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Short Paper

Molecular characterization and phylogenetic analysis of VIII sub-genotype of avian orthoavulavirus 1 isolated from Eurasian magpie (*Pica pica*)

Khosravi, M.[1*]; Seifi, S.[2] and Tazeh, Z.[3]

[1]Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special of Modern Technologies, Amol, Iran; [2]Department of Clinical Science, Faculty of Veterinary Medicine, Amol University of Special of Modern Technologies, Amol, Iran; [3]BSc Student in Veterinary Laboratory Sciences, Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special of Modern Technologies, Amol, Iran

[*]***Correspondence:*** *M. Khosravi, Department of Pathobiology, Faculty of Veterinary Medicine, Amol University of Special of Modern Technologies, Amol, Iran. E-mail: m.khosravi@ausmt.ac.ir*

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Abstract

Background: Newcastle disease (ND) has been categorized as a highly contagious viral disease, remaining as a constant threat to both wild birds and commercial chickens. **Aims:** In this study, we recovered and characterized the avian orthoavulavirus 1 (AOaV-1) strain, nominated as EM1, from the Eurasian magpie (*Pica pica*). **Methods:** The nucleotide and amino acid sequence of the fusion protein (F protein) of EM1 were determined and its phylogenetic relationship was investigated with well-characterized AOaV-1 genotypes, which originated from wild bird species and chickens around the world. **Results:** Phylogenetic analysis and deduced amino acid sequences of the *F* gene revealed that EM1 virus belonged to VIII sub-genotype viruses with the characteristic multibasic amino acid sequences associated with the velogenic motif as ^{112}RRQKRF^{117} at the cleavage site of its precursor fusion protein. EM1 shared a high level of similarity to the other virus sub-genotypes in nucleotide and amino acid sequences of F protein. Furthermore, the evolutionary difference between the studied virus and viruses belonging to the VIII sub-genotype indicated that a close relatedness and the possibility of a common origin. **Conclusion:** These results show that the virulent AOaV-1 of sub-genotype VIII is circulating continuously in Iran, and is disseminating among wild and domestic bird species that can cause bidirectional spillover infection. Therefore, further epidemiological studies can be beneficial in the assessment of the evolution of AOaV-1 in its hosts and will help us to be well-equipped in facing the emergence of new sub-genotypes of this virus.

Key words: Avian orthoavulavirus 1, Bidirectional spillover infection, Eurasian magpie (*Pica pica*), VIII sub-genotype

Introduction

Newcastle disease (ND) has been known as one of the most serious infectious and fatal viral illnesses. Newcastle disease has a global distribution and leads to massive economic losses in the poultry industry (Alexander, 2011). The pathogen agent, avian orthoavulavirus 1 (AOaV-1; also known as ND virus) is classified as the genus *Orthoavulavirus* belonging to the family Paramyxoviridae within the order Mononegavirales (ICTV, 2018).

Avian orthoavulavirus 1 is a negative-sense, single stranded, non-segmented, enveloped RNA virus. Avian orthoavulavirus 1 genome designs in the order of 3´ NP-P-M-F-HN-L 5´ (Steward *et al*., 1993). Two surfaces enveloped glycoproteins, HN, and F proteins are thought to play a key role in virus cell entry (Sergel *et al*., 1993).

The amino acid sequences of AOaV-1 F cleavage-activation site are the main determinant of virus pathogenicity (Courtney *et al*., 2013). Virulent AOaV-1 strains possess two dibasic amino acids (^{112}R/K-R-Q-K/R-R^{116}) at the C terminus of the F2 protein and F (phenylalanine) at residue 117, while the avirulent strains exhibit a sequence of monobasic amino acids (^{112}G/E-K/R-Q-G/E-R^{116}) in the mentioned region and L (leucine) at residue 117 (Alexander, 2011).

Based on genetic analyses of F protein, AOaV-1 has been divided into two major genotype classes, class I and II. Class I with nine genotypes (1-9) includes avirulent strains, while class II consists of either avirulent or virulent viruses. It divides into 18 genotypes (I-XVIII), which are further clustered in various sub-genotypes (Czegledi *et al*., 2006; Miller *et al*., 2010).

