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Original Article

Genetic Analysis of Avian Orthoavulavirus Type I (AOAV-1) Strains Isolated from Broiler Flocks

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Abstract

Background and Aims: Newcastle disease (ND) is one of the most serious diseases among many species of birds and causes devastating effects on the poultry industry. This disease is endemic in Iran and ND outbreaks occur unexpectedly and with high mortality and severe clinical signs. The sequence of the F protein cleavage site is that the major virulence determinant of Newcastle disease virus (NDV). This study aimed to understand the molecular characterization of three NDVs isolates from commercial broilers from Qazvin province during 2019-2020. Thus, we can design appropriate control programs by obtaining useful data.

Materials and Methods: The partial open reading frame sequences of the F gene and haemagglutinin-neuraminidase (HN) genes of three isolates were amplified and sequenced. All sequences were edited using BioEdit Package and phylogenic trees were created based on the partial F gene sequences of isolates attained in this study and other NDV sequences available in GenBank with MEGA 7 software.

Results: The phylogenetic analysis displayed that the viruses belonged to genotype VII and further clustered into sub-genotype VII.1.1. Analysis of the F protein showed polybasic amino acid motif and a phenylalanine at position 117 at the cleavage site.

Conclusions: The similarity of these strains could indicate the entry of migratory birds from neighboring countries into Iran. Also, the role of backyard poultry is very important in the epidemiology of ND. This study proclaims the urgency of effective control strategies.

Keywords: Newcastle disease virus, genetic characterization, genotype VII, commercial poultry.

Introduction

Newcastle disease (ND) is one of the highly infectious diseases affecting countless species of avian and leads to lethal outbreaks. It affects over 250 species of birds and causes significant losses to the poultry industry all over the world (1). The ND is caused by the Newcastle disease virus (NDV), a member of the family Paramyxoviridae of the genus Avian orthoavulavirus 1 (AOAV-1) (2). The viral genome is a negative sense, single-stranded RNA, contains six genes in order 3'-NP-P-M-F-HN-L-5'. The genome encodes nucleocapsid (N) protein, phosphoprotein (P), matrix (M) protein, fusion (F) protein, hemagglutinin-neuraminidase (HN) and large polymerase protein (L) (3). RNA editing of the P protein produces two nonstructural proteins of V and W in infected cells

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(4). The F and HN proteins are surface glycoproteins and are considered as protective antigens of NDV (5). The F protein directs the viral fusion activity, and amino acid sequence at the cleavage site (112-117 amino acids), has been specified as a major virulence determinant of NDV. While the HN protein is responsible for virus attachment. Based on pathogenicity, strains of NDV are subdivided into four main pathotypes including neurotropic velogenic, viscerotropic velogenic, mesogenic, lentogenic and asymptomatic enteric strains (5). The pathotype can be designated by *In Vivo* pathogenicity tests including mean death time (MDT), intracerebral pathogenicity index (ICPI), and intravenous pathogenicity index (IVPI).

Conversion of protein F0 to F1 and F2 in the cleavage site by cellular proteases is required for the entry of NDV into the host cells. The number of basic amino acids in the cleavage site determines the virulence of NDV strains. According to previous studies, there are at least five antigenic sites related to epitopes on the HN protein of NDV (3). several molecular classification systems of NDVs have been developed since 1980. According to updated seamlessly phylogenetic classification system and reconsidered nomenclature for Newcastle disease virus-based on complete sequences of the F gene, the NDV strains divide into two classes of I and II. The class I viruses contain one genotype. compared with class I, class II includes a range of non-virulent to virulent viruses and the complete analyses identified at least 20 distinguished genotypes (I to XXI) (2). Currently, genotype VII strains are responsible for the most outbreaks in the Middle East and Asia. These viruses cause notable economic losses in vaccinated poultry flocks (6). Despite the widespread use of vaccination, outbreaks of NDV are observed in Iranian commercial poultry with high mortality and severe clinical signs (7). In the current study, the partial sequence of F gene of three Iranian NDVs, circulating in broilers commercial poultry in Qazvin provinces from 2019 to 2020, were sequenced and the sequences were analyzed phylogenetically and molecularly based on the data available in GenBank.

