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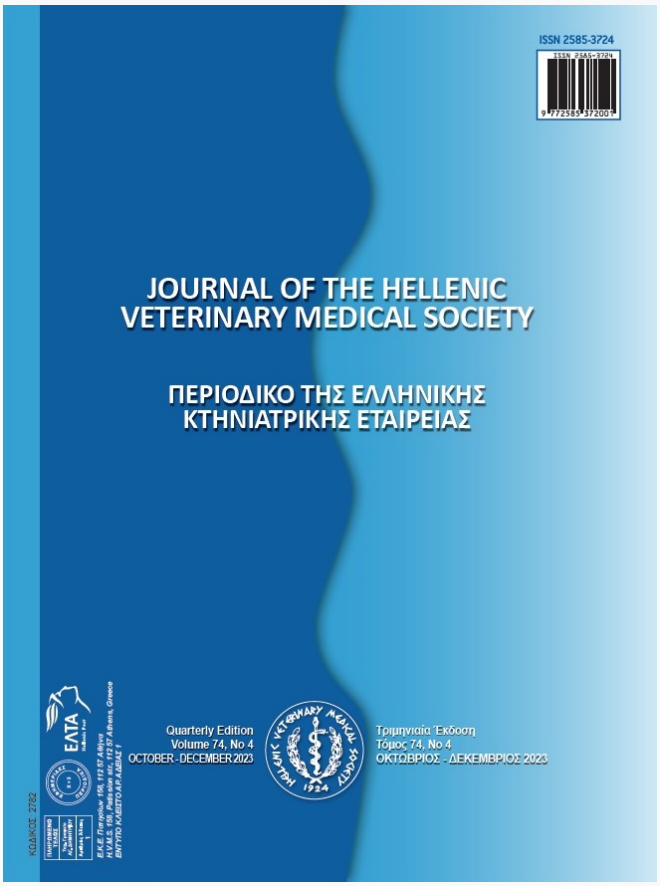

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The Molecular detection and pathotypic characterization of Genotype VII.2 of Newcastle Disease Virus Isolated from Imported Cockatiels in Iran

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Abstract: Genotype VII of Newcastle disease virus (NDV), the most prevalent genotype of avian paramyxovirus I (APMV-1) in Asia, is rapidly spreading worldwide. The emergence of new sub-genotype VII.2 in different countries raises questions about the evolutionary patterns of these isolates. Despite the devastating effects of NDV on endangered parrot species and the major role of the psittacines in the cross-species transmission of the virus, there have not been any phylogenetic studies on the NDVs circulationin these populations in Iran. In this regard, a brain sample obtained from three dead cockatiels of a suspected NDV flock with an 80% mortality rate was implemented for further molecular, pathogenicity, and phylogenetic analysis of the fusion gene and deduced amino acid sequences. Pathogenicity indices and Cleavage site investigation revealed the high virulence (^{112}RRQKRF^{117}) of the virus. Phylogenetic studies clustered our isolate (SR0077) among VII.2 sequences from Pakistan, Indonesia, China, Jordan, and Malaysia. Moreover, the nucleotide distances between the studied isolate and VII.2 strains reported from Pakistan were less than 0.01. However, non-VII.2 isolates previously reported from Iran were phylogenetically distinct from our isolate. Taken together, these findings, along with some identical substitutions at functional domains of the F protein, highlight the risk of introducing VII.2 strains to other countries and the possible incidence of new panzootics. Finally, based on history and molecular analyses, it seems that bird trade from Pakistan is the main cause of the development of new VII.2 NDV strains in Iran.

Keywords: Cockatiel; F gene; Illegal trade; Iran; Newcastle disease virus

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INTRODUCTION

Newcastle disease (ND) is devastating disease, and it places huge economic burdens on the poultry industry (Ghorbankhani et al., 2022) worldwide. The causative agent of this global infectious disease is Newcastle disease virus (NDV), also known as avian paramyxovirus, Type 1 (APMV1). It is an enveloped, negative-sense, single-stranded RNA virus belonging to the *Paramyxoviridae* family and *Avulavirus* genus (Ravindra et al., 2008; Shafaati et al., 2022). The 15 kb genome of the virus, with 3′ -NP-P-M-F-HN-L-5′ sequence, encodes six major proteins. NDV has a broad host range, including 241 species from 27 orders of birds (Morovati et al., 2022).

Based on pathogenicity criteria including mean-death time (MDT), intracerebral pathogenicity (ICPI), and intravenous pathogenicity (IVPI) indexes, NDV strains have been categorized into velogenic, mesogenic and lentogenic (Mehrabanpour et al., 2014). The amino acid sequence of the cleavage site of the fusion (F) protein determines the virulence of the virus. According to the World Organization for Animal Health (OIE), more than two basic amino acids at positions 113, 115, and 116 followed by a phenylalanine (F) residue at position 117 classified the virus as a notifiable virulent NDV (OIE, 2005). In addition, the other regions of the F protein, including heptad repeats, hypervariable regions, neutralizing epitopes, fusion peptides, transmembrane domains, N-glycosylation sites, and conserved cysteine residues, have important roles in the fusogenicity efficiency of NDV (Neyt et al., 1989; Sergel-Germano et al., 1994; Toyoda et al., 1988; Umali et al., 2013; Wang et al., 2016; Yusoff et al., 1989).

As per the latest classification, the NDV strains can be categorized into two classes:Class I NDVs, which are mostly isolated from wild birds, have less virulence and diversity compared to class II viruses. They include only a single genotype. In contrast, class II NDVs encompass a range of virulent and avirulent viruses and compromise 21 genotypes (I to XXI) (Dimitrov et al., 2019).