During the recent decade, new emergence of genotypes of Class II is largely expanded in wild bird populations. For example, several virulent strains of genotypes VI, VII, and XIII have been reported within the members of Phasianidae, Ardeidae, Accipitridae, Anatidae, and Spheniscidae families (Rahman *et al*., 2018). Therefore, the circulation of virulent AOaV-1 may pose severe threats to commercial poultries.

There are few studies investigating the presence and

characterization of AOaV-1 strains in wild birds in Iran. This study aimed to perform a genotyping and phylogenetic analysis of a distinct AOaV-1 isolate, named EM1, isolated from the Eurasian magpie (*Pica pica*). In addition, the sequence of F protein was analyzed to elucidate any potential evolutionary mutation in comparison with vaccine and other AOaV-1 reference viruses.

Materials and Methods

The experimental protocol was approved by Animal Care Committee of Amol University of Special Modern Technologies [ir.ausmt.rec.1399.02.16], Mazandaran, Iran.

Sample preparation and virus propagation

Briefly, the whole brain and spleen of fresh Eurasian magpies' carcasses were extracted. Two hundred µL of homogenized tissues were inoculated into the allantoic cavity of 8-day-old embryonated chicken eggs (ECEs) in three times passaging process. The positive haemagglutination assay (HA) allantoic fluid was stored at -70°C.

RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

Viral RNA extracted using viral gene-spin™ viral DNA/RNA extraction kit (iNtRON, South Korea), according to the manufacturer's instructions. The single strand-cDNA was synthesized using murine leukemia virus reverse transcriptase (M-MLV RT) and random hexamer-primer (Yektatajhiz, cDNA Synthesis Kit, Iran). PCR reaction analysis was done as described previously by Kant *et al*. (1997).

Sequencing analysis

The complete extracellular domain protein of the *F* gene was amplified by primer sets that were designed by Qin *et al*. (2008) and subjected to sequencing. The sequence was trimmed, assembled, and aligned using Geneious version 6.1.2 (https://www.geneious.com) (Qin *et al*., 2008). The newly generated sequence was submitted to GenBank (accession No. MK659700). The sequences aligned using MAFFT Plug-in in Geneious with the default setting. The best substitution model for each alignment was determined K2 + G using MEGA7 under the default setting.

Phylogenetic analysis

Phylogenetic trees were constructed and interpreted with well-characterized AOaV-1 genotypes, according to the criteria proposed by Diel *et al*. (2012). Analyses were performed using the maximum likelihood method (ML) based on the Kimura 2-parameter model using the MEGA7 with 500 bootstrap replicates. Bootstrap >60% was applied for defining node, and different genotypes were designated an average distance per site >10% and the gamma distribution model (shape parameter = 5). Initial phylogenetic analyses were conducted by the coding sequences of *F* gene associated with the studied virus and sequences applying in the Diel classification system (n=82) (named data set 1). To ensure validity of the initial tree, the second tree was constructed with the date set 2 including 61 well known sequences from different sub-genotypes VII (n=61) and EM1 (named data set 2). The evolutionary divergence among studied virus and several sub-genotype VII isolates, as well as, the evolutionary difference between EM1, and AOaV-1 isolates of sub-genotype VIIl were assessed using the maximum composite likelihood method with 500 bootstrap replicates in MEGA7 software (Kimura, 1980; Kumar *et al*., 2016).

Results

Virus isolation and HA test

In this study, the isolated virus was confirmed by RT-PCR. This isolate was named Eurasian magpie/Iran/EM1/2017 (abbreviated as EM1).

Preliminary analysis of nucleotide and deduced amino acid sequences

BLAST and clustal omega analysis revealed that a high value of nucleotide (*nt*) and amino acid (*aa*) identity was between EM1 and Iranian AOaVs-1 *F* gene sequences available in GenBank, ranged between 83.61-99.81% and 87.94-99.61%, respectively (Table 1).