Methods

Three NDVs isolates from the trachea and brain samples of dead birds, from broiler farms of Qazvin province, were isolated in 2019–2020. Affected chickens showed respiratory signs, neurological signs and digestive system lesions. Tissue samples were transferred with ice to the virology laboratory of veterinary medicine faculty of Tehran University. Virus isolation was performed in the allantoic cavity of 10-day-old specific-pathogen-free (SPF) embryonated hen's eggs as a standard method. NDV-positive embryonated chicken eggs were identified by hemagglutination (HA) and hemagglutination inhibition (HI) tests of allantoic fluids and stored at -70 °C for further analysis. The pathogenicity of NDVs was determined by mean death time (MDT) in 10-day-old pathogen-free embryonated chicken eggs. Viral RNA was extracted from the virus-infected allantoic fluids using the RNX^{TM}-Plus Kit (CinnaGen, Tehran, Iran) according to the manufacturer's protocol. Random Hexamer Primer and RevertAid first- strand cDNA synthesis kit (RevertAid™ first-strand cDNA synthesis kit; Thermo Scientific, MA, USA) was used to synthesis of The first-strand cDNA (cDNA), according to the manufacturer's recommendations (8). Kant's primer sets were used to amplify cDNA (A: 5'-TTG ATG GCA GGCCTC TTG C-3' B: 5'-GGA GGA TGTTGG CAG CAT T-3') (9).

PCR products were purified with a gel extraction kit (GeneJET™ gel extraction kit; Thermo Scientific, MA, USA) and sequenced by a commercial DNA sequencing company (Bioneer, Daejeon, South Korea) in both directions by Sanger dideoxy sequencing technology.

All sequences were edited using BioEdit Package (version 7.2.5) (10). Nucleotide sequence alignments were conducted with ClustalW, and phylogenic trees were created based on the partial F gene sequences of isolates attained in this study and other NDV sequences available in GenBank with MEGA7 software using the maximum likelihood method with the general time-reversible model. Naming genotypes and sub-genotypes was based on Dimitrov et al.'s classification (2).

The three nucleotide sequences for the F and HN genes of NDV isolates were submitted to GenBank.

Results

In this study, we focused on the isolation of NDVs from separated ND outbreaks in broiler flocks with total mortality of more than 30 percent from Qazvin province of Iran during 2019 and 2020 .

Isolates of this study were velogenic strains. All three NDV isolates had MDT of <60 h and had Multibasic amino acid sequence motif at the C-terminus of the F2 protein (Table 1).

F genes phylogenetic analyses were carried out according to Dimitrov et al.'s study (2).

The studied viruses were closely related to Newcastle disease virus isolate 5 (JQ344319) (Figure 1).

Analysis of the HN sequence of the viruses showed that isolates were associated with Avian avulavirus 1 strain chicken/Iran/SMV-3/2011 (KU201418) (Figure 2)

These results indicate that the tested isolates in our study were classified as NDV subgenotype VII.1.1 (Table 2,3).

Table 1. The pathogenicity indices and cleavage site motif of studied isolates.

Sample name	Accession number (F gene)	Accession number (HN gene)	Cleavage site	MDT
AVG8	MN931809	MN931799	112RRQKR↓F117	50
AVG9	MN931810	MN931800	112RRQKR↓F117	49.5
AVG10	MN931802	MN931792	112RRQKR↓F117	51.8

Table 2 Percent identity of partial nucleotide sequences of the Fusion glycoprotein genes