Cockatiels (*Nymphicus hollandicus*), as popular pets with global distribution, are a member of the *Cacatuidae* family and *Psittaciformes* order (Al-Shammari et al., 2020). These birds, which are threatened with extinction, are sensitive to the ND infection and considered as a source of velogenic NDV (Erickson et al., 1977).

ND was first identified from chicken in Iran in 1951 (Sohrab, 1973). This coincided with the expansion of the poultry industry in the country. The disease spread gradually to different parts of the Iran so that it became an endemic disease in this region. Since then, the periodic occurrence of ND has been reported from all provinces (Boroomand et al., 2016; Bouzari & Mousavi, 2006; Ghalyanchilangeroudi et al., 2018; Ghiamirad et al., 2010; Hadipour, 2009; Hassanzadeh & Bozorgmeri Fard, 2004; Hosseini et al., 2014; Kianizadeh et al., 1999, 2002; Madadgar et al., 2013; Mehrabanpour et al., 2007; Molouki et al., 2019; Momayez et al., 2007; Rezaeianzadeh et al., 2011; Sabouri et al., 2018; Samadi et al., 2014). Despite the intensive immunization programs and several studies in the poultry field, there is insufficient knowledge about the circulating NDVs in psittacine populations in Iran. It is believed that psittacine species are more resistant to NDV and show less clinical signs during the infection. Hence, they can spread virulent NDVs to more susceptible avian domestic species and lead to the evolution of new genotypes (Panigrahy et al., 1993; Senne et al., 1983). Based on a study for characterizing NDV in exotic caged birds in Iran, 10% of the samples were negative based on clinical signs and serological tests, but positive by RT-PCR (Madadgar et al., 2013). Illegal trade across vast common borders of Iran and different countries, especially those with low biosecurity levels, is an important issue that may well contribute to new outbreaks and should be considered more strictly (Ghalyanchilangeroudi et al., 2018).

This study is the first attempt at molecular characterization and phylogenetic analysis of NDV in psittacine species in Iran. Here, we have represented the molecular sequence of an NDV isolated from the brain samples of three cockatiels. The analyses were performed based on the *F* gene and deduced amino acid full-length sequences. The information obtained from this study could serve as an introduction for NDVs infecting psittacine species in Iran. We have concentrated on finding the relationship of the studied isolate and the sequences reported so far and explain the potential of NDVs of *Psittaciformes* to cause new outbreaks.

MATERIALS AND METHODS

Case history

A flock of 150 cockatiels kept at a live bird market in the Alborz province, Iran was referred to bird

clinic of Veterinary School-University of Tehran. Severe neurological and enteric clinical signs including weakness, green diarrhea, ataxia, torticollis, wing tremors, and limb paralysis accompanied by nodding and shaking of the heads manifested in 100 birds. Progressive mortality rate of up to 80% was seen in the affected population. Succumbed birds were necropsied. Hyperemia and petechial hemorrhages along with multifocal necrosis at proventriculus and spleen mucosa were the most significant pathological lesions.

Virus isolation and identification

The brain samples aseptically collected from three dead cockatiels were pooled and homogenized and inoculated into 9-day-old, specific-pathogen-free (SPF), embryonated chicken eggs, according to previously described guidelines for studying APMV-1 (Yusoff et al., 1989). Embryos found dead 48 hours post-inoculation (P.I) were considered NDV-infected, and their allantoic fluids were harvested for further hemagglutination assay (HA) pathogenicity tests and molecular characterizations.

Hemagglutination properties of inoculated embryonated eggs allantoic fluids were evaluated by making a two-fold serial dilution and 1% (v/v) erythrocyte in a 96-well microtiter plate. After 30 minutes of incubation at room temperature, the highest dilution giving complete agglutination was considered the virus HA titer (Alexander, 2000). Moreover, based on the previously standard protocols (OIE, 2005; Alexander, 2000), the pathogenicity tests, including ICPI and MDT, were performed in one-day-old chickens and 9-day-old embryonated SPF eggs.

RNA isolation and molecular diagnosis

Total RNA was extracted from allantoic fluids using High Pure Viral RNA Isolation Kit (Roche, Germany), according to the manufacturer's guideline. Extracted RNA was transcribed into cDNA and amplified using One-Step RT-PCR Kit (biotechrabbit, Germany). Subsequently, for F gene polymerization the F-Fwd-middle and F-Rev-middle nested to F-Fwd and F-Rev (Bioneer Corporation, South Korea) (Table 1) as previously described (Molouki et al., 2019). Since the unknown sequence was attributed to NDV strains, the PCR products containing the complete nucleotide sequence of F gene were gel-purified using S-1050-1 kitTM (Dena Zist, Iran). The products were then characterized by Dye-terminator Sanger sequencing method (Macrogen Company, South Korea) using the primers presented in Table 1. Four overlapping PCR reads were edited and assembled by Clustal X (version 2.1) (Thompson et al., 1997) and BioEdit (version 7.2.6) (Hall et al., 2011). Consequently, a 1662-bp product was constructed covering the full-length of the F gene. In the next step, the NDV nature of the acquired sequence was confirmed using the Blast tool of the National Center for Biotechnology Information (NCBI) database. The sequence data of the studied isolate (SR0077) was submitted to the GenBank database under accession number MT254060.

Sequence analysisand phylogenetic studies

Using the MEGA 7 (version 7.0.26) program (Kumar et al., 2016), the phylogenetic tree was generated based on the open reading frame (ORF) of nucleotide sequences of F gene of more than 100 curated isolates obtained from an NDV dataset (https://github.com/NDVconsortium). The isolates were selected so that they cover various hosts and all (sub) genotypes of NDV. The evolutionary history was indicated by utilizing maximum likelihood, GTR+G+I model, and 1000 bootstrapping. Three class I of APMV-1 were considered outgroups.

