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Original Article

Characterization of Pigeon Paramyxovirus Type 1 Viruses (PPMV-1) Isolated in Iran

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Abstract

Background and Aims: Newcastle disease (ND) is one of the contagious viral diseases in avian species. Recently, several ND outbreaks in pigeon caused by pigeon paramyxovirus serotype-1 (PPMV-1) have been reported din limited numbers from Iran and phylogenetic studies have been conducted on partial sequence of NDV fusion (F) gene.

Materials and Methods: In the present study, ten PPMV-1, named Pigeon_paramyxo-virus1_isolate_pigeon/Iran/UT_EGV1-10/2018, isolated from infected pigeons, and were subjected to partial sequencing. All isolates showed MDT of 74-80 hours, thus categorizing them as mesogenic.

Results and Conclusion: The phylogenetic analysis based on the F gene sequence revealed the isolates belong to XXI.1.1 subgenotype. According to BLAST results, the partial genome of UT-EGV1-10 had high homology with some Russian and Egyptian strains (the highest was 96.55%). The information obtained from this study can be useful in preventive measures for ND caused by PPMV-1 in pigeons.

Keywords: Pigeon paramyxovirus serotype-1 (PPMV-1), Partial sequencing, Fusion gene, Phylogenetic Study, Epidemiology, Iran

Introduction

Avian orthoavulavirus 1 (AOAV-1), commonly known as Newcastle disease virus (NDV, used hereafter in this paper) causes Newcastle disease (ND) which is one of the most serious infectious and contagious viral diseases in various avian species. NDV is an enveloped virus carrying a negative sense, non-segmented and single-stranded RNA genome of 15Kb in size, which encodes six proteins in the order of (3'NP-P-M-F-HN-L'5) and two non-structural proteins (V and W) generated by P-gene mRNA editing [1, 2]. The two glycoproteins, namely F and HN, are considered key antigens of NDV in inducing neutralizing antibody [3]. The F protein directs the viral fusion activity, and the amino acid sequence of its cleavage site (112-117 amino acids) has been known as a major determinant of NDV virulence, furthermore,

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the HN protein is responsible for the virus attachment to the host cell [4]. According to the latest NDV classification provided by NDV consortium, NDV divides into two major classes of class I and class II [5]. Class I viruses, isolated from wild birds and chicken worldwide, includes one genotype, and are nonvirulent for poultry such as chickens. while, class II viruses have been classified into at least twenty one (I-XXI) distinct genotypes, containing virulent and nonvirulent strains and groups for different bird species. Undoubtedly, the pigeon-derived group (VI, XX and XXI) is the largest NDV group [5]. As the result, the Iranian PPMV-1 isolates in the large NDV dataset available at the GitHub page of the NDV consortium are only two groups of XXI.1.1 (formerly VIg) and XXI.2 (formerly VIi). Over the last few years, the outbreaks of ND caused by genetic variants of NDV adapted to host species other than chickens have been reported, and it has been shown that the isolates circulated among several bird species such as pigeon [6, 7]. So far, there have been five panzootic of ND [8]. It is believed that an antigenic variant of NDV caused the 3rd panzootic to be adapted to pigeons [9]. It is speculated that event was originated from the Middle East (the first report from Iraq) in the late 1970s [10, 11]. Clinical symptoms of PPMV-1 infection in pigeons are neurological signs such as wings and legs paralysis, torticollis, and gastrointestinal injuries, including watery to hemorrhagic diarrhea [12].

While PPMV-1 infect a range of hosts including poultry [11], they are identified as mild or low pathogenic for chicken, with some being completely non-virulent [13].

Furthermore, several studies reported that PPMV-1 isolates increased their virulence after some passages in chickens through the mutation in L and P proteins, revealing mild respiratory signs and inducing a humoral response against a standard antigen of NDV (La Sota) in the HI test [14]. Owing to such historical evidence proving the ability of some PPMV-1 to cause clinical disease in commercial poultry flocks, there is a concern that these strains might spread from pigeons to commercial poultry flocks.