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	AVG8(MN931809)													
2	AVG9(MN931810)	99.14												
3	AVG10(MN931802)	98.28	98.28											
4	Newcastle_disease_virus_isolate5(JQ344319)	96.47	96.47	97.80										
5	chicken/Iran/SMV-5/2012(KU201412)	96.43	95.45	95.61	98.92									
6	Newcastle_disease_virus_isolate1(JQ344315)	95.27	95.27	96.68	98.93	97.83								
7	Chicken/China/Jilin/YJ06/2015_(KU200253.1)	92.43	92.43	92.71	95.40	91.65	94.23							
8	UT-PCR_2017(MG871466)	92.31	91.18	92.59	89.87	89.22	88.56	92.71						
9	Avian_avulavirus_1_isolate_Peacock/AJK/AW-pc45/2018(MH717069.1_)	92.16	91.00	92.45	89.61	89.00	88.27	92.57	100.00					
10	Avian_avulavirus_1_strain_Okara_(MH607122_)	87.23	84.44	85.71	88.24	85.71	88.24	94.74	100.00	100.00				
11	NDP95(KT343678)	84.66	84.66	77.07	80.28	83.21	80.28	80.28	83.21	82.47	83.21			
12	NDP25(KT343670)	82.08	82.08	77.59	80.70	83.56	80.70	80.70	83.56	82.85	83.56	98.33		
13	LaSota.71.IR(KU665482)	78.10	74.61	70.25	71.03	77.46	69.15	73.56	70.25	69.49	69.23	80.28	74.18	

Table 3. Percent identity of partial nucleotide sequences of the hemagglutinin-neuraminidase (HN) genes

		1	2	3	4	5	6	7
1	AVG8(MN931799)							
2	AVG9(MN931800)	99.57						
3	AVG10(MN931792)	100.00	99.57					
4	chicken/Iran/SMV-3/2011_(KU201418)	98.28	97.84	98.28				
5	UT-PCR_2017_(MG871466)	90.46	90.00	90.46	91.04			
6	parakeet/Pak/R-Pindi/SFR-16/2016_(KX791183.1)	90.26	89.80	90.26	90.83	99.42		
7	backyard/Pakistan/Lahore/SFR144C/2016(KX791186.1)	89.68	89.22	89.68	90.26	99.13	98.57	