In addition, the assembly was aligned against the mentioned sequences (Clustal X, version 2.1) (Thompson et al., 1997). The identity and distance analyses were carried out using the BLAST tool and MEGA 7 software (version 7.0.26). Moreover, different genotypes of the virus previously reported from Iran and sequences from other countries with a high degree of homology with our isolate were investigated for residue substitutions at substantial regions of F protein. These regions included cleavage site (aa residues 112-117), Fusion peptide (aa residues 117-141), HRa (aa residues 143-185), HRb (aa residues 268-299), HRc (aa residues 471-500), hypervariable

Table 1 List of primer set sequences used in this study

Primer Name	Primer Sequences	Position
F-Fwd	5′-YTGCTTATAGTTAGTTYACCTGTC-3′	4490-5635
F-Rev	5′-ACCCGTGTATTGCTYTTYGG-3′	5239-6285
F-Fwd-middle	5′-GCAACCAATGAAGCTGTGCATGA-3′	5053-6183
F-Rev-middle	5′-ACAGCTTCTCCATAATTTTGCGA-3′	4668-5437

regions (aa residues 4, 10, 11, 13, 20, 21, 27, 52, 63, 78, 93, 101, 121), neutralizing epitopes (aa residues 72, 74, 75, 78, 79, 157-171, 343), and transmembrane domain (aa residues 501-521).

RESULTS

Primary virus identification

Results of HA confirmed the hemagglutinating activity of the studied isolate. The titre was 6 HA units per 25 µl. The MDT and ICPI of the studied isolate were calculated 50.4 h and of 1.9, respectively. These values indicated the velogenic nature of the virus.

Genetic and phylogenetic characterizations

The 1662-bp *F* gene bands were successfully detected by specific RT-PCR in presumptive samples with NDV. The phylogenetic tree grouped the studied isolate into sub-genotypes VII.2 (former VIIi) sequences reported from Iran, Pakistan, Indonesia, Jordan, China, and Israel (Fig. 1). Furthermore, other strains from Iran belonging to sub-genotypes and genotypes II, VII.1.1, XIII, and XX1 (MF417546, MG456676, MK592884, AY928933, KX268351, JQ267584, and JQ267579)formed the separate clusters to our isolate. Although a VII.2 sequence (MG871466) with 97.41% identity had the highest degree of similarity to our isolate among reported strains from Iran, the other sequences from Iran belonging to other (sub) genotypes shared < 90% identity (Table 2). Moreover, two vaccine strains including LaSota and B1 (ANW12438, ANQ45239) with 83% identity were genetically distinct from the studied isolate. The alignment of the complete coding region of the *F* gene (1662 nt.s) of the studied isolate with other sequences revealed more than 98% identity to theVII.2 (former VIIi) sequences reported from Pakistan, Israel, Indonesia, China, Malaysia, and Jordan. Pakistani sequences from parakeets (KX268689, KX268690, and KX268691) and chicken (KY076035) had more than 99% identity with our isolate. Compared to recently reported Indonesian strains isolated from chickens (HQ697254 and MN557411), three other ancestral sequences from Indonesia (KF767104 from cockatoo, KF767106 from parrots, JX393313 from parrots) exhibited a lower level of similarity to our isolate (92% identity). Distances between our isolate and different sequences were calculated and documented in Table 2. Despite the high nucleotide distances of the studied isolate from non-VII.2 strains previously reported from Iran, the low distances (<0.01) were observed compared to VII.2 Pakistani

