLAST matches may reflect the limited number of Iranian sequences in public databases rather than a true absence of related viruses. Additionally, the lack of complete genome data limits our ability to evaluate mutations in other viral genes, such as the HN gene, which also contributes to antigenicity and virulence. Future studies involving whole-genome sequencing and in vivo pathogenicity testing are warranted to better understand the evolutionary dynamics and biological significance of these isolates.

5- Conclusion

Two NDV isolates, one from a broiler breeder flock in Khorasan Razavi Province and one from a broiler flock in Mazandaran Province, carried a virulent-type cleavage site and were assigned to sub-genotype VII.1.1. Both carried I513 and A520 in the F protein transmembrane domain, which differed from the Iranian VII.1.1 strains included in the comparison. The close phylogenetic relationship with Egyptian strains suggests a possible transboundary introduction, distinct from previously described Iranian lineages. These findings reinforce the necessity of continuous molecular surveillance, strict biosecurity, and the consideration of genotype-matched vaccines to effectively control Newcastle disease in the region.

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Data availability

The complete genome sequences generated in this study have been deposited in GenBank under accession numbers PX452495 and PX452496. The protein sequences are available under accession numbers YBG53389 and YBG53390.

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Authors' Contribution

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Study concept and design: Sanaz Majidi, Kobra Razavi Pashabeyg, Seyed Ali Pourbakhsh; Acquisition of data: Sanaz Majidi, Seyed Ali Pourbakhsh; Analysis and interpretation of data: Sanaz Majidi; Drafting of the manuscript: Sanaz Majidi, Kobra Razavi Pashabeyg, Seyed Ali Pourbakhsh; Critical revision of the manuscript for important intellectual content: Sanaz Majidi, Seyed Ali Pourbakhsh, Mohammad Ebrahimi; Statistical analysis: Sanaz Majidi, Seyed Ali Pourbakhsh; Administrative, technical, and material support: Nima Keshan Zare

Conflict of Interest

The authors declared no conflict of interest.

Compliance with ethical guidelines

This study used carcasses and tissue samples submitted to the diagnostic laboratory for routine disease investigation. No live animals were experimentally infected, handled, or euthanized for research purposes. The study protocol was reviewed and approved by the SANA institutional ethics committee under approval number SIAHDR-VIR-REC2024=002.

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