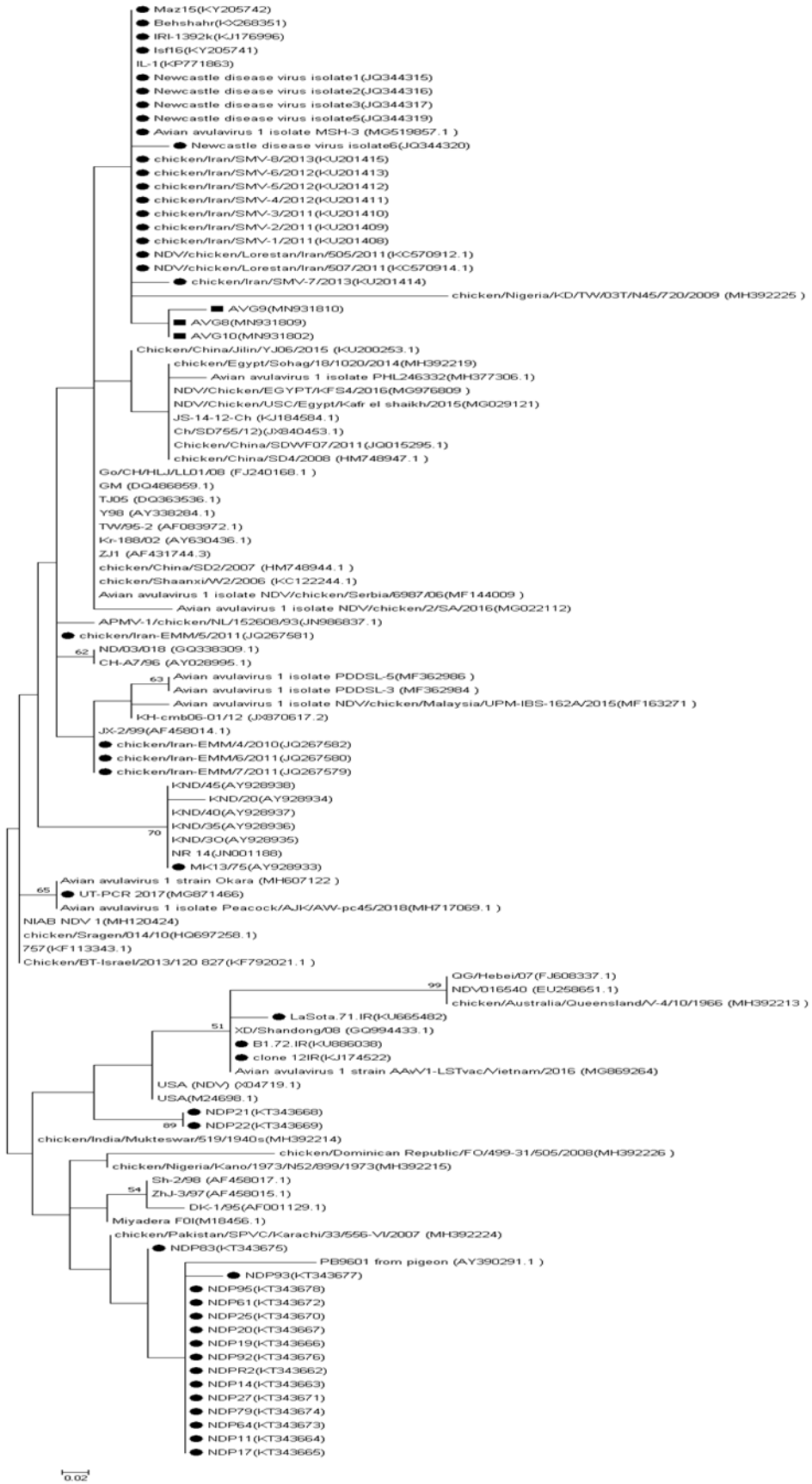

Maz15(KY205742)
Behshahr(KC268351)
IRI-1392k(KJ176996)
Isf16(KY205741)
IL-1(KP771863)
Newcastle disease virus isolate1(JQ344315)
Newcastle disease virus isolate2(JQ344316)
Newcastle disease virus isolate3(JQ344317)
Newcastle disease virus isolate5(JQ344319)
Avian avulavirus 1 isolate MSH-3 (MG519857.1)
Newcastle disease virus isolate6(JQ344320)
chicken/Iran/SMV-8/2013(KU201415)
chicken/Iran/SMV-6/2012(KU201413)
chicken/Iran/SMV-5/2012(KU201412)
chicken/Iran/SMV-4/2012(KU201411)
chicken/Iran/SMV-3/2011(KU201410)
chicken/Iran/SMV-2/2011(KU201409)
chicken/Iran/SMV-1/2011(KU201408)
NDV/chicken/Lorestan/Iran/505/2011(KC570912.1)
NDV/chicken/Lorestan/Iran/507/2011(KC570914.1)
chicken/Iran/SMV-7/2013(KU201414)
chicken/Nigeria/KD/TW/03T/N45/720/2009 (MH392225)
AVG9(MN931810)
AVG8(MN931809)
AVG10(MN931802)
Chicken/China/Jilin/YJ06/2015 (KU200253.1)
chicken/Egypt/Sohag/18/1020/2014(MH392219)
Avian avulavirus 1 isolate PHL246332(MH377306.1)
NDV/Chicken/EGYPT/KFS4/2016(MG976809)
NDV/Chicken/USC/Egypt/Kafr el shaikh/2015(MG029121)
JS-14-12-Ch (KJ184584.1)
Ch/SD755/12)(JX840453.1)
Chicken/China/SDWF07/2011(JQ015295.1)
chicken/China/SD4/2008 (HM748947.1)
Go/CH/HLJ/LL01/08 (FJ240168.1)
GM (DQ486859.1)
TJ05 (DQ363536.1)
Y98 (AY338284.1)
TW/95-2 (AF083972.1)
Kr-188/02 (AY630436.1)
ZJ1 (AF431744.3)
chicken/China/SD2/2007 (HM748944.1)
chicken/Shaanxi/W2/2006 (KC122244.1)
Avian avulavirus 1 isolate NDV/chicken/Serbia/6987/06(MF144009)
Avian avulavirus 1 isolate NDV/chicken/2/SA/2016(MG022112)
APMV-1/chicken/NL/152608/93(JN986837.1)
chicken/Iran-EMM/5/2011(JQ267581)
62
ND/03/018 (GQ338309.1)
CH-A7/96 (AY028995.1)
63
Avian avulavirus 1 isolate PDDSL-5(MF362986)
Avian avulavirus 1 isolate PDDSL-3 (MF362984)
Avian avulavirus 1 isolate NDV/chicken/Malaysia/UPM-IBS-162A/2015(MF163271)
KH-cmb06-01/12 (JX870617.2)