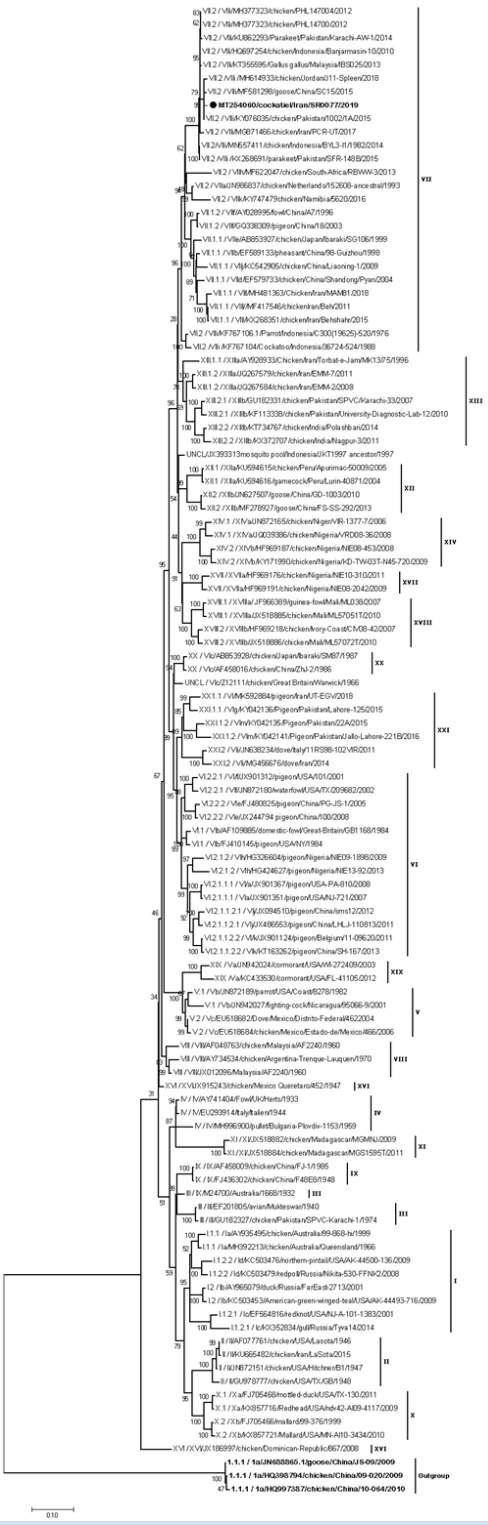

Figure 1. Phylogenetic tree based on F gene ORF sequences of the studied isolate and more than 100 NDV isolates with different genotypes submitted in GenBank. The maximum likelihood and TN93+G+I model were implemented for tree construction. The number of nodes represents the bootstrap values of 1000 replicates. The studied sequence is in bold and highlighted with a black circle. New nomenclature (Dimitro et al) of sequences is displayed by vertical lines. Three class I paramyxoviruses were considered outgroups.

Table 2 Nucleotide identities (%) and distances of the studied isolate with some other similar sequences. The identities are shown above the diagonal. The distances are observed in the lower left of the table and codon positions included were 1st+2nd+3rd+Noncoding. The sequences with the highest level of identity and the lowest distances to the studied isolate are shown in bold. The most identical Iranian isolate is bold and underlined

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	VII.2/MT254060/cockatiel/Iran/2019	-	**99.22**	**99.10**	97.83	92.66	92.96	98.13	92.12	97.59	98.13	98.13	**97.41**	98.44	98.68	97.89	90.44	90.20	90.26	83.53	83.90	90.92	90.61	88.15	86.95
2	VII.2/KX268691/parakeet/Pak/2015	**0.008**	-	99.51	98.50	93.44	93.74	99.21	92.03	98.50	98.92	98.85	98.30	99.04	99.34	95.61	90.80	90.51	90.69	83.96	84.32	91.23	91.40	86.50	86.22
3	VII.2/KY076035/chicken/Pak/2015	**0.009**	0.001	-	98.62	93.56	93.86	99.24	92.10	98.54	99.04	98.98	98.37	99.31	99.46	97.56	90.88	90.69	90.81	83.96	84.32	91.41	91.40	86.55	86.28
4	VII.2/KU862293/Parakeet/Pak/2014	0.022	0.015	0.014	-	93.98	94.16	99.58	93.26	99.04	99.58	99.58	98.74	97.83	98.32	99.34	91.52	91.16	91.34	84.29	84.71	92.12	91.76	88.53	87.27
5	VII.2/KF767104/Cockatoo/Indo/1987	0.073	0.066	0.064	0.060	-	98.50	94.28	95.79	94.46	94.28	94.16	93.80	93.02	93.32	94.04	92.84	92.36	92.78	85.19	85.37	93.62	93.26	90.13	88.03
6	VII.2/KF767106/Parrot/Indo/1976	0.070	0.063	0.061	0.058	0.015	-	94.58	96.45	94.77	94.58	94.46	93.98	93.32	93.62	94.34	92.90	92.42	92.72	85.19	85.37	94.28	94.16	90.67	88.63
7	VII.2/HQ697254/chicken/Indo/2010	0.019	0.011	0.010	0.004	0.057	0.054	-	92.52	99.29	100	99.88	99.03	98.62	98.74	96.36	91.22	90.53	91.52	84.35	84.77	92.11	91.94	86.80	86.53
8	VII.2/JX393313/culex/Indo/1979	0.079	0.071	0.070	0.067	0.042	0.035	0.064	-	92.41	93.56	93.13	91.94	91.68	92.78	90.85	92.05	91.52	92.12	85.75	85.81	93.86	94.89	89.33	88.76
9	VII.2/MN557411/chicken/Indo/2014	0.024	0.016	0.015	0.010	0.055	0.052	0.005	0.064	-	99.46	99.04	98.37	97.96	98.19	95.84	91.11	90.58	91.58	84.34	84.76	92.34	92.12	86.70	86.40
10	VII.2/KF792018/chicken/Israel/2011	0.019	0.011	0.010	0.004	0.057	0.054	0.000	0.064	0.005	-	99.88	99.16	98.26	98.74	99.76	91.70	91.34	91.52	84.35	84.77	92.54	91.94	88.95	87.45
11	VII.2/MH377323/chicken/Israel/2012	0.019	0.012	0.011	0.004	0.058	0.055	0.001	0.066	0.007	0.001	-	98.92	98.50	98.62	99.19	91.65	90.53	91.52	84.23	84.65	91.99	91.82	87.47	87.75
12	VII.2/MG871466/chicken/Iran/2017	0.026	0.019	0.018	0.013	0.062	0.060	0.008	0.070	0.013	0.008	0.010	-	97.82	97.89	97.44	90.75	89.75	91.04	84.23	84.65	91.58	91.34	86.39	86.27
13	VII.2/MH614933/chicken/Jordan/2018	0.016	0.008	0.008	0.022	0.070	0.067	0.017	0.075	0.023	0.017	0.019	0.025	-	98.68	97.05	90.49	90.17	90.26	83.47	83.84	91.00	91.10	86.28	86.03
14	VII.2/MF581298/goose/China/2015	0.013	0.007	0.005	0.017	0.067	0.064	0.013	0.072	0.018	0.013	0.014	0.021	0.013	-	98.50	90.69	90.44	90.50	83.91	84.27	91.58	91.76	88.45	86.95
15	VII.2/KT355595/chick/Malaysia/2013	0.021	0.013	0.012	0.007	0.060	0.057	0.002	0.067	0.007	0.002	0.004	0.010	0.020	0.015	-	89.44	90.36	91.28	84.35	84.77	91.93	91.76	86.44	86.19
16	VII.1.1/MF417546/chicken/Iran/2011	0.096	0.092	0.091	0.085	0.072	0.071	0.083	0.077	0.082	0.083	0.083	0.088	0.096	0.094	0.085	-	98.72	99.82	84.05	84.48	90.59	90.02	86.73	86.25
17	VII.1.1/MH481363/Chicken/Iran/2018	0.099	0.094	0.093	0.088	0.076	0.076	0.087	0.079	0.086	0.087	0.087	0.093	0.099	0.096	0.088	0.013	-	98.50	83.63	84.06	90.30	89.48	87.35	85.20
18	VII.1.1/KX268351/chicken/Iran/2015	0.098	0.094	0.093	0.087	0.072	0.073	0.085	0.079	0.084	0.085	0.085	0.090	0.098	0.096	0.087	0.002	0.015	-	83.93	84.36	90.68	89.84	88.69	85.98
19	II/KU665482/chicken/Iran/2015	0.167	0.162	0.162	0.159	0.148	0.150	0.158	0.146	0.158	0.158	0.159	0.159	0.167	0.164	0.158	0.162	0.166	0.163	-	98.92	85.86	85.14	84.11	84.02
20	II/KU886038/chicken/Iran/2014	0.163	0.159	0.159	0.155	0.145	0.148	0.154	0.145	0.154	0.154	0.156	0.156	0.164	0.160	0.154	0.159	0.163	0.160	0.011	-	86.10	85.38	84.71	84.52
21	XIII.1.1/AY928933/Chicken/Iran/1996	0.091	0.084	0.082	0.079	0.064	0.057	0.075	0.060	0.073	0.075	0.076	0.080	0.087	0.085	0.077	0.092	0.095	0.094	0.144	0.142	-	94.52	88.54	87.31
22	XIII.1.2/JQ267579/chicken/Iran/2011	0.094	0.086	0.086	0.082	0.067	0.058	0.081	0.051	0.079	0.081	0.082	0.087	0.089	0.088	0.082	0.100	0.106	0.102	0.150	0.148	0.055	-	89.13	87.98
23	XX1.1/MK592884/pigeon/Iran/2018	0.120	0.114	0.114	0.116	0.099	0.093	0.111	0.085	0.111	0.111	0.111	0.117	0.118	0.117	0.112	0.113	0.117	0.113	0.161	0.155	0.111	0.110	-	90.17
24	XXI.2/MG456676/dove/Iran/2014	0.132	0.129	0.130	0.129	0.120	0.114	0.127	0.110	0.125	0.127	0.127	0.129	0.134	0.132	0.126	0.140	0.139	0.140	0.170	0.166	0.122	0.121	0.095	-