Also, the highest level of identity was observed with those strains recently characterized by Mouloki *et al*. (2019) and Sabouri *et al*. (2017) particularly, Ck/IR/MAM68/17 (99.81%) and Ck/IR/MAM72/18 (98.61%) in levels of *nt* and *aa*, respectively Supplementary Table 1 (ST1) (Sabouri *et al*., 2017; Molouki *et al*., 2019). The comparison of *F* gene sequences between EM1 and AOaV-1 strains isolating from other countries such as Israel and China, displayed identity up to 96.95% (nt) and 97.28% (aa) (Table 1).

Phylogenetic analysis

The phylogenetic analysis revealed that EM1 isolate was clustered with the genotype VII and segregated into novel VIIl sub-genotypes that was distinguished from all other sub-genotype VII strains (Fig. 1). In addition, a phylogenetic tree generated with data set 2 again confirmed our preliminary observation Supplementary Figure 1 (SF1).

The evolutionary divergence between EM1 and other sub-genotypes VII showed the distinct variation rate, ranging from 0.0095 to 0.1160, with the lowest and highest distances levels versus VIIl and VIIk (Table 2).

As shown in the Supplementary Table 2 (ST2), 15 sub-genotype VIIl viruses and EM1 were a divergence with 0 to 1.9% within the cluster, whereas the lowest and highest divergence toward Ck/IR/MAM68/17 (0.2%) and Ck/IR/SMV-3/11(1.8%), respectively.

Table 1: Identities of *F* gene in level of nucleotide and amino acid between EM1 and AOaV-1 strains isolated from Iran and other countries

Virus	Less similar to		More similar to	
	Nucleotide	Amino acid	Nucleotide	Amino acid
EM1 vs Iranian strains	Ck/IR/NDP27/12 (KJ174522.1) (83.61%)	Ck/IR/12IR/12 (KJ174522.1) (87.94%)	Ck/IR/MAM68/18 (MH481362) (99.81%)	Ck/IR/MAM72/18 (MH247186) (99.61%)
EM1 vs other countries strains	Turk/IS/PHL24317/07 (MH377276) and Adygea/MD/12/08 (KP189357) (96.05%)	CK/CH/SGM01/05 (DQ227248) (96.30%)	Ck/IS/242/01 (JF795621) (96.95%)	Go/CH/JSG0210/02 (JF340367) and CK/CH/SD883/13 (97.28%)

Table 2: Estimates of evolutionary divergence between EM1 and representatives of other sub-genotypes of VII

	VIIl	**VIId**	**VIIe**	**VIIf**	**VIIg**	**VIIi**	**VIIj**	**VIIk**	**VIIh**	**VIIb**	**EM1**
VIIl (n=15)		(0.0043)	(0.0048)	(0.0058)	(0.0082)	(0.0084)	(0.0059)	(0.0092)	(0.0079)	(0.0054)	(0.0017)
VIId (n=35)	0.0399		(0.0035)	(0.0049)	(0.0070)	(0.0075)	(0.0044)	(0.0086)	(0.0071)	(0.0038)	(0.0049)
VIIe (n=14)	0.0473	0.0329		(0.0045)	(0.0077)	(0.0070)	(0.0050)	(0.0082)	(0.0070)	(0.0045)	(0.0055)
VIIf (n=5)	0.0568	0.0412	0.0372		(0.0083)	(0.0070)	(0.0063)	(0.0085)	(0.0069)	(0.0058)	(0.0064)
VIIg (n=4)	0.1023	0.0838	0.0929	0.0956		(0.0102)	(0.0078)	(0.0105)	(0.0095)	(0.0079)	(0.0087)
VIIi (n=30)	0.0938	0.0802	0.0759	0.0709	0.1255		(0.0084)	(0.0081)	(0.0076)	(0.0080)	(0.0089)
VIIj (n=23)	0.0611	0.0441	0.0509	0.0607	0.1012	0.0968		(0.0100)	(0.0080)	(0.0029)	(0.0065)
VIIk (n=5)	0.1149	0.1036	0.0967	0.0936	0.1447	0.0928	0.1160		(0.0086)	(0.0094)	(0.0095)
VIIh (n=14)	0.0915	0.0836	0.0774	0.0707	0.1263	0.0830	0.0992	0.1045		(0.0077)	(0.0083)
VIIb (n=27)	0.0564	0.0383	0.0456	0.0527	0.1001	0.0902	0.0286	0.1114	0.0945		(0.0060)
EM1	**0.0095**	0.0429	0.0513	0.0607	0.1067	0.0971	0.0652	**0.1160**	0.0941	0.0607	