JX-2/99(AF458014.1)
chicken/Iran-EMM/4/2010(JQ267582)
chicken/Iran-EMM/6/2011(JQ267580)
chicken/Iran-EMM/7/2011(JQ267579)
KND/45(AY928938)
KND/20(AY928934)
KND/40(AY928937)
KND/35(AY928936)
70
KND/30(AY928935)
NR 14(JN001188)
MK13/75(AY928933)
Avian avulavirus 1 strain Okara (MH607122)
65
UT-PCR 2017(MG871466)
Avian avulavirus 1 isolate Peacock/AJK/AW-pc45/2018(MH717069.1)
NIAB NDV 1(MH120424)
chicken/Sragen/014/10(HQ697258.1)
757(KF113343.1)
Chicken/BT-Israel/2013/120 827(KF792021.1)
QG/Hebei/07(FJ608337.1)
99
NDV016540 (EU258651.1)
chicken/Australia/Queensland/V-4/10/1966 (MH392213)
LaSota.71.IR(KU665482)
51
XD/Shandong/08 (GQ994433.1)
B1.72.IR(KU886038)
clone 12IR(KJ174522)
Avian avulavirus 1 strain AAvV1-LSTvac/Vietnam/2016 (MG869264)
USA (NDV) (X04719.1)
USA(M24698.1)
NDP21(KT343668)
89
NDP22(KT343669)
chicken/India/Mukteswar/519/1940s(MH392214)
chicken/Dominican Republic/FO/499-31/505/2008(MH392226)
chicken/Nigeria/Kano/1973/N52/899/1973(MH392215)
Sh-2/98 (AF458017.1)
54
ZhJ-3/97(AF458015.1)
DK-1/95(AF001129.1)
Miyadera F0I(M18456.1)
chicken/Pakistan/SPVC/Karachi/33/556-V/2007 (MH392224)
NDP83(KT343675)
PB9601 from pigeon (AY390291.1)
NDP93(KT343677)
NDP95(KT343678)
NDP61(KT343672)
NDP25(KT343670)
NDP20(KT343667)
NDP19(KT343666)
NDP92(KT343676)
NDPR2(KT343662)
NDP14(KT343663)
NDP27(KT343671)
NDP79(KT343674)
NDP64(KT343673)
NDP11(KT343664)
NDP17(KT343665)
0.02

Fig 1. Phylogenetic analysis of F genes of Iranian NDVs. This tree shows only class II. The phylogenetic tree was generated by using maximum likelihood method based on the Kimura 2-parameter model. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 (version 7.0.14). The classification system in this figure is according to Dimitrov et al. Viruses identified in this study are marked with a black square, Other Iranian NDVs are marked with a black circle.