(KX268691 and KY076035) sequences isolated from mosquito pool and chicken populations. However, distances of approximately 0.02 between our isolate and other VII.2 strains reported from chicken farms of Iran (MG871466) and other Asian countries were computed.

Amino acid substitutions

The pathogenicity results obtained from the primary evaluations were confirmed by sequencing of the cleavage site of *F* protein. The sequence of ^{112}RRQKRF^{117} revealed the high-virulence of the studied isolate. Other sequences from Iran had the same sequences as our isolates, excluding one genotype XIII (AFD50427) and two vaccine strains (ANW12438 for LaSota and ANQ45239 for B1) (Table 3).

Compared to prevalent VII.1.1, XIII, and XXI NDV genotypes reported from Iran, our isolate along with other VII.2 strains had a different pattern of substitutions in hypervariable regions (Table 4). The V residue at position 11 in VII.2 isolates was replaced by A in other Iranian sub (genotypes) (Table 3). The M20V and C27R were two common substitutions among the VII.1.1 (except VIIi strains) and VII.2isolates. However, vaccine strains (ANW12438, ANQ45239) with seven different substitutions were less identical to the studied isolates.

Amino acid sequences of the neutralizing epitopes were almost conserved in different isolates. However, position 170 was the most variable residue among different strains. Contrary to another VII.2 strain isolated from a chicken flock, the studied isolate and VII.1.1 strains previously reported from Iran, contained D→N substitution at this position (Table 4).

Compared to the consensus sequence, the number of amino acid substitutions in heptad repeat sequences at our isolate and other VII.2 strains was fewer than other (sub) genotypes (Table 5). Vaccine strains (ANW12438, ANQ45239) with nine substitutions were the most variable sequences compared to the studied isolate. Moreover, the common A482T substitution was observed at sequences of our isolate and other VII.2 isolates from Iran, Pakistan, China, Malaysia, and Israel.

The data obtained from the comparison of fusion peptide sequences were broadly consistent with other results obtained in this study. With three substitutions at positions 117, 121, and 124, the frequency of amino acid mutations in vaccine strains (ANW12438 and ANQ45239) was more than other sequences. According to the consensus sequence, almost all strains showed in table 3 represented highly conserved sequences.

The studied isolate contained two substitutions of V→A and V→Fat positions 506 and 513. The vaccine strains (ANW12438and ANQ45239) with three replacements of G and V by residue were the most phylogenetically distant isolates to our strain at transmembrane domain (Table 3).