Therefore, continuous circulation of PPMV-1 among pigeons can be considered a serious threat to the poultry industry [13, 15, 16]. Recently, several PPMV-1 infections in Iranian pigeon populations have been reported [15, 17, 18], and phylogenetic studies have been conducted based on the partial sequence of the F gene of these isolates. However, the information on the complete genome characterization of Iranian PPMV-1 is not available so far. In the present study, in addition to a complete genome sequence of a recent PPMV-1 isolate, we study five partial F gene sequences that we isolated previously.

The five partially sequences were submitted by our group to GenBank a few years ago, but surprisingly, we had not realized that they could belong to a novel subgenotype, until now.

Methods

Virus isolation and identification. During 2017-2018, fifty brain tissue samples of ND-suspected pigeon flocks (from different parts of Iran) with nervous symptoms were referred to the Pet Bird Clinic of the University of Tehran. The samples were collected aseptically and stored at -70°C. According to OIE guidelines [19], 0.2 ml of the homogenized brain tissue was inoculated into the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated eggs, then incubated at 37 °C and monitored for seven days. The allantoic fluids of the isolates that caused embryo mortality within 3 to 4 days post-inoculation (PI) were collected and examined separately for haemagglutination activity (HA). Six HA-positive allantoic fluids were further checked for hemagglutination inhibition (HI) activity using reference antisera against NDV (provided by Razi Vaccine and Serum Research Institute, Karaj, Alborz, Iran). The isolates were also subjected to PCR and partial F gene sequencing, as described previously [20].

Pathogenicity study. According to the OIE guidelines, the pathogenicity of all studied viruses was determined using the mean death time (MDT) in 10-day-old pathogen-free embryonated chicken eggs [19].

RNA extraction and RT-PCR. RNA was extracted from all the positive allantoic fluids using RNX^{TM}-Plus Kit (CinnaGen, Tehran, Iran) according to the manufacturer's recommendations and stored at -70°C until use. cDNA synthesis was synthesized using random hexamer primers, as well as a gene-specific primers (GSP) 5´-GACCGCTGACCACGAG-GTTA-3' [21] using Revert Aid First Strand cDNA Synthesis Kit (Thermo, Burlington, ON, Canada) according to manufacturer's manual and purified with a Direct-zol kit (Zymo Research, USA).

Partial genome sequencing and bioinformatics analysis. The five EGV group (EGV 01 to EGV10) samples were selected for partial sequencing of the F gene. The amplified products were detected on SYBR® Green-stained (Invitrogen, USA) 1% agarose gel after electrophoresis and UV illumination. PCR products were purified using the AccuPrep® DNA Gel Purification Kit (Bioneer, Korea) according to the manufacturer's recommendation and submitted for automated sequencing in both directions at the Sequetech company (USA) using PCR primers as sequencing primers NDcre F: GGTGAGTCT-ATCCGGARGATACAAG and NDcre R: TCATTGGTTGCRGCAATGCTCT. The nucleotide (nt) sequences of the F gene were aligned and analyzed by BioEdit software (Hall, 1999) with selected AOAV-1sequences available in GenBank. Phylogenetic trees were constructed using Muscle Distance-based neighbor-joining trees made by the Tamura 3-parameter model available in the program MEGA7 (Kumar et al., 2016) using bootstrap 1000.

GenBank accession numbers. The sequence data from the present study were deposited to the GenBank database with the accession numbers shown in Table 1.

Result

All the allantoic fluids of the isolates of this study were positive in HA and HI tests with 2^6 and 2^4 titers, respectively. The EGV group was partially sequenced. All the isolates were considered mesogenic PPMV-1 based on the MDT results (Table 1).

The latest large and pilot NDV datasets were downloaded from the GitHub webpage of the NDV consortium, as explained [5]. Using the pilot file, a phylogenetic tree based on the F gene sequences of the EGV group was made (Fig 1). Based on EGV group shared 95.75%-100% homology with together and five previously reported Irainan PPMV-1isolates (GH group) in NCBI center (Table 2). The analysis placed them next to the XXI.1.1 subgenotype. Generally EGV group was similar to Egyptian and Russian PPMV-1 strains. Based on phylogenetic analysis EGV01 had high identity (98.98%) and Egyptian PPMV-1 and also 98.46% with Russian (Table 2).

Analysis for the deduced 112-116 aa sequence of the F protein cleavage sites of all viruses showed that they carried multiple basic amino acids and phenylalanine at position 117, thus identified as virulent based on the OIE guidelines (Table 1).