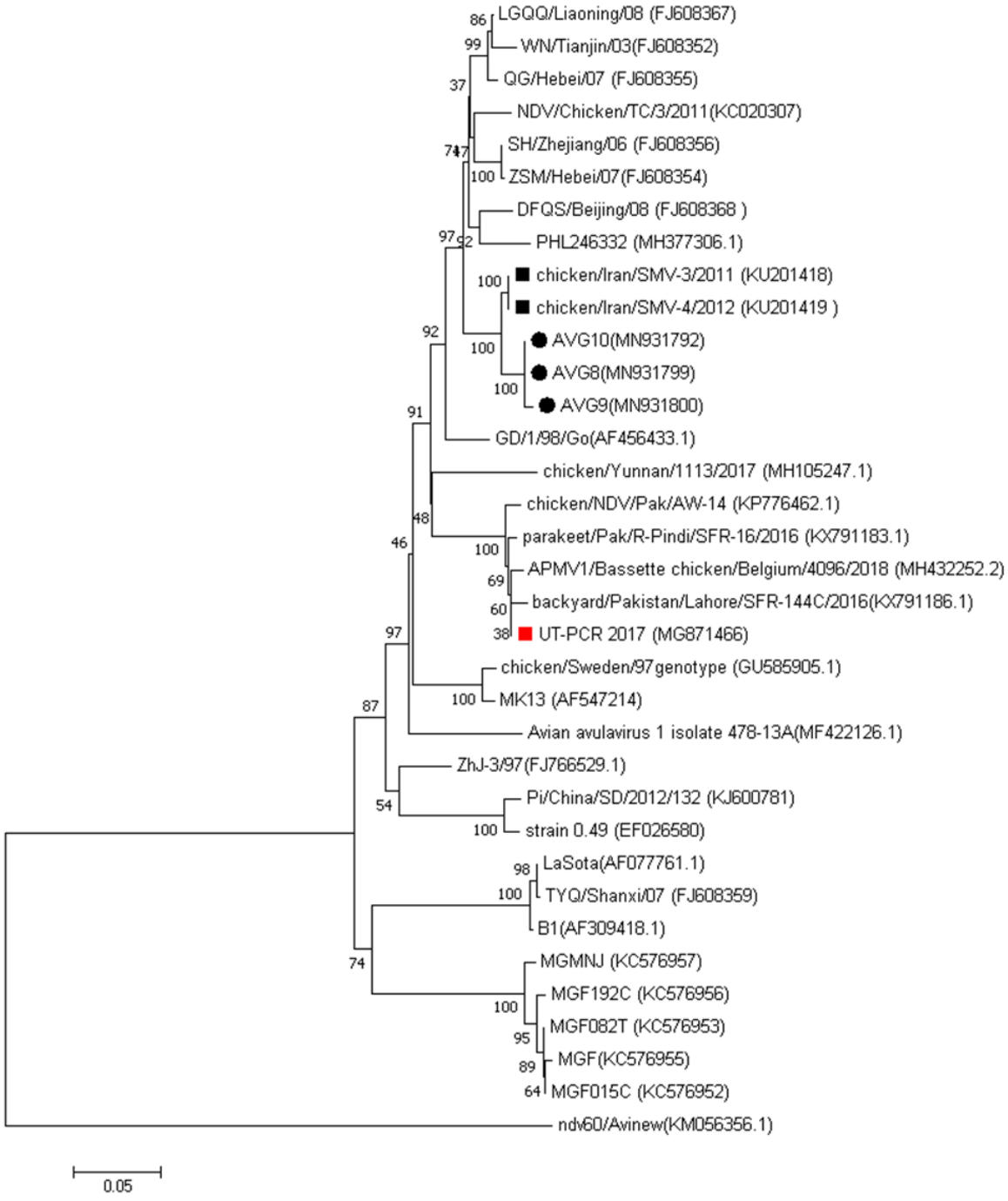


Fig 2. Phylogenetic analysis of HN genes of Iranian NDVs. The phylogenetic tree was generated by using maximum likelihood method based on the Kimura 2-parameter model. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. Evolutionary analyses were conducted in MEGA7 (version 7.0.14). The classification system in this figure is according to Dimitrov et al. Viruses identified in this study are marked with a black circle, Other Iranian NDV s are marked with a black square**.**

Discussion

Newcastle is a virulent disease in the poultry industry in many countries. The outbreak of ND constantly happened in commercial broilers and layers flocks in Iran and circulation of a novel genotype of NDVs.