Six N-glycosylation motifs (Asn-X-Ser/Thr that X is any amino acid except proline or aspartic acid)

Table 3 The amino acid substitutions in fusion peptide, transmembrane domain and cleavage site of F protein in the studied isolate and some other similar sequences submitted in GenBank

Strains	Genotype (Dimitroet al)	Fusion peptide (117-141)				Transmembrane domain (501-521)								Cleavage site (112-117)
		117	121	124	125	506	509	510	511	513	516	517	520	
Consensus[a]		F	V	S	V	V	V	I	S	V	V	L	G	RRQKRF
cockatiel/Iran/2019	VII.2	-[b]	-	-	-	A	-	-	-	F	-	-	-	-
AWL80007/chicken/Iran/2017/VIIi	VII.2	-	-	-	I	A	-	-	-	F	-	-	-	-
APY26422/parakeet/Pak/2015/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
ARE67974/chicken/Pak/2015/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
AOM52877/Parakeet/Pak/2014/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
AEV40792/chicken/Indo/2010/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
AHH91522/Cockatoo/Indo/1987/VIIi[C]	VII.2	-	-	-	-	I	-	V	-	-	A	-	-	-
AHH91524/Parrot/Indo/1976/VIIi[C]	VII.2	-	-	-	-	I	-	V	-	-	A	-	V	-
AGL44387/Culex/Indo/1979/VIIi[C]	VII.2	-	-	-	-	-	-	-	-	-	-	-	V	-
ASU55432/goose/China/2015/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	V	-
AXK59831/chicken/Jordan/2018/VIIi	VII.2	-	-	-	-	-	-	-	-	F	-	-	-	-
AXE74278/chicken/Israel/2012/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
AHI07431/chicken/Israel/2011/VIIi	VII.2	-	-	-	-	-	-	-	-	F	-	-	-	-
AMD82623/chick/Malaysia/2013/VIIi	VII.2	-	-	-	-	A	-	-	-	F	-	-	-	-
AFD50426/chicken/Iran/2011/XIIId	XIII.1.2	-	-	-	-	-	-	-	-	-	A	-	-	RR**R**KRF
AEZ36085/Cockatoo/India/1982/XIII	XIII.1	-	-	-	-	-	-	-	-	-	A	-	V	-
ANS53884/chicken/Iran/2015/VIIj	VII.1.1	-	-	-	-	-	-	-	-	-	A	-	V	-
QBA31041/chicken/Iran/2018/VIIl	VII.1.1	-	-	-	-	-	-	-	-	-	A	-	-	-
QBA17051/chicken/Iran\/2011/VIIl	VII.1.1	-	-	-	-	-	-	-	A	-	A	-	-	-
APQ40662/chicken/Iran/2018/VIIl	VII.1.1	-	-	-	-	-	-	-	-	-	A	-	-	-
ABQ85424/chicken/China/2007/VIId	VII.1.1	-	-	-	-	-	-	-	-	-	A	-	-	-
AAX31653/Chicken/Iran/1996/XIIIa	XIII.1.1	-	-	-	-	-	-	-	-	-	I	-	V	-
QDE10569/pigeon/Iran/2018/VIg	XXI.1.1	-	I	-	-	-	-	-	-	-	A	-	I	-
AYA42676/dove/Iran/2014/VIi	XXI.2	-	I	-	I	I	-	-	-	-	A	-	A	-
AWL30643/chicken/Iran/2011/VIId	VII.1.1	-	-	-	-	-	-	-	-	-	A	-	-	-
ANW12438/chicken/Iran/2015/II	II	L	I	G	-	-	I	-	-	-	I	-	I	**G**RQ**G**R**L**
ANQ45239/chicken/Iran/2014/II	II	L	I	G	-	-	I		-	-	I	-	I	**G**RQ**G**R**L**

Table 4 The amino acid substitutions in hypervariable regions and neutralizing epitopes of F protein in the studied isolate and some other similar sequences submitted in GenBank

Strains	Genotype (Dimitro et al)	Hypervariable region													Neutralizing epitopes						
		4	10	11	13	20	21	27	52	63	78	93	101	121	72	74	75	78	79	157-171	343
Consensus[a]		K	P	V	L	M	L	C	V	V	K	T	K	V	D	E	A	K	A	SIAATNEAVHEVTDG	L
cockatiel/Iran/2019	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AWL80007/chicken/Iran/2017/VIIi	VII.2	[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
APY26422/parakeet/Pak/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ARE67974/chicken/Pak/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AOM52877/Parakeet/Pak/2014/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
AEV40792/chicken/Indo/2010/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHH91522/Cockatoo/Indo/1987/VIIi[C]	VII.2	-	-	-	-	V	-	-	I	-	-	-	-	-	E	-	-	-	-	-	-
AHH91524 /Parrot/Indo/1976/VIIi[C]	VII.2	-	-	-	-	-	-	-	I	-	-	-	R	-	-	-	-	-	-	-	-
AGL44387/Culex/Indo/1979/VIIi[C]	VII.2	-	-	-	-	-	-	-	I	-	-	-	R	-	-	-	-	-	-	-	-
ASU55432/goose/China/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AXK59831/chicken/Jordan/2018/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	G	-	-	-	-		-
AXE74278/chicken/Israel/2012/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
AHI07431/chicken/Israel/2011/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-
AMD82623/chick/Malaysia/2013/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AFD50426/chicken/Iran/2011/XIIId	XIII.1.2	-	-	-	-	-	-	-	I	-	-	-	R	-	-	-	-	-	-	-	-
AEZ36085/Cockatoo/India/1982/XIII	XIII.1	-	-	-	-	-	-	-	I	-	-	-	R	-	-	-	-	-	-	-	-
ANS53884/chicken/Iran/2015/VIIj	VII.1.1	-	-	A	-	V	-	R	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
QBA31041/chicken/Iran/2018/VIIl	VII.1.1	-	-	A	-	V	-	R	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
QBA17051/chicken/Iran\/2011/VIIl	VII.1.1	-	-	A	-	V	-	R	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
APQ40662/chicken/Iran/2018/VIIl	VII.1.1	E	-	A	-	V	-	R	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
ABQ85424/chicken/China/2007/VIId	VII.1.1	-	-	A	-	-	-	H	-	-	-	-	-	-	-	-	-	-	-	-	-
AAX31653/Chicken/Iran/1996/XIIIa	XIII.1.1	-	-	-	-	-	-	R	I	-	-	-	R	-	-	-	-	-	-		-
QDE10569/pigeon/Iran/2018/VIg	XXI.1.1	-	-	A	-	T	-	-	I	I	R	N	R	I	-	-	-	R	-		-
AYA42676/dove/Iran/2014/VIi	XXI.2	-	-	T	P	-	-	-	I	-	-	S	R	I	-	-	-	-	-		-
AWL30643/chicken/Iran/2011/VIId	VII.1.1	-	-	A	-	V	-	R	-	-	-	-	-	-	-	-	-	-	-	SIAATNEAVHEVT**N**G	-
ANW12438/chicken/Iran/2015/II	II	R	-	A	M	A	-	-	I	-	-	-	R	I	-	-	-	-	-	-	-
ANQ45239/chicken/Iran/2014/II	II	R	-	A	M	A	-	-	I	-	-	-	R	I	-	-	-	-	-	-	-