Discussion

According to previous studies ND is endemic in Iran (WAHIS 2018) and outbreaks routinely occur in commercial poultry and other avian species such as pigeons [15, 17, 18, 22-25]. This is probably due to the lack of proper control strategies, including poor vaccination and hygienic status in commercial poultry farms. In addition, PPMV-1 adversely affects the nervous and digestive system in pigeons and can spread to commercial poultry, causing considerable economic losses, including drop-in egg production and increase in mortality rate [26, 27]. Therefore, studying PPMV-1 status in Iran is important.

In this study, the sequences of ten EGV samples of XXI.1.1 (formerly known as VIb/2

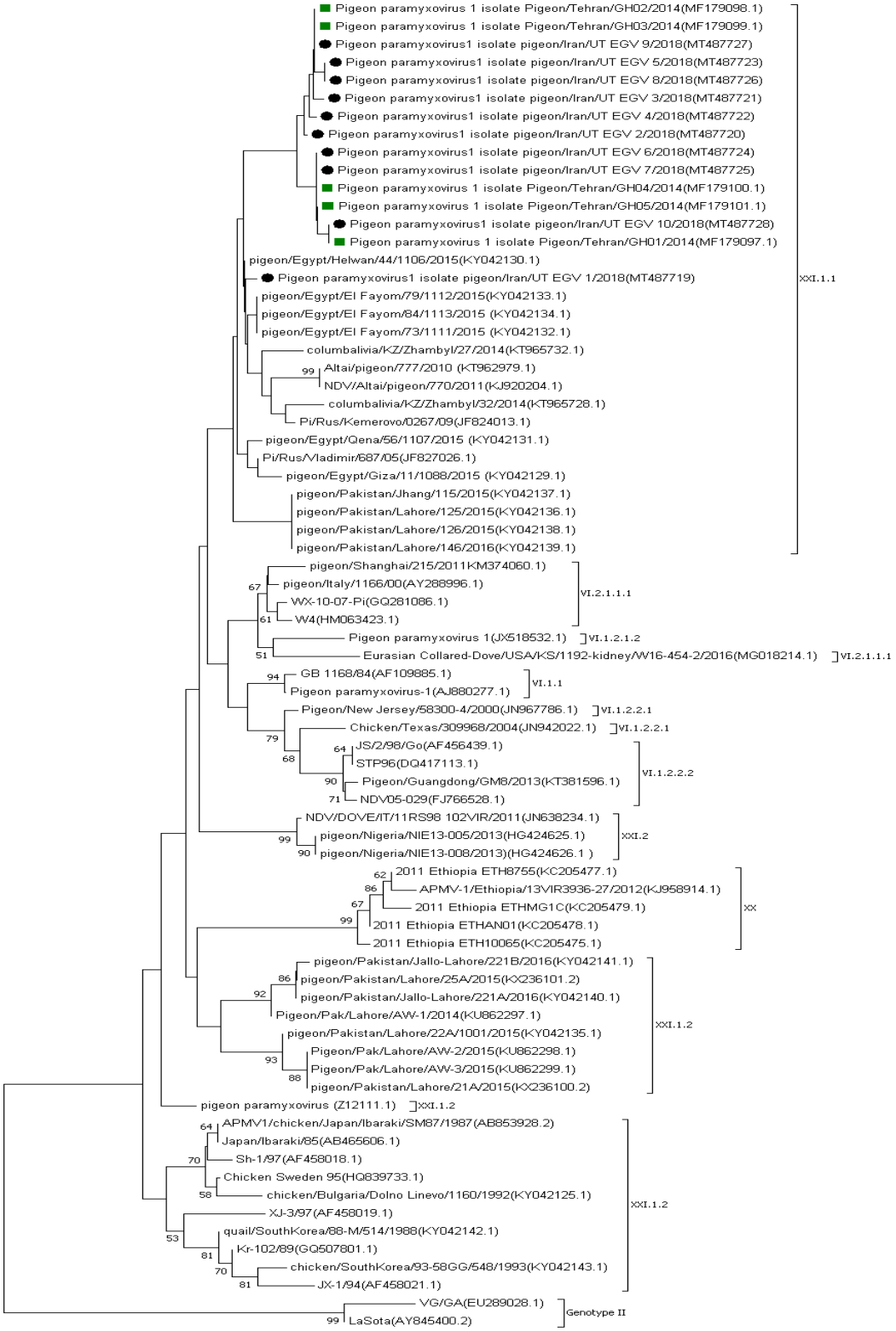

Fig. 1. The evolutionary history was inferred using the Neighbor-Joining method (Saitou N. & Nei M., 1987). The optimal tree with the sum of branch length = 1.17454199 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J. 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al, 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 74 nucleotide sequences of Fusion gene. All positions containing gaps and missing data were eliminated. There were a total of 201 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al, 2016). UT-EGV group is indicated with a ● and previous Iranian PPMV-1 isolates (GH group) with a ■.

Table 1: Information on the isolates studied in this article.

Isolates	F gene accession number	F Cleavage site	MDT (Hour)
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_1/2018	MT487719	112 KRQKRF117	74
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_2/2018	MT487720	112KRQKRF117	85
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_3/2018	MT487721	112KRQKRF117	83
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_4/2018	MT487722	112KRQKRF117	88
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_5/2018	MT487723	112KRQKRF11	78