For this reason, monitoring of this disease helps to plan for its control, sequencing and phylogenetic analysis are considered the best choice for characterization of novel NDV strains circulating in the field (2). This disease has been reported in Iran since 1950. Based on data collected from a phylogenetic study, viruses of genotype VI, VII and XIII have circulated in Iran since 1995 (11).). In current study, NDVs were isolated from broilers. The infected birds indicated nervous, respiratory and enteric clinical signs. The isolated strains, named AVG (8-10), were identified as genotype VII due to phylogenetic analysis on the F gene sequence. Homology between tested isolates was 96.47%-99.14%. Most strains were more closely relevant to Iran isolates.

Because of the remarkable genetic diversity of NDVs, increase the speed of phylogenetic conclusion, a unified classification system has been designed by Dimitrov et al (2). For these purposes, the complete nucleotide sequence of the F gene of all accessible class I and class II NDV isolates were used for classification (12). Class I viruses contain one genotype, and there are three subgenotypes within genotype 1 of class I. compared with class I, class II strains include a rang of non-virulent to virulent viruses and the complete analyses identified at least 20 distinguished genotypes (I to XXI) (2). In this system, no subgenotypes were identified in genotypes II, III, IV, VIII, IX, XI, XVI, XIX and XX. Using the classification criteria updated substantially impacted genotype VII. Former subgenotypes VIIb, VIId, VIIe, VIIj and VIIl were classified into a single genotype (VII.1.1). Former subgenotype VIIf that was classified as a separate subgenotype (VII.1.2). VIIh, VIIk and VIIi were merged into subgenotype VII.2. Former subgenotypes VIIh and VIIi are responsible for the fifth Newcastle panzootic in Indonesia, Asia, the Middle East, Europe, and Africa (13, 14). Genotype XIII included four subgenotypes (XIII.1.1, XIII.1.2, XIII.2.1 and XIII.2.2). Viruses isolated from Iran between 2008 and 2011 formed subgenotype XIII.1.2 (2, 11). Furthermore, based on partial sequence analysis of F genes, the studied isolates were classified into subgenotypes VII.1.1. Former sub-genotype VIIj (VII.1.1) was isolated from Iran and China in commercial poultry in 2016 (15, 16).

Subgenotype VII.1.1 was predominant in China and has been identified in 11 provinces of china during 2011-2015 (16). This sub-genotype has also been identified from Markazi and Mazandaran provinces in 2016 And this highlights the important role of migratory birds in transferring subgenotypes between countries.

Backyard poultry are regarded as an influential factor and potential reservoirs of NDVs (17).

Previous studies have shown that only a few point mutations increase NDV virulence (18).

In addition, contact between poultry and backyard poultry provides an opportunity to transmit avian viruses to new hosts and increase the hazard of viral evolution (19).

Qazvin province is an important center for the Iranian poultry industry and is considered as the hub of the laying poultry industry.

Therefore, knowledge of the Newcastle virus circulation status and under common genotypes in the region can be of great help in designing different strategies to control the disease.

Generally, the outbreaks of ND are entirely ruinous with mortality rate up to 100%. Many previous reports are indicating the risk of substantial mortality even in vaccinated flocks (20). In Iranian poultry industry, ND is still an endemic disease, Vaccine failure may be due to inadequate dosing and mismatch of the vaccine strain with circulating strains.There are several reasons that cause infection in vaccinated flocks including dose, interval between vaccination and challenge, and type of vaccine. The most important reason may be that the vaccine can protect birds against clinical consequences and mortality, but it cannot prevent the virus from spreading to post-vaccinated healthy birds, which is a major cause of the outbreak.

There is a need for time to characterize our native viral strains and develop new vaccine strategies to better control this devastating poultry disease soon.

Conflict of Interest

The authors declare that they have no conflict of interest.

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