Table 5 The amino acid substitutions in Heptad repeat domains of F protein in the studied isolate and some other similar sequences submitted in GenBank

Strains	Genotype (Dimitro et al)	HRa (143-185)					HRb (268-299)			HRc (471-500)								
		145	146	153	170	176	270	272	288	474	479	480	482	486	489	491	494	498
Consensus[a]		N	Q	R	D	S	T	Y	N	I	D	K	A	S	D	V	R	T
cockatiel/Iran/2019	VII.2	-[b]	-	-	N	-	-	-	-	-	-	-	T	-	-	I	-	-
AWL80007/chicken/Iran/2017/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
APY26422/parakeet/Pak/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
ARE67974/chicken/Pak/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
AOM52877/Parakeet/Pak/2014/VIIi	VII.2	-	-	-	N	-	-	-	-	-	-	-	T	-	-	-	-	-
AEV40792/chicken/Indo/2010/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
AHH91522/Cockatoo/Indo/1987/VIIi[C]	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHH91524 /Parrot/Indo/1976/VIIi[C]	VII.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AGL44387/Culex/Indo/1979/VIIi[C]	VII.2	-	-	-	-	A	-	H	-	-	-	-	-	-	-	-	-	-
ASU55432/goose/China/2015/VIIi	VII.2	-	-	-	-	-	-	-	-	D	-	-	T	-	-	-	-	-
AXK59831/chicken/Jordan/2018/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
AXE74278/chicken/Israel/2012/VIIi	VII.2	-	-	-	N	-	-	-	-	-	-	-	T	-	-	-	-	-
AHI07431/chicken/Israel/2011/VIIi	VII.2	-	-	-		-	-	-	-	-	-	-	T	-	-	-	-	-
AMD82623/chick/Malaysia/2013/VIIi	VII.2	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-
AFD50426/chicken/Iran/2011/XIIId	XIII.1.2	-	-	-	-	A	-	H	T	-	-	-	-	-	-	-	-	A
AEZ36085/Cockatoo/India/1982/XIII	XIII.1	-	-	-	-	A	A	-	-	-	-	-	-	-	-	-	-	-
ANS53884/chicken/Iran/2015/VIIj	VII.1.1	-	-	Q	N	-	-	-	-	-	-	R	-	-	E	-	-	-
QBA31041/chicken/Iran/2018/VIII	VII.1.1	-	-	Q	N	-	-	-	-	-	-	-	-	-	E	-	-	-
QBA17051/chicken/Iran\/2011/VIII	VII.1.1	-	-	Q	N	-	-	-	-	-	-	R	-	-	E	-	-	-
APQ40662/chicken/Iran/2018/VIII	VII.1.1	-	-	-	N	-	-	-	-	-	-	R	-	-	E	-	-	-
ABQ85424/chicken/China/2007/VIId	VII.1.1	-	K	-	-	-	-	-	-	-	-	-	-	-	E	-	-	-
AAX31653/Chicken/Iran/1996/XIIIa	XIII.1.1	-	-	-	-	-	-	H	T	-	-	-	E	-	-	-	-	-
QDE10569/pigeon/Iran/2018/VIg	XXI.1.1	-	-	-	-	-	-	H	-	-	-	R	-	-	-	-	K	-
AYA42676/dove/Iran/2014/VIi	XXI.2	-	-	-	-	-	-	H	-	-	-	R	-	-	-	-	K	-
AWL30643/chicken/Iran/2011/VIId	VII.1.1	-	-	Q	N	-	-	-	-	-	-	R	R	-	-	E	-	-
ANW12438/chicken/Iran/2015/II	II	K	-	-	-	A	-	N	T	-	N	-	E	R	-	-	K	-
ANQ45239/chicken/Iran/2014/II	II	K	-	-	-	A	-	N	T	-	N	-	E	R	-	-	K	-

[†]The consensus sequence was obtained from 100 sequences represented in GenBank [‡]Identical amino acid as the consensus sequence

were conserved and detected in all isolates (Tables 2-4). These sequences, which have main roles in pathogenicity of NDV, were located at residues 85 to 87, 191 to 193, 366 to 368, 447 to 449, 471 to 473, and 541 to 543.