The number of base substitutions per site is shown that is from averaging all sequence pairs between all sub-genotypes VII. Standard error estimates are shown above the diagonal and were obtained by a bootstrap procedure (500 replicates). Analyses were conducted using the maximum composite likelihood model Kimura (1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The analysis involved 173 nucleotide sequences. Codon positions included 1st + 2nd + 3rd + Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1540 positions in the final data set. Evolutionary analyses were conducted in the MEGA7 Kumar *et al.* (2016). The highest and lowest average evolutionary distance of NDV isolates are shown in bold obtained in the present study with other sub-genotypes of genotype VII

Table 3: F protein comparison of EM1 with other AOaV-1 from both chicken and non-chicken origins

Isolate	Cleavage site (^{112}RRQKRF^{117})	Cysteine residue	Glycosylation site	Neutralizing epitopes							
				72	74	75	78	79	157-171	343	378
EM/IR/EM1/17/VIII/MK659700	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471	D	E	A	K	S	SIAATNEAVHEVTNG	L	A
Chicken-origin poultry											
CK/CN/Hicken/04VIId/DQ485269	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
CK/CN/SWS03/05VIIf/DQ227254	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
CK/CN/H2/07/VIIe/EF589134	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
CK/CN/TZ060107/08/VIId/FJ011448	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
CK/CN/XD/08/VIIg/GQ994433	GRQGRL	76,199,338,347,362,370,394,399,401,424,523	85,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
CK/IR/SMV-1/11/VIII/KU201408	RRQKRF	199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/IR/ SMV-5/12/VIII/KU201409-15/	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/IR/MSH-1/15/VIId/MG519855	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/IR/Isf16/16/VIIj/KY205741	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/IR/MAM68/17/VIII/MH481361-3	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/IR/MAM55/17/VIII/MH247184-7	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTNG	L	A
CK/US/Lasota-AF/46/II/AY845400	GRQGRL	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
FO/US/B1/47/II/AF309418	GRQGRL	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
Non chicken-origin poultry											
GO/CN/QY97-1/97/VIIe/AF162714	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
Pe/CN/BP01/99/VIId/DQ080015	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
GO/CN/SF02/02/VIId/AF473851	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
Ma/CN/GD/1/05/VIId/FJ480824	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
KE/KR/SNU-5070/05/VIId/EU140949	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
WB/CN/HLJ001/06/VIId/FJ480788	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
BU/CN/HLJ009/06/VIIb/FJ480774	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
DU/CS/749/07/VIId/GU227738	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
DU/CN/LC12/12/VIIj/KF771883	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	V	SIAATNEAVHEVTDG	L	A
TURK/ZA/N2057/13/VIIf/KR815908	RRRKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A
WP/PK/20A/996/15/VIIi/KX496962	RRQKRF	76,199,338,347,362,370,394,399,401,424,523	85,191,366,447,471,541	D	E	A	K	A	SIAATNEAVHEVTDG	L	A