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_6/2018	MT487724	112KRQKRF11	80
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_7/2018	MT487725	112KRQKRF11	82
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_8/2018	MT487726	112KRQKRF11	84
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_9/2018	MT487727	112KRQKRF11	85
Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_10/2018	MT487728	112KRQKRF11	78

[17] or VIg [18]were studied. All of these viruses caused neurological signs with a high mortality ratio in infected pigeon flocks. Based on the phylogenetic analysis, the EGV group forms a nearest cluster to subgenotype XXI.1.1 (VIg), however, Partial sequences are not recommended for placing an isolate in specific subgenotype [5].

PPMV-1 has been mostly isolated from pigeons and doves [5]. It was suggested that trade of racing pigeons, as well as dispersion of free-ranging pigeons and doves, were one of the most frequent reasons of geographical extension for PPMV-1 all over the world [28].

Genotype VI viruses have been almostly isolated from pigeons included fourteen sub-genotypes (VIa to VIn). But some viruses were taken out of genotype VI, after utilizing the recently updated criteria suggested by Dimitrov [5]. However, this genotype is still found to include seven sub-genotypes. The earliest known members of the genotype VI, VIa, and the last one VIn were merged into sub-genotype VI.2.1.1.1 [29]. Genotype VI.1.1 (previously known as subgenotype VIb viruses) were isolated from Iraq for the first time in late 1978 and were responsible for the 3rd panzootic of ND during the 1980s [2, 9, 30]. A few VI.1.1 viruses have been isolated from some ND outbreaks in poultry in the United Kingdom during 1997 [31].

Subgenotype VIc viruses were regrouped as genotype XX [32]. Previously known subgenotypes VIe, VIf, VIh, VIi, VIl, and VIm viruses were reassigned as VI.1.2.2.2, VI.1.2.2.1, VI.1.2.1.2, XXI.2, XXI, and