The studied isolate (brain samples from affected cockatiels) contained 12 absolutely conserved cysteine (C) residues at positions 27, 76, 199, 338, 347, 362, 370, 394, 399, 401, 424 and 523. Three ancestral Indonesian isolates (AHH91522, AHH91524, and AGL44387) along with two vaccine strains (ANW12438 and ANQ45239) and XIII sequences reported from Iran (AFD50427) had an additional C residue at position 25. The other Iranian strains with VII.1.1 genotype included fewer C residues following C→R and C→Y substitutions at positions 27 and 76.

DISCUSSION

The main purpose of the study is to draw attention to the importance of NDV characterization in psittacine populations in Iran. In this regard, the molecular characterization of an NDV isolate recovered from three dead cockatiels kept in a live bird market in Iran was assessed based on the *F* gene complete sequence. Next, the possible relation between the studied isolate and sequences reported from Iran and other countries were evaluated. To our knowledge, this is the first study to investigate the phylogenetic and molecular aspects of NDV in psittacine species in Iran.

Molecular analysis revealed a high degree of homology between the studied isolate and several sequences from Pakistan belonging to the sub-genotype VII.2. Iran, with an area of 1,648,000 square kilometers and vast common borders with vulnerable countries, especially those with documented velogenic NDV outbreaks in commercial/backyard poultry or even pet birds, is exposed to various viral infections like ND. Illegal bird trade at Iran borders with other countries, such as Pakistan, provides the opportunity of the disease transmission through these countries (Ghalyanchilangeroudi et al., 2018). This finding is broadly consistent that of a recent study on NDV in broiler farms of Iran (Ghalyanchilangeroudi et al., 2018). According to this study, which was conducted in 2018, the similarity between Iranian isolates and VIIi (now designated as VII.2) Pakistani strains could be related to illegal trade across the Pakistan-Iran border. Although the geographical location of Iran within the African-western Eurasian migratory pathway is considered as a major cause of dissemination of VII.1.1 strains, it is more probable that illegal trade of pet birds and lack of effective biosecurity manage-

ment along borders would be the main cause of the introducing of VII.2 sub-genotype in Iran.

The VII.2 isolates reported from different countries such as Indonesia, China, Jordan, Israel, and Malaysia represented approximately 98% identity to our isolate. Moreover, according to the phylogenetic tree, these sequences all clustered together with the studied isolate. The high expansion rate of VII.2 NDVs among different bird species and several countries and the similar substitutions at substantial motifs (Table 3-5) highlighted the potential of velogenic VII.2 viruses to cause another ND panzootic, as explained by Miller et al (Miller et al., 2015).

Despite the high similarity of our strain to another VII.2 sequence (MG871466), there was less identity between the studied isolate and other NDV (sub) genotypes previously reported from Iran (Table 2). LaSota and B1, which are the most common vaccine strains used in Iran, are genomically distinct from our isolate (Fig.1 and Table 2). The sub-genotype VII.1.1 is the most prevalent NDVs in the poultry fields of Iran. Nevertheless, a study conducted in 2018 showed that VIIi viruses (now identified as VII.2) were introduced in a chicken flock which is consistent with our finding in a cockatiel population. Ebrahimi *et al.* (2012)analyzed the genetic variations of 51 isolates of NDV obtained from chickens during 2008-2011. He concluded that Sub-genotypes VIIb of the Indian subcontinent and some other Pakistani genotypes are changing due to positive selection or recombination of *the F* gene. In this line, Kianizadeh *et al*. (2002) predicted that the sequences of the cleavage site of *F* protein of NDV isolates were changing gradually in Iran. Furthermore, studies indicated the emergence of newly mutated strains (Ebrahimi et al., 2012; Esmaelizad et al., 2017; Molouki et al., 2019; Sabouri et al., 2018), showing that genetic changes in NDV strains in Iran are still happening. Generally, it does not seem that genotype VII.2 NDVs are evolved directly from vaccine strains (Fig.1 and Table 2) or other sub-genotypes of VII (34). Moreover, it is believed that VIIb, VIId, and XIII NDVs in Pakistan and Israel have been replaced gradually by VIIi (now identified VII.2) isolates in recent years.

The lack of a standard vaccination program for immunization of psittacine species in Iran places huge economic burdens on the exotic pet bird industry. However, the vaccination of captive birds should be conducted with more caution. Further investigation for finding and using vaccine strains homologous to the circulating isolates that do not lead to evolving of new virulent viruses (Miller et al., 2007, 2010)is needed.

The limited information about the molecular characteristics of the virus in neighboring countries makes it difficult to present a comprehensive molecular analysis of the circulating NDV in the region. Furthermore, contrary to numerous studies on chicken farms in Iran, the features of NDVs in psittacine species are not clear. So further molecular and phylogenetic studies on psittacine' and avian wild birds' NDV are necessary to extend our knowledge about the potential roles of these birds in the evolution and cross-species transmission of the virus.

On the other hand, considering the high prevalence of ND in Asia, the governments need to perform the strict standard quarantine rules and apply for extreme monitoring and control programs against the trade of bird and poultry products across borders in Iran.

CONCLUSIONS

In summary, the isolate obtained from cockatiels in our study is closely related to VII.2 NDV strains reported from Pakistan. It seems that the emergence of this new sub-genotype in Iran could be related to the illegal trade of birds and avian products across Iran-Pakistan borders. The virulence of this virus highlights the importance of precise surveillance of the disease in psittacine species that can facilitate NDV cross-species transmission and emergence of new outbreaks of ND potentially affecting the local and neighboring chicken industries. Regarding the heterogeneity of NDV isolates in Iran, applying homologous vaccines with the currently prevailing strains can help to perform more effective prevention programs against the studied disease not only in commercial chickens but also in exotic pet birds.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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