The modified epitope position is highlighted by dark square. BU: Buzzard, CI: Cote D'Ivoire (Ivory Coast), Ck: Chicken, CO: Cockatoo, CO: Cormorant, CRIB: Crested ibis, DU: Duck, EM: Eurasian magpie, EG: Egret, FA: Fantail, FO: Fowl, Go: Foose, JAQU: Japanese quail, KE: Kestrel, Ma: Mallard, MDU: Muscovy duck, OW: Owl, Pe: Penguin, Pc: Peacock, PG: Pigeon, PH: Pheasant: RUTU: Ruddy turnstone, RPG: Racing pigeon, SP: Sparrow, TDO: Turtle dove, TURK: Turkey, UN: Unknown, VW: Village weaver, WB: Wild bird, WPG: Wild pigeon, RPG: Racing pigeon, CS: Serbia, AU: Australia, CN: China, IN: India, ID: Indonesia, IR: Iran, IE: Ireland, IT: Italy, KR: South Korea, MG: Madagascar, MX: Mexico, PK: Pakistan, TU: Turkey, TW: Taiwan, UAE: United Arab Emirates, UK: United Kingdom, US: United States, and ZA: South Africa

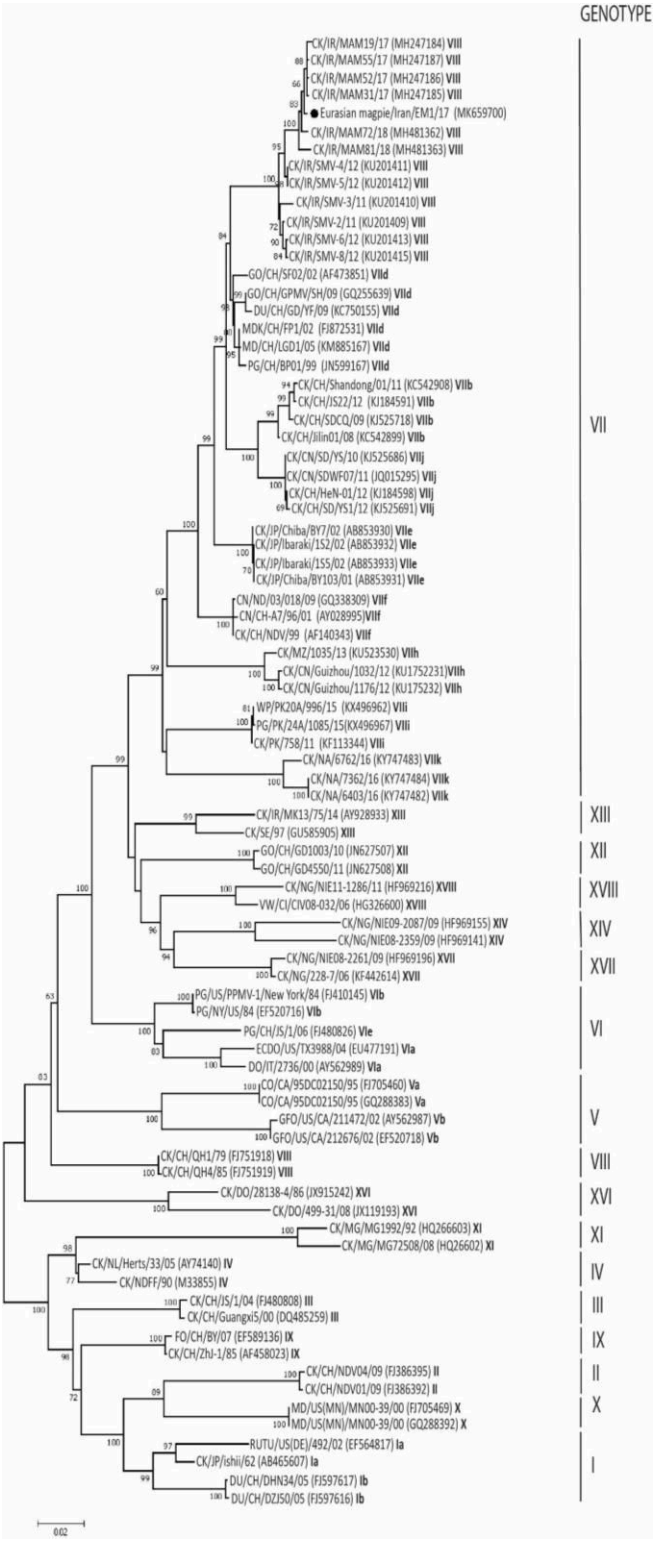

Fig. 1: Molecular phylogenetic relationship of AOaV-1 *F* gene (nt 52-1599) from Eurasian magpie with other widely accepted AOaV-1 genotypes. The analysis involved 82 nucleotide sequences. EM1 isolate in this study is indicated by black circle. Abbreviations are shown in the legend of Table 3