Table 2. The homology between EGV group and another NDVs. The isolates were selected from the large dataset available at the GitHub webpage of NDV consortium. The number of base substitutions per site from between sequences is 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 (Tamura et al. 2004). The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_1/2018(MT487719)	####																								
2	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_2/2018(MT487720)	96.82																								
3	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_3/2018(MT487721)	95.69	98.99																							
4	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_4/2018(MT487722)	96.82	98.99	98.99																						
5	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_5/2018(MT487723)	95.69	98.99	98.99	98.99																					
6	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_6/2018(MT487724)	96.26	98.47	98.47	98.47	98.47																				
7	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_7/2018(MT487725)	96.26	98.47	98.47	98.47	98.47	100.00																			
8	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_8/2018(MT487726)	95.69	98.99	98.99	98.99	100.00	98.47	98.47																		
9	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_9/2018(MT487727)	96.26	99.50	99.50	99.50	99.50	97.95	97.95	99.50																	
10	Pigeon_paramyxovirus1_isolate_pigeon/Iran/UT_EGV_10/2018(MT487728)	95.69	97.95	97.95	97.95	97.95	99.50	99.50	97.95	97.42																
11	Pigeon_paramyxovirus_1_isolate_Pigeon/Tehran/GH02/2014(MF179098.1)	96.26	99.50	99.50	99.50	99.50	97.95	97.95	99.50	100.00	97.42															
12	Pigeon_paramyxovirus_1_isolate_Pigeon/Tehran/GH01/2014(MF179097.1)	95.69	97.95	97.95	97.95	97.95	99.50	99.50	97.95	97.42	100.00	97.42														
13	pigeon/Egypt/Helwan/44/1106/2015 (KY042130.1)	99.50	97.37	96.26	97.37	96.26	96.82	96.82	96.26	96.82	96.26	96.82	96.26													
14	pigeon/Egypt/El_Fayom/79/1112/2015 (KY042133.1)	98.99	96.82	95.69	96.82	95.69	96.26	96.26	95.69	96.26	95.69	96.26	95.69	99.50												
15	Pi/Rus/Vladimir/687/05(JF827026.1)	98.47	96.26	95.11	96.26	95.11	95.69	95.69	95.11	95.69	95.11	95.69	95.11	98.99	98.47											
16	pigeon/Egypt/Giza/11/1088/2015_ (KY042129.1)	97.42	95.11	93.93	95.11	93.93	94.52	94.52	93.93	94.52	93.93	94.52	93.93	97.95	97.42	98.99										
17	pigeon/Pakistan/Lahore/146/2016 (KY042139.1)	96.85	94.47	93.25	94.47	93.25	93.87	93.87	93.25	93.87	93.25	93.87	93.25	97.39	96.85	97.39	96.29									
18	W4(HM063423.1)	94.58	92.00	90.71	92.00	90.71	91.36	91.36	90.71	91.36	90.71	91.36	90.71	95.16	94.58	93.99	93.99	93.32								
19	Pigeon/New_Jersey/58300-4/2000 (JN967786.1)	94.05	92.71	91.45	91.45	91.45	90.81	90.81	91.45	92.09	90.15	92.09	90.15	94.63	94.05	93.46	92.24	92.78	95.16							
20	pigeon/Pakistan/Jallo-Lahore/221A/2016 (KY042140.1)	93.45	90.80	89.49	89.49	89.49	88.81	88.81	89.49	90.15	88.13	90.15	88.13	92.85	92.24	91.62	91.62	90.90	90.90	92.85						
21	Eurasian_Collared-Dove/USA/KS/1192-kidney/W16-454-2/2016(MG018214.1)	92.63	89.83	88.44	88.44	88.44	87.73	87.73	88.44	89.14	87.01	89.14	87.01	92.00	91.36	90.71	90.71	91.45	93.93	91.36	90.05					
22	Pigeon/Guangdong/GM8/2013(KT381596.1)	92.16	90.71	89.37	89.38	89.37	88.69	88.69	89.37	90.05	88.00	90.05	88.00	92.78	92.16	91.54	91.54	89.49	90.81	96.29	90.90	90.61				
23	2011_Ethiopia_ETH8755(KC205477.1)	88.81	87.15	85.69	85.69	85.69	84.94	84.94	85.69	86.43	84.18	86.43	84.18	88.13	87.43	89.49	88.12	88.69	85.11	86.72	88.13	84.00	82.83			
24	LaSota(AY845400.2)	75.30	76.51	74.64	74.64	76.51	75.58	75.58	76.51	75.58	74.64	75.58	74.64	74.37	73.44	75.30	75.30	73.08	75.90	72.48	71.52	71.34	66.00	68.69		
25	VG/GA(EU289028.1)	71.52	72.72	70.73	70.73	72.72	71.73	71.73	72.72	71.73	70.73	71.73	70.73	70.54	69.54	71.52	71.52	69.12	72.11	68.53	67.51	67.17	61.63	64.42	97.95	

XXI.1.2 respectively. Previously identified subgenotype VIg viruses were grouped as XXI.1.1 and have been isolated from pigeons and doves in Nigeria, Russia, Ukraine, Kazakhstan, Pakistan, Egypt and Iran from 2005 until now. These viruses are circulating currently in Asia such as Iran (EGV groups and several PPMV-1 isolates reported previously by some authors) and Africa and [17, 33]. Previously classified sub-genotypes VIj and VIk viruses were reassigned VI.2.1.1.2.1 and VI.2.1.1.2.2, respectively.