Molecular characterization of the F protein

Table 3 illustrates that the majority of the strains of genotype VII and EM1 shared the same cleavage sites motif (^{112}RRQKRF^{117}) which is the characteristics of the virulent virus.

The analysis of amino acid sequences of neutralizing epitope profiles of F protein revealed that EM1 relatively has conserved amino acids in comparison to other representative AOaV-1 strains. Exceptionally, EM1 contains an amino acid S (serine) at residue 79 than AOaV-1 vaccine strains (Lasota and B1) and other sub-genotypes VII strains (Table 3). Furthermore, EM1 and other references AOaV-1 strains genotype VII exhibited conserved patterns of potential glycosylation sites and the same number of cysteine amino acid residues (Table 3).

Discussion

In this study, the isolate EM1 was recovered from Eurasian magpie and genotypically characterized. To the best of our knowledge, this is the first report of isolation and characterization of AOaV-1 from the Eurasian magpie in Iran.

Genetic investigation indicated that the Eurasian magpie isolate belongs to the novel sub-genotype VIIi viruses, which recently has been identified from commercial and backyard chickens in Iran (Fig. 1 and Supplementary Figure 1 (SF1), Supplementary Table 1 (ST1)) (Sabouri *et al*., 2017; Molouki *et al*., 2019). The evolutionary difference analysis illustrated that EM1 has a close relatedness with strains Ck/IR/MAM68/17, which was classified into clusters VIIi (Supplementary Table 2 (ST2)). These results suggested the presence of an epidemiological association and the modes of dissemination of virus between wild birds and the chicken population.

Molecular pathotyping exhibited that AOaV-1 isolated from the Eurasian magpie is a velogenic one. This finding is in agreement with the data indicating that the majority of the genotype VII viruses isolated from both chicken and non-chicken origins were virulent (Table 3).

In most of AOaV-1 isolates, the neutralizing epitopes contain no deletions or insertions and were comparably conserved. Isolate EM1 possesses S (serine) at residue 79 in comparison with vaccine strains (Lasota and B1) and other strains of genotype VII (A79). This substitution is followed by changing the amino acid from the state of non-polar to polar mode (Table 3). Although the effect of this mutation in the pathogenicity of EM1 requires further analysis, this antigenic variant could be related to the virus during infection of a new host and the virus virulence (Dimitrov *et al*., 2016).

Based on our data, the close genetic similarity between AOaV-1 isolated from Eurasian magpie and other Iranian sub-genotype VIIi strains proposed the rapid circulation of this sub-genotype among different species of birds in distinctive geographical locations of the country. Isolation of the virulent strains in wild birds has been increasing the concern of new epizootics of ND in poultry (Courtney *et al*., 2013). Bidirectional spillovers occasionally disseminate virulent strains among wild bird species and chicken populations

(Shengqing *et al*., 2002). Therefore, highly mobile infected wild bird reservoirs such as Eurasian magpies may have crucial roles in introducing AOaV-1 to susceptible hosts.

Several major potential factors such as increasing the awareness of farmers, adopting strict biosecurity, developing new antigens vaccine, and wider vaccination can protect avian species more from the entry of AOaV-1 into chicken farms and can prevent the infection of other avian species. Overall, the data indicate that there are very few boundaries for virulent AOaV-1 to infect new vulnerable hosts. Hence, constant epidemiological studies would have a benefit for us to be well-equipped in the emergence of a new genotype of the virus and tracing its possible origin.

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Conflict of interest

None of the authors has any conflicts of interest to declare.

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