Our results showed that EGV group had high similarity with Egyptian and Russian isolates belonging to the subgenotype XXI.1.1. This high identiy can be attributed to the role of migratory aquatic birds in the transmission of pathogenic viruses from these countries to Iran through the flyways [34]. Based on the prtial F gene sequence analysis, EGV group was more similar to previously reprted Iranian PPMV-1 isolates (GH group). According to new classification system, almost of another previouys Iranian PPMV-1 strains were also classified in XX.1.1 subgenotype [5, 15, 17]. This suggests that Iranian circulating isolates must have shared common ancestors, and this may be due to possible trading between these distinct geographic areas and also due to the migration of aquatic birds as the main reservoir of many diseases [34].

Our finding is consistent with that of Mayahi and Esmaelizad's study, which concludes that some PPMV-1 isolates in Iran have been classified in XXI.1.1 genotype since 2012 [18]. Rezaei Far *et al.* classified 15 of 17 PPMV-1 isolates into XXI.1.1 (formerly named VIg or VIb/2) and 2 of 17 isolates belonging to VI.2.1.1.1 (formerly named VIa sub-genotype) based on the partial sequence of the F gene. However, the last two isolates were placed in VI.2.1.1.2.1 (sub-genotype VIj) in Mayahi and Esmaelizad's study [17, 18]. Similar to our results, Rezaei Far *et al.,* concluded that almost all of the PPMV-1 isolates (15 of 17) exhibited high identity with Russian isolates prevalent from 2000 to 2010. The F protein's cleavage site sequence (motif 112-117) of our PPMV-1 isolates were ^{112}KRQKRF^{117}, which is similar to the 15 PPMV-1 isolates of Rezaie Far et al and all of the 12 PPMV-1 isolates in Mayahi's study; therefore, these findings further emphasize the hypothesis of existence of common ancestor for Iranian XXI.1.1 PPMV-1 isolates [15, 17].

For the first time in 1984, some ND outbreaks were reported from the UK with a drop in egg production, nervous signs, and increased mortality, which were attributed to infection with PPMV-1 [35]. Later, the same outbreak occurred in other regions of the world, such as Africa. Contamination of feed with pigeon feces and carcasses was suggested as the main reason for most of these outbreaks [31, 35]. There are several ND outbreaks in the poultry industry in Iran, and their major causative NDV belongs to VII genotype [36]. VII.1.1 viruses are endemic in Iran and many Asian countries, but a VII.2 genotype (formerly VIIi sub-genotype) has been recently reported in Iran as well [22]. Therefore, at least three different genotypes (VI, VII and XXI), which are the causative agents of 3rd, 4th and 5th panzootic are co-circulating in Iran. Some of these genotypes include distinct subgenotypes that may lead to emergence of more novel genotypes/sub-genotypes [5, 17, 22, 24, 25].

Non-vaccinated pigeons infected with PPMV-1 may spread the disease to other pigeons as well as commercial poultry flocks, so that, biosecurity measures must be strictly implemented and all the pigeon populations should be vaccinated against pigeon-derived NDV or PPMV-1 strains. There are two types of commercial inactivated vaccines applied to protect against the NDV in pigeons, including Colombovac® PMV containing La Sota strain (ATCVet Code: QI01EA01), and Nobilis Paramyxo P201 containing P201 strain (ATCVet Code: QI 01 EA 01). Our findings revealed that all the EGV group in the current study had a 25.3% and 29.27% distance with La Sota and VG/GA respectively as vaccinal strains. Therefore, the use of this strain against the PPMV-1 may not be useful, and it is recommended that the PPMV-1 strains, isolated from pigeons may be used to provide better protection against ND in pigeons [37]. The Stone's study [37] indicated that the virus shedding after challenge with PPMV-1 was

considerably reduced in pigeons already vaccinated with a PPMV-1 isolate compared to other vaccines containing La Sota and Ulster strains, while all the vaccines protected pigeons against morbidity and mortality.

We are hopeful that the information provided in this study will help implement strategies and the generation of vaccines effective in protecting pigeons and other avian species against PPMV-1.

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